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OpenArray profiling reveals no differential modulation of miRNA by positive and negative CD4+ T-cell immunoselection

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Blood cell subsets contain unique constellations of microRNAs [1,2], short oligonucleotide effectors of post-transcriptional gene regulation that are important from differentiation to disease [3]. In addition to the promise of small RNA-based therapeutics, expression profiles of these molecules may be useful for diagnosis, prognosis, and monitoring of response to therapy of diseases that affect cells of the blood. Whereas many thousands of messenger RNA species may be present in a given cell type, only several hundred distinct miRNAs, at most, are found within a single cell type, permitting relatively facile measurement and analysis. The conservation and relative lack of diversity of canonical miRNA variants may also allow high-level multiplexing in high throughput sequencing [4], lowering the cost barrier to development of clinically useful assays.

However, selection of specific cell populations could conceivably confound miRNA profiling. Positive selection could stimulate target cells, altering small RNA profiles and thus masking disease-specific information or compromising the use of cells in downstream assays. Negative or “untouched” selection might better preserve native miRNA profiles but achieve less than complete removal of non-target cells, resulting in lower purity.

We conducted a study to identify any large fold differences in miRNAs of CD4+ T-isolated by positive and negative immunoselection. CD4+ T-cells were isolated from de-identified human blood (New York Blood Center) using negative or positive selection (Miltenyi Biotec, #130-096-533 or #130-045-101, following depletion of CD14+ cells with #130-50-201, respectively). Cell purity was determined by flow cytometry as greater than 97% for positively and ~95% for negatively selected cells. Total cellular RNA was isolated using the mirVana protocol, and RNA integrity was verified by Agilent BioAnalyzer (RIN>9 for all samples). For each sample, 100 ng RNA was reverse-transcribed, pre-amplified, and profiled using miRNA OpenArray (Life Technologies) per manufacturer’s

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protocol. Quantile normalization was done using R/Bioconductor. Raw and quantile normalized data are available on request and will also be uploaded to the Gene Expression Omnibus. Three additional normalizations were to the geometric mean of all miRNA with average Crt >30 (“global geometric mean”); to the geometric mean of RNU48, RNU44, and snRNA U6 Crts; and to miR-16.

Approximately 110 medium- to high-abundance miRNAs were detected in all samples [defined as amplification earlier than a relative threshold cycle (Crt) of 30]. Hierarchical clustering of quantile-normalized data revealed little support for global expression changes induced by positive selection of CD4⁺ T-cells (Figure 1A). For individual miRNAs, changes were not statistically significant following multiple comparison corrections. This outcome applied as well when data were normalized to the geometric mean of all miRNA with average Crt >30 (“global geometric mean”), the geometric mean of RNU48, RNU44, and snRNA U6 Crts, or to miR-16 alone. Evidence for influence of selection method was strongest for miR-130b (Figure 1B). In global geometric mean-normalized data, miR-130b was 1.8- to 3.7-fold more abundant in positively selected cells, and nominal “significance” of $p < 0.05$ by paired t-test was recorded for this and for miR-16-normalized data. For results normalized by quantiles but not for the other methods, miR-128a appeared to be slightly downregulated in all positively selected samples, also with a nominally significant p value (Figure 1B). Importantly, however, no miRNA was significantly changed when significance tests were corrected for multiple comparisons.

Confirmatory quantitative stem-loop RT-PCR was done with Life Technologies assays and reagents as described previously [5]. These data were normalized to the average of small RNAs RNU44 and U6. miRs-128a and -130b were tested based on the array results, along with several miRNAs with previously reported modulation in response to stimulation of T-cells or T-cell subsets: miRs-21 [1,6–8], -26a [1,6–9], -342-3p [1,7,9], and let-7g [6]. miR-16 and small RNAs RNU44 and snRNA U6 were also assayed. Cq values were normalized to the average of RNU44 and snRNA U6. Although very slight apparent upregulation of miR-130b and downregulation of miR-128a was again observed, no tested RNA species was significantly differentially expressed (Figure 1C).

To confirm that the cells we used were capable of miRNA differential regulation upon stimulation, and that our approach could detect changes in the levels of miRNA in these cells, we stimulated CD4⁺ T-cells or not with anti-CD3 and anti-CD28 antibodies [10]. miRNAs were assayed by stem-loop RT-qPCR as described above. There was no differential regulation of miRs-16 and -128a or RNU44 and U6. Levels of miRs-21 and -130b increased, as reported elsewhere [1,6–8], while miRs-26a, -342-3p, and let-7g were downmodulated (Figure 1D). These results confirm the findings of other investigators as regards stimulation of T-cells and suggest that our system and techniques were appropriate for detection of real changes in miRNA levels.

We conclude that brief exposure to anti-CD4 antibodies during positive cell selection did not stimulate large fold changes in miRNA expression when compared with a matched negative selection method. These results may bolster confidence that the judicious use of positive

immunoselection, which may result in higher purity of cell populations, does not immediately or drastically alter the miRNA profile of target cells.

Several limitations should be considered when interpreting this study. First, because of the small number of samples, small (i.e., less than two-fold) but consistent fold changes may have been overlooked. Investigators concerned that small fold changes of specific miRNAs could affect results might wish to pursue similar but larger studies. Second, we did not assess the behavior of positively and negatively selected cells in subsequent *in vitro* studies. Third, other cell types, selected using different surface markers, could be more or less sensitive to selection. Nevertheless, our findings offer reassurance that positive selection methods—which are relatively inexpensive on a per-cell basis and may achieve greater purity of isolated cells—may not unduly influence miRNA profiles and may thus be appropriate for isolation of cells for development of clinical assays.

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Figure 1.

(A) CD4⁺ T-cells were isolated from PBMC of three donors (1–3) with positive (+) or negative “untouched” (–) bead-based selection. OpenArray profiling data for miRNAs amplifying before relative threshold cycle (Ct) of 30 were quantile normalized and clustered using Pearson correlation and average linkage. Correlation scale on right indicates relatedness of samples. (B) No individual miRNA was significantly differentially regulated following application of multiple comparison correction to paired t-tests (OpenArray). Shown are two miRNAs with uncorrected and nominally “significant” p values (uncorrected $p < 0.05$ in data normalized by at least one technique of quantile normalization, geometric mean of all $Ct < 30$ (“GM All”), geometric mean of U6, RNU44 and RNU48 (“GM 3”) or miR-16. (C) Individual qPCR assays confirmed a lack of significant differences between positively and negatively selected CD4⁺ T-cells for candidate miRNAs (from OpenArray) or for miRNAs previously reported to be differentially expressed following cell activation. (D) As a positive control, significant differences were confirmed for several miRNAs following activation of CD4⁺ T-cells anti-CD3/anti-CD28 antibodies. For C and D, statistical significance was assessed for positively and negatively selected cells or stimulated versus unstimulated cells by Students t-test and indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). All features marked with multiple asterisks had statistically significant differences following Bonferroni correction for multiple tests. Raw data and quantile normalized processed data have been uploaded to the Gene Expression Omnibus.