Integrative Analysis of mRNA and miRNA Array Data Reveals the Suppression of Retinoic Acid Pathway in Regulatory T Cells of Graves' Disease

Zhe Wang,* Xiaohua Fan,* Rongrong Zhang, Zongwei Lin, Ting Lu, Xue Bai, Wenchao Li, Jiajun Zhao, and Qunye Zhang

Division of Endocrinology and Metabolism (Z.W., J.J.Z., R.R.Z., T.L., W.C.L.) and Division of Rehabilitation Medicine (X.H.F.), Shandong Provincial Hospital affiliated to Shandong University, Jinan 250021, China; National Glycoengineering Research Center (X.B.), Shandong University, Jinan 250100, China; Central Research Laboratory (Q.Y.Z.), The Second Hospital of Shandong University, Jinan 250033, China; and Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Ministry of Public Health (Q.Y.Z., Z.W.L), Qilu Hospital, Shandong University, Jinan 250100, China

Context: It is well known that regulatory T cells (Tregs) are abnormal in Graves' disease (GD) and play crucial roles in the breakdown of immune tolerance and GD development. However, there are controversies about whether the quantity and/or function of Tregs is aberrant in GD. The molecular mechanism of Tregs abnormality and its effects on GD development was still unclear, until now.

Objective: MiRNAs play important roles in the function and development of the immune system including Tregs. To reveal the Tregs abnormality and its molecular mechanism in GD, we systematically studied the quantity and immunosuppressive function as well as the differential expression profiles of miRNA and mRNA of Tregs in newly diagnosed patients with GD using TaqMan miRNA array and mRNA microarray.

Results: Our results showed that the quantity and immunosuppressive function of Tregs in initial patients with GD was significantly decreased. More importantly, the retinoic acid (RA) pathway was markedly suppressed and its agonist, all-trans retinoic acid, could notably improve the quantity and immunosuppressive function of Tregs from patients with GD in vitro. In addition, many other pathways including protein ubiquitination and circadian rhythm were also significantly regulated in Tregs of GD.

Conclusions: This integrative study first revealed the expression profiles of mRNA/miRNA in Tregs of initial GD and RA pathway might play important roles in GD development. Our results implied that all-trans RA, which had been used for a long time in the clinical setting, had potential value in the treatment of GD and was worthy of additional study. **(*J Clin Endocrinol Metab* 99: E2620–E2627, 2014)**

Graves' disease (GD) is the main type of autoimmune thyroid disease and thyroid-specific autoantibody is one of the most prominent pathological features of GD (1). The pathogenesis of GD is very complex. It is generally accepted that the interactions of many genetic and environmental factors lead to imbalance of immune homeostasis and the disruption of immune tolerance to thyroid antigens resulting in the production of autoantibodies, such as thyroid stimulating hormone receptor (TSHR), receptor antibody (TRAb), and GD development (1, 2). However, the mechanisms involved in the breakdown of immune tolerance to TSHR have not yet been fully elucidated.

Regulatory T cells (Tregs) are the important CD4+ T cell subset and their distinctive markers include CD25, FOXP3, and CD127. Tregs are essential in immunological

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received March 28, 2014. Accepted September 11, 2014.
First Published Online September 18, 2014

* Z.W and X.H.F. contributed equally to this work.

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; GD, Graves' disease; iuGD, initial patients with Graves' disease, without any treatment; LNA, locked nucleic acid; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; RA, retinoic acid; Tregs, regulatory T cells.

homeostasis and tolerance to self antigens (3). Numerous studies have demonstrated that abnormalities in quantity and/or function of Tregs could lead to many immune-mediated pathologies, including autoimmunity, chronic infection, and graft rejection. The mutation of *FOXP3*, a transcription factor that was crucial in the development and function of Tregs, could cause severe dysfunction of Tregs and result in multisystem autoimmune disease. Tregs were also significantly abnormal in multiple sclerosis (MS), rheumatoid arthritis (RA), and many other autoimmune diseases (3–5). Animal experiments suggested that removal of Tregs could significantly increase the incidence and severity of autoimmune diseases including autoimmune thyroiditis in mice (6). However, studies of peripheral Tregs in patients with GD were inconsistent. Many studies showed that the quantity and/or function of Tregs decreased markedly, but other studies demonstrated that Tregs were normal in patients with GD and it was the microenvironment or other cells that led to the dysfunction of Tregs (5, 7). Until now, most of the studies of Tregs in GD focused on delineating the abnormality of amount and/or function of Tregs. To understand the roles and their mechanism of Tregs in GD, it is critical to study the molecular changes (such as mRNAs/miRNAs) that were related to the dysfunction of Tregs in patients with GD. However, this kind of study has rarely been reported.

MiRNAs are small, noncoding RNA molecules that regulate gene expression by base pairing with their target genes and play crucial roles in the development and function of T cells. Dicer is the key enzyme in miRNA maturation and the Dicer knockout mice had severe systemic autoimmune diseases that were similar to those in depleting mice of Tregs (8). Moreover, miR-155 had pivotal roles in Tregs development and T cell–dependent antibody response. The quantity and function of Tregs were decreased significantly in miR-155 knockout mice. MiR-106b could regulate TGF-β pathway by targeting *CDKN1A/p21* and then regulate the differentiation and maturation of Tregs (9, 10). Lots of studies showed that the expression of many miRNAs altered significantly in autoimmune diseases and miRNA was one of the key factors in the occurrence and development of autoimmune diseases including systemic lupus erythematosus, MS, and type I diabetes. Some studies have investigated the expression of miRNAs in peripheral blood mononuclear cells (PBMC), CD4/CD8+T cells, serum, and thyroid tissue from autoimmune thyroid disease (10–14). However, the study of miRNAs in Tregs of patients with GD has not been reported to date. Therefore, additional studies are still needed to reveal the changes of miRNA expression of Tregs in patients with GD and their significance to the development of GD.

In this article, we found that the quantity and immunosuppressive functions of Tregs were significantly decreased in initial patients with GD compared with those in healthy individuals. To reveal the mechanism, we studied the differentially expressed miRNAs and mRNAs of Tregs in initial GD using microarray technology. The integrative analysis of these data suggested that many pathways including protein ubiquitination and circadian rhythm were obviously abnormal. More importantly, the retinoic acid (RA) pathway was markedly inhibited and its agonist, all-trans RA, could significantly improve the quantity and immunosuppressive function of Tregs from patients with GD in vitro. This result implied that all-trans retinoic acid, which had been used for long time in the clinical setting, might have the potential to treat GD and it is worth further study.

Materials and Methods

Patients and ethical statement

All initial patients with Graves' disease, without any treatment (iuGD), were recruited from the clinic of Shandong Provincial Hospital and diagnosed through standard laboratory and clinical tests. The controls were healthy volunteers with normal thyroid function and were excluded for autoimmune diseases and other diseases. The clinical data are shown in Table 1. The Ethics Committee of Shandong University has approved this study. Informed consent was obtained in accordance with the Declaration of Helsinki.

In vitro Tregs suppression assay and flow cytometry

The PBMCs were obtained from EDTA-anticoagulant blood by Ficoll gradient centrifugation. The CD4+CD25+Foxp3+ Tregs were analyzed using Tregs staining kit (eBioScience) on FACSCalibur. The viable CD4+CD25+CD127low Tregs were sorted by MoFlo XDP using Tregs sorting kit (BD Biosciences). The untouched CD4+ T cells were isolated from PBMCs using MACS isolation kit (Miltenyi-Biotec) and then were sorted to

Table 1. Clinical Characteristics of Patients with Graves' Disease

	Normal Control, Mean ± SD	Graves' Disease, Mean ± SD
n	51	51
Gender, F/M	47/4	48/3
Age, y	37.6 ± 6.1	38.2 ± 5.32
TSH, mIU/L	2.01 ± 0.64	0.025 ± 0.012
FT3, pmol/L	4.86 ± 0.43	21.69 ± 5.35
FT4, pmol/L	14.29 ± 1.63	54.69 ± 8.73
TRAb, U/L	0.71 ± 0.26	9.32 ± 6.57
TGAb, IU/ml	6.84 ± 0.81	162.38 ± 42.65
TPOAb, IU/ml	3.18 ± 0.79	320.72 ± 81.28

Abbreviations: FT3, free T3; FT4, free T4; TRAb, Thyroid stimulating hormone receptor antibody; TGAb, thyroglobulin antibody; TPOAb, Thyroperoxidase antibody.

collect untouched CD4+CD25− effector T cells. 1×10^5 effector T cells were labeled with 2 μM carboxyfluorescein succinimidyl ester (CFSE) and the sorted viable Tregs were cocultured with CFSE-effector T cells at graded or single concentrations (Teffect:Treg = 1:2, 1:1, 1:0.5, 1:0). Cells were cultured with 10 μg/ml anti-CD3 and 5 μg/ml anti-CD28-coated Dynal beads (Invitrogen) in Tregs induction medium (RPMI 1640 containing 10% serum and 1 ng/ml TGF-β1). All-trans RA (pan-RA receptor agonist) and AGN193109 (pan-RA receptor antagonist) were purchased from Sigma-Aldrich and Santa Cruz. In some experiments, all-trans RA and/or AGN193109 were added at final concentration of 2μM and 5μM respectively (15, 16). After 3 days, CFSE dilution was analyzed with flow cytometer and the suppressive capacity of Tregs to effector T cells was calculated (17). The purity of all sorted cells was > 95%. All experiments were repeated at least three times.

TaqMan miRNA array and mRNA microarray

The Tregs purified from 21 iuGD and 21 controls were respectively averaged into three sample pools (without significant difference). Total RNA was extracted from each pool using Trizol and the miRNAs were isolated using mirVana Isolation Kit (Ambion). The RNA samples were labeled by Cy3 and Cy5 dye and hybridized on Affymetrix Human Genome U133 Plus 2.0 Array. The slides were digitized by GenePix 4000B scanner. The miRNA samples were assayed using TaqMan Low Density Arrays on a 7500 Real-Time PCR system (Invitrogen) and the ΔΔCT values were calculated. All experiments were repeated three times.

Microarray data analysis

The differentially expressed miRNAs/mRNAs (Benjamini-Hochberg corrected $P < .05$ and fold changes ≥ 2) were identified by significant analysis of microarrays. Genes predicted by TargetScan and miRanda were combined as the potential target genes of differentially expressed miRNAs (18, 19). Because most miRNAs inhibit their target genes, the genes in which expression trends were opposite to those of miRNAs were selected from the intersection of differentially expressed mRNAs and the potential target genes of altered miRNAs. If a gene was regulated by two or more miRNAs and the changes of these miRNAs were inconsistent, this gene would be excluded. The selected genes and differentially expressed mRNAs were used to perform functional/pathway enrichment analysis and function prediction (z score at least 2 represents activation, z score ≤ -2 means inhibition) by DAVID (the Database for Annotation, Visualization and Integrated Discovery), Panther, and IPA (Ingenuity Pathway Analysis) trials (20).

qPCR and Western blot

Some differentially expressed miRNAs/mRNAs were confirmed, respectively, by locked nucleic acid (LNA) SYBR Green qPCR (Exiqon, Denmark), and TaqMan qPCR in another 30 patients with iuGD and 30 controls. The primers/probes were bought from Exiqon and Invitrogen. The human *RNU6B* or *GAPDH* was used as a reference gene. The fold changes were calculated using ΔΔCT values. The total protein was extracted from Tregs of each sample and 40 μg protein was separated by 10% SDS-PAGE. Then, the proteins were transferred and incubated with the corresponding primary antibody: anti-RARA, anti-RARB, anti-TGFBR1, anti-FOXP3, and anti-β-actin (Abcam). After washing, the polyvinylidene difluoride membranes were incubated with secondary antibodies and detected using an ECL Kit (GE Healthcare). The β-actin was used as an internal control.

All-trans RA concentration in plasma

The concentration of all-trans RA in plasma was determined by HPLC as described with some modifications (21). Briefly, 5 ng/ml internal standards (all-trans acitretin) were added into plasma and then 50 μl plasma was mixed with 150 μl acetonitrile/isopropanol (1:1, v/v) to precipitate proteins. After 3 minutes vortex mixing and 5 minutes centrifugation at 6000 rpm at 4°C, 100 μl supernatant was injected into the HPLC system and were separated on a reverse phase C18 column (2.1 mm × 150 mm × 3μm, Waters). The linear gradient was from 50% buffer A (1:1 acetonitrile:water, containing 60mM ammonium acetate; pH, 5.8) and 50% buffer B (19:1 acetonitrile:water, containing 60mM ammonium acetate; pH, 7.5) to 20% A and 80% B in 25 minutes. All-trans RA were detected at 340 nm. The sample preparation was performed in amber containers under red light.

Statistics

The data were expressed as means ± SEM or means ± SD. Student t test was used to assess significance between two groups. To determine the significance of differences in multiple groups, one-way ANOVA with Bonferroni post test was used. SPSS 15.0 (SPSS) was used for all calculations.

Results

Down-regulation of quantity and immunosuppressive function of peripheral Tregs in iuGD

To study the disagreement reported previously, the quantity and function of peripheral Tregs in 51 patients with iuGD was analyzed. The results showed that the av-

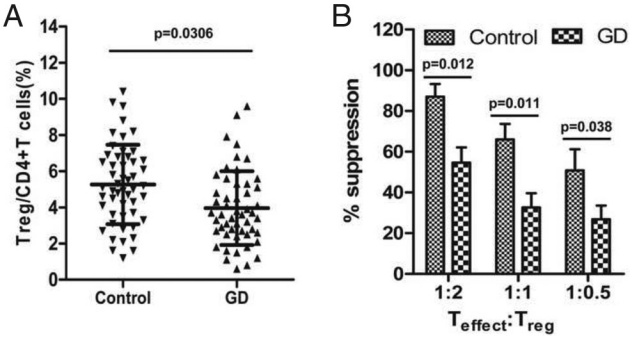

Figure 1. Down-regulation of quantity and immunosuppressive function of peripheral Tregs in patients with GD. A, Single-cell suspensions of hPBMC from 51 patients with GD and 51 healthy controls were stained by Tregs staining kit (CD4, CD25, and Foxp3) and the proportion of CD25+FoxP3+ Tregs cells in CD4+T cells was analyzed by FACSCalibur. Data were presented as mean ± SD (n = 51). Statistical significance was tested by t test. B, The immunosuppressive function of CD4+CD25+CD127low Tregs in GD and control was assayed as described in Materials and Methods. Data were presented as mean ± SEM (n = 10). t test was performed to determine the statistical significance between GD and control at the same ratio of Teffect/Treg. P values are shown in figure.

erage proportion of CD4+CD25+Foxp3+ Tregs to peripheral CD4+T cells in patients with iuGD was 3.97% and significantly decreased compared with those in age- and sex-matched controls (Figure 1A). Meanwhile, the immunosuppressive function of peripheral Tregs in iuGD was also significantly impaired and the suppressive effect of them to the proliferation of CD4+CD25− effector T cells induced by anti-CD3 and anti-CD28 coated beads was markedly decreased (approximately 50% of normal) (Figure 1B).

Profiles and functional enrichment analysis of miRNAs/mRNAs microarray data

For revealing the mechanism of the abnormality of Tregs in GD, the expression profiles of miRNAs/mRNAs were investigated. The results showed that there were 23 up-regulated and 14 down-regulated miRNAs (Figure 2A). The LNA qPCR of four top-ranked altered miRNAs and miR-155 confirmed that they were all significantly modulated in Tregs of iuGD and it was consistent with the results of microarray (Figure 2B). The results of mRNA microarray showed that 1907 genes (66.59%) were down-regulated and 957 genes (33.41%) were up-regulated notably (Figure 2C) in Tregs of GD. The expression feature of mRNAs was coordinated with it of miRNAs that most miRNAs were up-regulated because the up-regulation of numerous miRNAs was likely to result in the down-regulation of their corresponding target genes.

The integration of mRNA and miRNA expression data was performed according to the procedure described in methods (Supplemental Figure 1). The results of enrichment analysis of the integrated data revealed that the biological processes of T cells quantity and function, development, and proliferation of hematopoietic progenitor cells, activation-induced cell death, cell cycle, and protein ubiquitination were inhibited significantly in Tregs of GD (Figure 3A, Supplemental Figure 2A). Moreover, many pathways were also significantly enriched and most of the involved genes were down-regulated in patients with GD, such as RA pathway, circadian rhythm, and IL-10 pathway (Figure 3B, Supplemental Figure 2B). However, the roles and mechanisms of these pathways in dysfunction of Tregs in patients with GD were not clear and need to be studied in depth.

Suppression of RA pathway in Tregs of patients with iuGD

The above-discussed enrichment analysis discussed that RA pathway was enriched significantly in Tregs of patients with GD (Bonferroni-corrected $P = .0008$). Moreover, the regulated genes/miRNAs in RA pathway were more than in any other pathways (Figure 3B). The key genes of RA pathway (*RARA*, *RARB*, and *RXRA*) were down-regulated markedly, whereas miR-636 and miR-30a-5p that targeted the aforesaid three genes were up-regulated significantly. General transcription factors were the crucial transcription factors in RA pathway and many of them were also down-regulated in Tregs of GD. Moreover, miR-30a-5p and miR-181a, which targeted *GTFH1* and *GTFH2*, were up-regulated significantly. In contrast, *SAP130*, *SIN3A*, and *SAP18*, which inhibited the transcription induced by RA pathway, were up-regulated markedly. *CYP26B1*, which hydrolyzed all-trans RA, was increased and could decrease the amount of all-trans RA in Tregs and then inhibit RA pathway at ligand level (Figure 3C, Supplemental Figure 3). All the results implied that RA pathway was suppressed in Tregs of

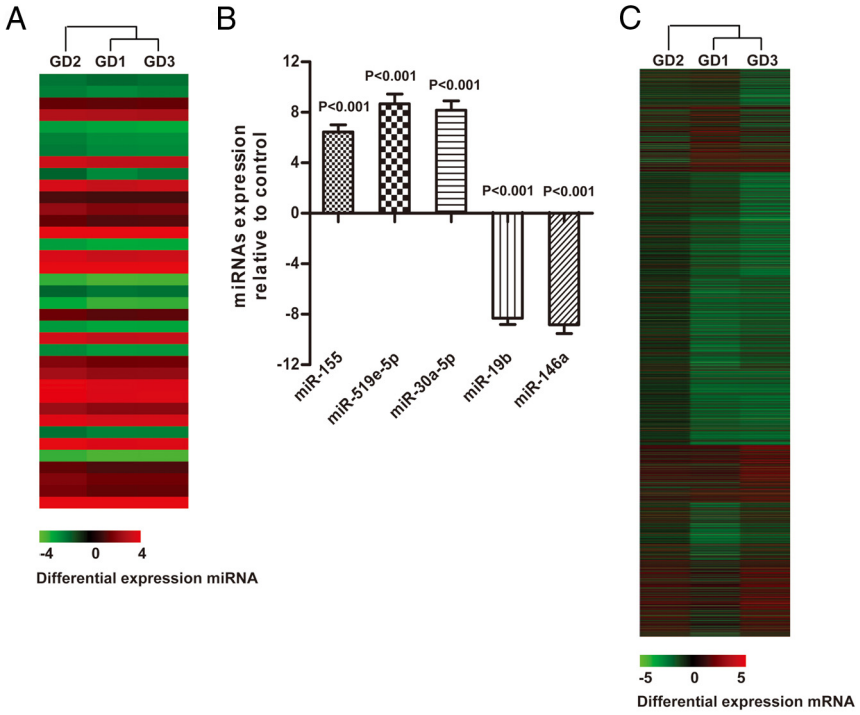

Figure 2. Expression profiles of miRNAs and mRNAs in peripheral Tregs of patients with GD. A, The cluster of 37 differentially expressed miRNAs between three GD sample pools and control sample pools. The color bar indicates expression ratios. B, The four top ranked differentially expressed miRNAs (miR-519e–5p, miR-30a–5p, miR-19b, miR-146a) and miR-155 were validated by LNA qPCR in another 30 patients with iuGD and 30 controls (n = 30). The results of the *t* test indicated they were all significantly modulated in Tregs of GD. The positive fold change indicated up-regulation and negative fold change indicated down-regulation. *P* values are shown in figure. C, The cluster of 2864 differentially expressed mRNAs between three GD sample pools and control sample pools. The color bar indicates expression ratios.

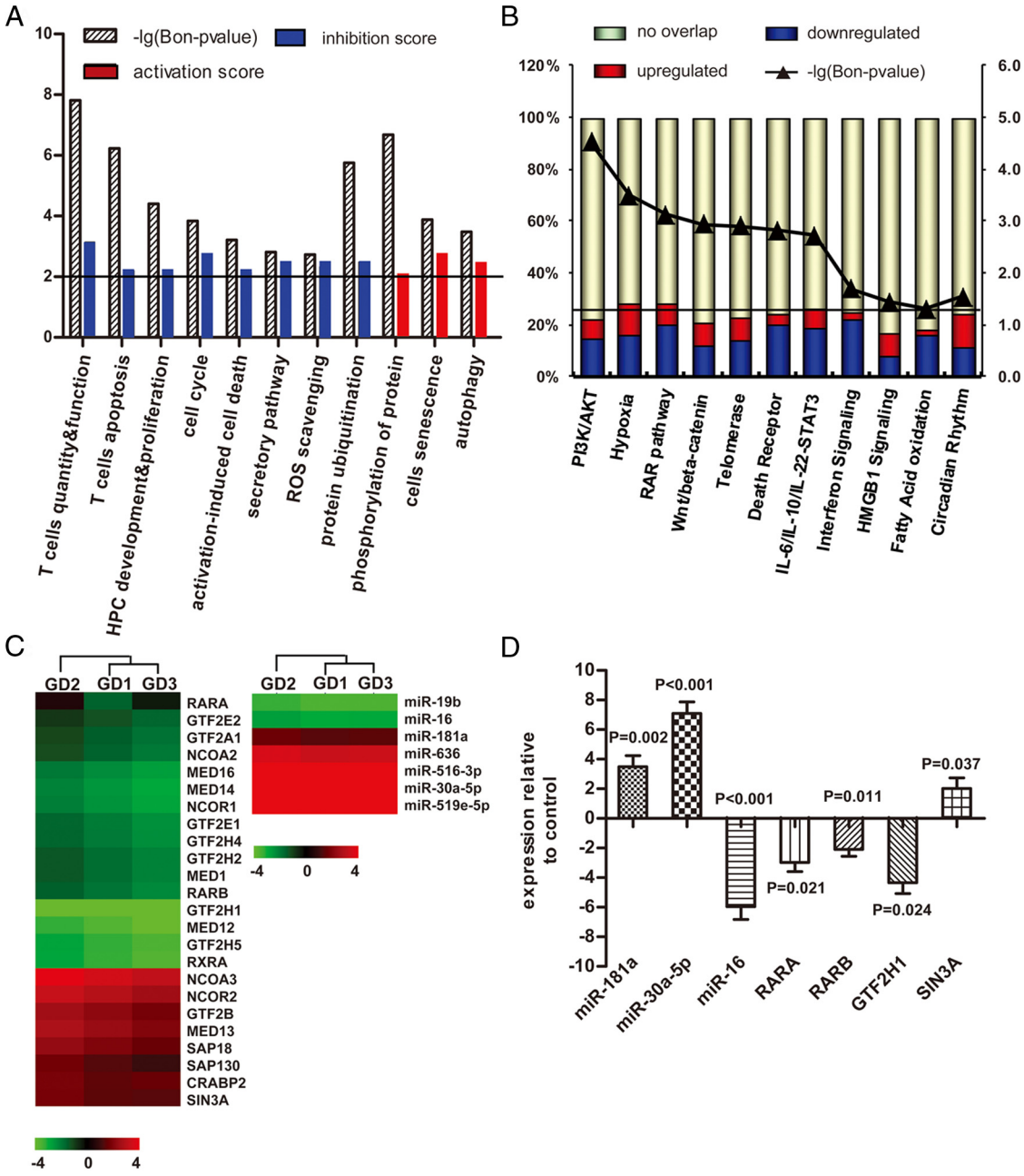

Figure 3. Enrichment analysis of biological processes and pathways. A, The result of enrichment analysis of biological processes. Blue indicates suppression and red indicates activation. The black horizontal line represents the significant threshold of *z* score = 2. B, The result of enrichment analysis of pathways. Blue (red) indicates the down-regulation (up-regulation) of genes. The left y-axis indicates the percentage of different genes (down-regulation, up-regulation, and nonoverlapping) in all genes of the corresponding pathways in database. The right y-axis indicates the −lg(Bon-*P*). The black horizontal line represents the significant threshold of −lg(0.05) = 1.301. C, The clusters of the enriched miRNAs/mRNAs associated with RA pathway. It shows that the RA pathway was down-regulated. The color bar indicates expression ratios. D, The validation of some key genes/miRNAs in RA pathway by TaqMan qPCR and LNA qPCR in another 30 patients with iuGD and 30 controls. The data are presented as mean ± SEM (n = 30). *t* test was performed to determine the statistical significance of each gene/miRNA between GD and control. *P* values were shown in figure. Bon-*P*, Bonferroni-corrected *P* value.

patients with GD and could contribute to the dysfunction of Tregs. To confirm this conclusion, changes of some key genes/miRNAs in RA pathway were validated by qPCR (Figure 3D).

Moreover, further functional studies suggested that all-trans RA could significantly increase the ratio of CD4+CD25+Foxp3+ Tregs to CD4+T cells and the immunosuppressive effects of CD4+CD25+CD127low Tregs on CD4+CD25− effector T cells (>70%), whereas AGN193109, a pan-antagonist of RA pathway, could effectively offset the effects of all-trans RA (Figure 4A, 4B). This result implied that all-trans RA might restore to some extent the function of Tregs in patients with GD. Although lone treatment of AGN193109 could further decrease the

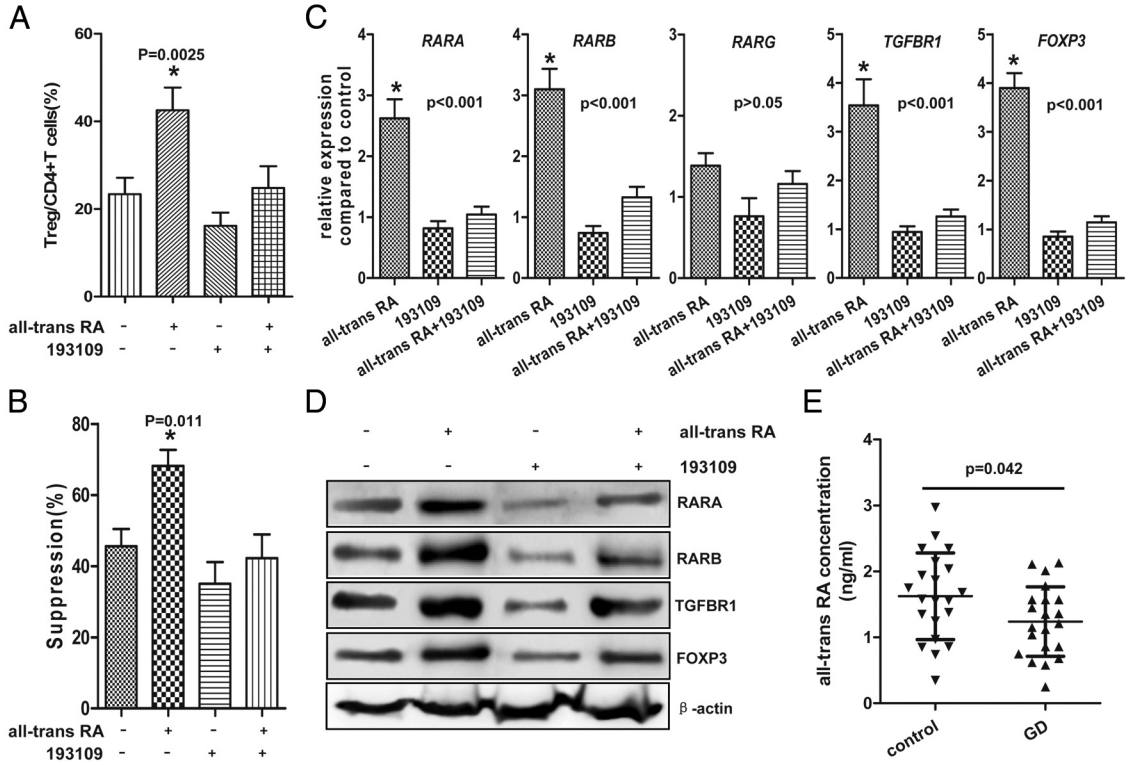

Figure 4. All-trans retinoic acid could restore the quantity and immunosuppressive function of Tregs in patients with GD in vitro. A, CD4+T cells were isolated from patients with GD by magnetic-activated cell sorting and were cultured with anti-CD3- and anti-CD28-coated Dynal beads in the presence of all-trans retinoic acid (2μM) and/or AGN193109 (5μM). After 3 days, the proportion of CD25+FoxP3+ Tregs in CD4+T cells was tested. B, CD4+CD25− effector T cells were labeled with CFSE and were cocultured with CD4+CD25+CD127low Tregs from patients with GD (Treg:Teffect = 1:2) in the presence of all-trans retinoic acid (2μM) and/or AGN193109 (5μM) as described in Materials and Methods. After 3 days, the immunosuppressive function of Tregs was analyzed. C and D, CD4+CD25+CD127low Tregs were sorted from patients with GD and cultured as described above in the presence of all-trans retinoic acid and/or AGN193109. After 3 days, some key genes of RA pathway were detected using C, TaqMan qPCR (*RARA*, *RARB*, *RARG*, *TGFBR1*, and *FOXP3*); and D, Western Blot (RARA, RARB, TGFBR1 and FOXP3). The data from all above-discussed experiments are shown as mean ± SEM (n = 6). One-way ANOVA followed by Bonferroni's post-hoc test was performed. *, significant differences in the groups compared with all other groups ($P < .05$). E, The levels of all-trans retinoic acid in plasma from patients with GD and healthy controls were determined by HPLC. Data are shown as mean ± SD (n = 21). Statistical significance was analyzed by *t* test. $P = .042$.

quantity/function of Tregs, it was not significant. The reason might lie in the fact that the activity of the RA pathway in Tregs of GD had been low and the suppressive effect of antagonist on it was not obvious. In addition, results of qPCR and Western Blot suggested that all-trans RA could significantly increase the expression of *RARA* and *RARB* (the critical genes of RA pathway), *TGFBR1* (the important target gene of RA pathway), and *FOXP3* (crucial gene in Tregs). However, all-trans RA had no significant effect on the *RARG* expression. Similarly, the antagonist AGN193109 could also inhibit the above-discussed effects of all-trans RA (Figure 4, C and D). Interestingly, the all-trans RA assay showed that the levels of all-trans RA in the plasma of patients with GD were significantly decreased compared with the normal controls ($P < .05$) (Figure 4E).

Discussion

Until now, it was widely accepted that the quantity and/or function of Tregs was anomalous in patients with GD. However, there was still controversy about the details of the anomaly. Some studies suggested that the quantity and function of peripheral Tregs in GD were significantly decreased (22), which was consistent with our results. Other studies showed that the quantity of peripheral Tregs in GD was reduced and their functions were normal, but other studies were contrary to them (5, 7). There might be many reasons for the inconsistency. First, there was disparity in sex, race, stages of GD (initial or relapse), and drug treatment of the samples in different studies. Second, selected markers for Tregs were different. Some studies considered CD4+CD25+T cells as Tregs and their results showed the ratio of peripheral Tregs to CD4+T cells in GD was not changed (5). However, other studies that selected CD4+CD25+FOXP3+T cells as Tregs demonstrated the ratio was significantly decreased (7). In addition, the difference in selection of effector T cells (CD4+T cells or CD4+CD25− T cells) might also affect the results of immunosuppressive function analysis of Tregs. Moreover, different conclusions were drawn even from the studies with the

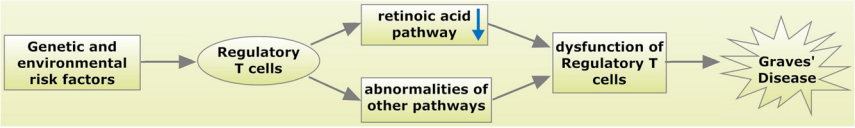

Figure 5. Schematic diagram of the pathogenesis of GD. Some genetic and environmental factors acted on the Tregs of patients with GD and resulted in the abnormalities of various pathways including the suppression of the RA pathway in Tregs. These abnormalities damaged the function of Tregs and might lead to the development of GD.

same experimental conditions (7, 22, 23). In our study, the initial and untreated patients with GD were selected to exclude the effect of disease stage and treatment on the results. More specific markers (CD4+CD25+FOXP3+) were used for analyzing the quantity of Tregs. Nevertheless, FOXP3 located within cells and the permeabilization in the process of experiment would damage Tregs. Therefore, CD4+CD25+CD127low T cells were sorted as Tregs in the study of Tregs function. These markers were more specific than CD4+ and CD25+ for Tregs. However, considering the purposes of our study and the experimental cost, the sample size was relatively small in our study. So, to more clearly reveal the changes of Tregs in GD, a multicenter study including a larger number of different stages of patients with GD are ongoing.

In this article, we demonstrated that RA pathway was suppressed markedly in Tregs of GD. RA pathway is crucial in the development, differentiation, apoptosis, and function of immune cells. Therefore, it plays important roles in immune responses and the pathogenesis/treatment of many autoimmune diseases (24, 25). All-trans RA and TGF-β can cooperatively up-regulate FOXP3 expression in human CD4+cells (26, 27) and decrease the generation of Th17 cells (15). Vitamin A and its derivatives can significantly alleviate the symptoms of some autoimmune diseases, including autoimmune encephalomyelitis, arthritis, type 1 diabetes, and colitis (28–30). Accordingly, deficiency of vitamin A can result in exacerbation of experimental colitis and shift the immune response from Th1-type to Th2-type (31). All-trans RA has been widely used in the treatment of acute promyelocytic leukemia and autoimmune diseases (such as psoriasis) for a long time. The clinical safety and efficacy of all-trans RA have been proven (32, 33). Moreover, our study also showed that the levels of all-trans RA in plasma of patients with GD were decreased significantly. Therefore, all-trans RA might have the possibility to be used for the treatment of GD. However, the immune regulation of all-trans RA might be biphasic. Recent evidence showed that RA deficiency led to impaired immunity, whereas excess RA could potentially promote inflammatory disorders (24, 25). Moreover, further functional studies should be necessary to clarify some ambiguities. For example, which one or more RA receptors play roles? What are the key miRNAs that lead to the abnormality of the RA pathway? Is all-trans RA treatment effective in animal models of GD? In fact, our study of the last question is in progress.

This study also found that some other important pathways were regulated in Tregs of patients with GD. For example, many E3 ligases (such as *Cblb*, *RNF144B*, and *UBE3C*) were down-regulated in Tregs of GD. The ubiquitination signals played key roles in the development and function of Tregs (34, 35). Therefore, it was worthy to study whether the protein ubiquitination was involved in the development of GD. In addition, our results showed that *CLOCK*, the key gene in circadian rhythm, was significantly down-regulated in Tregs of patients with GD. The disruption of the circadian system can damage the function of Tregs and increase susceptibility to immune diseases (36). This might contribute to the development of GD in people who were accustomed to stay up late.

In summary, this was the first study, to our knowledge, that systematically analyzed the expression profiles of mRNA and miRNA in Tregs of GD. The study revealed that the quantity and immunosuppressive function of Tregs in iuGD was significantly decreased. RA pathway was also markedly suppressed and the abnormalities of the RA pathway and other important pathways would lead to Treg dysfunction and GD development (Figure 5). All-trans RA, a RA receptor agonist that has been used for a long time in the clinical setting, could improve the quantity and function of Tregs from patients with GD in vitro. The results implied that all-trans RA has potential value in the treatment of GD and was worthy of additional study. However, we should note that this study focused on the altered expression of miRNA/mRNA and regulated pathways of Tregs in GD. Additional studies on the mechanism of the effect of certain miRNAs on Tregs of GD are being conducted in another study.

Acknowledgments

Address all correspondence and requests for reprints to: 107 Wenhua Road, Jinan, Shandong, China 250021. E-mail: wz.zhangqy@gmail.com or jjzhao@medmail.com.cn.

This work was supported by grants from the Chinese National Key Basic Research Project (2011CB503906); National Natural Science Foundation of China (81070625, 81370890), the Program for New Century Excellent Talents in University

(NCET-10–0555), and the Shandong Provincial Natural Science Foundation (2009ZRB14207).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Weetman AP.** Graves' disease. *N Engl J Med*. 2000;343(17):1236–1248.
2. **Wang Z, Zhang Q, Lu J, et al.** Identification of outer membrane porin f protein of Yersinia enterocolitica recognized by antithyrotopin receptor antibodies in Graves' disease and determination of its epitope using mass spectrometry and bioinformatics tools. *J Clin Endocrinol Metab*. 2010;95(8):4012–4020.
3. **Bilate AM, Lafaille JJ.** Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu Rev Immunol*. 2012;30:733–758.
4. **Wan YY, Flavell RA.** Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature*. 2007;445(7129):766–770.
5. **Glick AB, Wodzinski A, Fu P, Levine AD, Wald DN.** Impairment of regulatory T-cell function in autoimmune thyroid disease. *Thyroid*. 2013;23(7):871–878.
6. **Ellis JS, Wan X, Braley-Mullen H.** Transient depletion of CD4+ CD25+ regulatory T cells results in multiple autoimmune diseases in wild-type and B-cell-deficient NOD mice. *Immunology*. 2013;139(2):179–186.
7. **Mao C, Wang S, Xiao Y, et al.** Impairment of regulatory capacity of CD4+CD25+ regulatory T cells mediated by dendritic cell polarization and hyperthyroidism in Graves' disease. *J Immunol*. 2011;186(8):4734–4743.
8. **Cobb BS, Hertweck A, Smith J, et al.** A role for Dicer in immune regulation. *J Exp Med*. 2006;203(11):2519–2527.
9. **Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E.** Cutting edge: The Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol*. 2009;182(5):2578–2582.
10. **De Santis G, Ferracin M, Biondani A, et al.** Altered miRNA expression in T regulatory cells in course of multiple sclerosis. *J Neuroimmunol*. 2010;226(1–2):165–171.
11. **Liu R, Ma X, Xu L, et al.** Differential microRNA expression in peripheral blood mononuclear cells from Graves' disease patients. *J Clin Endocrinol Metab*. 2012;97(6):E968–E972.
12. **Bernecker C, Halim F, Lenz L, et al.** microRNA Expressions in CD4+ and CD8+ T-cell Subsets in Autoimmune Thyroid Diseases. *Exp Clin Endocrinol Diabetes*. 2014;122(2):107–112.
13. **Yamada H, Itoh M, Hiratsuka I, Hashimoto S.** Circulating microRNAs in autoimmune thyroid diseases. *Clin Endocrinol (Oxf)*. 2014;81(2):276–81.
14. **Bernecker C, Lenz L, Ostapczuk MS, et al.** MicroRNAs miR-146a1, miR-155_2, and miR-200a1 are regulated in autoimmune thyroid diseases. *Thyroid*. 2012;22(12):1294–1295.
15. **Wang G, Zhong A, Wang S, Dong N, Sun Z, Xia J.** Retinoic acid attenuates acute heart rejection by increasing regulatory T cell and repressing differentiation of Th17 cell in the presence of TGF-β. *Transpl Int*. 2010;23(10):986–997.
16. **Konta T, Xu Q, Furusu A, Nakayama K, Kitamura M.** Selective roles of retinoic acid receptor and retinoid x receptor in the suppression of apoptosis by all-trans-retinoic acid. *J Biol Chem*. 2001;276(16):12697–12701.
17. **Di Ianni M, Falzetti F, Carotti A, et al.** Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*. 2011;117(14):3921–3928.
18. **Friedman RC, Farh KK, Burge CB, Bartel DP.** Most Mammalian mRNAs Are Conserved Targets of MicroRNAs. *Genome Research*. 2009;19(1):92–105.
19. **John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS.** Human MicroRNA targets. *PLoS Biol*. 2005;3(7):e264.
20. **St-Pierre C, Brochu S, Vanegas JR, Dumont-Lagacé M, Lemieux S, Perreault C.** Transcriptome sequencing of neonatal thymic epithelial cells. *Sci Rep*. 2013;3:1860.
21. **Muindi JR, Frankel SR, Huselton C, et al.** Clinical pharmacology of oral all-trans retinoic acid in patients with acute promyelocytic leukemia. *Cancer Res*. 1992;52(8):2138–2142.
22. **Hu Y, Tian W, Zhang LL, et al.** Function of regulatory T-cells improved by dexamethasone in Graves' disease. *Eur J Endocrinol*. 2012;166(4):641–646.
23. **Marazuela M, García-López MA, Figueroa-Vega N, et al.** Regulatory T cells in human autoimmune thyroid disease. *J Clin Endocrinol Metab*. 2006;91(9):3639–3646.
24. **Hall JA, Grainger JR, Spencer SP, Belkaid Y.** The role of retinoic acid in tolerance and immunity. *Immunity*. 2011;35(1):13–22.
25. **Zhou R, Horai R, Mattapallil MJ, Caspi RR.** A new look at immune privilege of the eye: Dual role for the vision-related molecule retinoic acid. *J Immunol*. 2011;187(8):4170–4177.
26. **Mucida D, Park Y, Kim G, et al.** Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. 2007;317(5835):256–260.
27. **Nolting J, Daniel C, Reuter S, et al.** Retinoic acid can enhance conversion of naive into regulatory T cells independently of secreted cytokines. *J Exp Med*. 2009;206(10):2131–2139.
28. **Osanai M, Nishikiori N, Murata M, Chiba H, Kojima T, and Sawada N.** Cellular retinoic acid bioavailability determines epithelial integrity: Role of retinoic acid receptor alpha agonists in colitis. *Mol Pharmacol*. 2007;71:250–258.
29. **Zunino SJ, Storms DH, and Stephensen CB.** Diets rich in polyphenols and vitamin A inhibit the development of type I autoimmune diabetes in nonobese diabetic mice. *J Nutr*. 2007;137:1216–1221.
30. **Miyagawa N, Homma T, Kagechika H, Shudo K, and Nagai H.** Effect of synthetic retinoid, TAC-101, on experimental autoimmune disease. *Pharmacology*. 2003;67:21–31.
31. **Reifen R, Nur T, Ghebermeskel K, Zaiger G, Urizky R, Pines M.** Vitamin A deficiency exacerbates inflammation in a rat model of colitis through activation of nuclear factor-kappaB and collagen formation. *J Nutr*. 2002;132:2743–2747.
32. **Mi JQ, Li JM, Shen ZX, Chen SJ, Chen Z.** How to manage acute promyelocytic leukemia. *Leukemia*. 2012;26(8):1743–1751.
33. **Njar VC, Gediya L, Purushottamachar P, et al.** Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases. *Bioorg Med Chem*. 2006;14(13):4323–4340.
34. **Chen Z, Luo X, Lu Y, et al.** Ubiquitination signals critical to regulatory T cell development and function. *Int Immunopharmacol*. 2013;16(3):348–352.
35. **Harada Y, Harada Y, Elly C, et al.** Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. *J Exp Med*. 2010;207(7):1381–1391.
36. **Yu X, Rollins D, Ruhn KA, et al.** TH17 cell differentiation is regulated by the circadian clock. *Science*. 2013;342(6159):727–730.