Differential expression of microRNAs in human dendritic cells at different functional states

Dendritic cells (DC's) are the most potent antigen-presenting cells of the immune system. Using different stimuli, *in vitro*-derived immature dendritic cells can be polarized towards immunity (Th1 or Th2 responses) or tolerance. We set out to profile the miRNA content of DC's at different functional states to identify potential novel biomarkers. Three microRNAs including hsa-miR-155 and hsa-miR-146a were validated to be up regulated in immunogenic DC's, and one microRNA was found to be more than two-fold up regulated in DC's polarized towards tolerance compared to immature DC's.

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Experimental setup

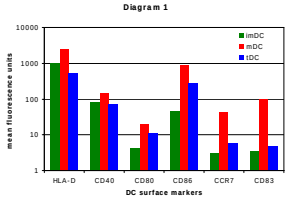
To explore the potential of using microRNA markers to characterize functional states of human dendritic cells (DC's), *e.g.* for immune therapeutic intervention, a total of 5 donors were enrolled in the study. From all 5 donors immature naive DC's were produced, and were included in the microRNA profiling study as individual reference points. For all donors immunogenic, *i.e.* mature, DC's were generated, and for 3 of the donors, in addition, a tolerogenic phenotype was established. MicroRNA profilng was conducted using the miRCURY™ LNA micro RNA array ver. 8.1, by employing a fluorescent dual-labeling approach with dye-swap. For each donor labeled total RNA was mixed accordingly: immature DC versus mature DC (with dye-swap), and immature DC versus tolerogenic DC (with dye-swap). This setup allowed for identification of differentially expressed microRNAs in either the mature or the tolerogenic DC's in relation to the immature state by performing a ratio analysis and statistical testing.

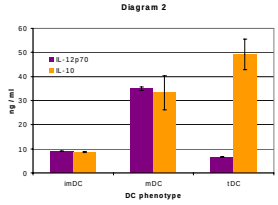
Selected microRNA that showed significant differential expression in either or both of the mature or tolerogenic DC's compared to the reference point were subsequently analyzed using TaqMan microRNA real-time PCR including a further subset of donors.

Establishing and characterizing different human DC phenotypes

Dendritic cells were generated from buffy coat-derived peripheral blood monocytes (PBMC) in the presence of stimuli to induce differentiation towards immunogenic (mature) or tolerogenic phenotypes. Monocytes were isolated by plastic adhesion and cultured in the presence of GM-CSF and IL-4 for 7 days. The resulting semi-adherent cells are termed immature DC (imDC). Immunogenic or mature DC's were established likewise except that on day 6 the cells were exposed to a DC maturation cocktail comprising TNFα, IL-1β, IL-6 and PGE_2. The culture on day 7 are then referred to as mature DC (mDC). The tolerogenic DC's (tDC) were generated by following the exact same procedure as for the mature DC's, except that on days 0, 3 and 5 of the culture, 100 nM 1α,25-dihydroxyvitamin D_3 was added.

The phenotypes of the different DCs of each individual donor were determined by FACS analysis and cell supernatants were collected for measuring levels of the cytokines IL-12p70 and IL-10 using ELISA. Below is shown the results of a representative experiment. Diagram 1 shows the expression of DC surface molecules in the three functional states (imDC, mDC and tDC). Diagram 2 shows the levels of IL-12p70 and IL-10 in imDC, mDC and tDC, respectively.

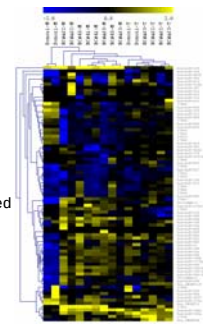


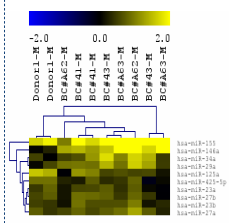


Summary of miRNA profiling

Unsupervised hierarchical clustering:

Unsupervised hierarchical clustering of $\log_2$ transformed ratio values from all microarray analyses including mature and tolerogenic DC's with immature DC's as reference was performed. The clustering was conducted on ratios that had the largest variance, filtering out microRNAs with ratio standard deviations across all samples less then 0.5. A total of 78 microRNAs fulfilled these filtering criteria. Unsupervised clustering clearly segregates all the tolerogenic miRNA expression profiles from the mature miRNA profiles, indicating differences in the microRNA expression pattern in mature (M) and tolerogenic (T) phenotypes



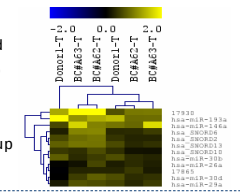


Differential expression in mature DC

Significance analysis of microarray (SAM) was conducted (using 1024 permutations and a false discovery rate (FDR) of 0%) to find significantly differentially expressed miRNAs in the mature DC's compared to the immature phenotype by selecting microRNAs across samples with $\log_2$ transformed ratios significantly different from 0. This analysis revealed that 10 miRNAs were significantly up regulated in the mature DC compared to immature.

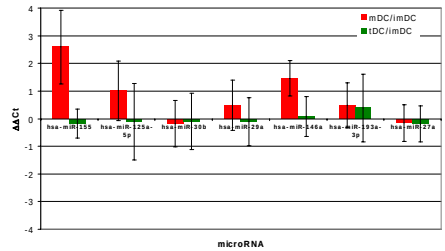
Differential expression in tolerogenic DC

Significance analysis of microarray (SAM) was conducted (using in this case 64 permutations and a FDR of 0%) to find significantly differentially expressed miRNAs in the tolerogenic DC's compared to the immature phenotype. This analysis revealed that 8 miRNAs were significantly up regulated in the tolerogenic DC compared to immature.



Validation of differentially expressed miRNAs using TaqMan based real-time PCR

Seven of the miRNAs selected from the statistical analysis of the microarray data to be differentially expressed in mature DC or in tolerogenic DC compared to immature were analyzed using TaqMan based real-time PCR to test the validity of the microarray data. The $\Delta\Delta Ct$ method was employed using U6 RNA as endogenous control and the reference immature DC *Ct* values as calibrator. Results are depicted below and represent average $\Delta\Delta Ct$ values including standard deviations from 9 donors (mDC) and 6 donors (tDC), respectively:



CONCLUSIONS

- Distinct microRNA profiles separate mature and tolerogenic DC-phenotypes
- A single microRNA (17930) was found to be more than two-fold up regulated in tolerogenic DC's compared to immature DC's and, hence, potentially represents a biomarker for tolerogenic DC's
- hsa-miR-155, hsa-miR-146a and hsa-miR-125a represent potential biomarkers for immunogenic human DC's
- Less than two-fold up or down regulations of miRNA determined by microarray analyses are difficult to validate using real-time PCR