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MicroRNA Regulation of Molecular Networks Mapped by Global MicroRNA, mRNA, and Protein Expression in Activated T Lymphocytes

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MicroRNAs (miRNAs) regulate specific immune mechanisms, but their genome-wide regulation of T lymphocyte activation is largely unknown. We performed a multidimensional functional genomics analysis to integrate genome-wide differential mRNA, miRNA, and protein expression as a function of human T lymphocyte activation and time. We surveyed expression of 420 human miRNAs in parallel with genome-wide mRNA expression. We identified a unique signature of 71 differentially expressed miRNAs, 57 of which were previously not known as regulators of immune activation. The majority of miRNAs are upregulated, mRNA expression of these target genes is downregulated, and this is a function of binding multiple miRNAs (combinatorial targeting). Our data reveal that coordination of this complex signature, rather than single miRNAs, is necessary to construct a full picture of miRNA-mediated regulation. Molecular network mapping of miRNA targets revealed the regulation of activation-induced immune signaling. In contrast, pathways populated by genes that are not miRNA targets are enriched for metabolism and biosynthesis. Finally, we specifically validated miR-155 (known) and miR-221 (novel in T lymphocytes) using locked nucleic acid inhibitors. Inhibition of these two highly upregulated miRNAs in CD4+ T cells was shown to increase proliferation by removing suppression of four target genes linked to proliferation and survival. Thus, multiple lines of evidence link top functional networks directly to T lymphocyte immunity, underlining the value of mapping global gene, protein, and miRNA expression. The Journal of Immunology, 2011, 187: 2233–2243.

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prediction algorithms, we demonstrated globally that targets of multiple upregulated miRNAs (combinatorial targeting) have decreased mRNA expression with activation. In validation, we showed that inhibition of two highly upregulated miRNAs in CD4+ T cells increased proliferation by removing suppression of four target genes involved in proliferation and survival. Thus, our studies provide novel evidence for a large number of functional molecular networks populated by downregulated targets of highly upregulated miRNAs.

Materials and Methods

T lymphocyte isolation

Blood draw for this study was accepted by our institution’s ethical commission, and all subjects gave their written consent according to review board guidelines. CD2+ T lymphocytes were purified from Ficoll-Hypaque density-separated PBMCs of seven healthy human donors. MACS CD2+ micromagnetic beads were used for the positive isolation of CD2+ T cells using an MACS separator with LS columns (Miltenyi Biotec). CD4+ T lymphocytes were isolated from PBMCs of three human blood donors by negative selection with the MACS CD4+ T Cell Negative Isolation Kit II (Miltenyi Biotec) according to the manufacturer’s protocol.

Lymphocyte activation, RNA, and protein isolation

Freshly isolated CD2+ T lymphocytes were resuspended in RPMI 1640 medium (HyClone) supplemented with 10% (v/v) FBS and 2 mM glutamine, with penicillin (100 U/ml) and streptomycin (100 U/ml), and activated with CD3/CD28 Dynal beads (Invitrogen) according to the manufacturer’s protocol. Cell activation was confirmed by flow cytometry for the following activation markers: CD134 (OX40), CD150 (SLAM), CD25 (IL-2Rα), CD69, and CD71 (transferrin receptor); and intracellular cytokines: IFN-γ, IL-10, IL-2, IL-4, and TNF-α for activated T cells as shown previously (1). The cells were harvested and stabilized in RNALater (Ambion) at 0, 24, 48, and 72 h postactivation. Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion), which also allows for the isolation of the total proteome fraction.

miRNA profiling

TaqMan stem-loop RT-PCR method (65) was performed on an ABI 7900HT Real-Time PCR system (Applied Biosystems) for 420 human miRNA primer/probes on 0.5 μg total RNA from 0-, 24-, 48-, and 72-h CD2+ T lymphocyte samples. A two-tailed Student t test with a p value threshold of 0.05 and false discovery rate (FDR)-adjusted p value (q-value) threshold of 0.1, for which q-value = p value × number tested/rank, between 0 and 48 h was used on the normalized data to identify differentially expressed miRNAs. A q-value of 0.1 implies that 10% of significant tests will result in false positives. miRNA expression following locked nucleic acid (LNA) nucleoporation in CD4+ T cells was measured with TaqMan MicroRNA Assays (Applied Biosystems) for hsa-miR-155 and -221 in accordance with manufacturers’ protocols. U6 was used as an internal control.

Microarray profiling

A total of 1.5 μg total RNA per sample was converted into labeled cDNA using the GeneChip WT Sense Target Labeling kit (Affymetrix). Labeled cDNA was hybridized to Affymetrix Human Exon 1.0 ST arrays (Affymetrix). Data for mRNA transcript profiles were generated in the form of CEL files using standard protocols.

Microarray data

Data have been deposited in the Gene Expression Omnibus under accession number GSE14352 and can be viewed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14352.

Differential gene expression analysis

Raw data expression values from CEL files were normalized by robust multivariate averaging algorithm provided through Affymetrix Power Tools (Affymetrix) and summarized in AltAnalyze software (http://altanalyze.org), retaining only probesets that align to a single Ensembl gene. Gene expression values were calculated based on the mean expression of all core probesets detected above background (p value thresholds for p < 0.01; FDR 0.01). Fold changes and p value (two-tailed t test assuming unequal variance) were calculated for each time point comparison. Statistics were performed for specified pairwise comparisons among all time points of activation. In parallel, we performed our own analysis of differential gene expression to corroborate AltAnalyze results. CEL files for each donor from the 1.0ST HuEx Arrays were normalized by robust multiarray averaging using a custom cumulative distribution function downloaded from the University of Michigan (http://brainarray.ncbi.nlm.nih.gov/Brainarray/DataBase/Custom/5D/genomic_curated_CDF.asp). Differential expression was measured with the Limma package (http://www.bioconductor.org/packages/2.6/bioc/html/limma.html) using a two-class model. All calculations were performed in R/Bioconductor. Genes were filtered using a fold-change filter of 1.5 (0.58 in log2) and an FDR filter of 0.01. Genes that were detected as significantly differentially expressed by both AltAnalyze and Limma analysis were then selected for further analysis.

The Multidimensional Protein Identification Tool proteinomics

The Multidimensional Protein Identification Tool (66) protocol was used as described previously (1). Protein fraction was denatured, alkylated, and trypsin digested. A total of 50 μg digested protein sample at 0 and 48 h was run in four technical replicates. Data were acquired using an LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific) interfaced inline with two-dimensional HPLC in a data-dependent manner in which each analytical full scan (mass spectrometry; range 200–2000 mass-to-charge ratio units) was followed by three fragmentation scans (tandem mass spectrometry) that targeted the three most abundant ions from the full scan. The 40-microsecond collision-induced dissociation pulses of 35% intensity were used for precursor ion fragmentation. A default exclusion list (Xcalibur 2.0; Thermo Fisher Scientific) of 180-s, 50 precursor ion members was used for data acquisition. Raw data were searched against the European Bioinformatics Institute database (12/01/2006 release) supplemented with a database in which all entries of the original protein contains its reversed sequence. Database searching used SEQUEST (v27), and outcomes were filtered using DTASelect version 2.0. Protein identifications were extracted, and a measure of normalized amino acid coverage was used as label-free quantification. Relative quantifications were done using spectral counts normalized to the median of the total spectral counts. Protein identifications across replicate experiments were pooled to represent unique peptides per category of 0 and 48 h postactivation. Proteins identified in two or more technical replicates per category were kept for further analysis. Relative protein abundance was compared between the 0- and 48-h postactivation for proteins present in more than one category. Proteins identified in more than one technical replicate in a single category and not in any other category were also considered for functional analysis as unique identifications. A two-tailed Student t test was used for hypothesis testing, and the significant differentially expressed proteins (p < 0.05) were considered for functional analysis.

miRNA target analysis

For prediction of target genes of differentially expressed miRNAs, three publicly available algorithms were used: PITA, MiRanda, and TargetScan 5.1. In the end, TargetScan predictions based on conservation scores were used to compute the 50th percentile targets in our expressed gene set.

Functional mapping

We used Ingenuity Pathways Analysis (IPA; https://analysis.ingenuity.com) to map molecular pathways and networks populated by predicted miRNA targets. The IPA Database is a constantly curated resource of published literature on gene functions and interactions. Canonical pathway and networks analysis was carried out by uploading the predicted downregulated genes targeted by the upregulated miRNAs. Significance of association between genes and pathway was measured by the Benjamini and Hochberg multiple testing corrected p value that can be interpreted as an upper bound for the expected fraction of falsely rejected null hypotheses among all functions with p values smaller than the threshold of 0.05. Network node genes were based on an especially high degree of links to other genes in the IPA database.

Electroporation

A total of 3 × 10^6 primary human CD4+ T lymphocytes were electro- porated in Nucleofector II instrument (Amaxa) using the Human T cell Nucleofector kit (VP-1002; Lonza) in duplicate with 50 nM miRCURY LNA microRNA Power Inhibitor or scrambled negative control probes (Exiqon) against hsa-miR-221 and -155 according to the manufacturer’s protocol. After electroporation, cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and 2 mM glutamine, and after 2 h, half of the medium was replaced with fresh medium. At 24 h after electro-
poration, the cells were activated with CD3/CD28 Dynal beads (Invitrogen) for 48 h.

**Cell proliferation assay**

Cell proliferation was measured using the Ziva Cell Proliferation Assay (Jaden BioScience). Electroporated CD4+ T lymphocytes were plated at $8 \times 10^5$/well in 96-well plates in duplicate for each condition and activated for 48 h with CD3/CD28 beads. Cells were pulsed with 10 μM BrdU/well 18 h before harvesting. Forty-eight hours after activation, cells were harvested, expander beads removed, and 100 cells/well were plated in 96-well Thermo Scientific Nuncl Plates (Fisher). Cell proliferation was measured with a chemiluminescent substrate to detect the presence of an anti-BrdU Ab labeled with alkaline phosphatase on the Insight-Mi Luminometer (Jaden BioScience). The signal was fully developed and measured 60 min after the addition of the substrate.

**Quantitative RT-PCR**

A total of 300 ng total RNA from CD4+ T cells was transcribed into cDNA using qScript cDNA Supermix (Quanta Biosiences) according to the manufacturer's guidelines, and gene expression for IRS2, IKKB, FOS, and PIK3R1 was quantitated with PrimeTime qPCR Assays (Integrated DNA Technologies) using Perfecta qPCR FastMix kit (Quanta Biosciences) on the ABI 7900HT Fast Real-Time PCR instrument (Applied Biosystems). Expression of 18S gene was set as endogenous control. For data analysis, the threshold cycle (Ct) value was determined and specific gene expression normalized to endogenous control using 2^-ΔΔCt method. The normalized ΔΔCt from LNA-transfected samples was then compared with the scrambled control to obtain the change (ΔΔCt) in gene expression.

**Statistics**

All statistical analyses used the Student t test of at least three independent experiments, unless stated otherwise. Differences with p values <0.05 are considered significant.

**Results**

**Activated T lymphocytes demonstrate a unique activation-induced miRNA signature**

We used a multidimensional approach to integrate genome-wide miRNA, mRNA, and protein expression (Fig. 1A). We activated human T lymphocytes via CD3/CD28 costimulation and harvested cells at 0, 24, 48, and 72 h. This activation strategy modeled T lymphocyte activation is marked by global gene upregulation (1). We therefore analyzed differential gene expression in parallel with miRNA expression. Genome-wide miRNA transcript analysis revealed 3798 differentially expressed mRNA transcripts between 0 and 48 h (p < 0.01; FDR 1%): 3362 upregulated (89%) and 436 downregulated targets that mapped to known functional pathways. In contrast, plotting the gene expression of targets predicted to bind multiple upregulated miRNAs (e.g., ≥4 or ≥7) revealed that combinatorial miRNA binding decreases miRNA expression (Fig. 1C, 1D). Combinatorial targeting benchmarked at the 50th percentile with both PITA and TargetScan gave the best predictions (Fig. 1E–J). As shown in Fig. 1I and 1J, TargetScan conservation predictions with combinatorial binding of four or more miRNAs show the best results correlating increased miRNA binding with decreased target gene expression.

**T lymphocyte activation is marked by global gene upregulation including miRNA-processing machinery**

We showed previously that T lymphocyte activation is dominated by widespread differential gene upregulation (1). We therefore analyzed differential gene expression in parallel with miRNA expression. Genome-wide miRNA transcript analysis revealed 3798 differentially expressed miRNA transcripts between 0 and 48 h (p < 0.01; FDR 1%): 3362 upregulated (89%) and 436 downregulated. Upregulation of the miRNA processing/biogenesis genes included: XPOS, EIF2CC2/AGO2, SIP1/GEMIN2, -4, -5, -6, and -7, RANGAP1, YBX1, and ADARB1 (Table III).

**Predicted targets of upregulated miRNAs populate networks associated with immunity, cell survival, and proliferation**

Using TargetScan conservation predictions, we identified 1640 candidate miRNA targets, of which 214 were downregulated (Supplemental Table II). Thus, half of all 436 downregulated genes are targets of upregulated miRNAs. Functional pathway and network enrichment analysis was done for the 182 out of 214 downregulated targets that mapped to known functional pathways and the 200 that mapped to molecular networks.
FIGURE 1. Combinatorial targeting by multiple upregulated miRNAs during T cell activation demonstrates decreased mRNA levels after activation with increased miRNA binding. A, Schematic of our experimental approach. B, miRNA signature of T cell activation: heat map of 71 statistically significant (p < 0.05; q < 0.1), differentially expressed miRNAs at 0 and 48 h. Heat map shows expression at 0, 24, 48, and 72 h across seven donors. Red represents positive change, cyan represents negative change, and white represents no change. C, A cumulative distribution function plot of relative fold change between 0 and 48 h of combined PITA, TargetScan/conservation, and TargetScan/context; top 50th percentile predictions in each, 2+ miRNAs targeting each gene. Target genes in red; nontarget genes in blue. D, Same as A with 4+ miRNAs targeting each gene. E, PITA, top 50th percentile predictions; 4+ miRNAs targeting a given gene. Target genes in red; nontarget genes in blue. F, PITA, top 50th percentile predictions with 7+ miRNAs targeting a given gene. Target genes in red; nontarget genes in blue. G, TargetScan/context score, top 50th percentile predictions; 4+ miRNAs targeting a given gene. Target genes in red; nontarget genes in blue. H, TargetScan/context score, top 50th percentile predictions; 7+ miRNAs targeting a given gene. Target genes in red; nontarget genes in blue. I, TargetScan/conservation score, top 50th percentile predictions; 4+ miRNAs targeting a given gene. Target genes in red; nontarget genes in blue. J, TargetScan/conservation score, top 50th percentile predictions; 7+ miRNAs targeting a given gene. Target genes in red; nontarget genes in blue.
to T lymphocyte activation, proliferation, and survival (Fig. 2).

We mapped the functional pathways enriched for these two classes of targets. The pathways linked to only downregulated miRNAs should have upregulated induced genes. A total of 487 genes were only targeted by induced miRNAs. A total of 487 genes were only targeted by downregulated miRNAs. In contrast, the majority (860) was also targeted by upregulated miRNAs, matching our observations on the apparent importance of combinatorial targeting. We predicted that targets of only downregulated miRNAs should have upregulated mRNA levels. Indeed, 410 (84%) were upregulated >1.5-fold.

We mapped the functional pathways enriched for these two classes of targets. The pathways linked to only downregulated miRNAs are predominantly cell metabolism and biosynthesis. Because miRNAs targeting these genes are downregulated with activation, the presumed regulation of their targets is removed or at least significantly decreased. The functional role of these genes in metabolism and biosynthesis, much like the genes we found were targeted by only downregulated miRNAs, supports the observation that activation-induced miRNAs are associated with proliferation and cell survival signaling networks. We identified 12 other target gene networks (Supplemental Table III).

Correlating global protein expression to predicted miRNA targets

Our hypothesis was that upregulated miRNAs regulate the immune response and should repress target proteins during T lymphocyte activation. Target protein repression can be accomplished by either inhibiting translation or enhancing mRNA degradation. Although it has been shown that most translational repression is coupled to regulated miRNAs. The central node gene PIK3R1 belongs to the phosphoinositide 3-kinase family that phosphorylates phosphatidylinositol-(4,5)-biphosphate to phosphatidylinositol-(3,4,5)-triphosphate to regulate cell proliferation, and cytokine production (75). PIK3R1 is a predicted target of four miRNAs (miR-155, -21, -21B, and -218, and -221). Thus, downregulated gene targets of upregulated activation-induced miRNAs are associated with proliferation and cell survival signaling networks. We identified 12 other target gene networks (Supplemental Table III).

Predicted targets of downregulated miRNAs are upregulated with activation

With respect to the impact of the 20 downregulated miRNAs, we predicted 1347 gene targets out of the total of 3798 activation-induced genes. A total of 487 genes were only targeted by downregulated miRNAs. In contrast, the majority (860) was also targeted by upregulated miRNAs, matching our observations on the apparent importance of combinatorial targeting. We predicted that targets of only downregulated miRNAs should have upregulated mRNA levels. Indeed, 410 (84%) were upregulated >1.5-fold.

We mapped the functional pathways enriched for these two classes of targets. The pathways linked to only downregulated miRNAs are predominantly cell metabolism and biosynthesis. Because miRNAs targeting these genes are downregulated with activation, the presumed regulation of their targets is removed or at least significantly decreased. The functional role of these genes in metabolism and biosynthesis, much like the genes we found were not targeted by miRNAs, supports the observation that activation-induced miRNAs target a functionally distinct class of genes. In contrast, the pathways linked to combinatorial targeting by both up- and downregulated miRNAs are enriched for signaling in immunity, growth, and cell proliferation (Fig. 3).

Pathway analysis revealed statistically significant enrichment for 71 canonical pathways (multiple test correction p value <0.05), with the top 30 pathways shown in Fig. 2A. Represented were primarily immune signaling pathways including IL-12, PI3K, IL-10, NFAT, sphingosine 1-phosphate, and TCR. Cell survival, growth, and proliferation pathways included prolatin, TNFR2, ceramide, thrombopoetin, and p70S6K signaling. In sharp contrast, differentially expressed genes that were not predicted targets of miRNAs were highly enriched for metabolism and biosynthesis pathways (Fig. 2B).

Molecular networks were constructed from the miRNA targets with downregulated expression. Network eligibility was based on connectivity to other genes with known interactions. Highly connected genes represent network nodes or hubs where closely connected genes are functionally similar. The top network was comprised of 21 genes significantly enriched for functions linked to T lymphocyte activation, proliferation, and survival (Fig. 2C).

The hub genes in this network are PIK3R1 with six connections and ATM, PARK2, HIP1R, and NCAM1 with three connections each. Members of this network are predicted targets for 17 up-
miRNA-mediated mRNA degradation (12, 14), we considered the possibility that some miRNA targets might be specifically repressed at the translational level without decreases in corresponding mRNAs. A focus exclusively on the downregulation of mRNAs would miss such targets. Therefore, a high-throughput shotgun proteomics protocol (66) was used to analyze global protein expression between 0 and 48 h.

A total of 589 differentially expressed proteins and another 876 proteins expressed uniquely at 0 or 48 h were identified. Correlating the predicted gene targets of miRNAs to expressed proteins, we identified 234 protein–mRNA transcript targets of upregulated miRNAs (Supplemental Table IV). Eighty-one of these proteins were enriched for only four pathways: RAN signaling; glycolysis/gluconeogenesis; tyrosine metabolism; and amino acid metabolism.

Because networks represent integration of multiple associations, we examined the top 3 networks to identify 19 downregulated genes for validation by qPCR: PIK3R1, FOS, IRS2, and CDKN1A. Among the predicted targets of miR-221 and miR-155, we chose four genes for validation by qPCR: PIK3R1, FOS, IRS2, and CDKN1A.

Several network proteins demonstrated increased mRNA but decreased protein levels (Fig. 4B). LMNB1, a predicted target of miR-218 and miR-7, showed the highest mRNA upregulation by 7-fold but was 1.5-fold down by protein. Inhibition of T lymphoblast proliferation is associated with downregulation of LMNB1 protein (78). CRKL is targeted by four miRNAs and involved in signal transduction through WIP, JNK, and ZAP70 (79). IQGAP1, targeted by two miRNAs, regulates lymphocyte cytoskeleton rearrangement in the immune synapse (80). Thus, these multiple lines of evidence linking the top functional networks directly to T lymphocyte immunity underline the value of such mapping based on global gene, protein, and miRNA expression.

**Table III. miRNA-processing machinery genes detected at 48 h postactivation**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Definition</th>
<th>Function</th>
<th>Fold Change</th>
<th>miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP05</td>
<td>Exportin-5</td>
<td>Mediates the nuclear export of mRNA precursors</td>
<td>4.30</td>
<td>miR-218, miR-24</td>
</tr>
<tr>
<td>RANGAP1</td>
<td>Ran GTPase-activating protein 1</td>
<td>mRNA processing and transport</td>
<td>1.60</td>
<td>ND</td>
</tr>
<tr>
<td>EIF2C2/AGO2</td>
<td>Protein argonaute-2</td>
<td>Provides endonuclease activity to RISC; cleaves siRNA/miRNA heteroduplexes bound to RISC</td>
<td>3.84</td>
<td>ND</td>
</tr>
<tr>
<td>SIP1/GEMIN2</td>
<td>Survival of motor neuron-interacting protein 1</td>
<td>Core component of the SMN complex, which plays an essential role in spliceosomal snRNP assembly in the cytoplasm and is required for pre-mRNA splicing in the nucleus</td>
<td>2.23</td>
<td>ND</td>
</tr>
<tr>
<td>GEMIN4</td>
<td>Gem-associated protein 4</td>
<td>Component of the SMN complex, which is required for pre-mRNA splicing in the nucleus</td>
<td>1.59</td>
<td>miR-155</td>
</tr>
<tr>
<td>GEMIN5</td>
<td>Gem-associated protein 5</td>
<td>Component of the SMN complex, which is required for pre-mRNA splicing in the nucleus</td>
<td>2.13</td>
<td>ND</td>
</tr>
<tr>
<td>GEMIN6</td>
<td>Gem-associated protein 6</td>
<td>Component of the SMN complex, which is required for pre-mRNA splicing in the nucleus</td>
<td>2.11</td>
<td>ND</td>
</tr>
<tr>
<td>GEMIN7</td>
<td>Gem-associated protein 7</td>
<td>Component of the SMN complex, which is required for pre-mRNA splicing in the nucleus</td>
<td>1.90</td>
<td>ND</td>
</tr>
<tr>
<td>YBX1</td>
<td>Nuclease-sensitive element-binding protein 1</td>
<td>Participates in different steps of mRNA biogenesis, including mRNA transcription, processing, and transport from the nucleus into the cytoplasm, binds to splice sites in pre-mRNA, and regulates splice site selection</td>
<td>1.53</td>
<td>ND</td>
</tr>
<tr>
<td>ADARB1</td>
<td>dsRNA-specific editase 1</td>
<td>Binds to siRNA without editing them and suppresses siRNA-mediated RNA interference</td>
<td>1.50</td>
<td>miR-218</td>
</tr>
</tbody>
</table>

**Table IV. miRNA-processing machinery genes detected at 48 h postactivation**

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Gene</th>
<th>Fold Change</th>
<th>miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-218</td>
<td>4.30</td>
<td>miR-218, miR-24</td>
<td></td>
</tr>
<tr>
<td>miR-7</td>
<td>1.60</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>miR-155</td>
<td>3.84</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*The FDR-adjusted p values of every gene are <0.007. Shown are genes implicated in miRNA processing/biogenesis that were significantly differentially expressed between 0 and 48 h, with relative fold changes.

ND, miRNA not detected; RISC, RNA-induced silencing complexes; siRNA, short interfering RNA; SMN, survival of motor neuron; snRNP, small nuclear ribonucleoprotein.

Knockdown of miR-221 and miR-155 increases T lymphocyte proliferation by removing negative regulation of target genes

The premise of target predictions and functional network mapping is that upregulated miRNAs regulate genes that populate critical networks in T lymphocyte activation. To validate our approach, we knocked down two of the highest upregulated miRNAs: miR-221 and miR-155. Validations were done using purified CD4+ T lymphocytes to simplify the cell subset composition and reflect our recent finding that CD4+ T lymphocytes are selectively activated in the early posttransplant period (81). We confirmed that miR-221 and 155 were significantly upregulated in CD4+ T cells at 48 h of activation (data not shown).

Although the function of miR-155 has been widely studied in CD4+ T lymphocytes (82), the function of miR-221 has not been studied in T lymphocytes (44) has not been studied in T lymphocytes.

The impact of inhibiting miR-221 and -155 on T lymphocyte proliferation was measured by transfecting cells with specific inhibitors or scrambled controls followed by activation. We obtained >60% knockdown of both miRNAs in three donors (n = 3) as measured by qPCR (Fig. 4C). Significantly increased proliferation resulted from inhibiting either miR-155 or -221 as compared with scrambled control (Fig. 4D).
IKBKE (Fig. 4E). FOS and IKBKE have been previously validated as targets of miR-221 (82) and -155 (83), respectively. At 48 h after activation, the expression of FOS and PIK3R1 is statistically increased by knocking down these two miRNAs. Although changes in IKBKE levels were not statistically significant, IRS2, a predicted target of miR-155, increased expression after miR-155...
knockdown. Upregulation of target genes following knockdown of miR-221 and -155 is consistent with the evidence above for mRNA repression mediated by miR-221 and/or -155 during T lymphocyte activation (Fig. 4F).

**Discussion**

We investigated genome-wide miRNA, mRNA, and protein expression following human T lymphocyte activation. T lymphocyte activation relies on signaling cascades that create a balance between activation, memory, and quiescence. This balance is modulated by mechanisms regulating gene expression including posttranscriptional miRNA regulation. In this study, we show a unique miRNA signature with a total of 71 differentially expressed miRNAs with 51 upregulated between 0 and 48 h. This signature comprises 57 miRNAs with no documented roles in T lymphocyte function. Additionally, our data validated previous findings for a number of miRNAs with known functions in T lymphocytes: upregulation of miR-155 can regulate the susceptibility of human and murine CD4+ T cells to natural regulatory T cell-mediated suppression (26), the miR-17–92 cluster inhibits T cell activation (30, 32), miR-106a is implicated in IL-10 regulation (31, 33), miR-24 can inhibit cell proliferation by targeting cell-cycle genes (6, 47), and miR-21, upregulated by STAT3, prevents CD4+ T cell apoptosis and is implicated in lymphocyte oncogenesis (18, 53). Also, consistent with the importance of activation-induced miRNA expression, we observed upregulation of 10 miRNA biogenesis/processing machinery genes.

miRNAs are known inhibitors of gene expression. The challenge is to map miRNAs to specific gene targets and the molecular networks they regulate. To address this challenge, we investigated the predictive values of four widely used computational algorithms. First, if the results from all of the algorithms are compared using a single miRNA hit/seed approach, the predicted targets are poorly correlated between methods to the extent that different methods will report very different results. Second, single hit/seed predictions did not correlate with mRNA repression. In contrast, combinatorial targeting (multiple seeds per target) gave the best predictions. TargetScan conservation predictions with combinatorial binding of four or more miRNAs showed the correlation between increased miRNA binding with decreased target gene expression.

By integrating activation-induced miRNA, mRNA, and protein expression changes with target predictions, we tested our hypothesis that target genes are involved in regulating immune activation, cell proliferation, and survival. Indeed, functional analysis demonstrated that downregulated miRNA targets populated signaling pathways highly enriched for immune response, proliferation, and survival. In contrast, activation-induced genes not predicted to be miRNA targets demonstrated significant enrichment for pathways of metabolism, DNA stability, and cell cycle. These novel results reveal that predicted targets of activation-induced miRNAs are functionally distinct from nontarget genes and presumably evolved with distinct selection pressures for such regulation. We also hypothesized that some targets might be specifically regulated by posttranscriptional mechanisms not coupled to mRNA decay. Based on our proteomics, we detected a number of such downregulated protein targets despite increased mRNA expression. Thus, inhibition of protein translation is not always coupled to corresponding mRNA degradation.

By investigating connectivity between predicted targets, we identified highly connected genes that function as network nodes, with closely connected genes being functionally similar. The top nodal gene PIK3R1 of one such network is a predicted target of
miR-221 and -155 and downregulated at both mRNA and protein levels. This gene is an adaptor kinase involved in TCR signaling and CD28 costimulation and regulates cell growth, proliferation, and T cell cytokine production (75). Thus, functional network analysis underlines the value of the mapping done in this study based on global gene, protein, and miRNA expression.

In validation, we focused on the functional roles of two top upregulated miRNAs in our data: miR-155, widely studied in T cells, and miR-221, not previously associated with T cell function. Knockdown of either miRNA produced a significant increase in proliferation of activated CD4+ T cells, confirming that these two miRNAs actually have antiproliferative roles during T cell activation.
activation. We identified four potential targets of miR-155 and/or -221, identified two induced genes that are not miRNA targets.

networking mapping that are clearly distinct from the activation-induced genes that are not miRNA targets.

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Disclosures

The authors have no financial conflicts of interest.

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