Genes and Immunity (2014) **15,** 303–312

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ORIGINAL ARTICLE

MiRNome and transcriptome aided pathway analysis in human regulatory T cells

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Owing to their manifold immune regulatory functions, regulatory T cells (Treg) have received tremendous interest as targets for therapeutic intervention of diverse immunological pathologies or cancer. Directed manipulation of Treg will only be achievable with extensive knowledge about the intrinsic programs that define their regulatory function. We simultaneously analyzed miR and mRNA transcript levels in resting and activated human Treg cells in comparison with non-regulatory conventional T cells (Tcon). Based on experimentally validated miR-target information, both transcript levels were integrated into a comprehensive pathway analysis. This strategy revealed characteristic signal transduction pathways involved in Treg biology such as T-cell receptor-, Toll-like receptor-, transforming growth factor-β-, JAK/STAT (Janus kinase/signal transducers and activators of transcription)- and mammalian target of rapamycin signaling, and allowed for the prediction of specific pathway activities on the basis of miR and mRNA transcript levels in a probabilistic manner. These data encourage new concepts for targeted control of Treg cell effector functions.

Genes and Immunity (2014) **15,** 303–312; doi:10.1038/gene.2014.20; published online 22 May 2014

INTRODUCTION

In the human immune system, several non-redundant regulatory mechanisms exist to maintain the delicate balance between pathogen defense, tumor surveillance and autoimmunity. Among them, regulatory T cells (Treg) are essential to preserve peripheral tolerance by moderating most types of immune responses and by preventing the activation of other T cells.[1] Taking their immune regulatory characteristics into account, Treg are promising candidates for various therapeutic interventions, including strategies to promote transplantation tolerance or to treat autoimmune disease or cancer.[2] The discovery that defects in *FOXP3* are causative for the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in humans due to the loss of Treg cells unravelled the transcriptional regulator FOXP3 as a critical modulator of Treg development and function.[3] Subsequently, great effort has been put into the identification of upstream signaling molecules of FOXP3 expression that result in Treg induction. Several signal transduction pathways, including T-cell receptor (TCR)-, Toll-like receptor (TLR)-, transforming growth factor-β (TGFβ)-, JAK/STAT (Janus kinase/signal transducers and activators of transcription)- and mammalian target of rapamycin (mTOR) signaling, and several key components of these pathways, for example, NFAT (nuclear factor of activated T cells), nuclear factor-κB (NF-κB), signal transducer and activator of transcription (STAT), SMAD have been associated with Treg biology.[4,5] In recent years, microRNA (miR) and mRNA profiling studies have been carried out to identify regulators of Treg development and function.[6] MiRs are short single-stranded non-coding RNA molecules, about 18–24 nucleotides in length and have an inimitable role in the regulation of gene expression.[7] They regulate genes by targeting mRNA for degradation or translational repression.[8]

Following their discovery in *Caenorhabditis elegans*,[9] many miRs have been identified in humans, with numbers constantly increasing. Every single miR can possibly regulate a huge variety of different mRNA transcripts and thereby has the capacity to alter entire signaling pathways.[7] Various miR[10–14] and mRNA[12,14–17] signatures have been established for human and mouse Treg cells. Based on these studies, many mRNAs, for example, the ones coding for CTLA-4 (CD152), HLA-DR, GARP (LRRC32) and CD39 (ENTPD1), and miRs, for example, miR-146a, miR-31 and miR-10a, have been identified as key regulators in Treg biology. In this study, we expanded this approach by simultaneously analyzing miR and mRNA expression in parallel and integrating information of both transcript levels for pathway description in human Treg cells. As another crucial aspect of this study, miR and mRNA profiles of Treg and Tcon were performed both under resting and stimulated conditions, as it has become clear that Treg need to be activated to exert their potent suppressive function.[18] This study reveals new miR and mRNA candidates who may have a role in Treg biology and expands existing knowledge by joint analysis of both transcript levels based on experimentally validated miR-target information. Comparative analysis further allows for the prediction of miR–mRNA interactions within Treg-associated signal transduction pathways. These results shed new light on the complex interplay between gene transcription and its regulation in defining the human Treg phenotype, and should open up new starting points for targeted control of Treg function.

RESULTS

MiR and mRNA profiling of resting and stimulated Treg

To gain insight into the specific molecular program that defines Treg, we compared their transcriptomic profile (miR and

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Received 13 February 2014; revised 24 March 2014; accepted 27 March 2014; published online 22 May 2014

mRNA) with that of non-regulatory Tcon cells (Tcon). The experimental concept of this study is graphically outlined in Figure 1.

Treg ($CD4^+CD25^+CD127^{low}$) and Tcon ($CD4^+CD25^-CD127^+$) were enriched by fluorescence-activated cell sorting (FACS) to high purity as shown by flow cytometric analysis of surface marker expressions and intracellular FOXP3 staining (Figure 2a). The Treg population effectively suppressed allogeneic induced proliferation of responder T cells in a mixed lymphocyte reaction demonstrating their suppressive functionality (Figure 2b). As stimulation via their TCR and adequate co-stimulation are required for effector function of Treg and Tcon, we investigated expression profiles of miR and mRNA in Treg and Tcon under resting and stimulated (Treg(s) and Tcon(s)) conditions in parallel. Activated Treg and Tcon cells were collected after 48 h of stimulation with CD3/CD28 beads (Figure 2c). Total RNA was isolated from resting and stimulated Treg and Tcon cells, which was each pooled in two entities comprising three independent sorting procedures to obtain miR and mRNA array data in replicates.

Treg and Tcon have distinct miR patterns

To identify differentially expressed miRs, we performed a Taqman low-density array suitable for the detection of 664 distinct human miRs. Of those, 14 miRs were differentially expressed between resting Treg and Tcon cells, and 21 miRs were differentially expressed after activation (Figure 3a and Supplementary Table S1). A total of six differentially expressed miRs, with two upregulated miRs (miR-135b and miR-146a) and four downregulated miRs (miR-31, miR-363, miR-424 and miR-455) were shared by resting and activated Treg (Figure 3a). To confirm the accuracy of the miR array data, we performed quantitative real-time reverse transcription–PCR of eight miRs that were differentially expressed in resting or stimulated Treg. As shown in Figure 3b, correlation was highly significant with $\rho = 0.96$ when estimated with Spearman's rank correlation ($P = 2.2 \times 10^{-16}$).

Treg and Tcon have distinct mRNA patterns

Differentially expressed mRNAs were detected using the Agilent human whole-genome oligo microarray kit. This revealed 2185

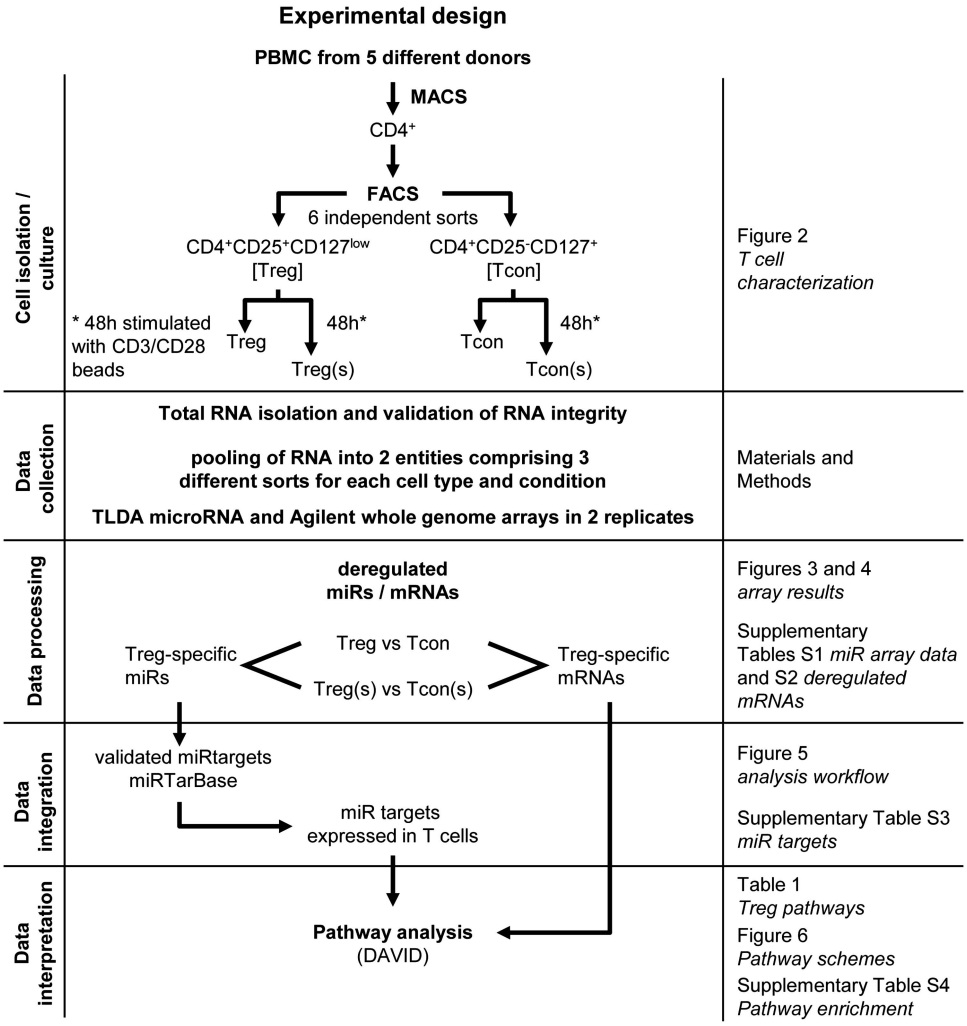

Figure 1. Schematic representation of experimental design. $CD4^+CD25^+CD127^{low}$ Treg cells and $CD4^+CD25^-CD127^+$ Tcon cells were isolated from human peripheral blood cells by subsequent enrichment with MACS and FACS sorting. One half of the sorted cells were directly subjected to RNA preparation, whereas the other half was activated for 48 h with CD3/CD28 beads to obtain RNA both from resting (Treg and Tcon) and stimulated (Treg(s) and Tcon(s)) T cells. Total RNA was then subjected to miR expression profiling using the TaqMan low-density array technique and whole-genome expression profiling with oligo single-color arrays from Agilent. Differentially expressed miRs and mRNAs were obtained by comparing resting Treg with Tcon cells, and stimulated Treg with stimulated Tcon cells. Experimentally validated miR targets were identified using miRTarBase.[21] Validated and expressed miR targets and mRNA expression profiles of resting and stimulated Treg cells were used to extract novel miR/mRNA interactions within Treg-driven pathways using DAVID (database for annotation, visualization and integrated discovery) bioinformatics resources.[19,22]

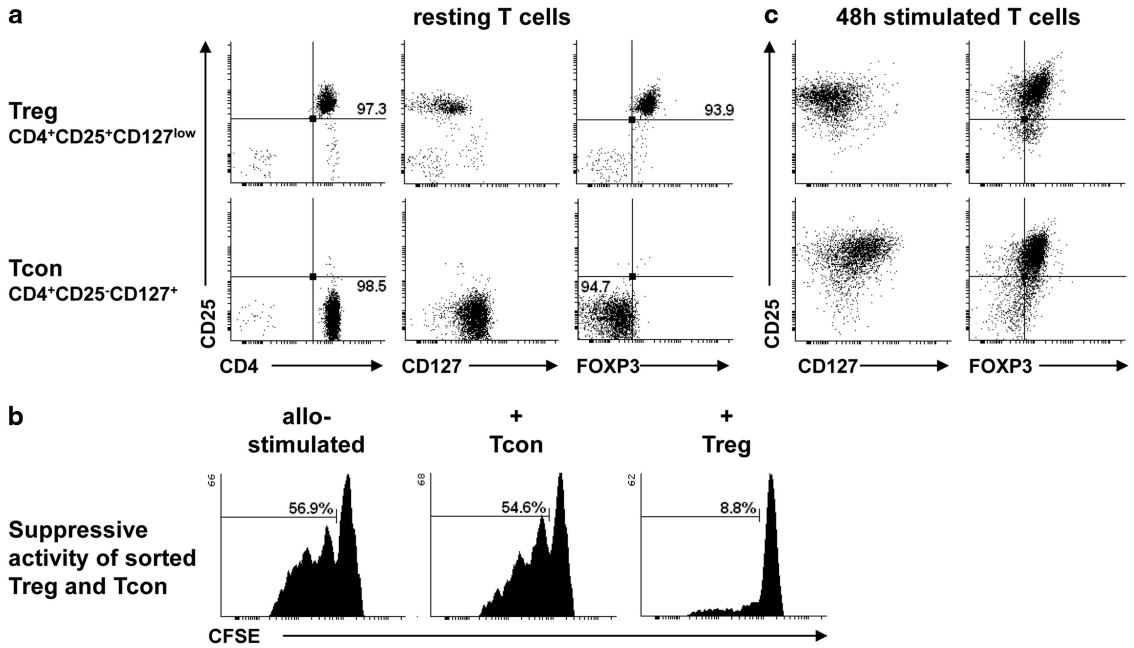

Figure 2. FACS-sorted Treg cells express FOXP3 and have suppressive function. (**a**) Purity of FACS-sorted Treg cells and Tcon cells was analyzed by flow cytometry based on expression of CD4, CD25, CD127 and FOXP3. Gates were set on live cells. (**b**) Suppressive activity of sorted T cells was determined in a mixed lymphocyte reaction with allogeneic activated carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled responder T cells at a ratio of 1:1. Histogram gates were set on live $CD4^+CFSE^+$ cells. (**c**) Flow cytometric analysis of CD25, CD127 and FOXP3 expression in Treg and Tcon 48 h after activation with CD3/CD28 beads. Gates were set on live cells.

a deregulated miRs

	microRNA	logFC Treg vs. Tcon	logFC Treg(s) vs. Tcon(s)	validated by single RT-PCR	exp. validated targets Treg vs. Tcon	exp. validated targets Treg(s) vs. Tcon(s)
up	miR-146a	3.74	3.28		27	28
up	miR-526b*	1.55		x	/	/
up	miR-135b	1.84	2.00	x	8	8
up	miR-145		5.20			87
up	miR-184		5.70			3
up	miR-874		4.00			/
up	miR-10a		3.07			43
up	miR-192*		3.06			/
up	miR-801		2.57			/
up	miR-511		2.05	x		1
up	miR-642		2.02			1
up	miR-342		1.82			3
up	miR-339		1.68			1
down	miR-100	-5.52			10	
down	miR-363	-4.96	-4.73		1	1
down	miR-502	-4.81			/	
down	miR-125b	-4.19			55	
down	miR-31	-3.63	-2.05		32	33
down	miR-99a	-3.54		x	5	
down	miR-107	-3.50		x	21	
down	miR-424	-3.25	-2.25	x	23	23
down	miR-193b	-3.15			86	
down	miR-365	-3.07			3	
down	miR-455	-1.54	-2.09	x	/	/
down	miR-218		-6.18			26
down	miR-422a		-2.52			1
down	miR-324		-2.04			2
down	miR-17		-1.60	x		42
down	miR-92a-1*		-1.52			/

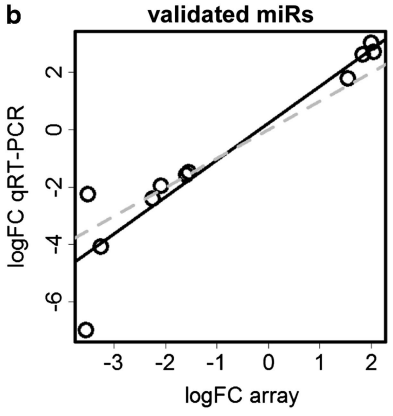

Figure 3. Differentially regulated miRs in resting and activated Treg cells. (**a**) Differentially regulated miRs in resting and activated Treg cells with $P<0.05$. MiRs shared within both groups are highlighted in gray. Experimentally validated miR targets of expressed genes in resting and stimulated Treg were retrieved from miRTarBase.[21] (**b**) The reliability of the microRNA array data was tested by Taqman-based quantitative real-time reverse transcription (qRT–PCR) with the original samples. qRT-PCR was performed for eight miRs (miR-17, miR-99a, miR-107, miR-135b, miR-424, miR-455, miR-511 and miR-526b*). Spearman's correlation coefficient: $\rho = 0.96$, $P = 2.2 \times 10^{-16}$.

($P<0.05$) differentially expressed mRNAs between resting Treg and Tcon cells. After stimulation, the number of differentially expressed mRNAs increased two-fold to a total of 4286 mRNAs ($P<0.05$), indicative for different downstream effects in Treg and Tcon cells after TCR engagement and CD28 co-stimulation. Among these differentially expressed genes, 911 mRNAs were shared both by resting and activated Treg cells. Based on array data of two previously published genomic studies on human Treg cells,[12,14] we performed Gene Set Enrichment Analysis to validate our array data. We observed significant gene enrichment in our data set of genes previously described by Pfoertner *et al.*[12] and Sadlon *et al.*,[14] including *FOXP3*, *CTLA-4* (*CD152*), *HLA-DR*, *GARP* (*LRRC32*) and *CD39* (*ENTPD1*), both in resting and stimulated Treg (Figure 4). The identification of important Treg marker genes in our expression list and the enrichment of Treg-associated biological pathways (Table 1) are consistent with criteria for high-quality data previously described by Huang da *et al.*[19]

In summary, these analyses confirm the validity of our array data.

Joint miR-mRNA analysis reveals key pathways in Treg biology

Integrating both miR and mRNA profiles of resting and activated Treg cells, we aimed to identify specific signaling pathways that account for their regulatory phenotype. It has been suggested that co-regulated miRs act together in the same pathways.[20] Furthermore, regulation of target genes by miRs occurs at the posttranscriptional level and, in principal, miRs can bias translation either by influencing the amount of mRNA or the level of translation itself.[8] Thus, we concluded that the integration of both levels of transcript information should considerably enhance the conclusiveness of pathway analysis. One fundamental challenge is the identification of miR targets. As target prediction algorithms are hampered by high false-positive and -negative rates, we retrieved miR targets from miRTarBase,[21] which provided the largest collection of experimentally validated miR-target interactions at the time we prepared the manuscript. We found 271 experimentally validated targets for the 14 differentially regulated miRs in resting and 303 targets for the 21 deregulated miRs in activated Treg cells (Figures 5a and c). The dedicated targets are shown in Figure 3a (The complete list of miR targets is available in Supplementary Table S3).

In order to detect enriched miR–mRNA networks affecting Treg biology, we combined experimentally validated and T-cell-expressed miR targets and significantly deregulated genes in resting and activated Treg cells for pathway analysis using DAVID bioinformatics resources with BioCarta and KEGG databases.[19,22] For further in-depth analysis of signal pathway-embedded miR–mRNA networks, only data-driven objectives were considered.[23] Although this restriction excluded many highly significant pathways ($P<0.0001$) with obvious implications in Treg biology, for example, diabetes mellitus, allograft rejection, graft-versus-host disease, asthma and autoimmune thyroid disease (Figure 5c, complete signal pathway list is available in Supplementary Table S4), we intended to focus specifically on data-driven intracellular signaling pathways with potential implication in T-cell fate determination rather than on broad

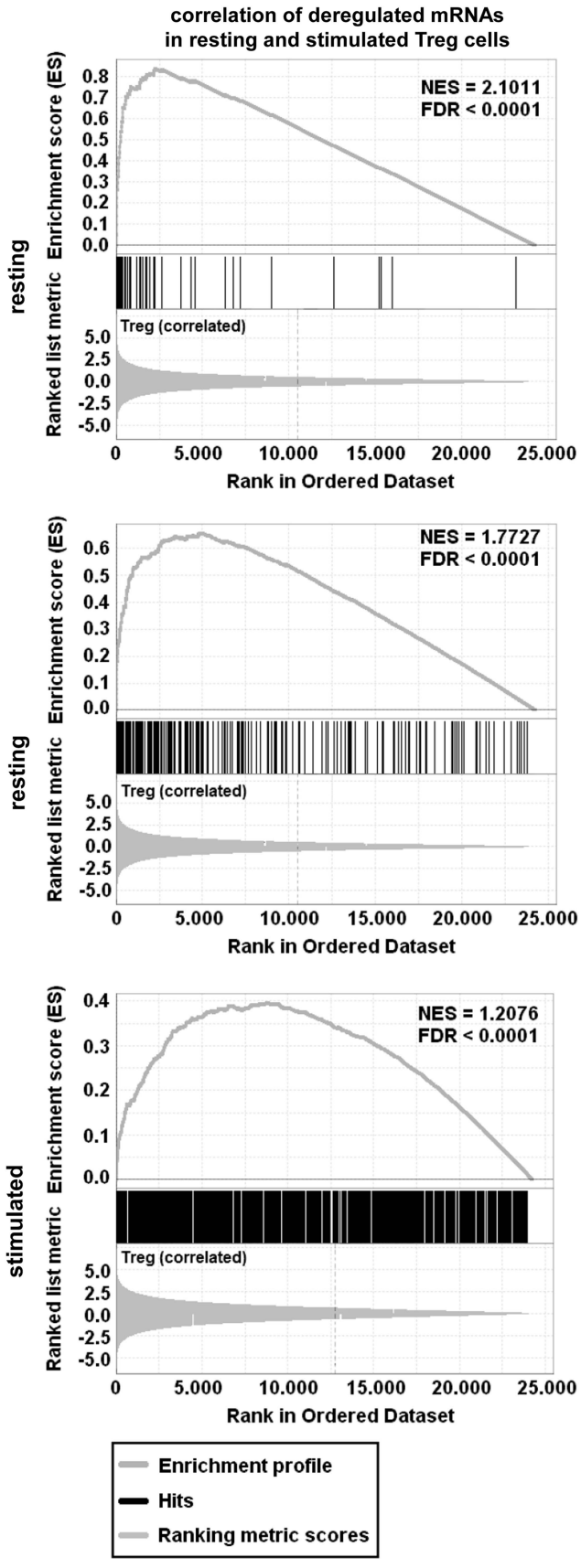

Figure 4. Differentially expressed genes in resting and activated Treg cells. Strong enrichment of genes previously reported in Treg cells. Gene expression array data was validated with the Gene Set Enrichment Analysis tool GSEA[50] using previously published gene sets. Enrichment analysis plots of deregulated mRNAs in resting Treg using data set published by Pfoertner *et al.*[12] (upper panel) and of deregulated mRNAs in resting and stimulated Treg using data set published by Sadlon *et al.*[14] (middle and lower panel). NES, normalized enrichment score; FDR, false discovery rate (*q*-value).

Table 1. Overrepresented pathways in resting and stimulated Treg cells

Category	*Term*	*Treg vs Tcon*		*Treg(s) vs Tcon(s)*	
		No of genes	P-*value*	*No of genes*	P-*value*
Apoptosis					
KEGG	Apoptosis	23	0.00018	31	0.00230
KEGG	p53 Signaling pathway	21	0.00003	32	0.00001
BIOCARTA	FAS signaling pathway (CD95)	10	0.07431	14	n.s.
BIOCARTA	Induction of apoptosis through DR3/DR4/5	9	0.06300	10	n.s.
BIOCARTA	D4-GDI signaling pathway	5	0.06000	7	0.03100
BIOCARTA	Apoptotic signaling in response to DNA damage	6	n.s.	10	0.05500
BIOCARTA	Granzyme A-mediated apoptosis pathway	3	n.s.	7	0.02400
BIOCARTA	RB tumor suppressor/checkpoint signaling	4	n.s.	7	0.01100
Cell cycle					
KEGG	Cell cycle	27	0.00170	39	0.02900
BIOCARTA	G1/S check point	10	0.08200	16	0.00510
BIOCARTA	G2/M check point	9	0.07400	10	0.08900
BIOCARTA	ATM signaling pathway	6	n.s.	12	0.01400
Cytoskeleton					
KEGG	Regulation of actin cytoskeleton	45	0.00012	40	n.s.
Immune regulation					
KEGG	Chemokine signaling pathway	33	0.02000	56	0.00790
KEGG	Toll-like receptor signaling pathway	23	0.00170	32	0.05200
KEGG	TGFβsignaling pathway	15	0.05200	22	n.s.
KEGG	Fc epsilon RI signaling pathway	14	0.04400	22	0.08900
KEGG	RIG-I-like receptor signaling pathway	13	0.01800	17	n.s.
KEGG	mTOR signaling pathway	12	0.06200	10	n.s.
BIOCARTA	Signal transduction through IL1R	9	0.02300	13	0.00110
BIOCARTA	CXCR4 signaling pathway	9	0.04200	8	n.s.
BIOCARTA	NF-κB signaling pathway	7	0.02000	10	0.03800
KEGG	JAK/STAT signaling pathway	22	n.s.	42	0.00880
KEGG	T-cell receptor signaling pathway	15	n.s.	34	0.03900
KEGG	Cytosolic DNA-sensing pathway	9	n.s.	22	0.00210
BIOCARTA	The co-stimulatory signal during T-cell activation	4	n.s.	10	0.08600
BIOCARTA	IL-6 signaling pathway	4	n.s.	9	0.05500
BIOCARTA	The 4-1BB-dependent immune response	5	n.s.	9	0.00310
BIOCARTA	Role of Tob in T-cell activation	3	n.s.	7	0.07500
BIOCARTA	IL-5 signaling pathway	4	n.s.	6	0.02300
Surface receptor signaling					
KEGG	Neurotrophin signaling pathway	29	0.00028	36	0.00039
KEGG	Insulin signaling pathway	21	0.07800	44	0.00240
KEGG	ErbB signaling pathway	16	0.01100	22	n.s.
KEGG	NOD-like receptor signaling pathway	15	0.01500	23	0.01000
BIOCARTA	Integrin signaling pathway	10	0.05800	12	n.s.
KEGG	MAPK signaling pathway	35	n.s.	73	0.08200
BIOCARTA	EGF signaling pathway	4	n.s.	11	0.05000
BIOCARTA	IGF-1 signaling pathway	4	n.s.	10	0.08100
Wnt/Shh signaling					
BIOCARTA	Sonic Hedgehog (Shh) Pathway	5	0.02000	5	n.s.
KEGG	Wnt signaling pathway	21	n.s.	41	0.01900
BIOCARTA	Nitric oxide signaling pathway	0	n.s.	5	0.06500

Abbreviations: ATM, ataxia telangiectasia mutated; EGF, epidermal growth factor; IGF-1, insulin-like growth factor 1; IL, interleukin; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; RB, retinoblastoma; TGFβ, transforming growth factor-β.
Enriched pathways were deduced by analyzing differently expressed mRNAs and expressed miR targets of differentially regulated miRs in Treg and Tcon cells under resting and stimulated conditions with the DAVID platform.[19,22] Enriched pathways (Fisher's exact *P*-value < 0.1) in resting and stimulated Treg cells were grouped by cellular function and listed in alphabetical order. Shared pathways enriched under resting and stimulated conditions are highlighted in gray.

knowledge-based intercellular networks of diseases, protein classification or metabolic pathways. This led to the identification of 24 enriched pathways in resting and 31 enriched signaling pathways in activated Treg cells with Fisher's exact *P*-value < 0.1 (Figure 5c). Among them, 14 pathways, defined by 6 miRs and 911 mRNAs, were shared both by resting and activated Treg cells (Figure 5d and Table 1).

As expected, most strongly represented were pathways associated with immune regulation, including TCR-, TLR-, TGFβ- and JAK/STAT signaling (Table 1). These pathways are presented in the discussion section in more detail. They are well known to have a role in regulation of FOXP3 expression and thus make them promising targets to control Treg cell function (Figures 6a–d and Supplementary Table S4). Along the same line, there is

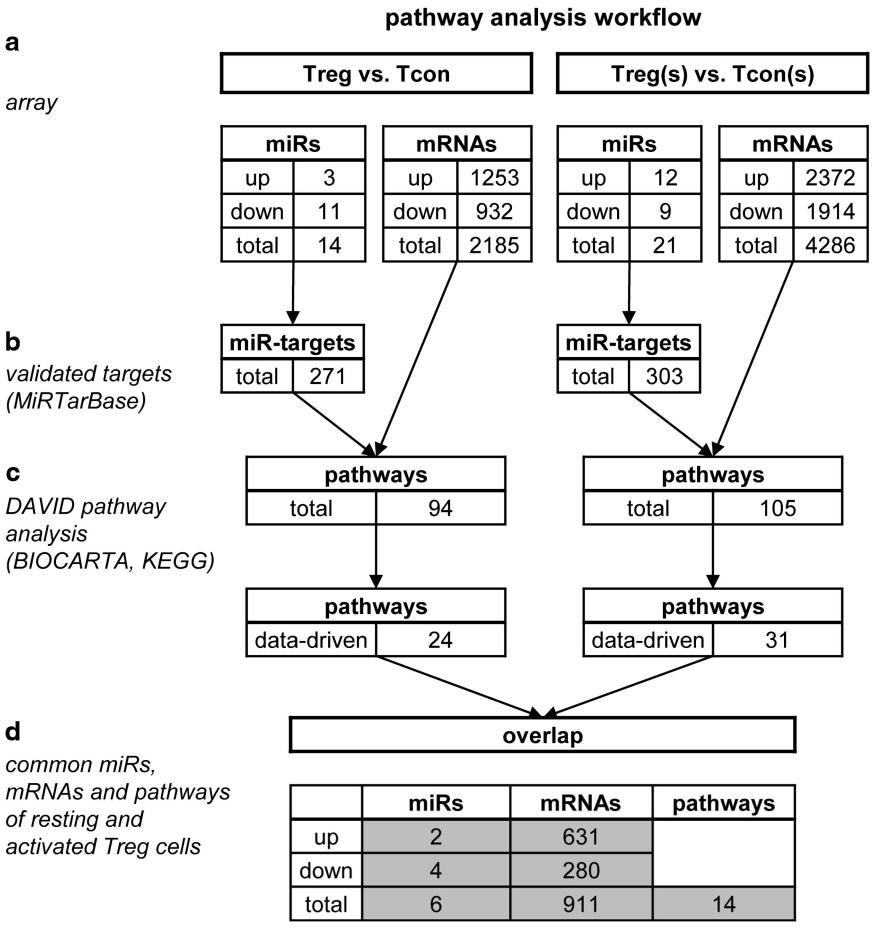

Figure 5. Comparative analysis workflow of miR and mRNA expression data sets of Treg and Tcon cells under resting and stimulated conditions. (**a**) Numbers represent deregulated mRNAs and miRs of resting Treg compared with resting Tcon cells (Treg vs Tcon) and stimulated Treg compared with stimulated Tcon cells (Treg(s) vs Tcon(s)) with P-value < 0.05. (**b**) Human miR targets of up- and downregulated miRs were retrieved from miRTarBase.[21] (**c**) Data sets of deregulated mRNAs and validated miR targets were combined and analyzed for pathway enrichment using DAVID bioinformatics resources.[19,22] Only pathways implying data-driven objectives were further considered. Comparative pathway list is shown in Table 1. The complete pathway list is available in Supplementary Table S4. (**d**) Overlap between resting and activated Treg cells defines Treg-specific mRNA and miR interactions in overrepresented signaling pathways (Ease Score < 0.2).

accumulating evidence for the involvement of mTOR signaling in Treg function and *FOXP3* transcription, wherefore we also describe a detailed miR–mRNA network concerning this pathway (Figure 6e).

No less interesting is the enrichment of diverse signaling pathways not directly involved in immune regulation. The enrichment of several apoptosis and cell cycle-related pathways further underscores the differential susceptibility to apoptosis and proliferative signals in Treg and Tcon cells. Particularly noteworthy are FAS (CD95) and CASPASE-8, which where both upregulated in Treg cells, likely rendering them sensitive to FAS-induced apoptosis. Differential expression of several key molecules of the mitogen-activated protein kinase (MAPK) cascade led to the enrichment of related growth factor signaling pathways, such as the ErbB-, insulin- and NOD-like receptor signaling pathway. In this context, the elevated levels of the stress-regulated MAPKs p38 (MAPK13) and MAPKAP2 in Treg cells should be mentioned, as they relay inflammatory signals to the nucleus. In relation to FOXP3 induction, the Sonic Hedgehog signaling pathway should be mentioned, as some key molecules, notably DYRK1A, PTCH1 and SMO, were differentially regulated in Treg cells, the last two being substrates of miR-125b, which we found to be downregulated in resting Treg cells.

The combined analysis of deregulated mRNAs and targets of deregulated miRs led to the identification of signaling pathways specifically used by Treg cells. Furthermore, the integrated analysis revealed new links between both transcript types in terms of FOXP3 induction and Treg cell function.

DISCUSSION

Although over the past decade Treg cells have received considerable scientific and medical attention because of their promise in immunotherapy, there is still a paucity of information regarding the complex signaling events that control Treg function and development. Based on miR and mRNA expression profiles of resting and stimulated human Treg cells and non-regulatory Tcon cells, we aimed to combine both data sets to gain a more detailed insight into the signaling pathways specifically used by Treg cells. The activation state of Treg and Tcon was accounted for in this study, as it has become clear that Treg cells need to be activated to exert their potent suppressive function. Thornton *et al.*[18] demonstrated that they acquire full suppressive capacity after pre-activation of 2 days. On the other side, Tcon cells also express peak levels of activation markers and also intracellular FOXP3 at this time point without acquiring suppressive function, making it an appropriate time point to study their transcriptomic differences.[24]

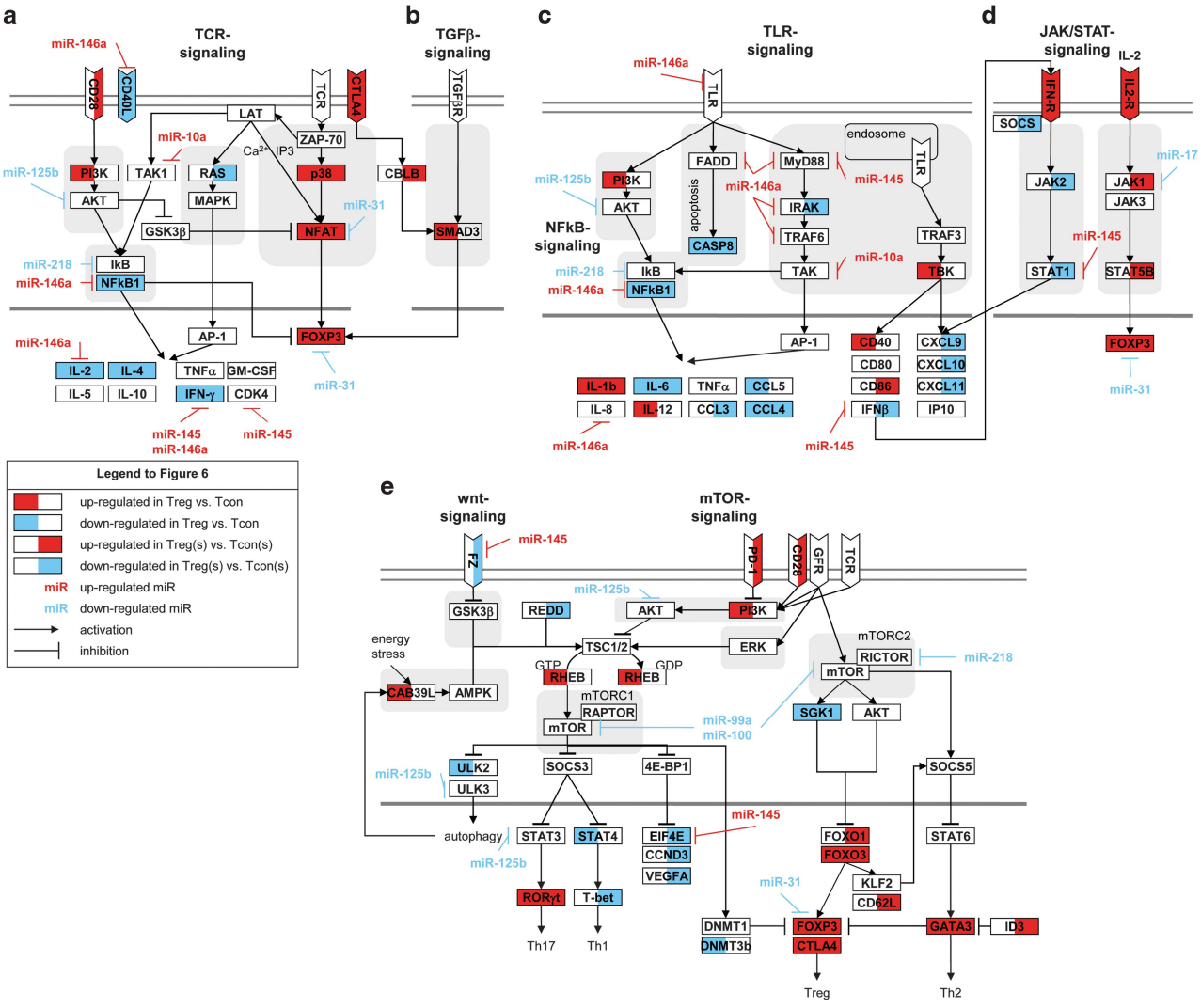

Figure 6. Interplay of Treg-specific miRs and mRNAs in immunological pathways. Protein signaling cascades of deregulated mRNAs and miR targets are shown for TCR signaling (**a**) TGFβ signaling (**b**) TLR signaling (**c**) JAK/STAT signaling (**d**) and mTOR signaling (**e**). Color coding of the pathways indicate over- (red) and under- (blue) expression of deregulated mRNAs (boxes) and deregulated miRs (miR). Left part of boxes indicates deregulation in resting and right part of boxes indicates deregulation in stimulated Treg cells. Joint miR/mRNA data are in Supplementary Table S4.

In recent years, a few studies have dealt with the acquisition of either miR[10–14] or mRNA[12,14–17] expression profiles of Treg cells from mice and humans. As expression of both miR and mRNA depend on each other across multiple interaction modes of transcription,[25] we acquired both transcript levels in parallel to optimize pathway detection. Considering further that Treg cells isolated from human peripheral blood comprise different subpopulations of naturally and adaptive/inducible Treg, we suggest that the parallel acquisition of miR and mRNA levels within the same cell preparation but from multiple donors should reduce inter- and intralaboratory variability. We also assessed the validity of our gene expression data by comparing them with previously defined criteria,[19] such as the presence of important Treg-associated marker genes in our gene lists and the enrichment of certain biological processes (Table 1), thus further solidifying the findings in this study.

Among the differentially regulated miRs identified in our study, miR-10a, miR-31, miR-145 and miR-146a have been previously described in Treg, probably indicating the robust role of these miRs in Treg biology. This is underlined by several studies, including one by Jeker *et al.*,[26] who described the expression of miR-10a to maintain Foxp3 expression selectively in mouse Treg cells. Likewise, direct interaction of human miR-31 with FOXP3 mRNA was recently described by Rouas *et al.*,[13] which is well in line with the reduced levels of miR-31 in resting and activated Treg cells described here. Elevated levels of miR-146a (previous ID: miR-146) in Treg were first described in an miR profiling study by Cobb *et al.*[27] Later on, it was demonstrated that miR-146a is critical for the suppressive function of Treg cells by targeting STAT1 (ref. 28) and associated with the pathogenesis of several autoimmune diseases by targeting NF-κB, TRAF6 and IRAK1.[29,30] MiR-145 was introduced by Fayyad-Kazan *et al.*[11] via profiling of magnetic cell-sorted peripheral blood Treg as a negative regulator of CTLA-4.

A combined pathway analysis of miR targets and deregulated mRNA revealed numerous enriched pathways in Treg cells. Several immunologically relevant pathways such as Chemokine-, JAK/STAT-, NF-κB-, TCR-, TGFβ- and TLR signaling were numerically most prominent, followed by cell cycle, growth factor and apoptosis-related signaling pathways (Table 1). Of note, many of these pathways have already been implicated in promoting Treg cell characteristics and in FOXP3 expression, but our data solidify

these findings with a combined miR and mRNA approach.[4,5] Within the TCR signaling pathway, we identified 28 significantly deregulated mRNAs and 14 miR targets (Figure 6a and Supplementary Table S4), highlighting the importance of TCR engagement in promoting Treg functions. TCR engagement triggers different pathway branches including LAT/PLCγ1, phosphoinositol 3-kinase/AKT and MAPK signaling, which process incoming signals. One critical target downstream of LAT/PLCγ1 signaling is NFAT that cooperate with SMAD3 to induce FOXP3 expression.[31] In this context, we found miR-31 inversely correlated with the induced expression levels of its targets NFAT[32] and FOXP3 (ref. 13) both in resting and activated Treg cells (Figure 6a). Regarding the co-stimulatory TCR pathway, we found CD28 to be upregulated in activated Treg cells, which is known to trigger FOXP3 expression via the phosphoinositol 3-kinase/AKT cascade along with NFAT. Further downstream of CD28 signaling, NF-κB regulates expression of several cytokines. Here we found NF-κB1 (p50) to be downregulated both in resting and activated Treg cells, which is in line with the expression of miR-146a. This is also concordant with the finding by Jana *et al.*,[33] that deletion of the p50 subunit enhances FOXP3 expression and Treg generation in $p50^{-/-}$ mice. Moreover, miR-10a and miR-218, both deregulated specifically in stimulated Treg, potentially influence NF-κB signaling by targeting TAK1 (MAP3K7) and IκB. SMAD3 and CBL-B, both of which we found to be upregulated on an mRNA level in resting or activated Treg cells, further connects TGFβ signaling and CTLA-4 engagement positively to the induction of FOXP3 expression (Figure 6b).

In addition to TCR signaling, the Toll-like receptor signaling pathway turned out to be enriched when comparing Treg with Tcon cells, both under resting and stimulated conditions (Table 1). This is in line with previous reports describing TLR-dependent immune modulation of Treg function.[34,35] Here we could identify several components of the MyD88-dependent TLR signaling as targets primarily for the upregulated miR-145 in stimulated Treg and miR-146a both in resting and activated Treg cells. The downregulated expression of the miR-146a target IRAK1, which we specifically observed in stimulated Treg cells, fits well with the model described by Liu *et al.*,[35] who reported transient suppression of FOXP3 expression and regulatory functions after TLR2 engagement. Furthermore, TLR2, MyD88 and TRAF6 have been described as functional targets of miR-146a,[29] although we did not observe changes at mRNA levels. MiR-10a and its target TAK1 together represent another synergistic negative regulator of TLR signaling, which is concordant with the observed decrease in expression of pro-inflammatory cytokines in Treg cells (Figure 6c). Seemingly in contrast, TLR5 engagement has been implicated in FOXP3 induction and enhancement of suppressive capacity in Treg.[34] In line with this observation, we found elevated mRNA levels of TLR5 in Treg cells.

Not surprisingly, key elements of JAK/STAT signaling, notably the cascade initiated by IL-2 including IL-2R, JAK1 and STAT5B, were upregulated in Treg cells. Upstream of FOXP3, we recognized miR-17 as a regulator of JAK1 (Figure 6d). MiR-17 has recently been introduced as an inhibitor of iTreg differentiation, probably by inhibiting CREB1 and TGFβRII receptor.[36]

The nutrition sensing mTOR signaling pathway has emerged as a critical regulator of immune function that integrates diverse micro-environmental signals to instruct T-cell differentiation.[37,38] In this study, we could recognize six validated targets of the miRs miR-31, miR-99a, miR-100, miR-107, miR-125b, miR-218 and miR-145, as part of the mTOR pathway (Figure 6e). Interestingly, the two members of the miR-99 family, miR-99a and miR-100, have been recently implicated in tumor growth and apoptosis directly by targeting mTOR.[39] Sun *et al.* further demonstrated that mTOR is regulated by these miRs via translational repression, which corresponds well with our finding that Treg and Tcon express equal mRNA levels of mTOR despite changes in miR-99a and miR-100 expression. Downstream signaling by mTORC1 is critical for Th1 and Th17 cell fate determination by regulating the lineage-specific transcription factors T-bet and RORγt. Here we observed elevated levels of the Th17-inducing factor RORγt in Treg, even though it is believed that FOXP3 antagonizes RORγt function in favor of Treg differentiation instead of Th17 commitment.[40] This may implicate that the counterbalance between RORγt and FOXP3 is tightly controlled to maintain a certain degree of $CD4^+$ T-cell plasticity, which becomes apparent in Treg that loose suppressive function on FOXP3 downregulation while gaining effector function.[41] Indeed, several upstream regulators of FOXP3, including DNA methyltransferase DNMT3b, the FOXO-regulators FOXO1 and FOXO3, GATA3 and ID3, were differentially regulated in Treg and Tcon cells (Figure 6e). With miR-145, whose target EIF4E is downregulated in stimulated Treg cells, and miR-125b with its targets STAT3 and ULK3, we could identify two more miRs with potential function in mTOR signaling. In conjunction with the decreased expression of ULK2 and the increased expression of CAB39L, the miR and mRNA expression characteristics of Treg provide further insights into the connection of autophagy and energy metabolism with Treg function (Figure 6e).

Being a 'master regulator' of Treg cells, the regulation of FOXP3 expression needs to be tightly controlled and coordinated by diverse signaling pathways. Importantly, these pathways are not unique for Treg but also function in effector T cells. Activation-dependent upregulation of FOXP3 in Tcon is well documented.[42] Here we provide evidence that the simultaneous plotting of miR and mRNA expression data of resting and activated T cells allows conclusions about different pathway activities between Treg and Tcon cells.

Although the simultaneous interpretation of miR data provides information at a second transcript level, the outcome strongly depends on the accuracy of prediction algorithms or on the description of experimentally verified miR–target interactions. Because of high false-positive rates accompanied by target prediction, we retrieved known miR–target interactions from miRTarBase.[21] Of note, when using a knowledge-based approach it is necessary to appreciate that the content of this database is a reflection of scientific interests at a given time, which will influence data interpretation and enrichment analysis to some degree.

This study was undertaken to enrich our knowledge of Treg-associated pathways with the aim of deciphering new potential targets for manipulating Treg function *in vivo*. Besides well-known complexes such as CTLA4, CD25 or mTOR, which can be pharmacologically influenced to inhibit or enhance Treg function,[43] we also identified other potentially valuable targets for which pharmacological compounds have already been described, such as JAK1, STAT5b, phosphoinositol 3-kinase, Serum/glucocorticoid-regulated kinase 1 and DNA methyltransferase (DNMT).[43–46]

Many of the pathways described here are involved in diverse physiological processes. In-depth biological analysis and profound understanding of these regulatory networks is a prerequisite for selective therapeutic intervention. This detailed analysis of the mutual relationship of miRs and mRNAs in Treg-specific pathway activities should aid in the future identification of pharmacological compounds or miRs to manipulate dysfunctional Treg activities in autoimmunity, transplantation, infection and cancer.

MATERIALS AND METHODS

Isolation and stimulation of human peripheral blood T-cell populations

Human peripheral blood mononuclear cells were isolated from heparinized blood samples from five different healthy donors, in accordance with the local ethical committee of the LMU München, over a Ficoll-Hypaque 1077 density gradient (Pharmacia, Uppsala, Sweden). Before FACS sorting, untouched $CD4^+$ T cells were separated using the $CD4^+$ T-cell Isolation

Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Selected $CD4^+$ cells were stained with FITC-anti-CD127 (eBiosciences, San Diego, CA, USA), PE-anti-CD25 and APC-anti-CD4 (Miltenyi Biotec) antibodies, and $CD4^+CD25^+CD127^{low}$ Treg cells and $CD4^+CD25^-$ Tcon cells were sorted using a FACS Aria II (Becton Dickinson, San Jose, CA, USA). The purity of both subpopulations was reanalyzed with a FACS Canto II cytometer (Becton Dickinson) based on surface expression of CD4, CD25 and CD127, and intracellular expression of FOXP3 with PerCPCy5.5-anti-FOXP3 (eBiosciences). Purity of sorted $CD4^+CD25^+CD127^{low}$ and $CD4^+CD25^-$ cells was always $>96\%$ based on CD25 expression and $>93\%$ based on FOXP3 expression. One half of the sorted cells was directly used for RNA isolation and the second half of the T cells was stimulated for 48 h with CD3/CD28 beads (Dynal Biotech, Oslo, Norway) at a ratio of 8:1 (cells:beads) in a 96-well round-bottom microplate in RMPI 1640 medium supplemented with 2 mM glutamine, 100 U ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin and 10% fetalcalf serum (PAA Laboratories, Pasching, Austria) at a density of 10^5 cells per well. Regulatory activity of sorted cells was determined in a mixed lymphocyte reaction as previously described.[24] Freshly isolated Treg and Tcon cells were added to a mixed lymphocyte reaction consisting of carboxyfluorescein diacetate succinimidyl ester-labeled responder $CD3^+$ cells from the original donor and allogeneic mitomycin-treated CD3-depleted stimulator APC at a ratio of 5×10^4 to 2.5×10^5 cells per 96-well in complete RPMI media. Proliferation of carboxyfluorescein diacetate succinimidyl ester-labeled effector T cells was measured after 5 days in culture. T cells that had undergone at least one cell division were designated as alloreactive T cells.

RNA preparation

Total RNA was isolated from T cells using the miRNeasy Mini kit (Qiagen, Hilden, Germany). RNA purity based on A260/A280 ratio was calculated using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and was always above 1.9. RNA integrity was confirmed using the Agilent Bioanalyzer with an RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA, USA) and only included for array studies when >9. RNA was subpooled in two entities comprising three different sorts to perform miR and mRNA array data in replicates.

TaqMan low-density array

Reverse transcription was performed with 100 ng of total RNA with the Taqman miR reverse transcription kit and cDNA was pre-amplified using the human Megaplex RT kit (Applied Biosystems, Carlsbad, CA, USA). MiR profiling was carried out using Taqman low-density arrays on an ABI Prism 7900HT Sequence Detection System according to the manufacturer's protocol (Applied Biosystems). Normalization was done with the cyclic LOESS method.[47]

Agilent human GE 4×44 k v2 microarray

Gene expression profiling was carried out with 90 ng Cyanine-3-labeled RNA hybridized on Agilent human whole-genome microarrays (4×44 k) at IMGM Laboratories (Munich, Germany). Statistical analysis was carried out on background-corrected data (gProcessedSignal) produced by Feature Extraction (v10.7.3.1, Agilent Technologies). The data discussed in this publication have been deposited in Gene Expression Omnibus[48] and are accessible through accession number GSE54953.

Statistical analysis

Differential miR and mRNA expression was analyzed in R (version 2.13.1) using the Limma (Linear Models for MicroArray) package.[49] MiRs, including those confirmed by quantitative real-time reverse transcription–PCR, and mRNAs with a *P*-value <0.05 of the moderated *t*-test were considered as differentially expressed.

Quantitative real-time PCR

Quantitative real-time PCR was performed on a StepOnePlus instrument with the original samples in triplicates using Taqman miR assays according to the manufacturer's protocol (Applied Biosystems). Relative expression of miR-17, miR-99a, miR-107, miR-135b, miR-424, miR-455, miR-511 and miR-526b* was calculated by the ΔΔCt method after normalization to RNU44 and RNU48.

Data mining

Human miR targets were retrieved from miRTarBase as of 7 August 2013.[21] Pathway enrichment analysis was performed with the database for annotation, visualization and integrated discovery (DAVID v6.7) with the databases of BioCarta and KEGG.[19,22] *P*-values were determined by the Ease Score algorithm, a modified Fisher's exact test. Gene lists containing deregulated genes and miR targets expressed in resting and activated Treg cells were uploaded from Supplementary Tables S2 and S3. Enriched pathways (Fisher's exact *P*-value <0.1) were identified using the Ease Score algorithm, a modified Fisher's exact test.[19,22] Gene-set enrichment analysis using log2 ratio of classes for metric ranking of genes was conducted to correlate gene expression array results with previously described gene sets.[50] Gene sets of deregulated mRNAs of resting and activated Treg were retrieved from Pfoertner *et al.*[12] and Sadlon *et al.*[14]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This project was supported in part by the Wilhelm-Sander-Stiftung and by the 'Mehr LEBEN fuer krebskranke Kinder—Bettina—Braeu-Stiftung' to MHA.

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Supplementary Information accompanies this paper on Genes and Immunity website (http://www.nature.com/gene)