MicroRNA-31 negatively regulates peripherally derived regulatory T-cell generation by repressing retinoic acid-inducible protein 3

Lingyun Zhang1, Fang Ke1, Zhaoyuan Liu1, Jing Bai1, Jinlin Liu1, Sha Yan1, Zhenyao Xu1, Fangzhou Lou1, Hong Wang1, Huiyuan Zhu1, Yang Sun1, Wei Cai1, Yuanyuan Gao1, Qun Li2, Xue-Zhong Yu3, Youcun Qian4, Zichun Hua5, Jiong Deng6, Qi-Jing Li7 & Honglin Wang1,8

Peripherally derived regulatory T (pTreg) cell generation requires T-cell receptor (TCR) signalling and the cytokines TGF-β1 and IL-2. Here we show that TCR signalling induces the microRNA miR-31, which negatively regulates pTreg-cell generation. miR-31 conditional deletion results in enhanced induction of pTreg cells, and decreased severity of experimental autoimmune encephalomyelitis (EAE). Unexpectedly, we identify Gprc5a as a direct target of miR-31. Gprc5a is known as retinoic acid-inducible protein 3, and its deficiency leads to impaired pTreg-cell induction and increased EAE severity. By generating miR-31 and Gprc5a double knockout mice, we show that miR-31 promotes the development of EAE through inhibiting Gprc5a. Thus, our data identify miR-31 and its target Gprc5a as critical regulators for pTreg-cell generation, suggesting a previously unrecognized epigenetic mechanism for dysfunctional Treg cells in autoimmune diseases.

1 Shanghai Institute of Immunology, Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai 200025, China. 2 Shanghai Institute of Hypertension, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai 200025, China. 3 Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425, USA. 4 Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences/Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai 200025, China. 5 State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University, Nanjing 210093, China. 6 Key Laboratory of Cell Differentiation and Apoptosis of Minister of Education, Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai 200025, China. 7 Department of Immunology, Duke University Medical Center, Durham, North Carolina 27710, USA. 8 Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai 200025, China. Correspondence and requests for materials should be addressed to H.W. (email: honglin.wang@sjtu.edu.cn).
cells serve as a central cellular player in adaptive immunity, and their activation and differentiation are elicited by signals from T-cell receptor (TCR), co-stimulatory receptors and various cytokines. Once activated by an antigen, naïve CD4+ T cells proliferate and differentiate into various T helper (Th) cell subsets, including Th1, Th2, Th17 and regulatory T (Treg) cells, that release different cytokines and exhibit distinct effector functions. Besides their critical role in driving immune responses against infections, Th1 and Th17 cells participate in the pathogenesis of autoimmune inflammatory diseases, such as experimental autoimmune encephalomyelitis (EAE). Moreover, naïve T cells differentiate into Treg cells exhibiting immunosuppressive capacity, and the transcriptional factor FoxP3 controls their development and function. According to their origins, Treg cells are divided into thymus-derived Treg (tTreg) cells derived from the thymus, peripherally derived regulatory T (pTreg) cells generated out of the thymus under various inductive signals, and in vitro-induced regulatory T (iTreg) cells. It is now clear that naïve CD4+ T cells sorted as FoxP3− in the mouse thymus possess full potential to differentiate into iTreg cells, thus are potential targets for therapeutic interventions for chronic inflammatory diseases. Independent on thymus, pTreg cells differentiate in secondary lymphoid organs and tissues, and require TCR signalling and the cytokines TGF-β and IL-2 (ref. 7), and only a low antigen dose of a high-affinity TCR ligand is optimal to generate a persistent population of iTreg cells in vivo. So far, dysfunctional Treg cells are identified in several autoimmune disorders including multiple sclerosis (MS). One of the failures of Treg-cell-mediated immunoregulation is inadequate numbers of Treg cells that may be due to defective induction of pTreg cells in the periphery. Thus, understanding of molecular mechanisms underlying pTreg cell generation might provide deeper insights into physiological and pathological immune responses in autoimmune inflammatory diseases.

MicroRNAs (miRNAs) are single-stranded, small noncoding RNAs located in introns or exons of protein-coding genes as well as in non-coding genes. miRNAs have been implicated in maintaining immune homeostasis during stress, such as inflammation, by regulating gene expression at post-transcriptional level. Several studies have reported that specific miRNA signatures were observed for specialized T-cell subsets, and these miRNAs are dynamically regulated during T-cell maturation. Dicer and Drosha are two essential components for the generation of miRNA, and loss of these factors leads to defects in lymphocyte differentiation and autoimmune inflammation. Recently, accumulating evidence has demonstrated that miRNAs are also crucial for Treg-cell development, function and stability. Treg cells display a set of miRNAs that is distinct from conventional T cells. However, intrinsic miRNAs involved in the polarization of Treg cells from naïve T cells in vitro and in vivo settings are largely undetermined.

In this study, we showed that miR-31 expression was triggered by TCR signalling, and downregulated by TGF-β1-induced FoxP3. The conditional deletion of miR-31 in CD4+ T cells led to enhanced induction of pTreg cells in the periphery, and decreased severity of EAE. Retinoic acid (RA) regulates the expression of genes required for cell proliferation, differentiation and survival by binding its nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Although RA has been shown to enhance pTreg-cell generation, the mechanism by which RA promotes pTreg-cell induction is ill-defined. Unexpectedly, we here identified Gprc5a as a direct target of miR-31. Gprc5a is also known as retinoic acid-inducible protein 3 harbouring the functional RAR/RXR binding sites of RA in its core promoter. Gprc5a was targeted by miR-31 through direct binding to its 3′-untranslated regions (3′-UTR), and its deficiency resulted in the impairment of pTreg-cell induction and increased EAE severity. Thus, our findings demonstrated that miR-31 negatively regulated pTreg-cell generation by targeting Gprc5a, suggesting a novel epigenetic mechanism for impaired pTreg-cell induction in autoimmunity.

Results

miR-31 expression is triggered by TCR signalling. Report of FoxP3 miRNA harbouring the target sequence of miR-31 promoted us to investigate its role in the induction and/or function of Treg cells which are vital for preventing autoimmune disease. We induced EAE, an animal model of MS, with myelin oligodendrocyte glycoprotein peptide (MOG35–55) in mice to investigate expression pattern of miR-31 in pathogenic T cells in the tissue-specific autoimmune inflammation. miR-31 expression was assessed in splenocytes and sorted CD4+ T cells at day 10 post immunization. We found that the expression of miR-31 was significantly increased in both splenocytes and pathogenic CD4+ T cells in EAE mice compared with healthy controls (Fig. 1a). We next stimulated the TCR of naïve T (CD4+ CD25− CD62Lhigh) cells with plate-coated anti-CD3- and soluble anti-CD28-specific antibodies, and we detected that the miR-31 expression was increased ~125-fold in activated CD4+ T cells compared with untreated naïve T cells (Fig. 1b). Together, these data suggest that TCR signalling induces miR-31 expression in CD4+ T cells.

Because the TCR signal coordinating with lineage-specific cytokines triggers naïve T cells to differentiate into specialized effector cells, we sought to examine miR-31 expression in different T-cell subsets. We differentiated naïve T cells in vitro under polarizing conditions for the generation of Th1, Th17 and iTreg cells in cultures as these T-cell subsets are critical in the pathology of EAE. At 4 days after activation, miR-31 expression was 29.5-fold higher in Th1 cells, 47.4-fold higher in Th17 cells, but there was 5.6-fold reduction in iTreg cells than that of naïve T cells (Fig. 1c), which suggested a possible regulatory role for miR-31 in CD4+ T-cell lineage differentiation. Because miR-31 has been implicated to negatively regulate FoxP3 expression in human Treg cells, we sought to investigate whether upregulation of miR-31 coincides with downregulation of FoxP3 during iTreg-cell induction. We polarized naïve T cells derived from FoxP3ΔF reporter mice into iTreg cells, and examined miR-31 expression in sorted CD25+FoxP3− and CD25+FoxP3+ cells. The miR-31 expression in CD25+FoxP3+ cells was ~90-fold lower than that in CD25+FoxP3− population (Fig. 1d,e). These data demonstrate that miR-31 is preferentially diminished in iTreg cells. Although the expression of miR-31 was slightly increased in iTreg and pTreg cells compared with iTreg cells, its expression in either iTreg or pTreg was not significantly different between control and EAE mice, suggesting that Treg cells maintain baseline miR-31 expression in vivo (Supplementary Fig. 1a). To further identify why iTreg cells exhibit diminished levels of miR-31, we activated naïve T cells with CD3- and CD28-specific antibodies in the absence or presence of TGF-β1, and measured the time-dependent appearance of miR-31. miR-31 abundance was gradually increased during the stimulation with CD3 and CD28 antibodies in the absence of TGF-β1, however, decreased at 12 h when FoxP3 was induced by adding TGF-β1 (Fig. 1f). Moreover, TGF-β1 dose-dependently decreased miR-31 expression in iTreg-cell differentiation (Supplementary Fig. 1b,c). Together, these data indicate that miR-31 expression might be downregulated by TGF-β1-induced FoxP3 during iTreg-cell induction in vitro. Database analysis revealed...
one potential FoxP3-binding site in the promoter element at −1919 upstream from the transcription start site (TSS) of mouse miR-31 (Fig. 1g upper panel). To establish the possible binding of FoxP3 to the putative binding site in the promoter element of miR-31, we carried out chromatin immunoprecipitation (ChIP) assays. These assays showed a significant recruitment of FoxP3 to the putative miR-31 promoter (Fig. 1g lower panel). Thus, our results suggest that FoxP3 possibly binds miR-31 promoter and downregulates its expression during iTreg cell in vitro differentiation.
T cells is a functionally relevant regulator for the development of autoimmune inflammation, we used homologous recombination to generate mice with a *miR-31* allele flanked by *loxP* sites (floxed; Fig. 2a upper panel). The germline-transmitted mice were crossed with *CD4<sup>Cre</sup>* transgenic mice to achieve a conditional knockout mouse model with a deleted *miR-31* allele in *CD4<sup>+</sup>* T cells (Fig. 2a lower panel). To verify a specific deletion of *miR-31* in *CD4<sup>+</sup>* T cells, we designed primers (P1 and P2) spanning the *loxP* sites (floxed allele, 1,195 bp; deleted allele, 474 bp) and genotyped mice using DNA of either splenocytes or sorted *CD4<sup>+</sup>* T cells derived from *miR-31<sup>fl/fl</sup>CD4Cre* (cKO) and *miR-31<sup>fl/fl</sup>* control mice (Fig. 2b). We detected both floxed and deleted alleles in splenocytes of cKO mice, while only a floxed allele in splenocytes of *miR-31<sup>fl/fl</sup>* control mice, a deleted allele in *CD4<sup>+</sup>* T cells of cKO mice and a floxed allele in *CD4<sup>+</sup>* T cells of *miR-31<sup>fl/fl</sup>* control mice (Fig. 2b). Quantitative real-time PCR (qPCR) analysis confirmed a specific *miR-31* ablation in *CD4<sup>+</sup>* T cells in cKO mice (Fig. 2c). These mice remained healthy without any detectable immune-mediated pathology at least for 32 weeks. By inducing EAE, we demonstrated that the specific *miR-31* ablation in *CD4<sup>+</sup>* T cells significantly decreased its severity accompanied by an evident prevention of weight loss in cKO mice compared with *miR-31<sup>fl/fl</sup>* controls (Fig. 2d,e). Moreover, the deletion of *miR-31* led to a marked decrease in infiltration of inflammatory cells and demyelination in spinal cord of cKO mice with EAE (Supplementary Fig. 2a–c). Thus, using genetic approach, we clearly demonstrated the significant impact of *miR-31* expressed by *CD4<sup>+</sup>* T cells on the development of autoimmunity.

**miR-31 skews the CD4 T-cell-mediated immune balance.** To assess how deletion of *miR-31* in *CD4<sup>+</sup>* T cells reduced the severity of progressive EAE, we analysed T-cell frequency and activation in non-immunized cKO mice. By flow cytometric analysis, we observed no substantial changes in T-cell numbers and activation status in the thymus in cKO mice compared with *miR-31<sup>fl/fl</sup>* controls (Supplementary Fig. 3a,b). T-cell proliferation in response to stimulation via TCR-CD28 was also similar in *miR-31<sup>fl/fl</sup>* and cKO T cells as determined by CellTrace Violet (CTV) fluorescence (Fig. 3a). These data suggest that *miR-31* is dispensable for T-cell development, activation and proliferation. We next analysed T<sub>reg</sub>-cell frequency in non-immunized mice, and found that *miR-31* deficiency did not change the proportion

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**Figure 2 | Alleviation of autoimmune disease in cKO mice.** (a) Schematic representation of the *miR-31* locus and targeting strategy. Cre-mediated recombination of *loxP* sites in mice. Primers (P1 and P2) were designed for genotyping floxed allele (1,195 bp) and deleted allele (474 bp). (b) PCR products of splenocytes and sorted *CD4<sup>+</sup>* T cells derived from either *miR-31<sup>fl/fl</sup>* control or *miR-31<sup>fl/fl</sup>CD4<sup>Cre</sup>* (cKO) mice. (c) qPCR analysis to confirm the deletion of *miR-31* in *CD4<sup>+</sup>* T cells derived from cKO mice. (d,e) Clinical scores and weight loss (mean ± s.e.m.) of *miR-31<sup>fl/fl</sup>* or cKO mice after the induction of EAE were assessed every day (n = 7 per group). ***P < 0.001, two-tailed Student’s *t*-test for **c**, one-way analysis of variance for **d** and **e**. Data are representative of two (c) or three (**d** and **e** independent experiments (mean ± s.e.m.).
of T_{reg} cells in the thymus and periphery, indicating that miR-31 had no impact on T_{reg} and pT_{reg}-cell development (Supplementary Fig. 4a–c). T_{H1} and T_{H17} cells are inflammatory cells that develop during tissue-specific inflammatory responses and play a critical role in enhancing tissue inflammation\textsuperscript{25,27}. Therefore, we investigated inflamed spleen and central nervous system (CNS) from \textit{miR-31^{fl/fl}} and cKO mice for the presence of IFN-\(\gamma\)-producing (T_{H1}) and IL-17-producing (T_{H17}) CD4\(^{+}\) T cells during EAE. In contrast to \textit{miR-31^{fl/fl}} controls, cKO mice showed a significant reduction of T_{H1} and T_{H17} cell proportion not only in inflamed spleen (Fig. 3b,c) but also in CNS (Fig. 3d,e) 14 days post immunization with MOG\textsubscript{35–55}. These data suggest that the development of inflammatory T_{H1} and T_{H17} cells in cKO mice is impaired during the induction phase of autoimmune disease. We next investigated whether the decreased encephalitogenic potential of CD4\(^{+}\) T cells in cKO mice was a
consequence of increased peripheral Treg-cell generation during EAE. On day 14 post immunization, we observed a marked increase in the proportion and absolute numbers of Treg cells in the periphery of cKO mice (Fig. 3f–h). Moreover, there was no significant difference of Treg frequency in miR-31fl/fl and cKO mice with EAE (Supplementary Fig. 4d). Together, these results demonstrate that miR-31 skews the balance between pathogenic T helper 1/17 cells and Treg cells in the periphery during autoimmune inflammation.

miR-31 limits pTreg-cell induction. Because miR-31 exhibited a distinct expression pattern in differentiated CD4+ T-cell subsets, we postulated that the intrinsic miR-31 may regulate their in vitro generation. We sorted naive T cells from miR-31fl/fl and cKO mice, and polarized them into Th1, Th17 and iTreg cells under lineage-specific conditions in vitro. After 4 days culture, we found no significant change for the differentiation of Th1 and Th17 cells from naive T cells of cKO mice compared with miR-31fl/fl control mice (Fig. 4a–d). Of note, the lack of miR-31 markedly induced iTreg-cell differentiation in culture (Fig. 4e). Thus, these data suggest that miR-31 deficiency preferentially enhanced the generation of TGF-β1-induced iTreg cells in vitro. Helios is a potent marker, which could distinguish iTreg cells from pTreg cells28. We injected intravenously bone marrow cells (5 × 10⁶) from either miR-31fl/fl or cKO mice into lethally irradiated C57BL/6 recipient mice to generate bone marrow chimeric mice. Eight weeks after bone marrow transplantation, EAE was induced in all chimeric mice. On day 14 post immunization, we analysed the frequency of Helios+ FoxP3+ pTreg cells and Helios+FoxP3+ iTreg cells in the spleen of chimeric mice by flow cytometry. By Helios staining, we demonstrated that the conditional deletion of miR-31 led to increased numbers of Helios+ pTreg cells, whereas Helios+ iTreg cells had no significant change in vivo (Supplementary Fig. 5). Together, our data indicate that miR-31 limits pTreg-cell induction in autoimmunity. We next examined the suppressive capacity of miR-31fl/fl and cKO Treg cells. CTX dilution determined that cKO Treg cells inhibited T-cell proliferation to the same extent as miR-31fl/fl Treg cells (Fig. 4f). pTreg cells had robust suppression and enhanced stability, suppressed ongoing EAE29. Given the fact that pTreg cells converted from conventional T cells play a critical role in the control of development of EAE or other autoimmune diseases30,32–34, we have provided strong evidence that promoting generation of pTreg cells by disrupting miR-31 was likely responsible for the observed phenotype.

Gprc5a is a target of miR-31. To elucidate the mechanisms, we combined microarray gene expression analysis and target prediction to look for putative targets of miR-31. Using a combination of these two approaches, we identified seven predicted target genes that were upregulated in polarized iTreg cells derived from cKO mice (Fig. 5a–c). To confirm accuracy of the microarray data, we validated these potential target genes by increasing sample numbers. We found one predicted target of miR-31, Gprc5a, was significantly upregulated at mRNA levels, and increased by more than 5.0-fold in cKO iTreg cells compared with miR-31fl/fl controls (Supplementary Fig. 6 and Fig. 5d). In contrast to miR-31fl/fl iTreg cells, Gprc5a protein level was also increased by 1.96-fold in cKO iTreg cells (Fig. 5e). Gprc5a was reported to be regulated directly by RA via its receptors, RARs and RXRs35,34. These nuclear RA receptors bind to the Gprc5a promoter for its transcriptional activity34. We next generated a reporter construct that includes the 3′-UTR of Gprc5a mRNA. In contrast to a control construct lacking the target sequence, miR-31 overexpression led to significantly decreased luciferase activity derived from the construct expressing the target sequence (Fig. 5f–g). Thus, our data demonstrate that miR-31 is capable of directly targeting a sequence within the 3′-UTR of Gprc5a mRNA and that Gprc5a is one of the key targets of miR-31 in Treg-cell differentiation.

Gprc5a is critical for pTreg-cell differentiation. Gprc5a was reported to be expressed preferentially in lung tissue and to be a putative lung tumour suppressor gene35. The functional analysis of Gprc5a in T-cell differentiation and autoimmunity is not yet performed. We induced EAE, and measured Gprc5a expression in inflamed spleen and sorted CD4+ T cells. In contrast with non-immunized controls, Gprc5a expression was significantly decreased in spleen and CD4+ T cells in EAE mice, and this might be the consequence of increased miR-31 under inflammatory conditions (Fig. 6a,b). Western blot analysis confirmed that expression of Gprc5a protein was much lower in spleen and CNS of EAE mice than those of healthy controls (Fig. 6c,d). We assessed the role of Gprc5a in pTreg-cell generation using Gprc5a−/− mice35. Gprc5a deficiency resulted in a marked decrease in the TGF-β1-mediated induction of iTreg cells, but had no impact on the induction of Th1 and Th17 cells (Fig. 6e and Supplementary Fig. 7a,b), suggesting that Gprc5a preferentially regulates FoxP3 expression. Thus, our data demonstrate that Gprc5a is a novel regulator in iTreg-cell generation. Interestingly, consistent with the observation in miR-31 cKO mice, we found that Gprc5a deficiency did not affect iTreg-cell generation in healthy mice (Fig. 6f). To test the impact of Gprc5a on the Treg-cell response during inflammation, we analysed the frequency of Treg cells in inflamed spleen in Gprc5a−/− mice after the induction of EAE. Gprc5a deficiency resulted in a significant decrease in the percentage of Treg cells compared with WT controls (Fig. 6g, h), suggesting that Gprc5a is critically required for pTreg-cell generation in vivo in autoimmune disease. Notably, Gprc5a−/− mice developed EAE not only much earlier, but also more severe than Gprc5a+/+ mice (Fig. 6i). Moreover, an excessive weight loss was displayed in Gprc5a−/− mice compared with Gprc5a+/+ mice (Fig. 6j). To further investigate whether miR-31 affects EAE development via regulating Gprc5a in vivo, we generated double knockout (DKO) mice by crossing miR-31−/− cKO mice with Gprc5a−/− mice. We demonstrated that the severity of EAE was significantly reduced in cKO mice compared with Gprc5a−/− mice, however, the disease phenotype was completely restored when Gprc5a was deleted in the cKO mice (Supplementary Fig. 7c). Collectively, our observations indicate that Gprc5a is regulated by miR-31, and functionally involved in the development of EAE. The beneficial effect of RA is possibly due to its stimulation of Gprc5a expression to promote pTreg-cell generation in tissue-specific autoimmune inflammation.

Discussion

Treg cells have been reported to be capable of controlling CNS autoimmunity in several CD4+ T-cell-driven EAE models. Treg-cell frequency within the CNS was increased during the recovery phase of actively induced EAE36,37, and the adoptive transfer of Treg cells ameliorated EAE symptoms36,38. Furthermore, depletion of Treg cells with anti-CD25 mAb has been demonstrated to exacerbate EAE38. More importantly, reduced Treg-cell proliferative potential and cloning frequency were identified in patients with MS12,39. Thus, regulators of Treg-cell generation are considered to harbour valuable potential for clinical applications in autoimmune disorders. Here, we report that miR-31 expression in CD4+...
T cells was triggered by TCR signalling, and downregulated by TGF-β-mediated FoxP3. Its conditional deletion substantially enhanced the pTreg-cell induction and ameliorated disease severity in the EAE model. Mechanistically, we have proven that by targeting Gprc5a, a known retinoic acid-inducible protein, miR-31 promoted the generation of pTreg cells in vivo. Gprc5a is a functional target of miR-31, and its deficiency resulted in impaired pTreg-cell generation and increased EAE severity. Antigen-specific stimuli delivered through the TCR cooperates with antigen-nonspecific cytokines to support proliferation and

![Figure 4](image-url)
Gprc5a expression in polarized iTreg cells derived from miRanda predicted 1,305 potential targets of miR-31. The overlapping seven genes were defined as ACCEPT genes. (microarray analysis (41,174 genes in total). Transcripts of top 63 genes were found to be upregulated in iTreg cells of cKO mice. (Mean ± s.e.m.)

Figure 5 | Gprc5a is directly targeted by miR-31. (a) Total RNAs of polarized iTreg cells derived from miR-31fl/fl controls and 3 cKO mice were used for a microarray analysis (41,174 genes in total). Transcripts of top 63 genes were found to be upregulated in iTreg cells of cKO mice. (b,c) TargetScan and miRanda predicted 1,305 potential targets of miR-31. The overlapping seven genes were defined as ACCEPT genes. (d,e) qPCR or western blot analysis of Gprc5a expression in polarized iTreg cells derived from miR-31fl/fl and cKO mice. (f) WT and point-mutated 3′-UTR reporter constructs. (g) Luciferase activity was determined in NIH3T3 cells that were transfected with miR-31 mimics and the indicated 3′-UTR reporter construct or with the indicated WT or point-mutated 3′-UTR reporter construct (WT UTR or mutant UTR). Results (d) are presented as the ratio of mRNA to the β-actin, relative to that in controls. *P < 0.05; ***P < 0.001, NS, not significant, two-tailed Student’s t-test. Data (d,e and g) are representative of at least two independent experiments (mean ± s.e.m.).

differentiation of distinct T<sub>H</sub> cell subsets<sup>40</sup>. However, it has become increasingly clear that miRNAs, post-transcriptional regulators, are involved in driving T<sub>H</sub> cell differentiation and lineage commitment<sup>41</sup>. A selective effect of miR-31 on pT<sub>T</sub>reg-cell differentiation could be explained by the differential requirement of TCR signalling in the induction of these T-cell lineages. A low antigen dose of a high-affinity TCR ligand favours to induce pT<sub>T</sub>reg cells in vivo<sup>9</sup>, whereas high doses of TCR stimulation prevents FoxP3 induction and pT<sub>T</sub>reg-cell generation through activating NF-κB signalling<sup>42</sup>. Indeed, we have determined that the activation of NF-κB induced miR-31 expression through a direct binding of p65 to its promoter (to be published elsewhere). Thus, it is possible that in the absence of TGF-β1, TCR stimulation at high doses elicits activation of NF-κB, which directly triggers the expression of miR-31 inhibiting FoxP3 levels in CD4<sup>+</sup> T cells. However, TCR stimulation at low doses induces FoxP3, which may downregulate miR-31 expression through binding to its promoter, providing a feedback loop during pT<sub>T</sub>reg-cell differentiation.

Several miRNAs have been reported to impact T<sub>T</sub>reg-cell development and function. miR-155 is highly expressed in T<sub>T</sub>reg cells, facilitates T<sub>T</sub>reg-cell homeostasis by repressing Socs1 and its deficiency results in decreased numbers of both tT<sub>T</sub>reg cells and pT<sub>T</sub>reg cells<sup>43</sup>. miR-21 indirectly acts as a positive regulator of human FoxP3 expression<sup>21</sup>. miR-146a is critical for Treg-cell-mediated control of T<sub>H</sub>1 responses via targeting Stat1 (ref. 44).
Figure 6 | Gprc5a deficiency decreases pTreg-cell differentiation and promotes autoimmune inflammation. (a,b) Splenocytes were prepared from unimmunized control mice (Ctr) or EAE mice 10 days after immunization (n = 3–4). qPCR analysis of Gprc5a expression in total splenocytes or in sorted CD4+ T cells. (c,d) Western blot analysis of Gprc5a in splenocytes and CNS infiltrating cells derived from healthy controls (Ctr) or EAE mice. (e) Naive T cells were sorted from Gprc5a+/+ and Gprc5a−/− mice. Flow cytometry of polarized iTreg cells in the presence of different concentrations of TGF-β. (f) Flow cytometry analysis of Treg cells in thymus from 6-week-old Gprc5a+/+ and Gprc5a−/− mice (gated on CD4+ T cells). (g,h) Flow cytometric analysis of Treg cells in the inflamed spleen and lymph nodes Gprc5a+/+ and Gprc5a−/− mice 14 days after the induction of EAE (gated on CD4+ T cells). Numbers adjacent to outlined areas indicate percent cells in each. (i,j) Clinical scores and weight loss (mean ± s.e.m.) of Gprc5a+/+ or Gprc5a−/− mice after the induction of EAE were assessed every day (n = 7). *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant, two-tailed Student’s t-test for a,b,f,g and h; one-way analysis of variance for i,j. Data are representative of at least two independent experiments (mean ± s.e.m.).

Despite that miR-17−92 is dispensable for the development of iTreg cells in vivo, miR-17−92 ablation reduces the frequency of MOG35−55-specific pTreg cells during EAE. miR-10a is induced by TGF-β1 and RA, and promotes the differentiation of pTreg cells through inhibiting Bcl-6 (ref. 46). Collectively, these Treg-cell-associated miRNAs are all enriched in Treg cells compared with conventional T cells, and function as positive regulators. Our data demonstrated that miR-31 was preferentially diminished in Treg cells, was downregulated by FoxP3, and negatively regulated naive CD4+ T cell differentiation into pTreg cells. The conditional deletion of miR-31 in CD4+ T cells resulted in enhanced induction of pTreg cells in the periphery, and decreased severity of autoimmune disease. Thus, we highlight miR-31 acts as a negative regulator for pTreg-cell generation in vivo. Although different targets of miR-31 were identified, its similar effect was also reported previously for human Treg cells. miR-31 regulates keratinocyte differentiation through inhibiting hypoxia-inducible factor 1 (ref. 49). Furthermore, in contrast to
other T-cell subsets, miR-31 has been shown to be downregulated in human Treg cells. This raises the intriguing possibility that miR-31 may be preferentially diminished in Treg cells and its upregulation in CD4+ T cells under inflammatory stress may limit pTreg cell induction in human autoimmune diseases.

RA has been proven to facilitate pTreg-cell generation. RA regulates Gprc5a transcriptional activity by binding to its receptors, RARs and RXRs. So far, the role of Gprc5a in the T-cell differentiation programme is not investigated. We here showed that Gprc5a deficiency led to a severe defect in in vivo- and in vitro-generation of Treg cells, as well as increased severity of inflammatory CNS phenotypes, indicating that this may be one of the mechanisms by which RA inhibits autoimmune reactions in vivo. However, the molecular mechanism by which Gprc5a promotes Treg-cell generation and suppresses autoimmune disease is subject to further investigation. Nevertheless, by generating miR-31 and its target Gprc5a DKO mice we clearly show that miR-31 promotes the development of autoimmune disease through inhibiting Gprc5a.

In summary, our results demonstrate that miR-31 inhibits pTreg-cell generation through directly targeting Gprc5a, a retinoic acid-inducible protein and promotes autoimmune, therefore, providing the first in vivo genetic evidence that miR-31 and its novel target Gprc5a are critical intrinsic factors for controlling physiological and pathological immune responses regulated by pTreg cells.

Methods

Mice. C57BL/6 (stock number: 000664), B6.Cg-FoxP3tmTch/J mice (stock number: 006772, designated as FoxP3GFP) and CD4Cre mice (stock number: 017336) were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129-Gprc5a−/− (Rag2tm1Fwa N12 (RAG2−/−) mice were purchased from Taconic Labs (Hudson, NY). Gprc5a−/− mice were generated as previously reported. Mice were kept under specific pathogen-free conditions in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval (SYXX-2003-0026) of the Scientific Investigation Board of Shanghai Jiao Tong University School of Medicine, Shanghai, China. To ameliorate any suffering of mice observed throughout these experimental studies, mice were euthanized by CO₂ inhalation.

Generation of miR-31fl/fl and miR-31fl/foxP3GFP mice. The miR-31 locus (mmu-mir-31 ENSMUSG00000065408, http://www.ensembl.org/index.html) is on chromosome 4 (Mus musculus) and encodes the miR-31 (mmu-mir31 ENSMUSG00000065408, http://www.ensembl.org/index.html) is on chromosome 4 (Mus musculus) and encodes the miRNA (GACTTGCAAACGTCAG-3’). To create loxP-miR-31-loxp mice, a targeting vector was designed to insert an f rt-flanked PGK-neo cassette and a loxp site upstream of miR-31, and a second loxp site downstream. Loxp site is a 34 bp length DNA sequence that can be recognized by Cre recombinase catalysts. If two loxp sites are introduced in the same orientation into a genomic locus, expression of Cre results in the deletion of the loxP-flanked DNA sequence. After linearization, the vector was electroporated into 129S-derived embryonic stem (ES) cells. The collected ES cells were screened with 300 μg ml⁻¹ G418 and 2 μM Ganc for 8 days and ascertained by PCR. The ES cells with right homologous recombination were injected into blastocyst. After birth, the chimeric mice were bred with 129S5 mice to generate the heterozygotes. At this point, mutant mice were bred with F1 recombinase-expressing mice to remove the f rt-flanked neo cassette. The resulting loxp-miR-31-loxp mice were backcrossed into C57BL/6 background for eight generations and bred with CD4Cre transgenic mice. P1 and P2 were used to genotype the miR-31 floxed allele (1,199 bp) and the miR-31 deleted allele (1,016 bp). The o bamboos GATTCAGACATCAGCATCGAGTGCAAAACGTCAG-3’. Excision by CRE was complete for all pups used in experiments. In some experiments, these mice were further crossed with FoxP3GFP mice to generate mice that express green fluorescent protein (GFP) in their Treg cells.

Induction of EAE. EAE was induced by complete Freund’s adjuvant (CFA)-MOG35-51 peptide immunization (China Peptides Biotechonology) and scored daily. Briefly, C57BL/6 mice were injected subcutaneously into the base of the tail with a volume of 200 μl containing 300 μg MOG35-51 peptide emulsified in CFA (Sigma-Aldrich). Mice were also injected intravenously with 200 ng of pertussis toxin (Merck-Calbiochem) on day 0 and 2 post immunization. All the reagents used for in vivo experiments were free of endotoxin. Mice were monitored daily for the development of disease which was scored according to the following scale: 0, normal; 1, tail tip hanging; 1.5, impaired righting reflex; 2, hind limb parale; 2.5, hind limb paralysis; 3, forelimb weakness; 4, complete paralyis; 5, moribund or death.

T-cell isolation and sorting. Peripheral T cells were obtained from the spleen and lymph nodes of 6-week-old mice. Naïve CD4+ T cells (CD4+ CD25− CD62Llo) were sorted by FACSAria III (BD Biosciences) after enrichment of CD4+ T cells by sorting with flow cytometry based on cell surface markers (CD4, CD25 and FoxP3GFP). Naïve CD4+ T cells were isolated from the spleen of mice by sorting with flow cytometry based on cell surface markers (CD4+ , CD25− and CD62Llo and...
FoxP3β). Spleenocytes from RAG2⁻/⁻ mice lacking mature T and B lymphocytes were used as antigen presenting cells. The purified naïve CD4⁺ T cells were labelled for 15 min at 37°C with 10 μM CTV (Life Technologies) and the CTV-labelled T cells (1 × 10⁵) were cultured in 96-well plates for 72 h together with an increasing ratio of sorted Treg cells in the presence of anti-CD3 (1 μg ml⁻¹) plus γ-irradiated antigen-presenting cells (1 × 10⁵). The suppressive function of Treg cells was determined by measurement of the proliferation of activated CD4⁺ effector T cells on the basis of CTV dilution.

**Generation of bone marrow chimeric mice.** Bone marrow cells were flushed from miR-31β/β or cko donor mice, and 5 × 10⁵ T-cell-depleted bone marrow cells were transplanted into each C57BL/6 host mouse with total-body irradiation of 950 cGy in two divided doses. Chimeric mice reconstituted with bone marrow cells derived from either miR-31β/β or cko were subjected for EAE induction 8 weeks after the transplantation.

**Histology.** Spleen cords from miR-31β/β or cko EAE mice were fixed in 4% paraformaldehyde and paraffin embedded. Paraffin-embedded 5-μm sections of spleen were stained with haematoxylin and eosin or Luxol fast blue and then examined by light microscopy (Axio scope A1, Zeiss).

**Luciferase reporter plasmid.** The Gprc5a 3′-UTR was amplified using primers Gprc5a Forward, 5′-AACCTGACCCTTGTTGAAAGATGGCGA-3′, Reverse, 5′-TCTGCGGCCGCAATGTTGTGACCACATCTTTATTG-3′. The Gprc5a 3′-UTR genomic fragment was digested with Xhol and NotI and inserted into the corresponding sites of the psiCHECK-2 Synthetic firefly luciferase reporter plasmid (Promega). This construct was also used to generate a miR-31-3′-UTR mutant plasmid. The mutagenic primers used for Gprc5a were Mutant Forward, 5′-AA TCTCGAGCTTGGAGGAAGTGGGACA-3′, Mutant Reverse, 5′-CAGCCCAC CGTCTCTGGGCGT-3′. The correctness of all the plasmids was confirmed by sequencing.

**Luciferase assays.** All 3′ UTR reporter vectors were prepared by amplifying the 3′ UTRs of Gprc5a, followed by insertion into the psiCHECK-2 vector (Promega). Site-specific mutants were generated by PCR in the psiCHECK-2 vector (Promega). NIH3T3 cells were maintained in DMEM (HyClone) supplemented with 10% fetal Site-specific mutants were generated by PCR in the psiCHECK-2 vector (Promega).

**MicroRNA microarray analysis.** Naïve T cells from 3 miR-31β/β mice and 3 miR-31β/β/CD4β mice were used to induce Treg in vitro. Total RNA was isolated using RNeasy Minikit (Qiagen). Mouse genome-wide cDNA microarray analysis was performed by Shanghai Biotechnology (Shanghai).

**Statistical analysis.** The data were analysed with GraphPad Prism 5 and were presented as the mean ± s.e.m. Student’s t-test was used when two conditions were compared, and analysis of variance with Bonferroni or Newman–Keuls correction was used for multiple comparisons. Probability values of <0.05 were considered significant; two-sided tests were performed.

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Author contributions

L.Z. and H.W. designed the research; H.W. supervised the research; L.Z. conducted the experiments; H.W. wrote the manuscript and revised the manuscript; F.K., Z.L., J.B., J.L., S.Y., Z.X., F.L., H.Z., Y.S., W.C., Y.G., Z.H. and J.D. helped with the experiments; Q.L., X.-Z.Y., Y.Q. and Q.-J.L. commented on the research.

Additional information

Accession codes: Microarray data have been deposited in the GEO database under accession code GSE61938.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications and accompanies this paper at http://www.nature.com/naturecommunications

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