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

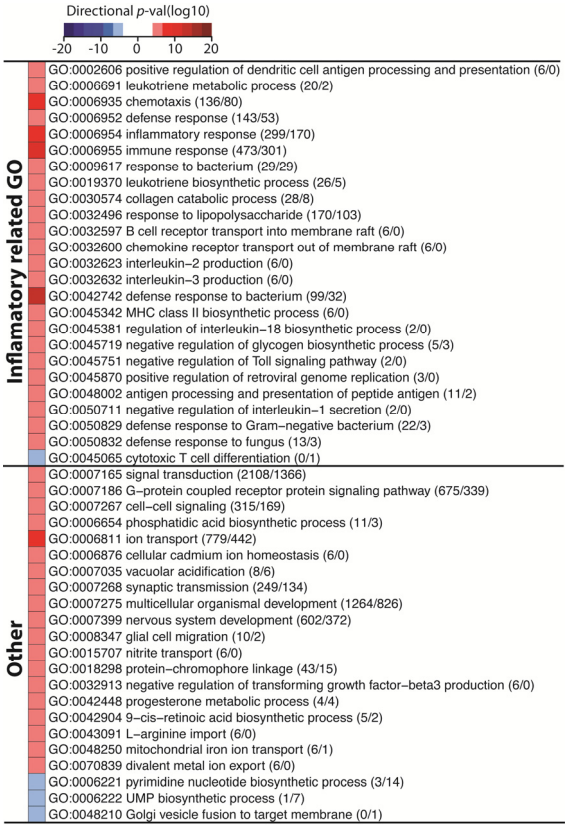
Supplementary figure 1

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.

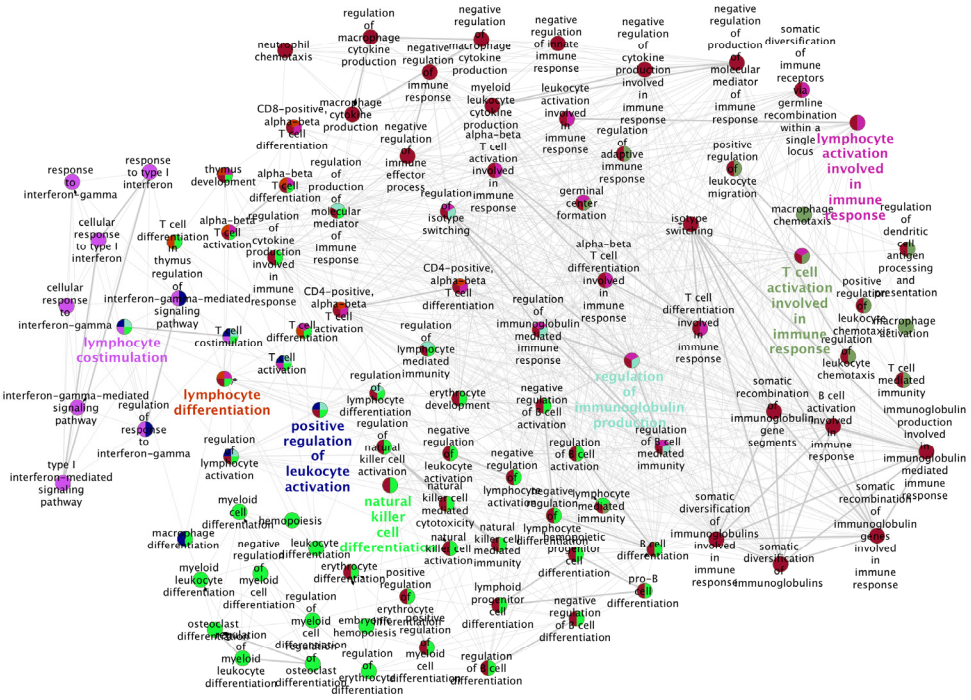
Supplementary figure 2

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