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Overexpression of microRNA-21 is associated with elevated pro-inflammatory cytokines in dominant-negative TGF- β receptor type II mouse

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Abstract

Dominant negative TGF- β receptor II (dnTGF- β RII) mice spontaneously develop an autoimmune cholangitis resembling human primary biliary cirrhosis (PBC). Interestingly, the dominant negative TGF- β receptor is expressed by both CD4⁺ and CD8⁺ T cells and leads to greatly reduced (but not absent) TGF- β signaling resulting in T cell intrinsic cell mediated autoimmunity. However, the mechanisms of the T cell dysregulation remain unclear. Recently it has been shown that TGF- β signaling is intimately involved with miRNA biogenesis and control. Herein we show that lack of T cell TGF- β signaling leads to down regulation of T cell miRNAs but upregulation of the key inflammatory miRNA 21. Furthermore, the expression of miR-21 from hepatic effector

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CD8⁺ T cells is significantly higher than in the same subsets isolated from spleen and mesenteric lymph nodes of the dnTGF- β RII mice. Previous studies indicate that miR-21 increases the synthesis of IFN- γ and IL-17A by T cells and suppresses apoptosis via programmed cell death protein 4 (PDCD4). Data presented herein demonstrate that transfecting w.t. B6 T cell subsets with miR-21 resulted in upregulation of the inflammatory cytokines TNF- α and IFN- γ , thus partly replicating the dnTGF- β RII T cell phenotype. In conclusion, these data suggest miR-21 plays a critical role in the production of pro-inflammatory cytokines in dnTGF β RII mice, which could be a contributing factor for the development of the organ-specific autoimmune cholangitis and colitis in this murine model of human PBC.

Keywords

Primary biliary cirrhosis; inflammatory bowel disease; autoimmunity; cholangitis; colitis; microRNA; miR-21

Introduction

Mice transgenic for directed expression of a dominant negative form of transforming growth factor beta receptor type II (dnTGF β RII), under the control of the CD4 promoter lacking the CD8 silencer, spontaneously develop an autoimmune biliary ductular disease with similarity to human primary biliary cirrhosis (PBC). In addition, dnTGF β RII mice spontaneously develop colitis similar to human inflammatory bowel disease (IBD) [1–5]. The utilization of adoptive transfer experiments have demonstrated that CD8⁺ T cells play a critical role in the autoimmune cholangitis, while CD4⁺ T cells are involved in colitis [2].

MicroRNAs (miRNAs) are small noncoding RNAs that control the translation of mRNAs by promoting the degradation of target mRNAs or preventing their translation [6–8]. Given the emerging roles of miRNAs in modulating both innate and adoptive immune responses, it is likely that any dysregulation of miRNA expression may contribute to the pathogenesis of autoimmune diseases and chronic inflammation [9]. Dysregulated miRNA expression has been associated with autoimmunity, specifically rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [10–12]. TGF- β receptor mediated signaling has been shown to directly regulate miRNA biogenesis by at least three mechanisms: 1) direct gene transcription of miRNA genes, 2) direct binding of TGF β receptor induced SMAD proteins to specific miRNAs, and 3) stabilization of miRNA processing machinery [13–15]. The net effect of TGF- β signaling is to upregulate miRNA biogenesis. Our goal was to investigate whether abrogation of TGF- β signaling in mouse CD4⁺ and CD8⁺ T cells affects miRNA expression and involvement in the pathogenesis of cholangitis. We demonstrate herein that, as expected, the overall effect of loss of TGF- β signaling resulted in the downregulation of many species of miRNA in T cells. Surprisingly, however, loss of TGF- β signaling resulted in the upregulation of several important miRNAs, including miR-21. The level of miR-21 was elevated in both CD4⁺ and CD8⁺ T cells compared with control mice. Furthermore, transfection of miR-21 in B6 T cells resulted in enhanced secretion of IFN- γ and TNF- α , recapitulating the dnTGF β RII T cell phenotype.

Materials and Methods

Animals

The dnTGF β RII colony on a C57BL/6 background (B6.Cg-Tg(Cd4-TGFBR2)16Flv/J) was maintained at the University of California at Davis animal facility [16]. B6 mice were purchased from The Jackson Laboratory. All mice were fed sterile rodent Helicobacter Medicated Dosing System (three-drug combination) diets (Bio-Serv, Frenchtown, NJ) and

maintained in individually ventilated cages under specific pathogen-free conditions. Sulfatrim (Hi-tech Pharmacal, Amityville, NY) was delivered through drinking water. At 9 weeks of age, animals were sacrificed to collect sera, spleen, liver, and colon tissues. In addition, at 2 weeks of age, animals were sacrificed to collect thymus and spleen. The liver and colon from sacrificed mice were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 4 μ m sections, deparaffinized, stained with hematoxylin and eosin (H&E), and evaluated using light microscopy [17]. All experimental protocols were approved by the University of California Animal Care and Use Committee.

Flow Cytometry

Mononuclear cells (MNCs) were isolated from liver, spleen, and mesenteric lymph node (mLN) [18, 19]. The cells were re-suspended in staining buffer (0.2% BSA, 0.04% EDTA and 0.05% sodium azide in PBS), divided into 25 μ l aliquots, and incubated with anti-mouse FcR blocking reagent (eBioscience) for 15 minutes at 4°C. Cells were washed and stained for 30 minutes at 4°C with a combination of fluorochrome conjugated mAb against the cell surface markers CD4, CD8 α , NK1.1 (Biolegend, San Diego, CA), and TCR- β (eBioscience, San Diego, CA). To define naïve versus effector T cell subpopulations, antibodies against CD62L and CD44 were used as described [1, 20, 21]. IgG isotype antibodies with matching conjugates were used in parallel as negative controls. The stained cells were washed with PBS containing 0.2% BSA before acquiring data on a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA) upgraded for detection of 5 colors by Cytex Development (Fremont, CA). The data were analyzed using Cellquest PRO software.

Isolation of cell and total RNA including small RNA—A MoFlo cell sorter (Beckman Coulter, Brea, CA) was used to isolate enriched populations of CD44⁺CD62L⁺ naïve or CD44⁺CD62L⁺ effector CD4 and CD8 T cells from liver, spleen, and mLN, and to isolate enriched populations of CD4 and CD8 T cells from thymic tissues of mice. CD4 (L3T4) MicroBeads and CD8 α (Ly-2) MicroBeads (Miltenyi Biotec, Auburn, CA) were used to enrich CD4 and CD8 T cells from spleen, liver and colon MNCs, respectively. Purity of isolated cells was re-analyzed on an aliquot of the enriched cells by flow cytometry. Post-purification purity of cells was >95%. For total RNA isolation (including small RNA), the isolated cells were washed three times with PBS. RNA extraction was performed using miRNeasy Mini kit (Qiagen, Valencia, CA) and chloroform (Sigma, St. Louis, MO).

miRNA PCR arrays—The microRNA Ready-to-Use PCR, Mouse & Rat panel I, V2.M (EXIQON) containing 376 mouse and rat miRNAs was utilized to obtain miRNA profiles.. Threshold cycle (Ct) number of each miRNA was acquired after the plates were run on a ViiA™ 7 Real-Time PCR System (Life Technologies, Carlsbad, CA). The results were analyzed as follows: we first defined very low expressing or non-expressing Ct (from either B6 or dnTGFbRII) as greater than or equal to 37 cycles and any miRNA with Ct greater than 37 in both strains was removed these from the dataset. In each strain, the remaining miRNAs were normalized to the global mean of miRNAs with Cts no greater than 35. Global mean normalization is preferred in screening studies where the dataset has a high number of expressed miRNAs (eg. >100) without assumptions about how many and/or which miRNAs are regulated. However, miRNAs expressed close to the cut-off tend to have greater technical variation than those expressed at lower Ct values, therefore only miRNAs with Cts less than or equal to 35 were used to calculate the global mean in our analysis. Cells from the B6 mice were used as a reference for each normalized miRNA. Fold changes (FC) of miRNA expression in dnTGFbRII compared to B6 splenic CD4⁺ and CD8⁺ T cells were calculated. Finally, FC values were converted to a log scale to obtain a normal distribution and plotted for each miRNA with Ct no greater than 37.

Biological pathway analysis of miRNA expression data—miRNAs with at least 2-fold increase or decrease in dnTGFβRII CD4 or CD8 T cells compared to B6 counterparts were inputted to the multiple microRNAs Analysis function of the web-based DIANA mirPath software (http://diana.cslab.ece.ntua.gr/pathways/index_multiple.php). TargetScan5 was selected for miRNA target prediction and the enrichment analysis of target genes was performed by the mirPath software by comparing the genes to all known pathways retrieved from KEGG (Kyoto Encyclopedia of Genes and Genomes, www.genome.jp/kegg/). A ranking score was calculated on the basis of the number of predicted target genes of all the miRNAs in the list as well as the position of these genes within the pathway. Higher scores mean a higher possibility of control by the selected miRNA. Pathways in which miRNAs are upregulated (or downregulated) in CD4 T cells are compared to those in which miRNAs are upregulated (or downregulated) in CD8 T cells to detect the pathway patterns controlled by these miRNAs in the two T cell populations.

Quantitative real time PCR for miRNAs—PCR primers for mmu-miR-21 and SNORD66 were purchased from Qiagen. miRNA was converted to cDNA using RT²miRNA First Strand kit (Qiagen). Real time PCR reactions were performed according to the manufacturer's instructions, using a ViiA^{TM} 7 Real-Time PCR System (Life Technologies). The measured levels of miR-21 were normalized to that of SNORD66 to obtain the relative levels of miR-21 in specific cell populations.

Overexpression of miR-21miRNA—Purified CD4⁺ and CD8⁺ T cells from spleens of the B6 mice were transfected with 25nM of miR-21 mimic (Qiagen) [22], or Allstar negative control siRNA (Qiagen) [23], using TransIT-TKO Transfection Reagent (Mirus, Madison, WI), following the manufacturer's instructions. The transfected cells were cultured overnight in RPMI 1640 medium with 10% FBS at 37°C in 5% CO₂, then stimulated with 0.5μg/ml of anti-CD3/CD28 antibodies (Biolegend) for 48 hours. The culture supernatant was collected for cytokine assay, while cell pellets were frozen at -80°C for miRNA analysis.

Cytokine Analysis

Total protein was extracted from 30 mg of frozen liver or colon tissues by homogenization in T-Per® tissue protein extraction buffer (Thermo, Rockford, IL) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). The homogenized tissue suspension was centrifuged at 12,000 g for 20 minutes at 4°C and the supernatant was stored at -80°C until use. The total protein concentration of each sample was measured by a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Levels of IL-17A, TNF-α, IL-6, and IFN-γ in sera, tissue lysates or culture media were measured by a mouse Th1/Th2/Th17 cytokine CBA kit (BD Biosciences).

Statistical analysis—Groups were compared using the non-parametric Mann-Whitney U-test or Student's t-test as appropriate. miRNA array data were analyzed using the SABiosciences web analysis portal.

Results

Cholangitis and Colitis in dnTGFβRII mice

Cholangitis and colitis were observed at 9 weeks of age (Fig. 1A). The levels of IFN-γ, IL-17A, TNF-α and IL-6 in serum, liver, and colon were significantly increased in the dnTGFβRII mice compared to age-matched B6 mice (Fig. 1B). The number of intrahepatic MNCs, CD4⁺ T cells, and CD8⁺ T cells were significantly higher in dnTGFβRII mice than that of B6 mice (Fig. 1C). In addition, the total number of MNCs in mLN, which correlates

with colitis severity [24, 25], was also significantly increased in dnTGFβRII mice compared to age-matched B6 mice (Fig. 1D).

miRNAs are predominantly down-regulated in dnTGFβRII splenic CD4⁺ and CD8⁺ T cells but miR-21 is upregulated

As shown in Fig. 2, the majority of miRNA are downregulated in dnTGFβRII CD4⁺ and CD8⁺ T cells, as expected given the biological role of TGF-β in miRNA biogenesis [13–15]. Thus, CD8⁺ T cells show a striking down regulation of miRNA with 142/285 (50%) miRNAs showing a more than 2-fold decrease in dnTGFβRII CD8⁺ T cells compared to B6 CD8⁺ T cells, and only 19/285 (7%) miRNAs with more than 2-fold increase in dnTGFβRII CD8⁺ T cells compared to B6 CD8⁺ T cells. A similar pattern is seen in CD4⁺ T cells with 86/320 (27%) miRNA showing more than a 2-fold decrease in dnTGFβRII CD4⁺ T cells, and 28/320 (9%) miRNAs showing more than 2-fold increase in dnTGFβRII CD4⁺ T cells compared to B6 CD4⁺ T cells. The smaller subset (~7–9%) of upregulated miRNAs was somewhat surprising given the role of TGF-β signaling in general to upregulate miRNA. We analyzed miRNA expression patterns of CD4 and CD8 T cells to see if the expression patterns (up or down) correlated between the two cell subsets. As shown in Figure 2b, the upregulated or downregulated miRNA's differed substantially between CD4 and CD8 T cell subsets, suggesting differential miRNA programming in response to TGFβ in these cell subsets. However, as shown in Figure 2c, we performed a pathway analysis of the biological pathways controlled by these miRNAs (either down or upregulated). Table one shows the top 20 pathways controlled by the miRNAs in each of the four cell subsets. Strikingly, the pathways controlled were largely conserved, with a majority of biological pathways overlapping in both the down- and up-regulated miRs from CD4 and CD8 cells (Fig 2c).

The most surprising result of the global miRNA analysis was the finding of an upregulation of a small number of miRNA, since TGFβ signaling is known primarily to upregulate miRNA and therefore loss of TGFβ signaling should downregulate miRNA (as it did for most miRNA). Thus, the reason why only a few miRNA were upregulated remains unclear. The miR-21 was of the greatest interest, since it has been published that TGF-β signaling upregulates miR21. Upregulation of miR-21 was also of great interest as miR-21 upregulation has been strongly correlated with the development of a variety of autoimmune diseases including T cell-derived psoriatic skin inflammation [26], SLE [27] and autoimmune type 1 diabetes (T1D) [28]. To understand how miR21 could be upregulated in the absence of TGF-β signaling, subsequent studies were focused on the analysis of the role of miR-21.

Levels of miR-21 expression in T cells of dnTGFβRII mice

To confirm the pattern of expression, we repeated microRNA screening using an individual miRNA PCR assay to directly compare the relative levels of miR-21 expression in total CD4⁺ and CD8⁺ T cells isolated from the spleen, liver and colon from 9-week old dnTGFβRII mice and age matched B6 mice. In spleen, liver, and colon, the relative levels of miR-21 were all significantly higher in dnTGFβRII than the B6 mice (Figure 3). Next we compared the relative miR-21 levels in purified naïve and effector CD4⁺ and CD8⁺ T cells between the two mouse strains. As shown in Fig. 4A, both effector CD4⁺ and CD8⁺ T cells expressed higher miR-21 than naïve cells. Again, miR-21 was expressed significantly higher in dnTGFβRII effector T cells compared to B6 effector T cells in all tissues that we tested. Of note, the relative miR-21 level of effector CD8⁺ T cells in liver was significantly higher than the spleen and mLNs (Fig. 4B). These PCR results therefore confirmed our paradoxical result that dnTGFβRII T cells show upregulated miR21 in the face of global miRNA downregulation in the previous PCR array.

miR-21 is increased at both early and late time points in dnTGFβRII disease

To begin to assess whether the elevated miR-21 level in the dnTGFβRII mice was a secondary effect of overall autoimmune disease in the dnTGFβRII mice, or an intrinsic characteristic unique to the abrogation of TGF-β signaling, we measured the miR-21 levels at the age of 2-weeks (before the onset of symptoms or significant pathology in the dnTGFβRII mice). As shown in Fig. 5A, the relative levels of miR-21 in both CD4⁺ and CD8⁺ T cells isolated from the thymus were significantly higher in the 2-week old dnTGFβRII mice than the 2-week old B6 mice. Next we examined if there is any difference in miR-21 levels before (2-week old mice) and after (9-week old mice) disease onset. The relative levels of miR-21 in splenic CD4⁺ and CD8⁺ T cells increased in both dnTGFβRII mice and B6 mice at 9 weeks compared to 2 weeks old. Further, at both ages, miR-21 levels were significantly higher in the dnTGFβRII mice than that of B6 mice (Fig. 5B). These results suggest that the elevated miR-21 levels in T cells of the dnTGFβRII mice start at an early age but are enhanced by the immune activation of T cells associated with the progression of disease.

Transfection of miR-21 into normal B6 T cells enhanced production of pro-inflammatory cytokines

To investigate the effects of elevated miR-21 on T cell function, we isolated CD4⁺ and CD8⁺ T cells from spleen of B6 mice and transfected them with a miR-21 mimic followed by stimulation with anti-CD3/CD28 mAb. After transfection, the cellular level of miR-21 expression was significantly increased in both CD4⁺ and CD8⁺ T cells transfected with miR-21 mimics as compared to that of control group (Fig. 6A). We next measured the pro-inflammatory cytokine production by these transfected cells. As shown in Fig. 6B, the amounts of IFN-γ and TNF-α in the supernatant of cells were significantly increased in miR-21 transfected group as compared with that of control group.

Discussion

Since it is well established that TGF-β signaling upregulates miRNA expression via multiple mechanisms [13–15], we investigated the levels of miRNA in dnTGFβRII with the hypothesis that we would see global miRNA downregulation which could contribute to the intrinsic pathogenicity of T cells from the dnTGFβRII mice. In fact, we did find overall miRNA down regulation in CD4⁺ and CD8⁺ T cells from the dnTGFβRII mice (Fig. 2). However, somewhat surprisingly, we also found an up-regulation of a small subset of miRNAs (Fig 2). Most interesting to us was the up-regulation of miR-21 in both CD4⁺ and CD8⁺ subsets. A fascinating parallel to this result is found in cancer biology, where it has been shown that while most miRNAs are downregulated, miR-21 is up-regulated [15]. Similar up-regulation of miR-21 has been found in other autoimmune conditions. Human IBD patients have colonic overexpression of miR-21 [29, 30] similar to the colonic inflammation in IL-10^{-/-} mice, an animal model of Th1-mediated IBD which is also accompanied by the up-regulation of miR-21 [31]. Since the elevated level of miR-21 is observed before the onset of clinical pathology, the miR-21 overexpression is likely to be a consequence of disrupted TGF-β signaling which is further increased by subsequent inflammation. We also demonstrate by *in vitro* transfection experiments that the experimental overexpression of miR-21 in the T cells derived from control mice results in enhanced production of pro-inflammatory cytokines.

Adoptive transfer of CD8⁺ T cells from dnTGFβRII into B6/Rag1^{-/-} mice leads to liver histopathology. In contrast, adoptive transfer of CD4⁺ T cells predominantly induces IBD in recipient mice [2]. It is of interest to note that whereas there is a global down regulation of miRNA in all sources of CD8⁺ T cells from the dnTGFβRII mice, there was a strikingly

higher level of miR-21 in intrahepatic effector CD8⁺ T cells as compared to that of spleen and mLN (Fig. 5B). These findings prompted us to perform miR-21 transfection experiments in efforts to specifically study the role of miR-21 expression. As shown above, the overexpression of miR-21 in normal mouse T cells resulted in an increased production of the pro-inflammatory cytokines IFN- γ and TNF- α upon TCR stimulation. These findings indicate that miR-21 might be a target of clinical treatment for inflammation in chronic autoimmune disease. IFN- γ secretion was higher in transfected CD8⁺ T cells than in CD4⁺ T cells; TNF- α secretion was higher in transfected CD4⁺ T cells than in CD8⁺ T cells (Fig. 6B). These findings indicate a differential role of the two cytokines in the pathogenesis of the two major clinical manifestations of disease.

The miR-21 promoter regions have binding sites for several transcriptional factors such as AP-1, STAT-3, MyD88, and NF- κ B [32–35]. Aberrant activation of NF- κ B has been linked to inflammatory and autoimmune disorders [36–39]. Given that TGF- β signal negatively regulates NF- κ B transcriptional activity [40–42], abrogation of TGF- β signaling in dnTGF β RII mice might cause an increase of NF- κ B activation, which induced miR-21 overexpression, independent of the global decrease in miRNA that we demonstrate. The combined effect of global decrease in miRNA induced regulation, plus specific up-regulation of a subset of deleterious miRNA such as miR21, could thus result in a profound increase in intrinsic T cell mediated immunity.

miR21 directly down regulates the expression of PDCD4 that encodes a protein that localizes to the nucleus in proliferating cells. The gene product of PDCD4 plays a role in pathogenic T cell apoptosis and cell proliferation [43–45]. miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression in human SLE [46], which suggest that induction of miR-21 may enable T cells to elude activated T cell apoptosis and enhance the potential of pro-inflammatory cytokine secretion, such as IFN- γ and IL-17, by its ability to repress the expression of PDCD4 [47].

miR-21 expression was highest in effector T cells and lowest in naive T cells suggesting that miR-21 may play an important role in maintaining effector phase of the T cells, which is consistent with a previous report [48]. However, the exact pathway linking miR-21 and the increased synthesis of IFN- γ and TNF- α (i.e. whether it is a direct gene effect or a downstream effect of increased cell cycling) remains unknown. There appears to be at least two major ways that miR-21 can be up-regulated in our dnTGF β RII mice (Fig. 7). First, miR 21 up-regulation could be a result of dysregulated gene expression normally controlled by TGF β (pathway on the right). In this scenario, loss of gene regulation leads to a decreased in SMAD factors (eg SMAD-3) which drives the inflammation leading to activation of pathways such as NF κ B and MAPK. The up-regulation of these pathways in turn has been shown to up-regulate mir21 directly. Thus, mir21 up-regulation can, via a positive feedback loop, up-regulate inflammation. In the second pathway (on the left), global down regulation of miR expression (as we demonstrate in figure two) leads to an up-regulation of multiple genes causing inflammation; this in turn could lead to the up-regulation of miR-21 with feedback up-regulation of inflammation. The two pathways may both be operative. Clarifying which pathway is primary in dnTGF β RII mice will be critical to our understanding of the pathogenesis of autoimmunity.

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Abbreviations

PBC	Primary biliary cirrhosis
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Crohn's disease
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
dnTGFβRII	Dominant negative form of transforming growth factor beta receptor type II
MNCs	Mononuclear cells
miRNA	microRNA
Ct	Threshold cycle
FC	Fold changes

References

1. Gorelik L, Flavell RA. Abrogation of TGF β signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity*. 2000; 12:171–181. [PubMed: 10714683]
2. Yang GX, Lian ZX, Chuang YH, Moritoki Y, Lan RY, Wakabayashi K, et al. Adoptive transfer of CD8(+) T cells from transforming growth factor beta receptor type II (dominant negative form) induces autoimmune cholangitis in mice. *Hepatology*. 2008; 47:1974–1982. [PubMed: 18452147]
3. Zhang W, Tsuda M, Yang GX, Tsuneyama K, Rong G, Ridgway WM, et al. Deletion of interleukin-6 in mice with the dominant negative form of transforming growth factor beta receptor II improves colitis but exacerbates autoimmune cholangitis. *Hepatology*. 2010; 52:215–222. [PubMed: 20578264]
4. Ando Y, Yang GX, Tsuda M, Kawata K, Zhang W, Nakajima T, et al. The immunobiology of colitis and cholangitis in IL-23p19 and IL-17A deleted dnTGF β RII mice. *Hepatology*. 2012
5. Tsuda M, Zhang W, Yang GX, Tsuneyama K, Ando Y, Kawata K, et al. Deletion of IL-12p35 induces liver fibrosis in dominant negative transforming growth factor beta receptor type II mice. *Hepatology*. 2012
6. Nelson P, Kiriakidou M, Sharma A, Maniataki E, Mourelatos Z. The microRNA world: small is mighty. *Trends Biochem Sci*. 2003; 28:534–540. [PubMed: 14559182]
7. Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. *Cell*. 2009; 136:642–655. [PubMed: 19239886]
8. O'Hara SP, Mott JL, Splinter PL, Gores GJ, LaRusso NF. MicroRNAs: key modulators of posttranscriptional gene expression. *Gastroenterology*. 2009; 136:17–25. [PubMed: 19049808]
9. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun*. 2009; 32:189–194. [PubMed: 19303254]
10. Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, et al. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum*. 2008; 58:1284–1292. [PubMed: 18438844]
11. Stanczyk J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, et al. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum*. 2008; 58:1001–1009. [PubMed: 18383392]
12. Dai Y, Huang YS, Tang M, Lv TY, Hu CX, Tan YH, et al. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus*. 2007; 16:939–946. [PubMed: 18042587]

13. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature*. 2008; 454:56–61. [PubMed: 18548003]
14. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. *Mol Cell*. 2010; 39:373–384. [PubMed: 20705240]
15. Hata A, Davis BN. Control of microRNA biogenesis by TGFbeta signaling pathway-A novel role of Smads in the nucleus. *Cytokine Growth Factor Rev*. 2009; 20:517–521. [PubMed: 19892582]
16. Oertelt S, Lian ZX, Cheng CM, Chuang YH, Padgett KA, He XS, et al. Anti-mitochondrial antibodies and primary biliary cirrhosis in TGF-beta receptor II dominant-negative mice. *J Immunol*. 2006; 177:1655–1660. [PubMed: 16849474]
17. Yoshida K, Yang GX, Zhang W, Tsuda M, Tsuneyama K, Moritoki Y, et al. Deletion of interleukin-12p40 suppresses autoimmune cholangitis in dominant negative transforming growth factor beta receptor type II mice. *Hepatology*. 2009; 50:1494–1500. [PubMed: 19676134]
18. Yang GX, Lian ZX, Kikuchi K, Liu YJ, Ansari AA, Ikehara S, et al. CD4-plasmacytoid dendritic cells (pDCs) migrate in lymph nodes by CpG inoculation and represent a potent functional subset of pDCs. *J Immunol*. 2005; 174:3197–3203. [PubMed: 15749849]
19. Lian ZX, Okada T, He XS, Kita H, Liu YJ, Ansari AA, et al. Heterogeneity of dendritic cells in the mouse liver: identification and characterization of four distinct populations. *J Immunol*. 2003; 170:2323–2330. [PubMed: 12594254]
20. Moritoki Y, Zhang W, Tsuneyama K, Yoshida K, Wakabayashi K, Yang GX, et al. B cells suppress the inflammatory response in a mouse model of primary biliary cirrhosis. *Gastroenterology*. 2009; 136:1037–1047. [PubMed: 19118554]
21. Yang GX, Wu Y, Tsukamoto H, Leung PS, Lian ZX, Rainbow DB, et al. CD8 T cells mediate direct biliary ductule damage in nonobese diabetic autoimmune biliary disease. *J Immunol*. 2011; 186:1259–1267. [PubMed: 21169553]
22. Ng R, Song G, Roll GR, Frandsen NM, Willenbring H. A microRNA-21 surge facilitates rapid cyclin D1 translation and cell cycle progression in mouse liver regeneration. *J Clin Invest*. 2012; 122:1097–1108. [PubMed: 22326957]
23. Garbacki N, Di Valentin E, Huynh-Thu VA, Geurts P, Irrthum A, Crahay C, et al. MicroRNAs profiling in murine models of acute and chronic asthma: a relationship with mRNAs targets. *PLoS One*. 2011; 6:e16509. [PubMed: 21305051]
24. Olson TS, Bamias G, Naganuma M, Rivera-Nieves J, Burcin TL, Ross W, et al. Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease. *J Clin Invest*. 2004; 114:389–398. [PubMed: 15286805]
25. Kim G, Levin M, Schoenberger SP, Sharpe A, Kronenberg M. Paradoxical effect of reduced costimulation in T cell-mediated colitis. *J Immunol*. 2007; 178:5563–5570. [PubMed: 17442938]
26. Lovendorf MB, Zibert JR, Hagedorn PH, Glue C, Odum N, Ropke MA, et al. Comparison of microRNA expression using different preservation methods of matched psoriatic skin samples. *Exp Dermatol*. 2012; 21:299–301. [PubMed: 22417307]
27. Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, et al. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. *J Immunol*. 2010; 184:6773–6781. [PubMed: 20483747]
28. Ruan Q, Wang T, Kameswaran V, Wei Q, Johnson DS, Matschinsky F, et al. The microRNA-21-PDCD4 axis prevents type 1 diabetes by blocking pancreatic beta cell death. *Proc Natl Acad Sci U S A*. 2011; 108:12030–12035. [PubMed: 21730150]
29. Wu F, Zikusoka M, Trindade A, Dassopoulos T, Harris ML, Bayless TM, et al. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha. *Gastroenterology*. 2008; 135:1624–1635. e24. [PubMed: 18835392]
30. Wu F, Zhang S, Dassopoulos T, Harris ML, Bayless TM, Meltzer SJ, et al. Identification of microRNAs associated with ileal and colonic Crohn's disease. *Inflamm Bowel Dis*. 2010; 16:1729–1738. [PubMed: 20848482]
31. Schaefer JS, Montufar-Solis D, Vigneswaran N, Klein JR. Selective upregulation of microRNA expression in peripheral blood leukocytes in IL-10–/– mice precedes expression in the colon. *J Immunol*. 2011; 187:5834–5841. [PubMed: 22043014]

32. Fujita S, Ito T, Mizutani T, Minoguchi S, Yamamichi N, Sakurai K, et al. miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. *J Mol Biol.* 2008; 378:492–504. [PubMed: 18384814]
33. Niu J, Shi Y, Tan G, Yang CH, Fan M, Pfeffer LM, et al. DNA damage induces NF-kappaB-dependent microRNA-21 upregulation and promotes breast cancer cell invasion. *J Biol Chem.* 2012
34. Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, et al. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nat Immunol.* 2010; 11:141–147. [PubMed: 19946272]
35. Bourguignon LY, Earle C, Wong G, Spevak CC, Krueger K. Stem cell marker (Nanog) and Stat-3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells. *Oncogene.* 2012; 31:149–160. [PubMed: 21685938]
36. Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clin Chem.* 1999; 45:7–17. [PubMed: 9895331]
37. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol.* 1996; 14:649–683. [PubMed: 8717528]
38. Finco TS, Baldwin AS. Mechanistic aspects of NF-kappa B regulation: the emerging role of phosphorylation and proteolysis. *Immunity.* 1995; 3:263–272. [PubMed: 7552992]
39. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med.* 1997; 336:1066–1071. [PubMed: 9091804]
40. Smythies LE, Shen R, Bimczok D, Novak L, Clements RH, Eckhoff DE, et al. Inflammation anergy in human intestinal macrophages is due to Smad-induced IkappaBalpha expression and NF-kappaB inactivation. *J Biol Chem.* 2010; 285:19593–19604. [PubMed: 20388715]
41. Haller D, Holt L, Kim SC, Schwabe RF, Sartor RB, Jobin C. Transforming growth factor-beta 1 inhibits non-pathogenic Gram negative bacteria-induced NF-kappa B recruitment to the interleukin-6 gene promoter in intestinal epithelial cells through modulation of histone acetylation. *J Biol Chem.* 2003; 278:23851–23860. [PubMed: 12672795]
42. You HJ, How T, Blobe GC. The type III transforming growth factor-beta receptor negatively regulates nuclear factor kappa B signaling through its interaction with beta-arrestin2. *Carcinogenesis.* 2009; 30:1281–1287. [PubMed: 19325136]
43. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdc4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene.* 2008; 27:2128–2136. [PubMed: 17968323]
44. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res.* 2008; 18:350–359. [PubMed: 18270520]
45. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem.* 2008; 283:1026–1033. [PubMed: 17991735]
46. Stagakis E, Bertsias G, Verginis P, Nakou M, Hatziaepostolou M, Kritikos H, et al. Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression. *Ann Rheum Dis.* 2011; 70:1496–1506. [PubMed: 21602271]
47. Iliopoulos D, Kavousanaki M, Ioannou M, Boumpas D, Verginis P. The negative costimulatory molecule PD-1 modulates the balance between immunity and tolerance via miR-21. *Eur J Immunol.* 2011; 41:1754–1763. [PubMed: 21469086]
48. Wu H, Neilson JR, Kumar P, Manocha M, Shankar P, Sharp PA, et al. miRNA profiling of naive, effector and memory CD8 T cells. *PLoS One.* 2007; 2:e1020. [PubMed: 17925868]

Figure 1.

Inflammatory changes in 9-week-old dnTGFβRII mice. A. Representative H&E staining of liver and colon sections. Note the presence of infiltrating lymphoid cells around the portal area, especially surrounding the bile duct. Lymphoid cells were diffuse infiltrating in colon. B. Comparison of proinflammatory cytokine levels in serum, liver and colon between dnTGFβRII mice (n=6) and B6 mice (n=6) at 9 weeks of age. C. Compare cell numbers of intrahepatic MNCs, CD4⁺ T cells, and CD8⁺ T cells in dnTGFβRII mice (n=6) with B6 mice (n=6) at 9 weeks of age. D. Analysis of MNC number isolated from the mLN of dnTGFβRII mice (n=7) as well as B6 mice (n=7) at 9 weeks of age. *, P < 0.05; **, P < 0.01; ***, P < 0.001; determined using two-tailed unpaired Mann-Whitney test. Horizontal bars represent standard deviation.

Figure 2. miRNAs are predominantly down-regulated in dnTGFβRII splenic CD4⁺ and CD8⁺ T cells but miR-21 is upregulated. A. 320 and 285 out of 376 miRNAs in splenic CD4⁺ and CD8⁺ T cells, respectively, passed the initial Ct cutoff (see methods). The FC of these miRNAs in dnTGFβRII compared to B6 cells was calculated using the delta delta Ct method and log₂FC was plotted for each of 320 miRNAs in CD4⁺ T cells and 285 miRNAs in CD8⁺ T cells. In CD4⁺ T cells, 86 miRNA have more than 2-fold decrease in dnTGFβRII CD4⁺ T cells with miR-300 expressed at the lowest FC, and 28 miRNAs have more than 2-fold increase in dnTGFβRII CD4⁺ T cells compared to B6 CD4⁺ T cells. In CD8⁺ T cells, 142 miRNAs have more than 2-fold decrease in dnTGFβRII CD8⁺ T cells compared to B6 CD8⁺ T cells,

while 19 miRNAs have more than 2-fold increase in dnTGF β RII CD8⁺ T cells compared to B6 CD8⁺ T cells. B. Venn diagram for differentially regulated miRNAs with fold change greater than 2 in dnTGF β RII CD4 and CD8 splenic T cells, generated using DIANA mirPath software C. A Venn diagram is illustrated for pathways in which differentially regulated miRNAs may be involved. Blue ellipse: pathways affected by down-regulated miRNAs in dnTGF β RII CD4 T cells. Yellow ellipse: pathways affected by down-regulated miRNAs in dnTGF β RII CD8 T cells. Green ellipse: pathways affected by up-regulated miRNAs in dnTGF β RII CD4 T cells. Red ellipse: pathways affected by up-regulated miRNAs in dnTGF β RII CD8 T cells. All venn diagrams are generated by web-based Venn diagram software Venny (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

Figure 3.

Relative expression levels of miR-21 in spleen, liver, and colon of dnTGFβRII mice (n=6) and B6 mice (n=6), Horizontal bars represent standard deviation. **, P < 0.01; ***, P < 0.001; determined using two-tailed unpaired Mann-Whitney test.

Figure 4.

Relative expression of miR-21 in naïve and effector T cells. A. naïve and effector CD4⁺ and CD8⁺ T cells were isolated from spleen, liver, and mLN by MoFlo cell sorter. Real-time PCR were performed for miR-21 expression. B. miR-21 expression levels in each organ (liver, spleen, mLN) of dnTGFβRII mice (n=6) were compared. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, determined using two-tailed unpaired Mann-Whitney test.

Figure 5.

Age related miR-21 expression in CD4⁺ and CD8⁺ T cells. A. Relative expression of miR-21 in thymic CD4⁺ and CD8⁺ T of 2 weeks old dnTGFβRII mice and B6 mice. B. miR-21 expression in splenic CD4⁺ and CD8⁺ T cells of 2 and 9 weeks old dnTGFβRII mice was compared with age-matched B6 mice.

Figure 6.

Transfection of miR-21 mimic into B6 CD4⁺ and CD8⁺ T cells. A. CD4⁺ and CD8⁺ T cells isolated from spleen of B6 mice were transfected with 25nM miR-21 mimics, negative control siRNA, or with TransIT-TKO reagent only. After overnight transfection, the levels of miR-21 expression were measured by real-time PCR. B. Transfected CD4⁺ and CD8⁺ T cells were stimulated with 0.5ng/ml anti-CD3/CD28 for 48 hours, cytokines in supernatant were measured by a mouse Th1/Th2/Th17 CBA kit. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, determined using two-tailed unpaired Mann-Whitney test.

Figure 7.
Proposed model for upregulation of miR-21 in dnTGFβRII T cells.

Table 1

	Pathways in which downregulated miRs may be involved		Pathways in which upregulated miRs may be involved	
	CD4	CD8	CD4	CD8
1	Adherens junction	Axon guidance	Adherens junction	Adherens junction
2	Axon guidance	Focal adhesion	Axon guidance	Glioma
3	Wnt signaling pathway	MAPK signaling pathway	Chronic myeloid leukemia	Regulation of actin cytoskeleton
4	Focal adhesion	Wnt signaling pathway	ErbB signaling pathway	MAPK signaling pathway
5	Colorectal cancer	Renal cell carcinoma	Glioma	Non-small cell lung cancer
6	Regulation of actin cytoskeleton	Adherens junction	Focal adhesion	Long-term depression
7	MAPK signaling pathway	Ribosome	Renal cell carcinoma	Chronic myeloid leukemia
8	Ribosome	Regulation of actin cytoskeleton	Non-small cell lung cancer	Colorectal cancer
9	TGF-beta signaling pathway	Metabolism of xenobiotics by	Colorectal cancer	GnRH signaling pathway
10	Chronic myeloid leukemia	cytochrome P450	MAPK signaling pathway	Long-term potentiation
11	Glioma	Linoleic acid metabolism	TGF-beta signaling pathway	TGF-beta signaling pathway
12	Renal cell carcinoma	Colorectal cancer	Wnt signaling pathway	Pancreatic cancer
13	ErbB signaling pathway	TGF-beta signaling pathway	Dorso-ventral axis formation	Focal adhesion
14	Prostate cancer	ErbB signaling pathway	Small cell lung cancer	Axon guidance
15	Metabolism of xenobiotics by	Long-term potentiation	Pancreatic cancer	Melanogenesis
16	cytochrome P450	Chronic myeloid leukemia	Regulation of actin	Renal cell carcinoma
17	Long-term potentiation	Arachidonic acid metabolism	cytoskeleton	Phosphatidylinositol signaling
18	Insulin signaling pathway	Autoimmune thyroid disease	ECM-receptor interaction	system
19	Non-small cell lung cancer	Prostate cancer	Acute myeloid leukemia	Insulin signaling pathway
20	Autoimmune thyroid disease	Glioma	Endometrial cancer	Prostate cancer
	Arachidonic acid metabolism	Melanogenesis	mTOR signaling pathway	ErbB signaling pathway