MicroRNA regulate immunological pathways in T-cells in immune thrombocytopenia (ITP)

Margareta Jernås,[1] Intawat Nookaew,[2] Hans Wadenvik,[1] and Bob Olsson[3]

[1]Department of Internal Medicine and [3]Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden, [2]Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden.

Running head: MicroRNA in ITP

Abstract count: 148

Word count: 1172

Tables: 1

Figures: 1

Corresponding author:

Margareta Jernås, Ph.D., Department of Internal Medicine, Institute of Medicine, Sahlgrenska Academy at University of Gothenburg, Vita Stråket 12, SE-413 45 Gothenburg, Sweden; E-mail: margareta.jernas@medic.gu.se, Phone no.: +46 31 3429433.

Key words

Autoimmunity, platelets, T-cell, cytotoxicity, microarray, microRNA

Key Point

MicroRNA and plasma levels of the target gene CXCL13 differ between ITP and controls indicating that microRNA may be important in ITP.

Abstract

MicroRNA are small non-coding RNA molecules that regulate gene expression. To investigate the role of microRNA in ITP, we performed genome-wide expression analyses of mRNA and microRNA in T-cells from ITP patients and controls. We identified 1,915 regulated genes and 22 regulated microRNA that differed between ITP patients and controls. Seventeen of the 22 regulated microRNA were linked to changes in target gene expression; 57 of these target genes were associated with the immune system, e.g. T-cell activation and regulation of immunoglobulin production. CXCL13 and IL-21 were two microRNA target genes significantly increased in ITP. We could demonstrate increased plasma levels of CXCL13 and others have reported increased plasma levels of IL-21 in ITP. Thus, regulated microRNA were significantly associated with both gene and protein expression of molecules in immunological pathways. We suggest that microRNA may be important regulatory molecules involved in the loss of tolerance in ITP.

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by low platelet count and increased bleeding tendency.[1] The pathophysiology of ITP is more complex than initially believed and includes both antibody-mediated and T-cell mediated platelet and/or megakaryocyte destruction.[2-4] An insufficient thrombopoietin production in ITP also contributes to the thrombocytopenia.[5]

MicroRNA are short (19-25 nucleotides) evolutionary conserved single stranded RNA molecules that regulate the expression of genes involved in diverse biological processes. The effect of microRNA on mRNA is mediated through the binding of the microRNA to the ribonucleoprotein complex RNA-induced silencing complex (miRISC) which in addition also bind to the 3´untranslated region (UTR) of complementary mRNAs.[6] The double stranded complex between the microRNA and mRNA are then degraded which leads to decreased protein translation.[7] Approximately 30% of the human genome is estimated to be regulated by microRNA and a single microRNA can potentially regulate hundreds of protein.[8,9] More than 1,000 microRNA have been identified in mammals and they have been implicated in a wide range of biological functions[10,11] and contribute to the pathophysiology of a number of important human diseases such as cancer,[12-15] cardiac and neurodegenerative diseases, diabetes, inflammation and diseases of the immune system.[16] However, the *in vivo* function of most microRNA is mainly unknown. This is the first report on microRNA as potential regulators of T-cell gene expression in ITP patients.

Methods

Patient characteristics and detailed methods are given in the Supplemental Methods.

In brief, T-cells isolation and extraction of RNA was performed as previously described.[2] Twenty ng RNA was reverse transcribed, amplified and labeled using the Ovation amplification system V2 (NuGEN Technologies Inc, San Carlos, CA) and the corresponding cDNA was fragmented and biotinylated using the Encore biotin module (NuGEN) and hybridized to Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. For the microRNA analysis 1000 ng T-cell RNA from each individual was biotin labeled using the FlashTag Biotin HSR kit (Genisphere, Hatfield, PA) according to the manufacturer's instructions and hybridized to microRNA 2.0 arrays (Affymetrix).

The DNA and microRNA microarrays were normalized using RMA and PLIER algorithms and significantly regulated genes and microRNA were essentially detected using Student's t-test (see supplemental information). To identify the global biological processes that differed between patients and controls a reporter algorithm was applied to the Gene Ontology (GO) network resulting in an enrichment score.[17] GO terms that had enrichment *P*-values <0.001, using the R software, were considered and selected in the construction of a heatmap (Supplementary Figure 1).

The mirBase (http://www.mirbase.org) was used to identify microRNA functions and microRNA target mRNA using TargetScan and Miranda algorithms. To achieve high confidence microRNA-mRNA associations and to evaluate the impact of each microRNA on the gene expression, the predicted target genes of each microRNA were identified and combined with the mRNA transcriptome from ITP patients and controls in an analysis using the Kolmogorov-Smirnov test (Table 1). The target genes from the microRNA identified as significant ($P<0.05$) in the Kolmogorov-Smirnov analysis were cross referenced against the list of significantly regulated mRNA between ITP patients and controls identified in the T-cell gene expression analysis. The resulting immune genes according to GO were classified

further according to functional enrichment based on Immune System Gene Ontology[18] by modular enrichment analysis (Supplemental Table 3 and Supplemental Figures 2 and 3).

Results and Discussion

Autoimmune diseases consist of more than 80 variable and serious illnesses that collectively affect more than 5% of the population, often with debilitating effects.[19] Loss of tolerance, where the immune system is misdirected and attacks organs or cells instead of protecting them, is the common denominator in these diseases. In order to better understand the genes and mechanisms involved in the organ specific autoimmune disease ITP we studied gene and microRNA expression in T-cells from chronic ITP patients and healthy controls.

Initially, we identified 1915 significantly regulated genes in peripheral blood T-cells between ITP patients and controls by DNA microarray analysis (P<0.05). The Gene Ontology (GO) project is a collaborative effort to address the need for consistent descriptions of gene products in different databases. One of these annotations is biological process which is defined as series of events accomplished by one or more ordered assemblies of molecular functions. Therefore, the significantly regulated genes were classified according to biological process in GO which demonstrated that ITP was associated with several significantly enriched biological processes involved in the immune system (Supplemental Figure 1). In the next step, we compared differences in expression of microRNA in peripheral blood T-cells and found that 22 microRNA differed significantly between ITP patients and controls (P<0.05). In addition, 16 small nucleolar RNA such as snoRNA and scaRNA also differed between ITP patients and controls (P<0.05; Supplementary Table 2).

Firstly, to better understand the role of the regulated microRNA in ITP patients we identified the predicted target genes of the significantly regulated microRNA using TargetScan and

Miranda algorithms. Secondly, to investigate the impact of the 22 significantly changed microRNA on gene expression we performed Kolmogorov-Smirnov test using the target genes identified in TargetScan and Miranda on all transcripts in the gene expression dataset. This resulted in 17 microRNA that were significantly associated with the expression of target genes (Table 1). Thirdly, the identified target genes, of the 17 significantly changed microRNA, were cross referenced against the significantly regulated mRNA that differed between ITP patients and controls resulting in 991 genes. Fourthly, the cross referenced genes were classified according to function by GO and the 57 genes classified as being involved in the immune system (Figure 1A) were analyzed using functional module enrichment based on Immune System Gene Ontology.[18] This resulted in 7 modules that were enriched with the following functions: T-cell activation involved in immune response, natural killer cell differentiation, regulation of immunoglobulin production, positive regulation of leukocyte activation, lymphocyte activation involved in immune response, lymphocyte differentiation, and lymphocyte costimulation (Supplemental Figures 2A-B). Regulation of immunoglobulin production is in agreement with one of the known mechanisms behind ITP, namely production of platelet autoantibodies seen in approximately 50-60% of all chronic ITP patients.[20] The other enriched processes such as T-cell activation involved in immune response, positive regulation of leukocyte activation, and lymphocyte co-stimulation further highlight the importance of T-cells in this disease. This supports previous findings such as proliferation of T-cells and production of cytokines in response to stimulation with whole platelets or fragments of GPIIb/IIIa and GPIIIa in ITP,[21-25] and that cytototoxic T-cells can lyse platelets in patients with ITP.[2,26-28] That both B-cell and T-cell mechanisms are important pathophysiologic mechanisms in ITP has also been shown in an elegant animal model of ITP.[26]

To test whether these changes in microRNA expression were accompanied by changes of corresponding proteins we determined plasma levels of CXCL13 in ITP patients and controls. The plasma level of this protein was found to be significantly increased in ITP patients compared with controls (Fig 1B). Our data also suggested that IL-21 was a target of the regulated microRNA and that IL-21 expression would be increased in patients with ITP. Indeed, increased plasma levels of IL-21 in ITP patients compared with controls has previously been demonstrated by Zhu et al, which supports our present data.[29]

In conclusion, regulated microRNA in ITP significantly affect both gene and protein expression in T-cells indicating that they may be important regulatory molecules involved in the loss of immune tolerance in ITP.

Acknowledgement

Work in the authors' laboratory is supported by grants from the Swedish Research Council (K2009-65X-15424-05-3, K2011-X-20401-05-6), the Swedish federal government under the LUA/ALF agreement, the Foundations of the National Board of Health and Welfare, Torsten and Ragnar Söderberg Foundation, Clas Groschinsky Foundation, the Arosenius Foundation, Åke Wiberg Foundation, Jeansson Foundation, Tore Nilsson Foundation for Medical Research, Magnus Bergvall Foundation, Wilhelm and Martina Lundgren Science Foundation. the Knut and Alice Wallenberg Foundation, Chalmers foundation.

Authorship Contribution

Dr. Jernås designed and coordinated the study, performed all laboratory work, analyzed data and wrote the paper. Dr Nookaew analyzed and interpreted data and wrote the paper. Dr Wadenvik collected the patient material, interpreted the data and wrote the paper. Dr Olsson designed the study, analyzed and interpreted data and wrote the paper.

Disclosure of Conflicts of Interest

None of the authors report any disclosures.

References

1. Cines DB, Blanchette VS. Immune thrombocytopenic purpura. *N Engl J Med*. 2002;346(13):995-1008.
2. Olsson B, Andersson PO, Jernas M, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat Med*. 2003;9(9):1123-4.
3. Harrington WJ, Minnich V, Hollingsworth JW, Moore CV. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med*. 1951;38(1):1-10.
4. Semple JW, Provan D, Garvey MB, Freedman J. Recent progress in understanding the pathogenesis of immune thrombocytopenia. *Curr Opin Hematol*. 2010;17(6):590-5.
5. Ballem PJ, Segal GM, Stratton JR, et al. Mechanisms of thrombocytopenia in chronic autoimmune thrombocytopenic purpura. Evidence of both impaired platelet production and increased platelet clearance. *J Clin Invest*. 1987;80(1):33-40.
6. Schwarz DS, Hutvagner G, Du T, et al. Asymmetry in the assembly of the RNAi enzyme complex. *Cell*. 2003;115(2):199-208.
7. Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. *Nature*. 2004;431(7006):343-9.
8. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005;120(1):15-20.
9. Grimson A, Farh KK, Johnston WK, et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*. 2007;27(1):91-105.
10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-97.
11. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006;34(Database issue):D140-4.
12. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A*. 2005;102(39):13944-9.
13. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005;353(17):1793-801.
14. Meng F, Henson R, Wehbe-Janek H, et al. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*. 2007;133(2):647-58.
15. Talotta F, Cimmino A, Matarazzo MR, et al. An autoregulatory loop mediated by miR-21 and PDCD4 controls the AP-1 activity in RAS transformation. *Oncogene*. 2009;28(1):73-84.
16. Tsai LM, Yu D. MicroRNAs in common diseases and potential therapeutic applications. *Clin Exp Pharmacol Physiol*. 2010;37(1):102-7.
17. Patil KR, Nielsen J. Uncovering transcriptional regulation of metabolism by using metabolic network topology. *Proc Natl Acad Sci U S A*. 2005;102(8):2685-9.
18. Diehl AD, Lee JA, Scheuermann RH, Blake JA. Ontology development for biological systems: immunology. *Bioinformatics*. 2007;23(7):913-5.

19. Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol*. 1997;84(3):223-43.
20. McMillan R, Wang L, Tani P. Prospective evaluation of the immunobead assay for the diagnosis of adult chronic immune thrombocytopenic purpura (ITP). *J Thromb Haemost*. 2003;1(3):485-91.
21. Semple JW, Freedman J. Increased antiplatelet T helper lymphocyte reactivity in patients with autoimmune thrombocytopenia. *Blood*. 1991;78(10):2619-25.
22. Semple JW, Milev Y, Cosgrave D, et al. Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity. *Blood*. 1996;87(10):4245-54.
23. Kuwana M, Kaburaki J, Ikeda Y. Autoreactive T cells to platelet GPIIb-IIIa in immune thrombocytopenic purpura. Role in production of anti-platelet autoantibody. *J Clin Invest*. 1998;102(7):1393-402.
24. Kuwana M, Kaburaki J, Kitasato H, et al. Immunodominant epitopes on glycoprotein IIb-IIIa recognized by autoreactive T cells in patients with immune thrombocytopenic purpura. *Blood*. 2001;98(1):130-9.
25. Sukati H, Watson HG, Urbaniak SJ, Barker RN. Mapping helper T-cell epitopes on platelet membrane glycoprotein IIIa in chronic autoimmune thrombocytopenic purpura. *Blood*. 2007;109(10):4528-38.
26. Chow L, Aslam R, Speck ER, et al. A murine model of severe immune thrombocytopenia is induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy. *Blood*. 2010;115(6):1247-53.
27. Zhang F, Chu X, Wang L, et al. Cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura. *Eur J Haematol*. 2006;76(5):427-31.
28. Zhao C, Li X, Zhang F, et al. Increased cytotoxic T-lymphocyte-mediated cytotoxicity predominant in patients with idiopathic thrombocytopenic purpura without platelet autoantibodies. *Haematologica*. 2008;93(9):1428-30.
29. Zhu X, Ma D, Zhang J, et al. Elevated interleukin-21 correlated to Th17 and Th1 cells in patients with immune thrombocytopenia. *J Clin Immunol*. 2010;30(2):253-9.

Table 1. Identification of microRNA that significantly affected the expression of the corresponding target genes in a combined analysis of ITP patients and controls. There were 22 microRNA that significantly differed between ITP patients and controls. To evaluate if these 22 microRNA were associated with changes in the mRNA expression of the target genes the TargetScan and Miranda algorithms were used and Kolmogorov-Smirnov statistics applied. This resulted in 17 microRNA that were significantly associated with mRNA expression.

microRNA	Targetscan	Miranda
miR-877	1.5×10^{-27}	5.3×10^{-27}
miR-671-5p	2.3×10^{-21}	2.2×10^{-11}
miR-494	7.3×10^{-131}	2.9×10^{-133}
miR-4270	NA	5.7×10^{-11}
miR-363	9.7×10^{-29}	9.5×10^{-54}
miR-3162	NA	3.2×10^{-14}
miR-30a	1.9×10^{-73}	5.8×10^{-138}
miR-197	2.8×10^{-63}	2.5×10^{-33}
miR-150-star	NA	2.6×10^{-53}
miR-149-star	NA	1.4×10^{-04}
miR-1280	4.3×10^{-18}	2.5×10^{-05}
miR-1275	1.5×10^{-11}	1.2×10^{-03}
miR-1268	7.6×10^{-06}	5.6×10^{-01}
miR-1260b	NA	2.9×10^{-09}
miR-125a-5p	7.4×10^{-12}	7.8×10^{-10}
miR-1207-5p	7.5×10^{-07}	3.2×10^{-01}
let-7b	5.6×10^{-21}	2.0×10^{-18}

NA: not available in the database.

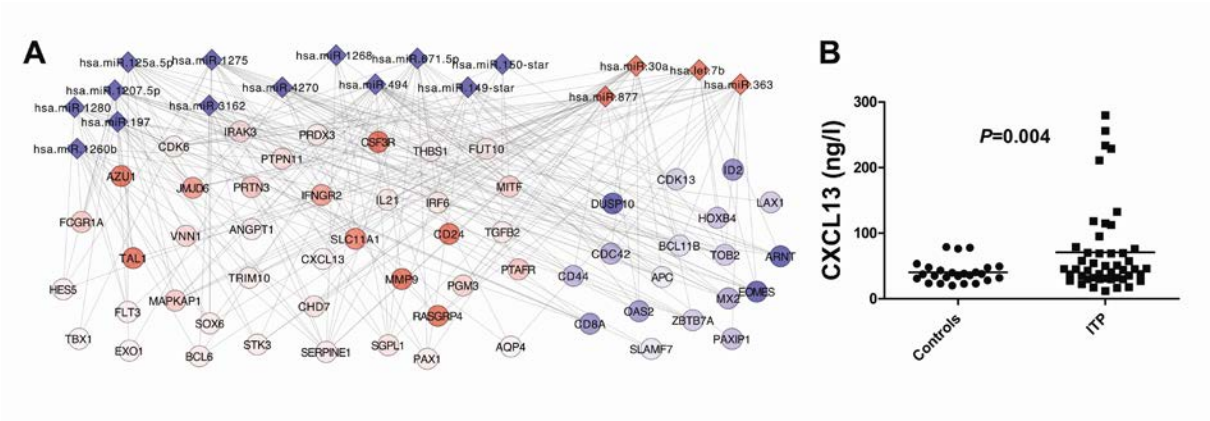
Table 1.

Figure legend

Figure 1

MicroRNA regulate mRNA and protein expression in T-cells. (A) A computational method to identify microRNA function and mRNA targets in T-cells. Target genes of significantly

regulated microRNA between ITP patients and controls, using TargetScan and Miranda algorithms, were compared with significantly regulated mRNA from peripheral blood T-cells between patients and controls. Diamonds are miRNA and circles are mRNA. Red indicate up-regulated and blue indicate down-regulated genes. (B) Plasma levels of CXCL13, which was a significantly regulated target gene of a microRNA that differed in expression between ITP patients and controls, were significantly increased in plasma from patients with ITP compared to controls.



Supplementary information

Methods

Patients

All individuals involved in this study gave informed consent. The study was approved by the regional ethics committee in Gothenburg, Sweden.

Subjects included in the DNA microarray analysis

The study subjects for the DNA microarray analysis consisted of 9 chronic ITP-patients (4 males and 5 females; mean age 47.8 years, range 22-86) and 10 healthy controls (4 males and 6 females; mean age 41.5 years, range 24-65). Clinical characteristics of the patients are shown in Supplemental Table 1.

Subjects included in the microRNA analysis

The study subjects for the microRNA analysis consisted of 9 chronic ITP-patients (6 males and 3 females; mean age 64 years, range 43-86) and 9 healthy controls (5 males and 4 females, mean age 44 years, range 28-63). Clinical characteristics of the patients are shown in Supplemental Table 1.

Subjects in the ELISA analysis of CXCL13

The study subjects for the analysis of plasma CXCL13 consisted of 47 chronic ITP-patients (21 males and 26 females; mean age 49 years, range 18-86) and 25 healthy controls (9 males and 16 females; mean age 38 years, range 19-61).

Isolation of T-cells and preparation of RNA

Heparin anti-coagulated blood was obtained from each study subject. The isolation of T-cells has previously been described in detail.[1] In brief, peripheral blood mononuclear cells (PBMCs) were separated from the blood immediately after collection, by density gradient centrifugation. After removal of $CD14^+$ cells by magnetic microbeads, T-cells were positively selected using $CD3^+$ magnetic microbeads, according to the manufacturer's recommendations (MACS, Miltenyi Biotec, Surrey, UK). The cells were stored frozen at −80°C until RNA preparation. RNA was isolated from the $CD3^+$ T-cells using the Chomczynski method.[2] For the microRNA analysis the RNA was precipitated using isopropanol overnight. For the DNA microarray analysis the RNA was further purified using RNeasy MinElute clean-up (Qiagen, Hilden, Germany). The RNA concentration was measured with a Nanodrop spectrophotometer and the A260/A280 ratio was 1.8–2.0 and the quality of the RNA was verified by agarose gel electrophoresis. The quantity of total RNA from isolated from the T-cells was in average 5.2 µg (range 1.2-10.3) for the microRNA analysis and in average 4.1 µg (range 1.7-8.7) for the DNA microarray analysis.

Labeling and hybridization to microRNA 2.0 microarrays

1000 ng T-cell RNA from each individual was biotin labeled using the FlashTag Biotin HSR kit (Genisphere, Hatfield, PA) according to the manufacturer's instructions. The labeling of the microRNA was checked using the Enzyme Linked Oligosorbent Assay (ELOSA) step (Genisphere), following the manufacturer’s instructions. All samples showed proper labeling and the labeled RNAs were hybridized to microRNA 2.0 arrays (Affymetrix, Santa Clara, CA), containing 15,644 mature microRNA sequences from 131 organisms included in the mirBASE miR database v15 (http://microrna.sanger.ac.uk). Besides the 1,105 microRNA that are human specific the arrays also contain human small nucleolar RNA such as snoRNAs and

small Cajal body-specific RNAs (scaRNAs) from the Ensembl Archive (www.ensembl.org/biomart/martview) and snoRNAbase (http//www.snorna.biotoul.fr/info.php). The arrays were washed and processed on a Fluidics Station 450 and scanned with a confocal laser scanner (GeneChip Scanner 3000, Affymetrix) according to the manufacturer's instructions.

Labeling and hybridization to U133Plus2.0 DNA microarrays

Twenty ng of purified RNA was reverse transcribed, amplified and labeled using the Ovation amplification system V2 (NuGEN Technologies Inc, San Carlos, CA) according to the manufacturer's instructions. Five μg of the generated cDNA was fragmented and biotinylated using the Encore biotin module (NuGEN) and hybridized to Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA), containing 54,675 transcripts. The arrays were washed and processed on a Fluidics Station 450 and scanned with a confocal laser scanner (GeneChip Scanner 3000, Affymetrix). These experiments comply with Minimum Information about a Microarray Experiment (MIAME).[3]

Data analysis of miRNA 2.0 microarrays

Data from the microRNA microarrays were analyzed with Affymetrix microRNA QC Tool. Robust multiarray (RMA) was used for normalization of the probe set intensities and Wilcoxon Rank Sum test was used to compare the size of the guanin-cytosin (GC) content for the microRNA probes. To be included in the analysis the probe sets had to be detected in more than 50% of the patients and controls. Student`s t-test (two-tailed) was employed to compare differences in mean values between ITP patients and controls. A $P<0.05$ was considered significant.

Data analysis of DNA microarrays U133Plus 2.0

All scanned output files were processed and normalized together using the Probe Logarithmic Intensity Error (PLIER) method.[4] Differential gene expression was identified by *P*-values derived from Student's t-test (two tailed) using linear models together with empirical Bayes.[5] To identify the global biological processes that differed between patients and controls a reporter algorithm was applied to the Gene Ontology (GO) network resulting in an enrichment score.[6,7] GO terms that had enrichment *P*-values <0.001 were considered and selected in the construction of a heatmap (Supplementary Figure 1). The analyses were performed using the R software.

Computational methods to identify microRNA functions and mRNA targets

The most commonly used microRNA database mirBase (http://www.mirbase.org), which includes over 19,000 mature microRNA products in 153 species, was used to identify microRNA functions and microRNA target mRNA. Multiple computational methods are developed that predict microRNA target sites[8] and in general, different target prediction algorithms focus varying on the complementarity between the microRNA and their potential targets around the "seed" sequence, heteroduplex free energy of binding, location and size of internal loops and bulges, and accessibility of the target site as predicted by RNA folding. Two of these algorithms are TargetScan (http://www.targetscan.org) and Miranda (www.microrna.org/miranda_new.html) which were used in the present study.[9,10] However, there are some evidence that perfect seed pairing may not necessarily be a reliable prediction for microRNA-mRNA interaction since these bioinformatics tools do not take into account the secondary structure of the mRNA which may affect the microRNA target recognition[11]. Therefore, to achieve high confidence microRNA-mRNA associations and to evaluate the impact of each microRNA on the gene expression, the predicted target genes of each

microRNA were identified and combined with the mRNA transcriptome from ITP patients and controls in an analysis using the Kolmogorov-Smirnov test (Table 1). The target genes from the microRNA identified in the Kolmogorov-Smirnov analysis were cross referenced against the list of significantly regulated mRNA between ITP patients and controls identified in the T-cell gene expression analysis. The resulting immune genes according to GO were classified further according to functional enrichment based on Immune System Gene Ontology[12] by modular enrichment analysis using Cytoscape[13] software equipped with ClueGO[14] (Supplemental Table 3 and Supplemental Figures 2 and 3).

Statistics

The specific tests are described above, under separate headings in the Methods section. *P*-values <0.05 were considered statistically significant.

Supplementary References

1. Olsson B, Andersson PO, Jernas M, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat Med*. 2003;9(9):1123-4.
2. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162(1):156-9.
3. Brazma A, Hingamp P, Quackenbush J, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat Genet*. 2001;29(4):365-71.
4. Therneau TM, Ballman KV. What does PLIER really do? *Cancer Inform*. 2008;6:423-31.
5. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.
6. Patil KR, Nielsen J. Uncovering transcriptional regulation of metabolism by using metabolic network topology. *Proc Natl Acad Sci U S A*. 2005;102(8):2685-9.
7. Oliveira AP, Patil KR, Nielsen J. Architecture of transcriptional regulatory circuits is knitted over the topology of bio-molecular interaction networks. *BMC Syst Biol*. 2008;2:17.
8. John B, Sander C, Marks DS. Prediction of human microRNA targets. *Methods Mol Biol*. 2006;342:101-13.

9. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19(1):92-105.
10. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res*. 2008;36(Database issue):D149-53.
11. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136(2):215-33.
12. Diehl AD, Lee JA, Scheuermann RH, Blake JA. Ontology development for biological systems: immunology. *Bioinformatics*. 2007;23(7):913-5.
13. Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T. Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics*. 2011;27(3):431-2.
14. Bindea G, Mlecnik B, Hackl H, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics*. 2009;25(8):1091-3.

Supplementary Table 1. Patient characteristics**,** denotes the experiment in which the patient participated.

Sex	Age (years)	Plc (10^9/l)	Treatment at time of blood sampling	Duration of disease(months)	mRNA	microRNA
M	81	10	Rituximab, Prednisolon, Azathioprine	15	x	x
F	61	64	Prednisolon	228		x
M	86	172	Eltrombopag	96		x
F	58	234	Eltrombopag, Splenectomy	15		x
M	61	61	None	96		x
F	73	243	None	7		x
M	67	39	Prednisolon, Splenectomy	15	x	x
M	43	16	None	7		x
M	46	83	Prednisolon	7		x
M	48	35	Splenectomy	332	x	
F	31	21	Splenectomy	228	x	
F	23	102	None	108	x	
F	87	91	Prednisolon	54	x	
M	22	44	Romiplostim, Splenectomy	156	x	
M	37	18	None	28	x	
F	35	69	Romiplostim	338	x	

Supplementary Table 2 Significantly regulated small nucleolar RNA in T-cells between ITP patients and controls.

Small nucleolar RNA	Mean Control	Mean ITP	t-test
ACA13	0.49	0.85	0.042
ACA4	-0.01	0.26	0.024
ACA41	0.25	0.68	0.028
ACA46	-0.18	0.31	0.007
ENSG00000200706	-0.14	0.22	0.044
ENSG00000206785	0.28	0.90	0.037
ENSG00000251992	0.40	0.04	0.009
HBII-276	4.31	4.61	0.040
HBII-295	0.15	0.55	0.039
HBII-85-4	0.45	0.16	0.031
hp_hsa-mir-1224	0.58	0.14	0.041
hp_hsa-mir-520h	0.23	-0.03	0.035
mgU12-22-U4-8	0.72	0.35	0.007
SNORD123	-0.05	0.28	0.022
U17b	5.54	4.55	0.037
U22	0.12	0.35	0.031

The mean control and ITP values are logarithmized with base 2.

Supplementary Table 3. Specification of the significantly regulated microRNA target genes belonging to the 7 modules based on Immune System Gene Ontology shown in Supplemental Figures 2A-B.

T cell activation involved in immune response	Natural killer cell differentiation	Regulation of immunoglobulin production	Positive regulation of leukocyte activation	Lymphocyte activation involved in immune response	Lymphocyte differentiation	Lymphocyte costimulation
AZU1	ANGPT1	APC	APC	APC	APC	AQP4
BCL6	APC	BCL11B	BCL11B	BCL11B	BCL11B	CD24
CD8A	ARNT	BCL6	BCL6	BCL6	BCL6	CD44
CXCL13	BCL11B	CD24	CD24	CD24	CD8A	CDC42
DUSP10	BCL6	CD8A	CD8A	CD8A	CHD7	FCGR1A
EOMES	CD24	CDC42	CDC42	CDC42	EOMES	IFNGR2
EXO1	CD8A	CHD7	CHD7	CHD7	FLT3	IRF6
IL21	CDC42	EOMES	EOMES	CXCL13	ID2	MAPKAP1
JMJD6	CDK13	FLT3	FLT3	EOMES	IL21	MX2
PAXIP1	CDK6	ID2	ID2	EXO1	JMJD6	OAS2
SERPINE1	CHD7	IL21	IFNGR2	FLT3	PAX1	PTAFR
SLAMF7	EOMES	IRAK3	IL21	ID2	TBX1	PTPN11
SLC11A1	EXO1	JMJD6	JMJD6	IL21	VNN1	SLC11A1
THBS1	FLT3	LAX1	LAX1	JMJD6		
	FUT10	MAPKAP1	MAPKAP1	LAX1		
	HES5	PAX1	MMP9	MAPKAP1		
	HOXB4	PAXIP1	PAX1	PAX1		
	ID2	PTPN11	PAXIP1	PAXIP1		
	IL21	SLAMF7	PTPN11	PTPN11		
	IRAK3	SLC11A1	SLAMF7	SLC11A1		
	JMJD6	TGFB2	SLC11A1	TBX1		
	LAX1	THBS1	THBS1	VNN1		
	MAPKAP1	VNN1	VNN1			
	MITF					
	MMP9					
	PAX1					
	PAXIP1					
	PGM3					
	PRDX3					
	PRTN3					
	PTPN11					
	RASGRP4					
	SGPL1					
	SLAMF7					
	SLC11A1					
	SOX6					
	STK3					
	TAL1					
	TBX1					
	TGFB2					
	THBS1					
	TOB2					
	TRIM10					
	VNN1					
	ZBTB7A					
	CSF3R					
	CXCL13					
	DUSP10					
	SERPINE1					

Supplementary Figure legends

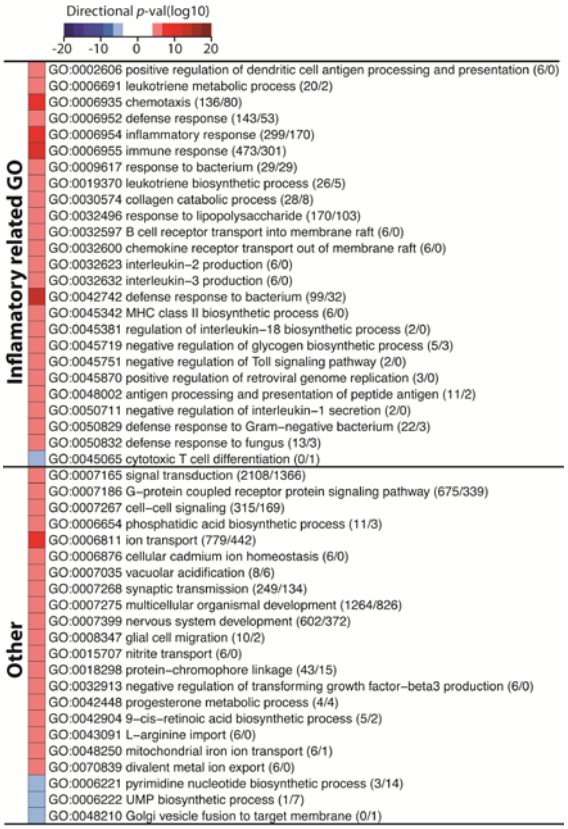
Supplementary figure 1

Gene ontology analysis of the significantly regulated genes in T-cells between ITP patients and controls. This analysis uses all genes in the U133Plus2.0 microarrays and the numbers after gene ontology term indicate number of up-regulated genes/number of down-regulated genes in ITP patients compared with controls. The enrichment *P*-values are shown in red for up-regulated gene ontology terms and blue for down-regulated gene ontology terms in patients compared with controls. All gene ontology terms that had *P*-values < 0.001 are shown.

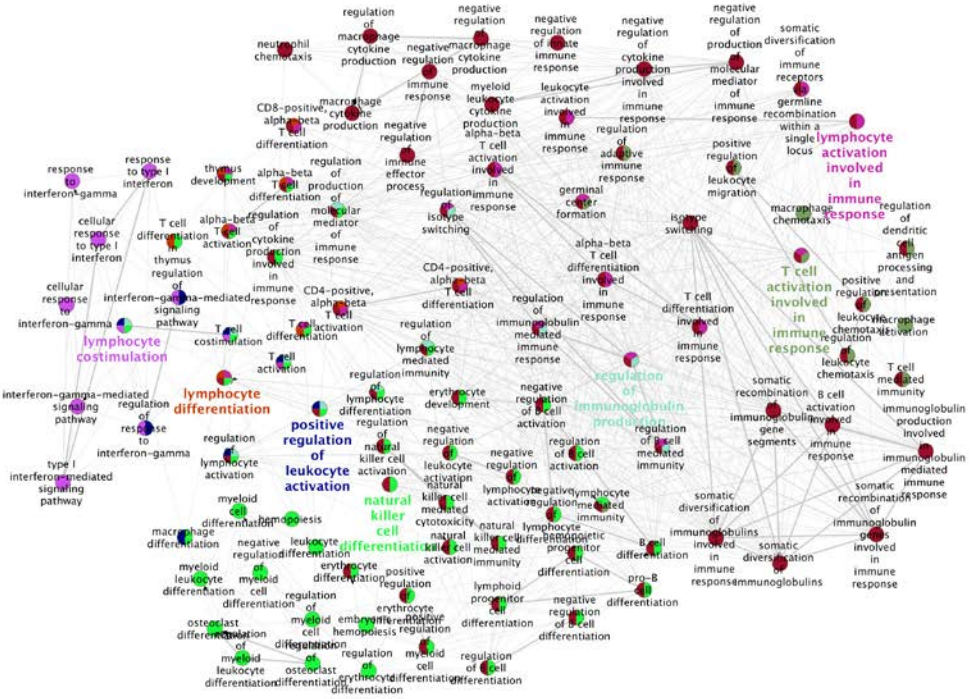
Supplementary figure 2

The 57 microRNA target genes that differed between ITP patients and controls and classified as being involved in the immune system according to Gene Ontology were analyzed using functional module enrichment based on Immune System Gene Ontology.[15] One circle represents one term and if the term has similar function as another it will be colored in the same way. The term in a group with the same color with the lowest *P*-value was reported in larger font. This resulted in 7 modules that were enriched with the following functions: T-cell activation involved in immune response, natural killer cell differentiation, regulation of immunoglobulin production, positive regulation of leukocyte activation, lymphocyte activation involved in immune response, lymphocyte differentiation, and lymphocyte costimulation. (A) Shows the result of the analysis in detail and (B) in a simplified form.

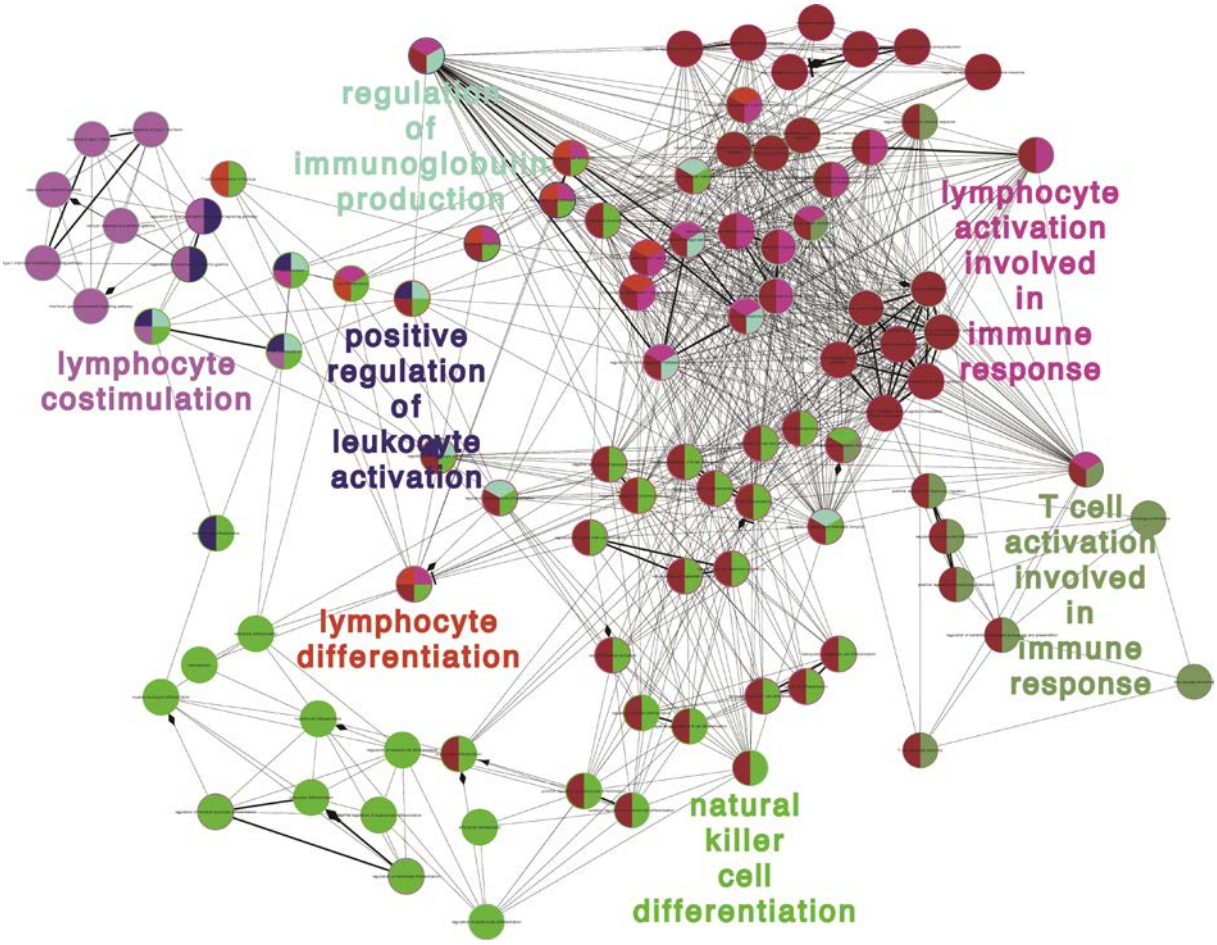
Supplementary figure 1

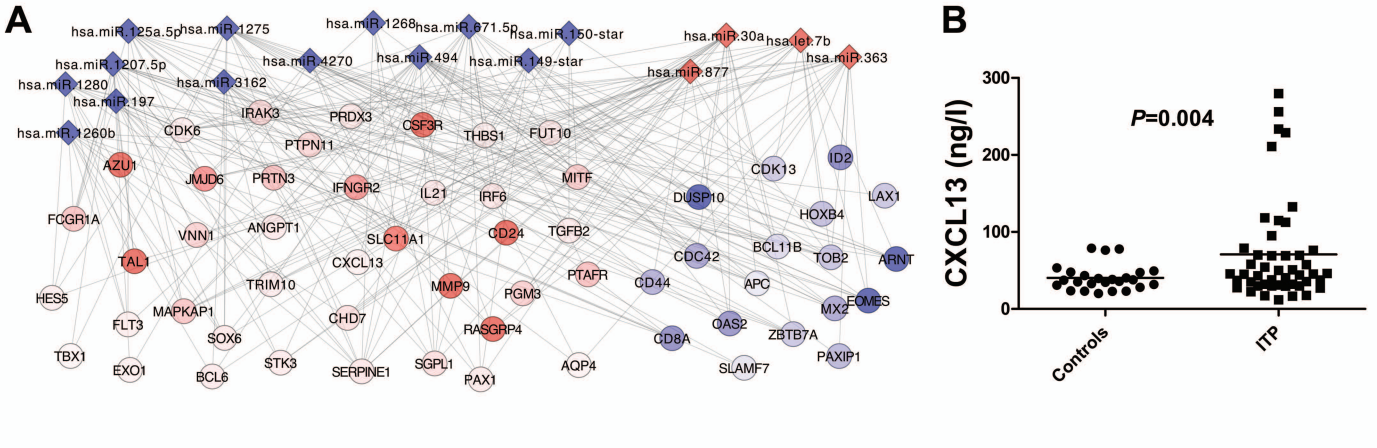


Supplementary figure 2A



Supplementary figure 2B




A
hsa.miR.125a.5p
hsa.miR.1275
hsa.miR.1268
hsa.miR.671.5p
hsa.miR.150-star
hsa.miR.30a
hsa.let.7b
hsa.miR.1207.5p
hsa.miR.4270
hsa.miR.494
hsa.miR.149-star
hsa.miR.877
hsa.miR.363
hsa.miR.1280
hsa.miR.3162
hsa.miR.197
IRAK3
PRDX3
CSF3R
THBS1
FUT10
hsa.miR.1260b
CDK6
PTPN11
AZU1
JMUD6
PRTN3
IFNGR2
IL21
IRF6
MITF
CDK13
ID2
LAX1
DUSP10
HOXB4
FCGR1A
VNN1
ANGPT1
SLC11A1
CD24
TGFB2
BCL11B
TOB2
ARNT
TAL1
CXCL13
CDC42
PTAFR
CD44
APC
TRIM10
MMP9
PGM3
HES5
MAPKAP1
CHD7
MX2
EOMES
FLT3
RASGRP4
OAS2
ZBTB7A
SOX6
CD8A
TBX1
STK3
SGPL1
AQP4
PAXIP1
EXO1
BCL6
SERPINE1
PAX1
SLAMF7
B
CXCL13 (ng/l)
300
200
100
0
P=0.004
Controls
ITP