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Functional network analysis reveals biological roles of lncRNAs and mRNAs in $MOG_{35–55}$ specific $CD4^+$T helper cells

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ABSTRACT

Long non-coding RNAs have the potential to regulate immune responses. Their impact on multiple sclerosis has remained elusive. For illustrating their roles in experimental autoimmune encephalomyelitis (EAE) pathogenesis, we investigated the differential expression of lncRNAs and mRNAs in $CD4^+$Th cells obtained from myelin oligodendrocytic $glycoprotein_{35–55}$($MOG_{35–55}$)-induced EAE and complete Freund's adjuvant (CFA) controls. We observed differential expression of 1112 lncRNAs and 519 mRNAs in $CD4^+$Th cells. The functional network showed lncRNAs had the capacity to modulate EAE pathogenesis via regulating many known EAE regulators such as Ptpn6. Predicting the function of lncRNAs demonstrated that dysregulated lncRNAs were closely associated with the development of EAE. These dysregulated lncRNAs may have function in EAE and they could be novel biomarkers and therapeutic targets of EAE. However, the precise mechanisms and biological functions of these specific lncRNAs in EAE pathogenesis require further study.

1. Introduction

Long non-coding RNAs (lncRNAs), which are > 200 nucleotides in length and lack protein-coding function [1], are characterized by tissue-specific expression [2],with lower expression level and less well conserved than those of protein-coding RNAs [3,4]. The regulatory mechanisms of lncRNAs are more complex than those of microRNAs; annotated functions have been established for only a few of lncRNAs. Recent studies suggest that lncRNAs perform diverse regulatory functions at the transcriptional or post-transcriptional level in multiple biological processes and diseases by interacting with DNA, RNA, and protein [5–10]. Many lncRNAs play crucial roles in modulating the innate and adaptive immune responses and contribute to the pathogenesis of autoimmune diseases by regulating immune cell differentiation [11–14].

Multiple sclerosis (MS) is one of the most prevalent autoimmune diseases [15].The condition is characterized by infiltrates of immune cells and plaques of demyelination in brain and spinal cord. MS is thought to be initiated and mediated by inflammatory autoreactive $CD4^+$T helper (Th) cells [16,17]. However, the complex, highly multicellular pathophysiological mechanism of MS has yet to be established [18]. Currently, with the development of next-generation sequencing (NGS) and molecular biology, several lncRNAs have been identified as key regulators of Systemic Lupus Erythematosus (SLE) [19], rheumatoid arthritis (RA) [20,21], and Type 1 Diabetes Mellitus (T1DM) [22,23]. Other lncRNAs regulate the differentiation and activation of T cells and B cells [24–28]. To elucidate the pathogenesis of MS and screen for specific biomarkers, it is essential to identify lncRNAs with specific expression to inflammatory self-reactive T cells.

Molecular biomarkers enable us to understand the epigenetic and molecular disorders of the disease and facilitate diagnosis and prognosis. Due to the convenience of collecting samples, one common approach to identify disease biomarkers is the detection of differentially expressed genes in peripheral blood mononuclear cells (PBMCs). Unpublished results from our laboratory showed that the differentially expressed genes observed in PBMCs of MS patients were similar to genes differentially expressed in other diseases associated with inflammation. In brief, differential gene expression in PBMCs of MS patients may reflect nonspecific inflammatory changes. These changes may be related to the altered proportion of immune cells among the

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PBMCs population.

In the present study, we chose myelin oligodendrocytic glycoprotein$_{35–55}$($MOG_{35–55}$)-induced experimental autoimmune encephalomyelitis (EAE) as the model of MS because the histopathologic and immunologic similarities [15,29]. Here, we first analyzed the expression profiling of lncRNAs and mRNAs in peripheral $MOG_{35–55}$ specific $CD4^+$Th cells from EAE and complete Freund's adjuvant (CFA) controls via lncRNA microarray. This approach, designed to rule out false-positive data resulting from nonspecific inflammatory changes, yielded 1112 specific lncRNAs and 519 dysregulated mRNAs. In addition, we constructed a pathway-network and signal-network for peak EAE. The crosstalk suggested that core mRNA Ptpn6 played a more important role in the development of EAE than previously thought. Here we have provided evidence that lncRNAs such as NONMMUT049403 participated in the pathogenic process of EAE through predicting their functions and gene ontology (GO) enrichment analysis. Our study established the utility of lncRNA as specific inflammatory biomarkers in the process of EAE. These findings should conduce to better understand the pathogenesis of MS and facilitate the search for new diagnostic and therapeutic targets of EAE and MS.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice, 6–8 weeks old, were purchased from Peking Vital River Laboratory Animal Ltd. (Beijing, China). All mice were kept in specific pathogen-free environments and all experiments were carried out according to protocols of the Care and Use of Laboratory Animals published by the China National Institute of Health.

2.2. EAE induction

C57BL/6 female mice were immunized subcutaneously in the axillary fossa with 200 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) peptide emulsified in complete Freund's adjuvant (Sigma, St.Louis, MO, USA) containing Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, USA) on 0d and then were injected intravenously with 200 ng pertussis toxin (LIST BIOLOGICAL LABORATORIES, INC.) both immediately after immunization and 2 days later. Disease severity was assessed as follows: 0, no clinical signs; 1, limp tail; 2, hind-limb weakness; 3, paraplegia; 4, quadriplegia; and 5, moribund or death, 0.5 was added to the lower score when clinical signs were intermediate between two grades of disease.

2.3. Preparation of mononuclear cells

Axillary lymph nodes of EAE and CFA mice were removed on the peak timing of EAE and minced into single-cell suspensions, then filtered through a 40 μm cell strainer (BD Biosciences). Subsequently, mononuclear cells were suspended in PBS for further analysis.

2.4. $CD4^+$T cells purification and sorting

$CD4^+$T cells isolated from draining lymph nodes of EAE and CFA mice were purified using the MojoSort™ Mouse $CD4^+$T Cell Isolation Kit (Biolegend, San Diego, CA) according to manufacturer's instruction. For achieving high purity of isolation, $CD4^+$T cells were negatively selected twice from total lymphocytes. Flow cytometric analysis revealed that the purity of isolated $CD4^+$T cells was > 90% (Supplementary Fig. 1). Each sample for microarray expression analysis contained 1 × 10^7 purified $CD4^+$T cells isolated from EAE and CFA mice. Each group had three individual samples.

2.5. RNA extraction

Total RNA from 1 × 10^7 sorted $CD4^+$T cells was extracted with TRIzol reagent RnaEx (GENEray) following the manufacturer's recommendations. RNA concentration was measured using NanoDrop 1000 Spectrophotometer. RNA purity and integrity for each sample were determined using spectrophotometer and gel electrophoresis. For spectrophotometer analysis, samples with OD260/OD280 ratio between 1.8 and 2.1 and OD260/OD230 ratio > 1.8 were only acceptable. For eletrophoresis analysis, samples should be free of genomic DNA contamination and ratio of 28S/18S band intensities should be > 2.0.

2.6. LncRNA microarray

The Mouse Transcriptome Assay 1.0 (MTA 1.0, Affymetrix, CA, USA), covering 55,000 lncRNAs and 23,000 mRNAs, was used for detecting the global expression profiling of mouse lncRNAs and mRNAs. Then, lncRNAs were carefully constructed using well-respected public transcriptome databases (i.e., Refseq, Ensembl, UCSC Known Genes, NONCODE, lncRNA db, Intergenic non-coding RNA from Luo H, et al.). The RNA labeling and microarray hybridized were carried out according to the Affymetrix expression analysis technical manual (Geneminix Informatics Ltd., Shanghai, China).

In brief, lncRNAs and mRNAs with differential expression were first filtered using the random variance model *t*-test ($P < 0.05$) for subsequent analysis, then, the results were presented as fold change. Moreover, Volcano Plot filtering and hierarchical clustering were performed to display the distinguishable lncRNAs and mRNAs expression patterns between the two groups.

2.7. Quantitative real-time PCR validation

To validate the microarray data, we randomly selected three up-regulated lncRNAs and mRNAs, (NONMMUT031096, NONMMUT057342, NONMMUT031095, Ifng, Tbx21, Il18rap) respectively. Meanwhile, we also randomly selected three down-regulated lncRNAs and mRNAs from abnormally expressed lncRNAs and mRNAs (NONMMUT028487, NONMMUT011309, and NONMMUT059037, Ccr8, Ptpn6 and Prg4). 1 μg of total RNA was reverse transcribed with an RT-PCR kit from Roche (Transcriptor First Strand cDNA Synthesis Kit, Roche, 04896866001) following recommendation of the supplier. Following first strand cDNA synthesis, qPCR reaction was carried out in a 10 μl reaction volume containing 1× SYBR Green PCR Master Mix (TransStart® Top Green qPCR SuperMix, Transgene,China), 0.2 μM of each specific forward and reverse primers, 1 μl of cDNA template and was performed in the CFX96 Real-Time system from Bio-Rad (C1000 Touch Thermal Cycler, Bio-Rad). Each sample was run in triplicate and the Δ Δ Ct method was used for relative quantification, with β-actin expression levels serving as internal control. The primers used are listed in Table S1 in Supplementary Material.

2.8. GO and pathway analysis

Gene ontology (GO) analysis was performed to illustrate the main function of the differentially expressed mRNAs. GO-map analysis was made to identify the interaction network of the significant GO terms of the differential expression genes.

Pathway analysis was used to discover underlying functions of the significantly differential expression mRNAs based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [30,31]. GO and pathways analyses of up-regulated and down-regulated mRNAs were performed by the Database for Annotation, Visualization and Integrated Discovery (The Database for Annotation, Visualization and Integrated Discovery, (https://david-d.ncifcrf.gov/home.jsp). The *P*-value denoted the statistical significance of GO terms enrichment and the pathways in

the differentially expressed mRNAs ($P < 0.05$).

2.9. LncRNA-mRNA co-expression network and LncRNA function annotation

The lncRNA-mRNA co-expression network was constructed between the differentially expressed lncRNAs and mRNAs according to the normalized signal intensity of specific expression levels of mRNAs and lncRNAs. We used Pearson's correlations, equal to or > 0.99, to calculate statistically significant associations. In basis of lncRNA-mRNA co-expression network, we drew conclusion about lncRNA biological functions.

2.10. Statistics

All statistical analysis was performed using SPSS11 statistical software and GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Statistical analyses included comparisons with the *t*-test, Fisher's exact test and the Pearson correlation, as appropriate; *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. LncRNA and mRNA expression profiles in peripheral CD4$^+$ Th cells isolated from EAE and CFA mice

Mouse Transcriptome Assay 1.0 was used to determine the expression levels of lncRNAs and mRNAs in peripheral CD4$^+$Th cells from three EAE mice and three CFA controls. After applying the RVM *t*-test, we observed significant differences in lncRNA and mRNA, with 1.2-fold change ($P < 0.05$) in two groups. Volcano plot analysis was applied to identify differential expression of lncRNAs (Fig. 1A) and mRNAs (Fig. 1B) in these two populations. Hierarchical clustering analysis enabled us to separate the EAE from CFA controls in terms of gene expression (Fig. 1C, D). Compared with CFA, a total of 1112 lncRNAs in peripheral $MOG_{35–55}$-specific CD4$^+$Th cells were specifically dysregulated, including 490 up-regulated lncRNAs and 622 down-regulated lncRNAs (Table S2). In addition, 519 mRNAs had differential expression in peripheral $MOG_{35–55}$-specific CD4$^+$Th cells as compared with CFA; of these, 341 were up-regulated, and 178 were down-regulated (Table 1, Table S3).

Table 1
Dysregulation of lncRNAs and mRNAs in peripheral CD4$^+$T helper cells of EAE.

	mRNA	lncRNAs
Upregulation	341	490
Downregulation	178	622
Total	519	1112

LncRNA, long non-coding RNA. EAE, experimental autoimmune encephalomyelitis.

3.2. Quantitative real-time PCR validation

To verify the reliability of the microarray data, we identified the up- and down-regulated lncRNAs and mRNAs selected randomly by quantitative real-time PCR. Comparison of EAE with CFA controls revealed six differentially expressed lncRNAs (NONMMUT031096, NONMMUT057342, NONMMUT031095, NONMMUT028487, NONMMUT011309, and NONMMUT059037) and six mRNAs with aberrantly differential expression (Ifng, Tbx21, Il18rap, Ccr8, Ptpn6 and Prg4) (Fig. 2). Among these random lncRNAs and mRNAs, lncRNA NONMMUT031096 was the most elevated (46.86-fold higher expression), followed by NONMMUT031095 (15.53-fold higher expression) and NONMMUT057342 (7.83-fold higher expression). For mRNA, IL-18Rap was the most elevated (12.46-fold higher expression), followed by IFN-γ (4.89-fold higher expression), and Tbx21 (3.08-fold higher

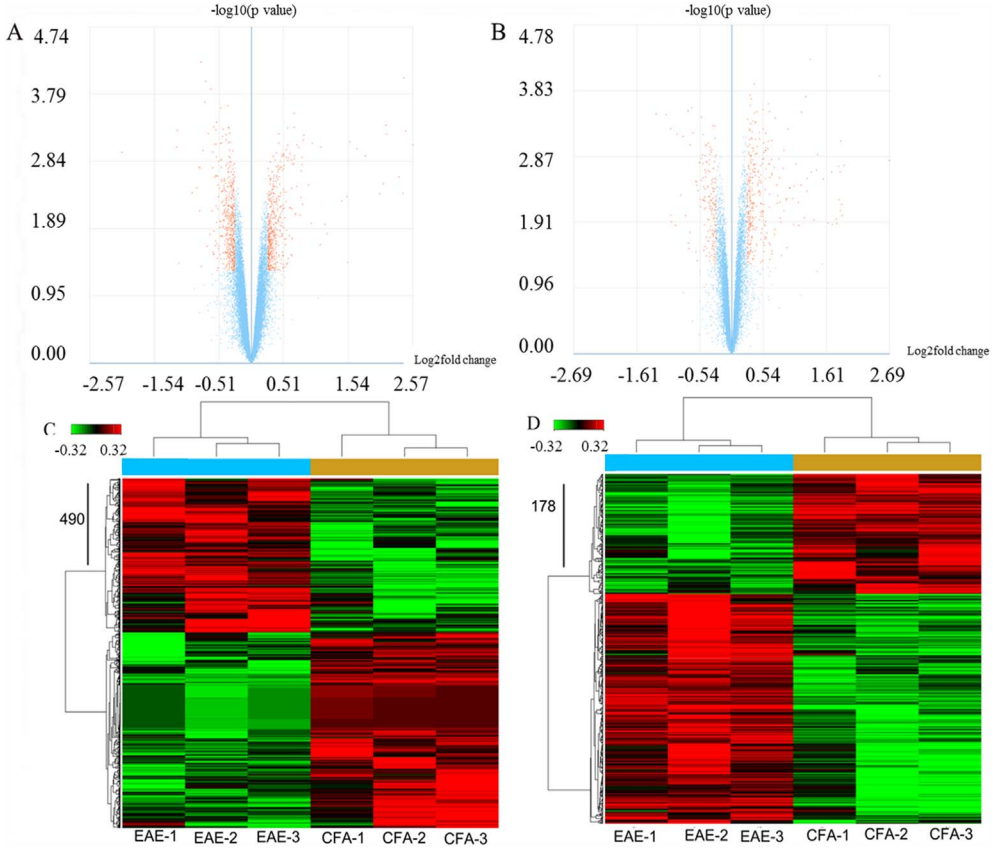

Fig. 1. LncRNA and mRNA expression profile for peripheral CD4$^+$T helper cells in EAE and CFA. (A–D)
Volcano plots were performed to distinguish differentially expressed lncRNAs (A) and mRNAs (B). Vertical lines represent 1.2-fold upregulation or downregulation. Horizontal lines represent $P = 0.05$. Red data points represent significantly differential expression of lncRNAs and mRNAs. Hierarchical clustering analyses show the differential expression patterns of lncRNA (C) and mRNA (D) between EAE (E1–E3) and CFA (C1–C3). Red and green represent high and low expression levels, respectively, among all samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

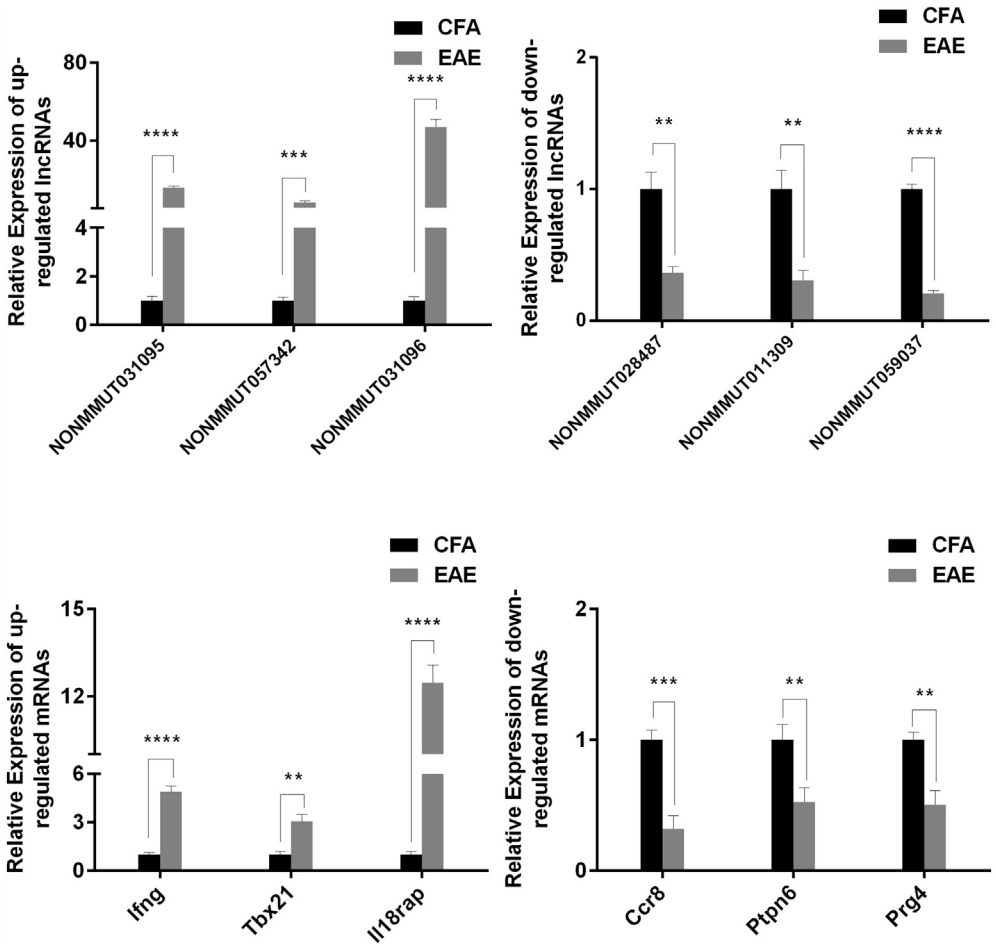

Fig. 2. Validation of lncRNA microarray data by real-time PCR.
Three upregulated and three downregulated lncRNAs as well as three upregulated and three downregulated mRNAs, were validated by real-time PCR of RNA extracted from peripheral $CD4^+$T cells of three EAE mice and three CFA controls. Relative expression levels of lncRNAs and mRNAs were normalized. Data displayed in histograms are expressed as mean ± SD; $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$ for EAE mice compared with CFA controls.

expression). LncRNA NONMMUT059037, NONMMUT011309, and NONMMUT028487 exhibited 4.85-, 3.68-and 2.76-fold lower expression, respectively. Ccr8, Ptpn6, and Prg4 exhibited 3.12-, 1.98- and 1.91-fold lower expression, respectively. Results of RT-PCR were consistent with the microarray data.

3.3. Functional exploration of dysregulated mRNAs

To investigate the comprehensive function of dysregulated mRNAs in biological processes, 519 differentially expressed mRNAs were submitted for GO and KEGG pathway analysis using DAVID. The negative logarithm of the *P*-value ($-\log_{10}P$) showed a positive correlation between gene expression and relevant biological processes ($P < 0.05$). GO analysis of biological processes based on dysregulated mRNAs showed that the up-regulated genes were enriched in immune system process, adaptive immune response, innate immune response, positive regulation of IFN-γ production, and negative regulation of inflammatory response, etc. (Fig. 3A, Table S4). Dissimilarly, down-regulated mRNAs were enriched in lipoprotein metabolic process, protein folding, lipid transport, immune response, and actin filament organization, etc. ($P < 0.05$) (Fig. 3B, Table S4). KEGG pathway analysis showed that aberrantly up-regulated genes were enriched in inflammatory bowel disease, cytokine-cytokine receptor interaction, tuberculosis, lysosome, primary immunodeficiency, etc. (Fig. 4A, Table S5), meanwhile, the down-regulated genes were enriched in measles, antigen processing and presentation, protein processing in endoplasmic reticulum, and influenza A ($P < 0.05$) (Fig. 4B, Table S5). Taken together, GO and pathway analysis indicated pro-inflammatory biological processes were predominant at the peak of EAE, as previously reported for MS and EAE [32]. These results verified the accuracy of microarray data.

The pathway-network was constructed according to KEGG database analysis to find the interaction among pathways enriched from mRNAs that were significantly dysregulated in the pathologic changes associated with EAE. Crosstalk involving the T cell receptor signaling pathway, cell adhesion molecules (CAMs), antigen processing and presentation may play an important role in the development of EAE (Fig. 5A, Table S6). Recent studies have indicated that the JAK-STAT pathway could regulate cells differentiation and cytokine secretion during EAE [33,34]. Our pathway-network suggested that the JAK-STAT pathway may participate in the disease as the target of natural killer cell mediated cytotoxicity and the toll-like receptor signaling pathway. To address the limited nature of interactions among genes in a single pathway, we constructed a signal-network by screening for mRNAs in an interaction repository between significantly regulated GO terms and pathways. Our data identified 36 core mRNAs in peripheral $CD4^+$Th cells during the development of EAE, according to the degree of gene interaction. The gene with the highest degree was Ptpn6 (Fig. 5B, Table S7). These data strongly support the inter-regulation of biological pathways and suggest that mRNAs may perpetuate the development of EAE.

3.4. LncRNA-mRNA co-expression network

To further illustrate the relationship between mRNAs and lncRNAs, a coding-noncoding gene co-expression (CNC) network was constructed between 1112 significantly dysregulated lncRNAs and 519 significantly dysregulated mRNAs, by calculating the Pearson correlation for each pair of RNAs. In total, 320 lncRNAs and 306 mRNAs (Pearson-correlation > 0.99) were included in the CNC network (Table S8). We selected mRNAs involved in an interactions repository based on significantly regulated GO terms and pathways that were relevant to the immune response. With the approach, the CNC network was able to identify closely correlated mRNAs and lncRNAs in peripheral $CD4^+$Th cells at the peak of EAE (Supplementary Fig. 2). The network showed that one mRNA was correlated with one or more lncRNAs, and vice versa. Furthermore, as Fig. 6 reveals, the network was operative in some meaningful pathways and GO terms related to the development of EAE. Seventeen lncRNAs interacted with 20 mRNAs in the "T cell receptor signaling pathway" (Fig. 6A); 10 lncRNAs interacted with 14 mRNAs in the "JAK-STAT signaling pathway" (Fig. 6B); 12 lncRNAs interacted with 8 mRNAs in the GO of "inflammatory response" (Fig. 6C); 8 lncRNAs interacting with 7 mRNAs in the GO of "immune response" (Fig. 6D).

3.5. Functional annotation and potential mechanism

To further elucidate the function of lncRNAs in EAE, we predicted lncRNA function based on the CNC network. Predicted function of lncRNAs was annotated using GO terms analysis of co-expressed mRNAs. For example, lncRNA NONMMUT028527 was associated with negative regulation of IL-2 production and negative regulation of inflammatory response (GO:0032695, GO:0050728). NONMMUT003168 was associated with positive regulation of T cell activation, positive regulation of IL-2 production, positive regulation of IFN-γ production, and positive regulation of T-helper 1 type immune response (GO:0050870, GO:0032743, GO:0032729, GO:00028270). NONMMUT049296 was associated with positive regulation of I-κB kinase/NF-κB cascade, toll-like receptor 2 signaling pathway, and interleukin-1-mediated signaling pathway (GO:0043123, GO:0034134, GO:0070498); NONMMUT004575 was associated with T cell costimulation, chemotaxis, and chemokine-mediated signaling pathway (GO:0031295, GO:0006935, GO:0070098). To systematically elucidate lncRNAs function, gene function enrichment analysis was performed for the annotated lncRNAs. Selecting the reliability prediction terms (according to *P*-value and enrichment) yielded 10 enrichment GO terms (Fig. 7A). The data showed that dysregulated lncRNAs were enriched in positive regulation of IL-8 production, Toll-like receptor 2 signaling pathway, I-κB phosphorylation, IL-1-mediated signaling pathway, and positive regulation of NIK/NF-κB signaling, etc.($P < 0.05$).

Based on the CNC network and predicted GO terms of differentially expressed lncRNAs, we chose lncRNA-Dleu2, which co-expressed with Foxo1 and might have function in acute-phase response (GO:0006953), for verifying a potential mechanism of lncRNAs in EAE. Detailed genomic region, products and transcription factors binding site analysis revealed that Dleu2 could bind with Foxo1 and produce pre-miR-15a

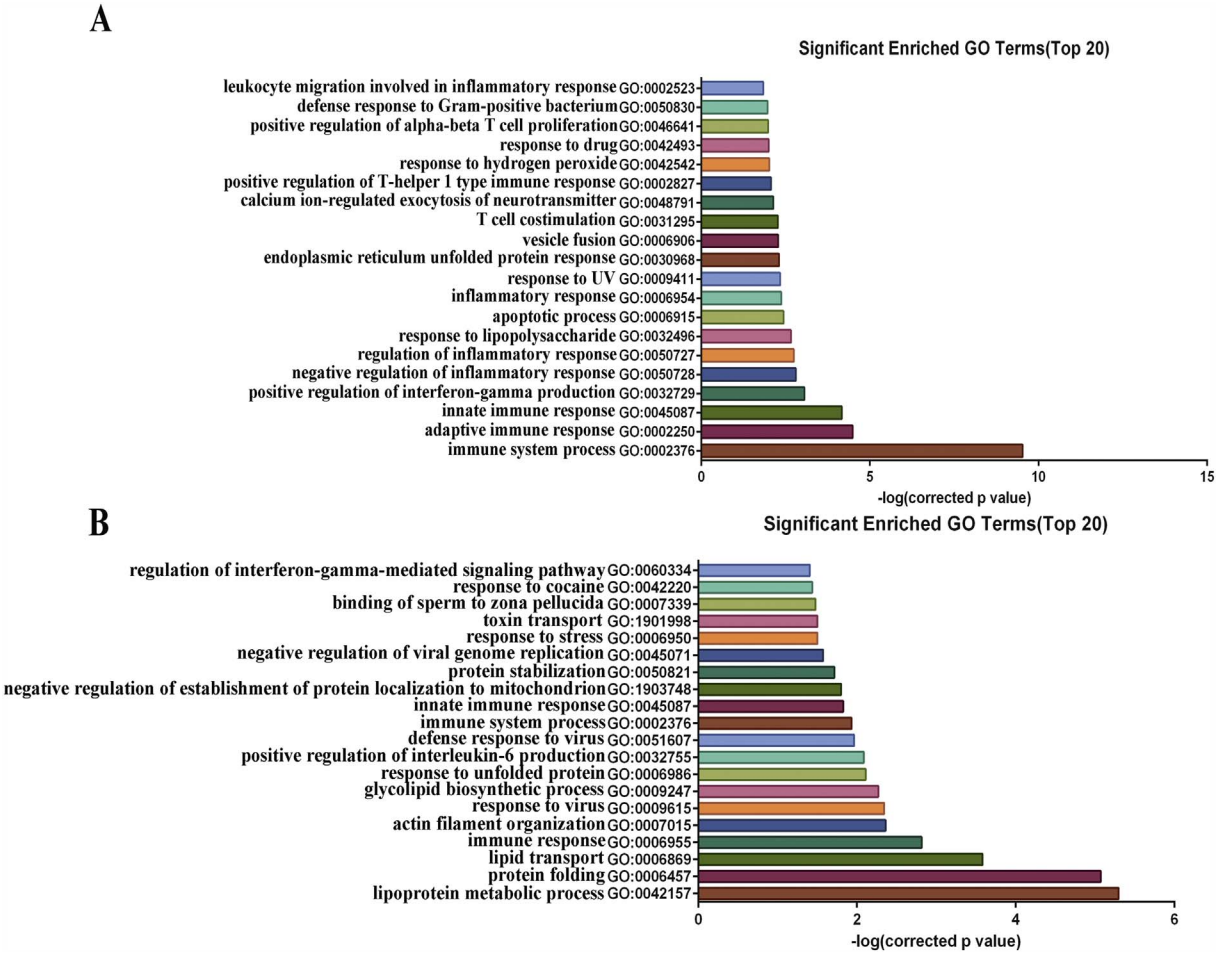

Fig. 3. Significant gene ontology analysis of differentially expressed mRNAs.
519 differentially expressed mRNAs were involved in GO analysis. A. The top 20 GO terms of upregulated mRNAs in EAE compared to CFA. B. The top 20 GO terms of downregulated mRNAs. Y-axis shows the GO category; x-axis shows the negative logarithm of the *P* value ($-\log10P$) representing the correlation between mRNAs and gene ontology ($P < 0.05$).

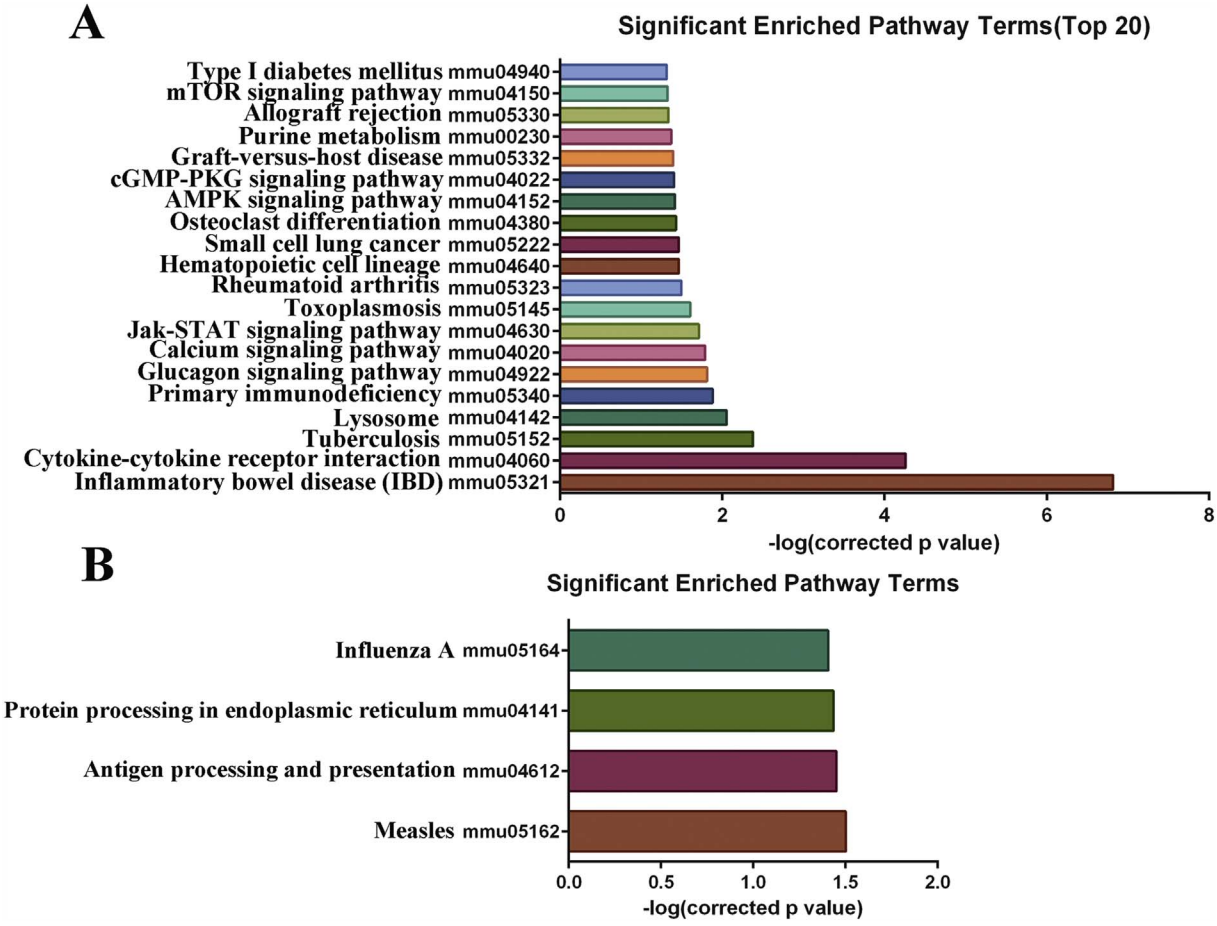

Fig. 4. Significant KEGG pathway analysis of differentially expressed mRNAs at the peak of EAE.
A total of 519 differentially expressed mRNAs were chosen in pathway analysis. A. The top 20 significantly enriched pathways of upregulated mRNAs in EAE and CFA. B. The top 4 significantly enriched pathways of downregulated mRNAs in EAE compared with CFA. Y-axis represents the pathway category; x-axis represents the negative logarithm of the *P*-value ($-\log10P$). A larger $-\log10P$ indicated a smaller *P*-value for the difference.

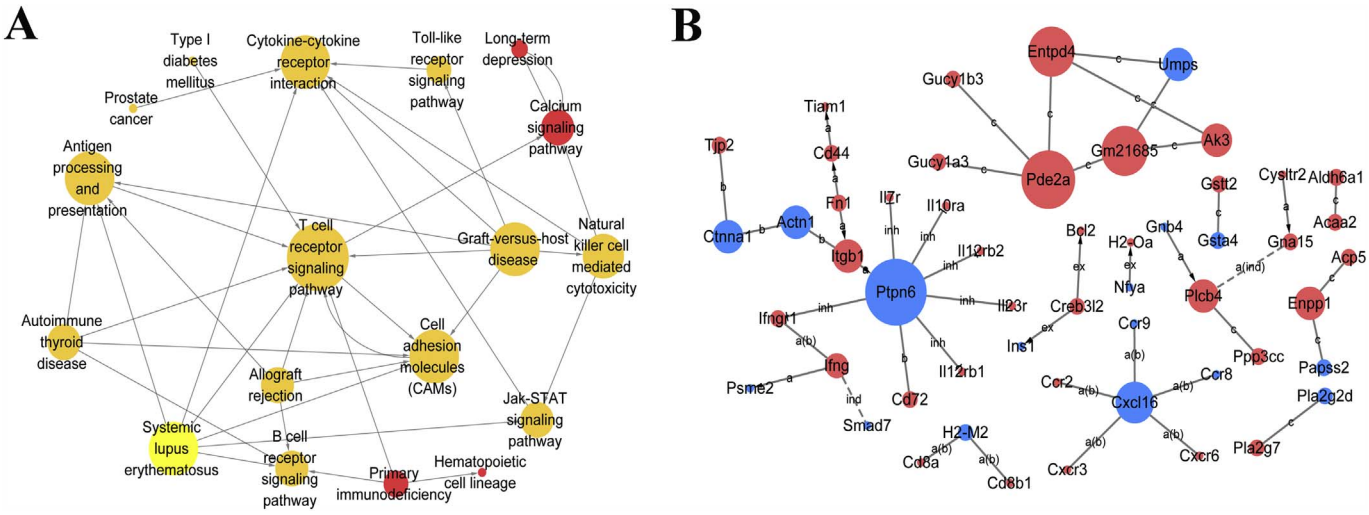

Fig. 5. Interaction network of significant pathways (Path-network) and differentially expressed mRNAs (Signal-network).
A. Pathway network. Counting connections of a pathway could evaluate the role of the pathway (a pathway with a high degree may play a pivotal role in the network). Red dot represents upregulated pathways and yellow represents up- and downregulated pathways. The lines represent consistency between pathways. B. The interaction network of differentially expressed mRNAs (Signal-network). Measuring the "betweenness centrality" of genes reflects the importance of a node relative to another. The ellipses represent the core mRNAs (Red: upregulated mRNAs; Blue: downregulated mRNAs). Size of the shape represents the degree of interaction, and lines represent the interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

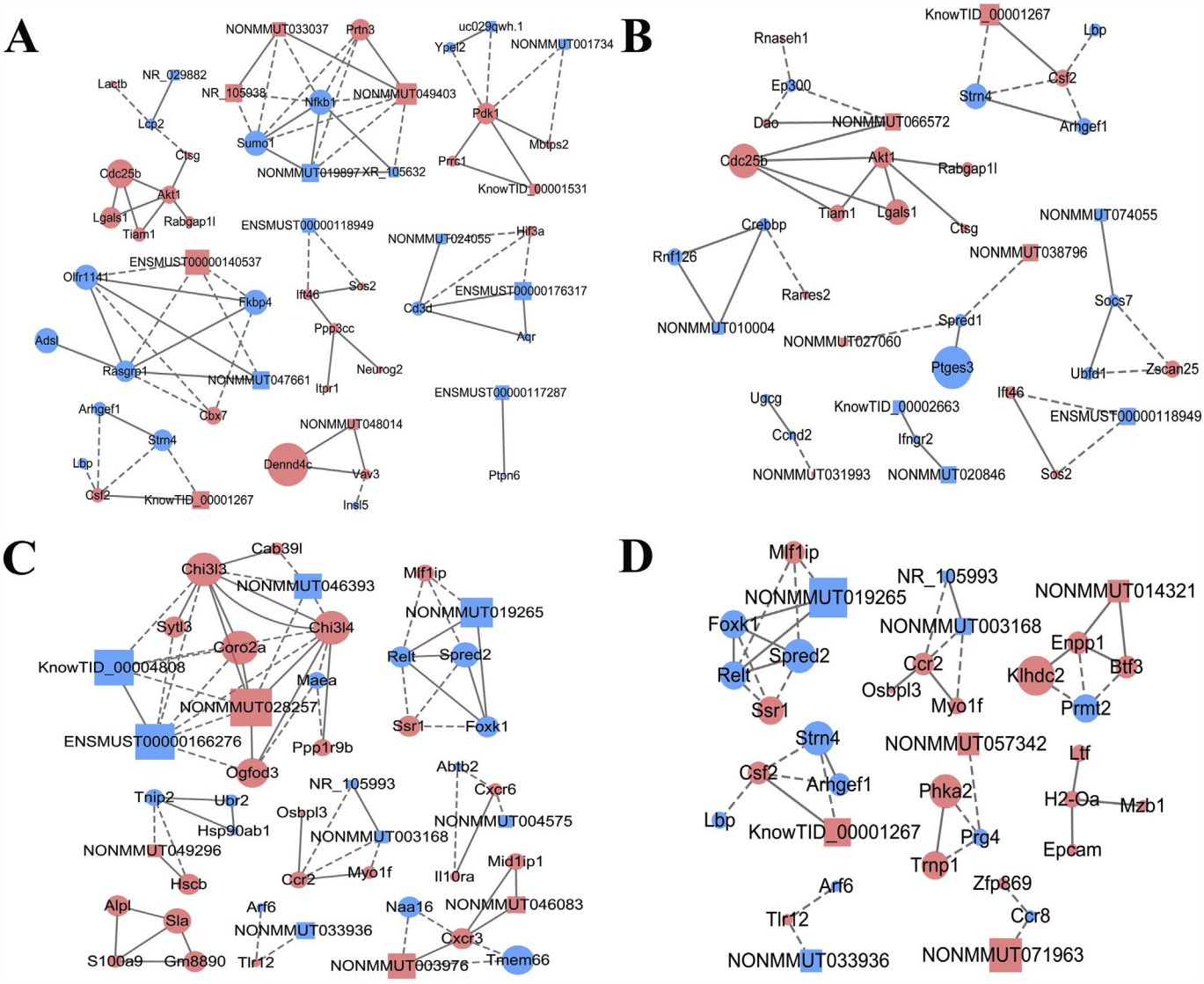

Fig. 6. LncRNA-mRNA co-expression network.
17 lncRNAs interacted with 20 mRNAs in the important "T cell receptor signaling pathway" (A); 10 lncRNAs interacted with 14 mRNAs in the meaningful "JAK-STAT signaling pathway" (B); 12 lncRNAs interacted with 8 mRNAs in the GO of "inflammatory response" (C); 8 lncRNAs interacted with 7 mRNAs in the GO of "immune response" (D). Ellipses represent upregulated (red) mRNAs. Blue represents doenregulated mRNAs in peripheral $CD4^+$T cells. Rectangles represent upregulated (red) lncRNAs, and downregulated (blue) lncRNAs. Lines represent the regulatory relationships between mRNAs and lncRNAs (solid lines represent positive correlation, dash lines represent negative correlation). Shape size represents the degree. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig.7B–C). On the basis of bioinformatic analysis, Dleu2 had the capacity to regulate Foxo1 and miR-15a expression. Detecting expression profile of Dleu2, Foxo1, miR-15a and Foxp3 in $MOG_{35–55}$ specific $CD4^+$T cells at peak EAE showed Dleu2 regulated Foxo1 expression negatively and reduced Foxp3 expression by increasing miR-15a expression (Fig.7D–G). These data illuminated that lncRNA could regulate mRNA expression directly or indirectly during EAE development.

4. Discussion

MS is a chronic autoimmune disease characterized by demyelination, inflammatory lesions, and axonal damage [35]; the etiology remains to be elucidated. Reportedly, lncRNAs involve in the process of MS and regulate the differentiation of $CD4^+$Th cells and B cells [27,28]. However, the association between specific lncRNAs expressed in $MOG_{35–55}$-specific $CD4^+$Th cells and MS remains unclear. Therefore, screening for lncRNAs abnormally expressed in $MOG_{35–55}$-specific $CD4^+$Th cells will facilitate the development of biomarkers. In this study, we detected, for the first time, the expression profiles of lncRNAs and mRNAs in peripheral $CD4^+$Th cells obtained from EAE models and CFA controls for eliminating the error resulted from the injection of CFA adjuvant itself [36].

The results identified 1112 lncRNAs and 519 mRNAs with differential expression in $MOG_{35–55}$-specific peripheral $CD4^+$Th cells, compared to the CFA group. The results of further bioinformatic analysis suggested that specific lncRNAs might be relevant to the pathology of MS and act as potential biomarkers for MS/EAE.

Increased incidences of Inflammatory Bowel Disease (IBD) among MS patients and the finding that gluten antibodies are remarkably effective in both diseases suggest a close association between MS and IBD [37]. The results of pathway analysis presented here demonstrated a close relationship between EAE and IBD. Significantly up-regulated expression of IL-18Rap suggested that IL-18Rap might be a novel shared gene locus between IBD and MS. IL-18Rap is reportedly involved in the pathogenesis of IBD via regulation of Th1-cell differentiation [38]. Hence, our data suggest that therapy targeting IL-18Rap might alleviate the symptoms of IBD in MS patients.

Previous studies have shown that Ptpn6 (SHP-1) limited Th1 cell

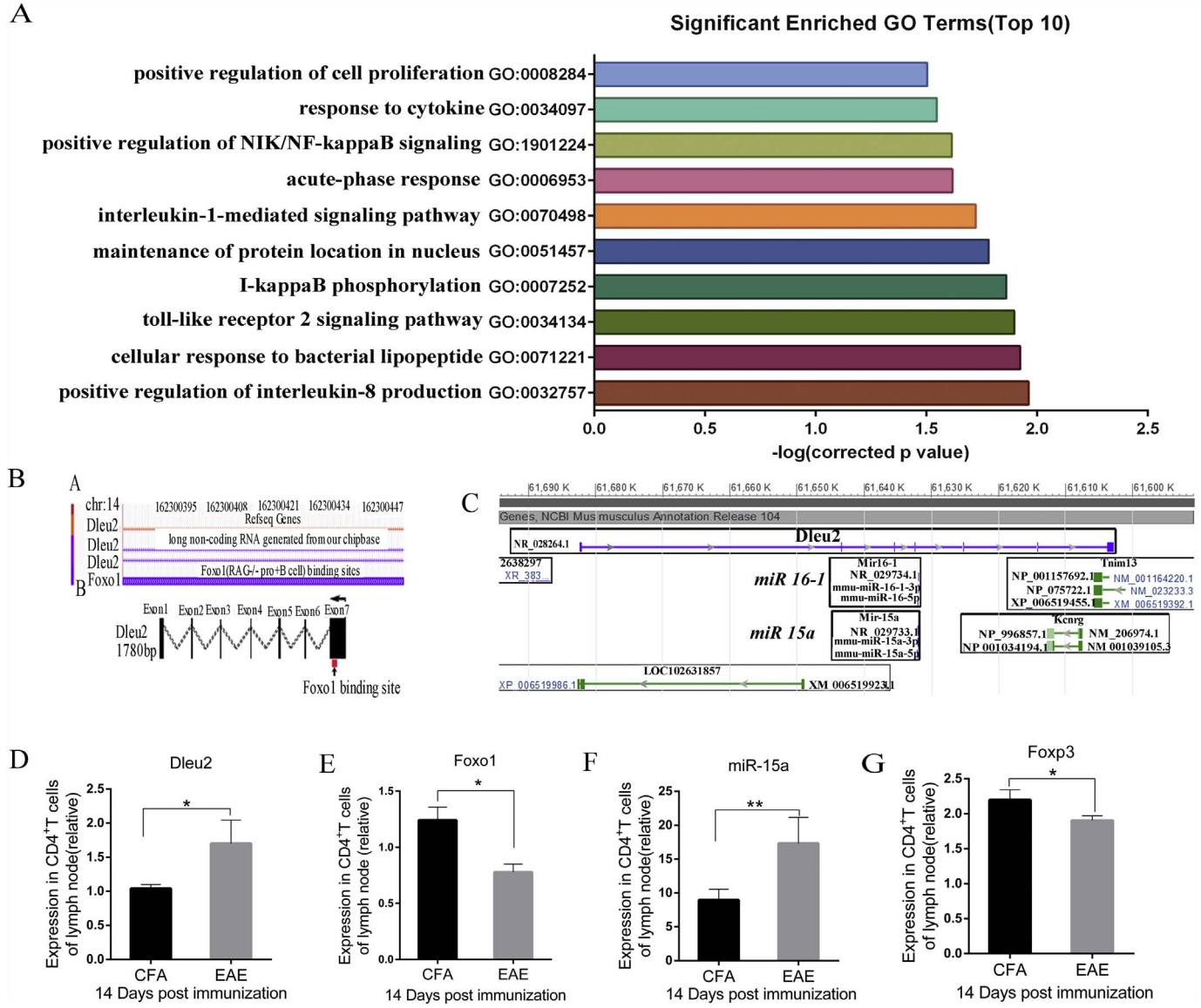

Fig. 7. Function annotation and preliminary mechanism exploration of lncRNAs.
(A) Top 10 significantly enriched GO terms of differentially expressed lncRNAs. Choosing the reliability prediction terms yielded the top 10 enrichment GO terms ($P < 0.05$). (B) Predicted binding sites of Foxo1 and lncRNA-Dleu2. (C) Genomic products of non-coding gene of Dleu2. (D–G) Expression profile of lncRNA-Dleu2, Foxo1, miR-15a and Foxp3 of MOG35–55 specific $CD4^+$T cells in EAE and CFA at peak EAE, $^*P < 0.05$, $^{**}P < 0.01$.

differentiation [39] and promoted the suppressive effects of TGF-β1 [40]. However, the precise mechanism by which Ptpn6 regulates T-cell development and differentiation during the development of EAE has remained elusive. We investigated several new hypotheses about regulatory mechanisms of Ptpn6 via our signal-network. In the signal-network, Ptpn6 acted as an upstream signal and inhibited downstream signals including IL7r, IL12rb1, IL12rb2, Ifngr1, IL23r, and IL10ra. We inferred Ptpn6 might negatively modulate Th1-cell differentiation by inhibiting activity of IL7r [41], IL12rb1, and IL12rb2 in the development of EAE [42,43]. Ptpn6 could regulate Th17-cell development by inhibiting IL23r and decreasing the tyrosine phosphorylation [44,45].Clearly, the results of network analysis illustrate the comprehensive function of dysregulated mRNAs in the process of EAE.

To accurately screen for biomarkers of EAE, we further analyzed the CNC network. The results showed that known regulators of EAE may interact with lncRNAs. Ifngr2, which is required for activating IFN-γ [46], may interact with lncRNAs KnowTID_00002663 and NONMMUT020846 in the JAK-STAT signaling pathway. In addition, Ptpn6 is likely to co-express with lncRNA ENSMUST00000117287 in T cell receptor signaling pathway. Nfkb1, a critical repressor of immune response that promotes Th2 differentiation and inhibits Th17 differentiation [47,48], may interact with lncRNAs XR_105632, NONMMUT049403, NONMMUT033307, NONMMUT019897, and NR_105938 in the TCR signaling pathway. Moreover, our preliminary study suggests lncRNA-Dleu2 may bind with the sequence of Foxo1 and negatively regulates its expression, in addition, lncRNA-Dleu2 reduced Foxp3 expression by producing mature miR-15a at peak EAE. Hence, we hypothesized lncRNA-Dleu2 participated the pathological process of EAE by regulating the key transcripition factors expression of $CD4^+$T cells. Based on the genomic products of Dleu2, miR-16-1 as miR-15a homologous RNA was likely to regulate the pathogenesis of EAE. Regulatory relationship between lncRNA-Dleu2 and miR-16-1 in the development of EAE will be studied subsequently. The bioinformatics suggest lncRNAs could regulate expression of co-expressed mRNA by multiple mechanisms such as RNA-protein complex or producing pre-miRNA molecules.These data strongly suggest that significantly dysregulated lncRNAs regulate the pathophysiology of EAE through affecting on mRNAs expression. Furthermore, these lncRNAs may serve as

biomarkers of EAE. The results of lncRNA GO terms analysis demonstrate the crucial role of specific lncRNAs in the process of EAE, as the most enriched GO terms were found to affect T cell differentiation and the pathophysiology of EAE [49–52].

5. Conclusions

In conclusion, the results presented above demonstrate the crucial role of lncRNAs and mRNAs in the process of EAE. Bioinformatic analysis of these results revealed a close association between IBD and EAE and certain genetic locus shared between the two diseases. Ptpn6 appears to regulate T cells differentiation through mRNA crosstalk. Further analysis of lncRNA function indicates a role in inflammation and EAE. In brief, our data clarify potential mechanisms by which mRNAs and lncRNAs regulate the process of EAE. Our results strongly support the possibility that lncRNAs acted as biomarkers of EAE. Nevertheless, the precise mechanisms and biological functions of these specific lncRNAs in the pathogenesis of EAE require further study.

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References

[1] S. Djebali, C.A. Davis, A. Merkel, A. Dobin, T. Lassmann, A. Mortazavi, A. Tanzer, J. Lagarde, W. Lin, F. Schlesinger, C. Xue, G.K. Marinov, J. Khatun, B.A. Williams, C. Zaleski, J. Rozowsky, M. Roder, F. Kokocinski, R.F. Abdelhamid, T. Alioto, I. Antoshechkin, M.T. Baer, N.S. Bar, P. Batut, K. Bell, I. Bell, S. Chakrabortty, X. Chen, J. Chrast, J. Curado, T. Derrien, J. Drenkow, E. Dumais, J. Dumais, R. Duttagupta, E. Falconnet, M. Fastuca, K. Fejes-Toth, P. Ferreira, S. Foissac, M.J. Fullwood, H. Gao, D. Gonzalez, A. Gordon, H. Gunawardena, C. Howald, S. Jha, R. Johnson, P. Kapranov, B. King, C. Kingswood, O.J. Luo, E. Park, K. Persaud, J.B. Preall, P. Ribeca, B. Risk, D. Robyr, M. Sammeth, L. Schaffer, L.H. See, A. Shahab, J. Skancke, A.M. Suzuki, H. Takahashi, H. Tilgner, D. Trout, N. Walters, H. Wang, J. Wrobel, Y. Yu, X. Ruan, Y. Hayashizaki, J. Harrow, M. Gerstein, T. Hubbard, A. Reymond, S.E. Antonarakis, G. Hannon, M.C. Giddings, Y. Ruan, B. Wold, P. Carninci, R. Guigo, T.R. Gingeras, Landscape of transcription in human cells, Nature 489 (2012) 101–108, http://dx.doi.org/10.1038/nature11233.

[2] T.R. Mercer, M.E. Dinger, S.M. Sunkin, M.F. Mehler, J.S. Mattick, Specific expression of long noncoding RNAs in the mouse brain, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 716–721, http://dx.doi.org/10.1073/pnas.0706729105.

[3] H. Bono, K. Yagi, T. Kasukawa, I. Nikaido, N. Tominaga, R. Miki, Y. Mizuno, Y. Tomaru, H. Goto, H. Nitanda, D. Shimizu, H. Makino, T. Morita, J. Fujiyama, T. Sakai, T. Shimoji, D.A. Hume, T. Arakawa, P. Carninci, J. Kawai, Y. Hayashizaki, Y. Okazaki, Systematic expression profiling of the mouse transcriptone using RIKEN cDNA microarrays, Genome Res. 13 (2003) 1318–1323, http://dx.doi.org/10.1101/gr.1075103.

[4] M. Guttman, M. Garber, J.Z. Levin, J. Donaghey, J. Robinson, X. Adiconis, L. Fan, M.J. Koziol, A. Gnirke, C. Nusbaum, J.L. Rinn, E.S. Lander, A. Regev, Ab initio reconstruction of cell type-specific transcriptomes in mouse reveals the conserved multi-exonic structure of lincRNAs, Nat. Biotechnol. 28 (2010) 503–510, http://dx.doi.org/10.1038/nbt.1633.

[5] S.A. Bhat, S.M. Ahmad, P.T. Mumtaz, A.A. Malik, M.A. Dar, U. Urwat, R.A. Shah, N.A. Ganai, Long non-coding RNAs: mechanism of action and functional utility, Non-Coding RNA Res. 1 (2016) 43–50, http://dx.doi.org/10.1016/j.ncrna.2016.11.002.

[6] J.J. Quinn, H.Y. Chang, Unique features of long non-coding RNA biogenesis and function, Nat. Rev. Genet. 17 (2015) 47–62, http://dx.doi.org/10.1038/nrg.2015.10.

[7] J.E. Wilusz, H. Sunwoo, D.L. Spector, Long noncoding RNAs: functional surprises from the RNA world, Genes Dev. 23 (2009) 1494–1504, http://dx.doi.org/10.1101/gad.1800909.

[8] R.J. Taft, K.C. Pang, T.R. Mercer, M. Dinger, J.S. Mattick, Non-coding RNAs: regulators of disease, J. Pathol. 220 (2010) 126–139, http://dx.doi.org/10.1002/path.2638.

[9] R. Ard, P. Tong, R.C. Allshire, Long non-coding RNA-mediated transcriptional interference of a permease gene confers drug tolerance in fission yeast, Nat. Commun. 5 (2014) 5576, http://dx.doi.org/10.1038/ncomms6576.

[10] M. G., Je-Hyun Yoon, Kotb Abdelmohsen, Post-transcriptional gene regulation by long noncoding RNA, J. Mol. Biol. 425 (2013) 3723–3730, http://dx.doi.org/10.1016/j.jmb.2012.11.024.

[11] M.K. Atianand, D.R. Caffrey, K.A. Fitzgerald, Immunobiology of long noncoding RNAs, Annu. Rev. Immunol. 35 (2017) 177–198, http://dx.doi.org/10.1146/annurev-immunol-041015-055459.

[12] D. Zemmour, A. Pratama, S.M. Loughhead, D. Mathis, C. Benoist, *Flicr*, a long noncoding RNA, modulates Foxp3 expression and autoimmunity, Proc. Natl. Acad. Sci. 114 (2017) E3472–E3480, http://dx.doi.org/10.1073/pnas.1700946114.

[13] M. Xia, J. Liu, S. Liu, K. Chen, H. Lin, M. Jiang, X. Xu, Y. Xue, W. Liu, Y. Gu, X. Zhang, Z. Li, L. Yi, Y. Qian, C. Zhou, R. Li, X. Zhang, Z. Li, X. Cao, Ash1l and lnc-Smad3 coordinate Smad3 locus accessibility to modulate iTreg polarization and T cell autoimmunity, Nat. Commun. 8 (2017) 15818, , http://dx.doi.org/10.1038/ncomms15818.

[14] J. Xu, F. Zhang, C. Gao, X. Ma, X. Peng, D. Kong, J. Hao, Microarray analysis of lncRNA and mRNA expression profiles in patients with neuromyelitis optica, Mol. Neurobiol. 54 (2017) 2201–2208, http://dx.doi.org/10.1007/s12035-016-9754-0.

[15] H. Batoulis, M.S. Recks, K. Addicks, S. Kuerten, Experimental autoimmune encephalomyelitis - achievements and prospective advances, APMIS 119 (2011) 819–830, http://dx.doi.org/10.1111/j.1600-0463.2011.02794.x.

[16] J. Goverman, Autoimmune T cell responses in the central nervous system, Immunology 9 (2009) 393–407, http://dx.doi.org/10.1038/nri2550.Autoimmune.

[17] L. Belbasis, V. Bellou, E. Evangelou, J.P.A. Ioannidis, I. Tzoulaki, Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses, Lancet Neurol. 14 (2015) 263–273, http://dx.doi.org/10.1016/S1474-4422(14)70267-4.

[18] C.A. Dendrou, L. Fugger, M.A. Friese, Immunopathology of multiple sclerosis, Nat. Rev. Immunol. 15 (2015) 545–558, http://dx.doi.org/10.1038/nri3871.

[19] T. Kino, D.E. Hurt, T. Ichijo, N. Nader, G.P. Chrousos, Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor, Sci. Signal. 3 (2010) ra8, http://dx.doi.org/10.1126/scisignal.2000568.

[20] B. Stuhlmüller, E. Kunisch, J. Franz, L. Martinez-Gamboa, M.M. Hernandez, A. Pruss, N. Ulbrich, V. a Erdmann, G.R. Burmester, R.W. Kinne, Detection of oncofetal h19 RNA in rheumatoid arthritis synovial tissue, Am. J. Pathol. 163 (2003) 901–911, http://dx.doi.org/10.1016/S0002-9440(10)63450-5.

[21] J. Song, D. Kim, J. Han, Y. Kim, M. Lee, E.J. Jin, PBMC and exosome-derived Hotair is a critical regulator and potent marker for rheumatoid arthritis, Clin. Exp. Med. 15 (2014) 121–126, http://dx.doi.org/10.1007/s10238-013-0271-4.

[22] H. Hakonarson, S.F. Grant, J.P. Bradfield, L. Marchand, C.E. Kim, J.T. Glessner, R. Grabs, T. Casalunovo, S.P. Taback, E.C. Frackelton, M.L. Lawson, L.J. Robinson, R. Skraban, Y. Lu, R.M. Chiavacci, C.A. Stanley, S.E. Kirsch, E.F. Rappaport, J.S. Orange, D.S. Monos, M. Devoto, H.Q. Qu, C. Polychronakos, A genome-wide association study identifies KIAA0350 as a type 1 diabetes gene, Nature 448 (2007) 591–594, http://dx.doi.org/10.1038/nature06010.

[23] V. Plagnol, J.M.M. Howson, D.J. Smyth, N. Walker, J.P. Hafler, C. Wallace, H. Stevens, L. Jackson, M.J. Simmonds, P.J. Bingley, S.C. Gough, J.A. Todd, Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases, PLoS Genet. 7 (2011), http://dx.doi.org/10.1371/journal.pgen.1002216.

[24] T.M. Aune, P.S. Crooke, C.F. Spurlock, Long noncoding RNAs in T lymphocytes, J. Leukoc. Biol. 99 (2016) 31–44, http://dx.doi.org/10.1189/jlb.1RI0815-389R.

[25] M.R. B., Sarah P. Collier, Patrick L. Collins, Christopher L. Williams, Thomas M. Aune, Influence of Tmevpg1, a long intergenic noncoding RNA, on the expression of Ifng by Th1 cells, J. Immunol. 189 (2012) 2084–2088, http://dx.doi.org/10.4049/jimmunol.1200774.

[26] K. Z., Gangqing Hu, Qingsong Tang, Suveena Sharma, Fang Yu, Thelma M. Escobar, Stefan A. Muljo, Jinfang Zhu, Expression and regulation of lincRNAs during T cell development and differentiation, 14 (2013) 1190–1198, http://dx.doi.org/10.1038/ni.2712.

[27] W. Huang, B. Thomas, R.A. Flynn, S.J. Gavzy, L. Wu, S.V. Kim, J.A. Hall, E.R. Miraldi, C.P. Ng, F. Rigo, S. Meadows, N.R. Montoya, N.G. Herrera, A.I. Domingos, F. Rastinejad, R.M. Myers, F.V. Fuller-Pace, R. Bonneau, H.Y. Chang, O. Acuto, D.R. Littman, Corrigendum: DDX5 and its associated lncRNA Rmrp modulate TH17 cell effector functions, Nature 533 (2016) 517–522, http://dx.doi.org/10.1038/nature16968.

[28] T.F. Braza, J.S. Johnson, M. Jennifer, Long noncoding RNAs in B-cell development and activation, 128 (2016) 10–20, http://dx.doi.org/10.1182/blood-2015-11-680843.

[29] H. Sakuma, K. Kohyama, I.K. Park, A. Miyakoshi, N. Tanuma, Y. Matsumoto, Clinicopathological study of a myelin oligodendrocyte glycoprotein-induced demyelinating disease in LEW.1AV1 rats, Brain 127 (2004) 2201–2213, http://dx.doi.org/10.1093/brain/awh260.

[30] M. Kanehisa, S. Goto, M. Furumichi, M. Tanabe, M. Hirakawa, KEGG for representation and analysis of molecular networks involving diseases and drugs, Nucleic Acids Res. 38 (2009) D355–D360, http://dx.doi.org/10.1093/nar/gkp896.

[31] T. Zhang, M. Jiang, L. Chen, B. Niu, Y. Cai, Prediction of gene phenotypes based on GO and KEGG pathway enrichment scores, Biomed. Res. Int. 2013 (2013), http://dx.doi.org/10.1155/2013/870795.

[32] T. Chitnis, The role of CD4 T cells in the pathogenesis of multiple sclerosis, Int. Rev. Neurobiol. 79 (2007) 43–72, http://dx.doi.org/10.1016/S0074-7742(07)79003-7.

[33] T.J. Harris, J.F. Grosso, H.-R. Yen, H. Xin, M. Kortylewski, E. Albesiano, E.L. Hipkiss, D. Getnet, M.V. Goldberg, C.H. Maris, F. Housseau, H. Yu, D.M. Pardoll, C.G. Drake, Cutting edge: an in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity, J. Immunol. 179 (2007) 4313–4317, http://dx.doi.org/10.4049/jimmunol.179.7.4313.

[34] J.J. O'Shea, R. Plenge, JAKs and STATs in immunoregulation and immune-mediated disease, Immunity 36 (2013) 542–550, http://dx.doi.org/10.1016/j.immuni.2012.03.014.JAKs.
[35] Y. Liu, A.T. Holdbrooks, P. De Sarno, A.L. Rowse, L.L. Yanagisawa, B.C. McFarland, L.E. Harrington, C. Raman, S. Sabbaj, E.N. Benveniste, H. Qin, Therapeutic efficacy of suppressing the JAK/STAT pathway in multiple models of experimental autoimmune encephalomyelitis, J. Immunol. 127 (2013) 358–366, http://dx.doi.org/10.4049/jimmunol.1301513.
[36] S. Kuerten, P.V. Lehmann, The immune pathogenesis of experimental autoimmune encephalomyelitis: lessons learned for multiple sclerosis? J. Interf. Cytokine Res. 31 (2011) 907–916, http://dx.doi.org/10.1089/jir.2011.0072.
[37] C.H. Lin, S. Kadakia, M. Frieri, New insights into an autoimmune mechanism, pharmacological treatment and relationship between multiple sclerosis and inflammatory bowel disease, Autoimmun. Rev. 13 (2014) 114–116, http://dx.doi.org/10.1016/j.autrev.2013.09.011.
[38] A. Zhernakova, C.C. van Diemen, C. Wijmenga, Detecting shared pathogenesis from the shared genetics of immune-related diseases, Nat. Rev. Genet. 10 (2009) 43–55, http://dx.doi.org/10.1038/nrg2489.
[39] W.-M. Yu, S. Wang, A.D. Keegan, M.S. Williams, C.-K. Qu, Abnormal Th1 cell differentiation and IFN-gamma production in T lymphocytes from motheaten viable mice mutant for Src homology 2 domain-containing protein tyrosine phosphatase-1, J. Immunol. 174 (2005) 1013–1019, http://dx.doi.org/10.4049/jimmunol.174.2.1013.
[40] I.-K. Park, L.D. Shultz, J.J. Letterio, J.D. Gorham, TGF-beta1 inhibits T-bet induction by IFN-gamma in murine $CD4^+$ T cells through the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1, J. Immunol. 175 (2005) 5666–5674, http://dx.doi.org/10.4049/jimmunol.175.9.5666.
[41] E. B., Carlos A. Arbelaez, Simon Glatigny, Rebekka Duhen, Gerard Eberl, Mohamed Oukka, IL-7/IL-7R signaling differentially affects effector $CD4^+$ T cell subsets involved in experimental autoimmune encephalomyelitis1, J. Immunol. 195 (2015) 1974–1983, http://dx.doi.org/10.4049/jimmunol.1403135.
[42] Y. Sharma, S. Bashir, P. Bhardwaj, A. Ahmad, F. Khan, Protein tyrosine phosphatase SHP-1: resurgence as new drug target for human autoimmune disorders, Immunol. Res. 64 (2016) 804–819, http://dx.doi.org/10.1007/s12026-016-8805-y.
[43] S.J. Szabo, A.S. Dighe, U. Gubler, K.M. Murphy, Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells, J. Exp. Med. 185 (1997) 817–824, http://dx.doi.org/10.1084/jem.185.5.817.
[44] I.S. Mauldin, K.S. Tung, U.M. Lorenz, The tyrosine phosphatase SHP-1 dampens murine Th17 development, Blood 119 (2012) 4419–4429, http://dx.doi.org/10.1182/blood-2011-09-377069.
[45] M.J. McGeachy, Y. Chen, C.M. Tato, A. Laurence, B. Joyce-Shaikh, W.M. Blumenschein, T.K. McClanahan, J.J. O'Shea, D.J. Cua, The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo, Nat. Immunol. 10 (2009) 314–324, http://dx.doi.org/10.1038/ni.1698.
[46] H.M. Schrijver, T. Hooper-Van Veen, M.J. Van Belzen, J.B.A. Crusius, A.S. Peña, F. Barkhof, C.H. Polman, B.M.J. Uitdehaag, Polymorphisms in the genes encoding interferon-γ and interferon-γ receptors in multiple sclerosis, Eur. J. Immunogenet. 31 (2004) 133–140, http://dx.doi.org/10.1111/j.1365-2370.2004.00456.x.
[47] R. A., J. Das, C. Chen, L. Yang, L. Cohn, P. Ray, A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation, Nat. Immunol. 2 (2001) 45–50.
[48] M. Chang, A.J. Lee, L. Fitzpatrick, M. Zhang, S.-C. Sun, N.F.- B1 p105 Regulates T Cell Homeostasis, Prevents Chronic Inflammation, J. Immunol. 182 (2009) 3131–3138, http://dx.doi.org/10.4049/jimmunol.0803637.
[49] T. Ishizu, M. Osoegawa, F.J. Mei, H. Kikuchi, M. Tanaka, Y. Takakura, M. Minohara, H. Murai, F. Mihara, T. Taniwaki, J.I. Kira, Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis, Brain 128 (2005) 988–1002, http://dx.doi.org/10.1093/brain/awh453.
[50] C.G. L., Borbala Gesser, Marianne Lund, Nicolai Lohse, Christian Vestergaard, Kouji Matsushima, Steen Sindet-Pedersen, Steen Lindkaer Jensen, Kristian Thestrup-Pedersen, IL-8 induces T cell chemotaxis,suppresses IL-4,and up-regulates IL-8 production by $CD4^+$ T cells, J. Leukoc. Biol. 59 (1996) 407–411.
[51] K.H.G. Mills, TLR-dependent T cell activation in autoimmunity, Nat. Rev. Immunol. 11 (2011) 807–822, http://dx.doi.org/10.1038/nri3095.
[52] J.M. Reynolds, B.P. Pappu, J. Peng, G.J. Martinez, Y. Zhang, Y. Chung, L. Ma, X.O. Yang, R.I. Nurieva, Q. Tian, C. Dong, Toll-like receptor 2 signaling in $CD4^+$ T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease, Immunity 32 (2010) 692–702, http://dx.doi.org/10.1016/j.immuni.2010.04.010.