Alteration of gene expression profile in CD3+ T-cells after downregulating MALT1

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Background: T cell immunodeficiency is a common feature in patients with different kinds of hematological disease such as T cell non-Hodgkin lymphoma (T-NHL), B cells NHL (B-NHL), NK/T cell NHL (NK/T-CL) and acute myeloid leukemia (AML). In our recent research, we found that significantly lower expression levels in MALT1 and NF-κB were related to suppression of T cell activation. Therefore, this study was conducted to further investigate the role of downregulating MALT1 in the development of immunodeficiency in T cells.

Methods: We induced activation inhibition in CD3+ T cells by MALT1 knockdown. Then we characterized the gene expression profile after MALT1 suppression by microarray analysis.

Result: The differentially expressed genes were ZAP-70, p65, MDM2, ATM, NFATC2 which participate in the NF-κ, p53, and NFAT pathways in CD3+ T cells after MALT1 downregulation.

Conclusion: MALT1 suppression may contribute to immunodeficiency in T cells via suppression of T cell activation and proliferation pathways. These data may help to explain some of the characteristics of immunodeficiency of T cells.

Keywords: CD3+ T-cells, MALT1, immunodeficiency, T-cell activation, microarray

Introduction

MALT1 has been gradually regarded as an important point of various signaling pathways in both innate and adaptive immune cells. MALT1 has an essential role in the activation of NF-κB by antigen receptors and in the control of lymphocyte activation and proliferation, but also including other receptors in regulating NF-κB with immunoreceptor tyrosine-based activation motifs and some G-protein coupled receptors expressed by both immune and nonimmune cells.1,2 Recently, we found that lower expression of MALT1, which results in lower T-cell activation, is a common characteristic in patients with T-cell non-Hodgkin lymphoma (T-NHL), B-cell NHL (B-NHL), NK/T-cell NHL (NK/T-CL), and acute myeloid leukemia, and it may play a critical role in the molecular mechanisms involved in T-cell immunodeficiency in such hematological malignances.3,4 Also, there are only a few studies focusing on the mechanism of immunodeficiency that may be induced by the deficiency of MALT1. So, in this study, we further analyzed the gene expression profile pattern related to T-cell activation and apoptosis in normal CD3+ T-cells which have been treated by MALT1-siRNA to confirm the role of MALT1 in immune regulation and its molecular mechanism.

Materials and methods

Cell culture

The human CD3+ T-cells were sorted from peripheral blood mononuclear cells (PBMCs) that were obtained from one healthy donor from our team by positive selection using...
CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The study was approved by the ethics committee of the First Affiliated Hospital of Jinan University. The donor was informed about this study, and written informed consent was obtained. The purified CD3+ T-cells were harvested in complete medium, which was supplemented with 200 IU/mL of IL-2 and 2 µg/mL of PHA and maintained in a 95% humidified incubator at 37°C and 5% CO2 for 6 days after stimulation and used for siRNA transduction using Nucleofector™ technology (Amaxa Biosystems, Cologne, Germany).

siRNA design and synthesis
MALT1-siRNA524 which targets domains between the eighth and ninth exons in the MALT1 gene (Accession: NM_006785.3) and a scrambled nonsilencing siRNA control (SC) were designed with online software (www.invitrogen.com) and synthesized by Invitrogen (Carlsbad, CA, USA).

Nucleofection
Human CD3+ T-cells were resuspended at 5×10^6 per 100 µL of Nucleofector solution from the human T-cell Nucleofector™ kit at room temperature,5,7 and were nucloected with 3 µg of MALT1-siRNA or a control SC siRNA using the T-020 program of the Nucleofection Device II (Amaxa Biosystems). Mock-transfected cells nucloected without siRNA were used as a negative control. After nucleofection, the cells were immediately mixed with 500 µL of prewarmed culture medium and transferred into culture plates. The treated cells were incubated at 37°C and were collected for RNA isolation.5

RNA isolation and real-time PCR
According to the results that the expression efficiency of siRNA was the highest and the viability of T-cells was the best, we chose to analyze the mRNA expression 48 hours posttransduction. Total RNA was extracted from the CD3+ T-cells according to the manufacturer’s instructions (TRIzol reagent; Invitrogen). The RNA quality was analyzed using 0.8% agarose gel electrophoresis with ethidium bromide staining. Expression levels of MALT1 and the β2-M reference gene were determined by SYBR Green I real-time PCR. PCR was performed as previously described.3,5 The sequences for the primers used in quantitative real-time PCR were as follows: MALT1: 5′-AAGCCCTATTCCTCACTACCAG-3′ (forward) and 5′-CCTCCACACTGCTCATGTTCC-3′ (reverse); β2-M: 5′-TACTGAAATCACCACCCAC-3′ (forward) and 5′-CATCCAAATCGCGCA-3′ (reverse).

Affymetrix microarray analysis
Total RNA (>3 µg) was sent for global gene expression profile analysis using the Affymetrix HG-U133 plus 2.0 gene chip (Shanghai Biochip Co. Ltd, Shanghai, People’s Republic of China). Affymetrix microarray analysis was performed using the Gene Spring GX11.0 software (Agilent Technologies, Santa Clara, CA, USA).8,9 Probe sets displayed a signal log ratio indicating an increase or marginal increase (ie, log ratio ≥1 (n)) and the detection of an experimental group displayed a signal change with P representing upregulated genes. Conversely, probe sets displayed a signal log ratio indicating a decrease or marginal decrease (ie, log ratio <−1(n)), and the detection of a control group displayed a signal change with P representing downregulated genes. The result data were analyzed using the SBC Analysis System (Shanghai Biotechnology Corporation, Shanghai, People’s Republic of China). After normalization and correction, the log fluorescence intensity value for each gene was obtained.8,9 A minimum 2-fold difference was considered significant. The fold change for all genes was calculated by comparing the MALT1-siRNAs- and SC-treated Human CD3+ T-cells.8,9

Statistical analysis
Unpaired t-test was used to analyze the MALT1 expression between different groups. A P<0.05 was considered to be statistically significant.

Results and discussion
Gene expression patterns of MALT1-siRNA-treated CD3+ T-cells
To determine the mechanisms of MALT1-mediated T-cell immunodeficiency and hematological malignances, we first downregulated MALT1 expression by MALT1-siRNA and analyzed its expression level by quantitative real-time PCR. The result indicated that there was a significantly lower expression of MALT1 in MALT1-siRNA-treated CD3+ T-cells (siRNA group: 0.10±0.01, SC group: 0.34±0.01; P<0.001) (Figure 1). Affymetrix microarray analysis was then performed. From the result we found that 339 genes (>2-fold: 17 genes; >4-fold: 1 gene) were upregulated and 854 genes (>2-fold: 104 genes, >4-fold: 1 gene) were downregulated at least 1.5-fold when the MALT1-siRNA and SC-treated expression data were compared. Then the genes which have at least 1.5-fold change and were involved in the apoptosis and proliferation pathway were selected to make heatmap (Figure 2A, Table 1).

Genes altered in TCR signaling pathway
The antigen-specific T-cell receptor (TCR) of the adaptive immune system triggers distinct signal transduction pathways.
Numerous genetic models in mice have confirmed the essential role of various transcription factors, like NF-κB, AP1, and NFAT, in promoting T-cell activation, expansion, and effector function in response to infection.\textsuperscript{10–12} In addition, abnormal pathway activation induced by TCR or abnormal activation of upstream regulators contribute to the development of autoimmunity, chronic inflammation, and malignances.\textsuperscript{13,14} Moreover, T-cell-induced immune function disorders are also associated with a risk for malignant transformation.\textsuperscript{15}

In this study, 12 genes were differentially expressed, including 3 upregulated genes and 9 downregulated genes, in the TCR signaling pathway (Figure 2B). The significant altered gene RelA (fold change: 0.49) which was downregulated after MALT1 suppression is the most important one of mammalian NF-κB family including Rel-A, NF-κB1, NF-κB2, c-Rel, and Rel-B.\textsuperscript{16} The c-Rel, Rel-B, and Rel-A pro-
teins also have a carboxyl-terminal nonhomologous transac-
tivation domain, which strongly activates transcription from
NF-κB-binding sites in target genes and phosphorylation of
Rel-A.11,16 Zap-70 is a cytosolic protein, and it is recruited at
the plasma membrane of T-cells following TCR stimulation,
and binds to phosphorylated CD3ζ ITAM; it plays a critical
role in activating downstream signal transduction pathways
in T-cells following TCR engagement.16,17 The results showed
that the downregulation of ZAP-70 and Rel-A contributed
to the NF-κB pathway inhibition on MALT1-siRNA-treated
CD3κ T-cells. As a result, the activation of CD3κ T-cells would
be inhibited.

Similarly, NFATC2 is important to the immune response
and regulates the growth and differentiation of multiple cell
types, and this gene was also downregulated,18,19 which can
also help identify that the activation and the sensitivity of
immune response of normal T-cells have been inhibited after
MALT1-siRNA treatment. Above all, the genes which are
closely related to activation and immune function of CD3κ
T-cells emerged to have a low expression level, which can
indicate that MALT1 may have an important role against
immune dysfunction.

Genes altered in the p53 signaling
pathway

TP53 (better known as p53) is a tumor suppressor gene that
controls response to several different cellular stresses including
DNA damage, hypoxia, and oncogene activation.20,21 In
response to DNA damage and other stress signals, p53 is
highly modified posttranslationally, which increases its stabil-
ity and promotes its activation and nuclear localization.20,22
While mutations in the p53 gene are present in approximately
50% of all cancers, in most that retain the WT1 gene, its tumor
suppressor ability may be reduced or eliminated by several
different mechanisms.

In this study, there were totally eleven genes differentially
expressed, including three upregulated genes and nine down-
regulated genes (Figure 2C). Among these genes, MDM2,
MDMX, ATM, and p53 were downregulated in MALT1-siRNA-
treated CD3κ T-cells. MDM2, which is an important negative
regulator of the p53 tumor suppressor, is an E3 ubiquitin ligase
that targets p53 protein for ubiquitin-dependent proteolysis in
the proteasome.23 In addition, decreased levels of MDM2 and
MDMX should result in high levels of p53 in cellular stresses,
which is opposite to our result. Besides, the ATM gene, a positive
regulator of p53 that triggers p53 Ser15 phosphorylation and
promotes its transcriptional activation,9 was also downregulated,
which contributes to the low level of p53 after siRNA-MALT1
treating. To sum up, the p53 signaling pathway also has been
inhibited after siRNA-MALT1. So, the ability of cells to inhibit
cancer development would be decreased, and the possibility for
tumorigenesis would be increased.

In conclusion, we characterized altered expression profile
of genes related to the TCR signaling pathway and p53 signal-
ing pathway in MALT1-siRNA-treated CD3κ T-cells from a
healthy individual. MALT1 suppression mediated inhibition
of normal activation in CD3κ T-cells related to these two
pathways through downregulation of Rel-A and NFATC2
could have an essential role in immunodeficiency, which is
correlated with the results we found. Also, downregulation
of p53 signaling pathway suggested that T-cells would have
limited ability to help fix DNA mutations; thus, the function
of T-cells would be unstable. However, further verification of
the altered genes and related proteins is needed.

| Gene symbol | NCBI accession | Fold change | Description | Pathway |
|-------------|----------------|-------------|-------------|---------|
| AKT1        | NM_005163.2    | –1.60       | v-akt murine thymoma viral oncogene homologue 1 (protein kinase B, γ) | TCR signaling pathway |
| AKT3        | NM_005465      | 0.60        | v-akt murine thymoma viral oncogene homologue 3 | TCR signaling pathway |
| CBLB        | NM_170662.3    | –1.72       | Cbl proto-oncogene, E3 ubiquitin protein ligase B | TCR signaling pathway |
| NFATC2      | NM_173091.3    | –2.56       | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 | TCR signaling pathway |
| RELA        | NM_021975.3    | –2.01       | v-relreticuloendotheliosis viral oncogene homologue A (avian) | TCR signaling pathway |
| ZAP70       | NM_207519.1    | –1.75       | Zeta-chain (TCR) associated protein kinase 70 kDa | TCR signaling pathway |
| ATM          | NM_000051      | –3.39       | Ataxia telangiectasia mutated | p53 signaling pathway |
| CASP8       | NM_033358.3    | –1.93       | Caspase 8, apoptosis-related cysteine peptidase | p54 signaling pathway |
| MDM2        | NM_002392      | –2.63       | Mdm2, p53 E3 ubiquitin protein ligase homologue | p55 signaling pathway |
| MDM4        | NM_002393.4    | –2.20       | Mdm4 p53 binding protein homologue | p56 signaling pathway |
| TP53        | NM_000546.5    | –1.58       | Tumor protein p53 | p57 signaling pathway |

Abbreviations: TCR, T-cell receptor; NCBI, National Center for Biotechnology Information.
Conclusion
In this study, by using microarray analysis to evaluate the effects of MALT1 knockdown, we identified a number of genes associated with the NF-κB, p53, and NFAT pathways in the CD3+ T-cells after MALT1 downregulation. These data suggest that MALT1 suppression may contribute to immu
nodeficiency in T-cells via suppression of T-cell activation and proliferation pathways.

Author contribution
YQL and CWZ contributed to the concept development and study design. XW performed the Nucleofection and analyzed the data. YK performed the T-cell sorting and real-time PCR, and prepared PBMCs, RNA, cDNA, and cell culture. YQL coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure
The authors report no conflicts of interest in this work.

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