# Gene expression analysis of CD14+ monocytes immunomagnetically separated directly from whole blood: adaptation of protocols towards clinical trial requirements

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# Introduction

Over the past decade microarray-based gene expression profiling has become a valuable and powerful tool for the identification of diagnostic/prognostic biomarkers. Microarray-based gene expression signatures have also been used for, e.g., improved classification of clinically relevant disease subgroups, as well as for the prediction of treatment responses or risk of metastasis formation. While in cancer research usually the tumor and/or metastasis are subjected to gene expression profiling, the choice of appropriate tissues in other settings, e.g., in transplantation or autoimmune diseases, is more challenging. Here, the vast majority of published transcriptome data is based on RNA derived from patients' blood, either as stabilized whole blood or peripheral blood mononuclear cells (PBMCs)[1]. A major advantage of using blood as the starting material is its easy accessibility and the possibility of repeated sampling. Furthermore, there is a realistic chance that the obtained gene expression profiles reflect the disease or progression stage, since blood cells are permanently in contact with the diseased tissue. However, one major disadvantage of this approach is that the detected transcriptional changes are mainly attributed to changes in the cellular composition rather than due to disease-related biology.

Monocytes represent circulating cells in the blood stream and play pivotal roles in the immune system. Several studies demonstrated altered function and changes in gene expression profiles of monocytes for a broad range of diseases. A recent publication already demonstrated that specific gene expression profiles of CD14+ monocytes purified from PBMCs in systemic lupus erythematosus (SLE) are superior to PBMC-derived profiles with respect to disease discrimination and biomarker identification[2]. Furthermore, it has been shown that such an approach is also applicable to other diseases[3].

In the study described here, we investigated whether CD14+ monocytes isolated directly from whole blood are superior to whole blood or PBMCs for transcriptional analysis. Although an automated workflow is highly preferred for improved reproducibility, this might be difficult to be realized in multicenter clinical trials. Therefore, we established standard operating procedures (SOPs) for manual and automated magnetic separation of CD14+ monocytes by MACS Technology. Our protocol with automated procedures has recently been successfully applied in a clinical phase 1 study[4].

# Methods

**Experimental design, cell separation, and gene expression analysis**

In total, 20.5 mL of whole blood from five different donors was collected. Three BD Vacutainer K2E (6 mL, Becton Dickinson) tubes were used for either PBMC isolation via Ficoll (Amersham) or CD14+ monocyte isolation via MACS Technology according to SOPs. Automated separation was performed with the autoMACS Pro Separator, manual separation via Whole Blood Columns (WBC) followed by MS Column purification steps. Both procedures were performed with Whole Blood CD14 MicroBeads (Miltenyi Biotec). For flow cytometric analysis, aliquots from whole blood and from purified CD14+ monocytes were stained with CD14-FITC and CD15-APC according to the manufacturer's instruction (Miltenyi Biotec).

Total RNA from ten CD14+ monocyte samples and five PBMC samples was isolated using standard RNA extraction protocols (NucleoSpin RNA II, Macherey-Nagel). For whole blood analysis, 2.5 mL of blood was collected into PAXgene Blood RNA tubes and five RNA samples were directly extracted using the PAXgene Blood RNA Kit (Qiagen). The quality and quantity of isolated RNAs was checked via an Agilent 2100 Bioanalyzer and ND-1000 Spectrophotometer (NanoDrop Technologies), respectively.

100 ng of each RNA was labeled and amplified according to the Low Input Quick Amp Labeling Kit (Agilent Technologies). Labeled cRNAs were hybridized on 4x44K Agilent Whole Genome Microarrays, One-Color, scanned with an Agilent Scanner and analyzed using Feature Extraction software (FES). FES-derived data files were used as input for downstream analysis using the RESOLVER (Rosetta Biosoftware), TIGR MeV (free open source software), or in-house software AgiRJoiner or Correlate (Miltenyi Biotec). A "between-subject" t-test was performed to identify monocyte-enriched probes. Whole blood and PBMC samples were assigned to group 1 and manually and automatically separated CD14+ monocytes were assigned to group 2. Probes were identified as differentially expressed, if their p-value was <0.01 and their expression more than twofold higher in CD14+ monocytes than in group 1.

# Results

## 1 Highly pure CD14+ monocytes isolated directly from whole blood

An overview of the experimental setup and workflow is shown in figure 1A. Gene expression profiles of isolated CD14+ monocytes from five healthy volunteers were compared to profiles of stabilized whole blood and PBMCs from the same donors. The average purity of CD14+ monocytes was >90%. The average recovery was ~1×10$^6$ cells/6 mL blood (fig. 1B) with RNA yields of ~3 µg (data not shown). In figure 1C one representative flow cytometric analysis of a single representative donor is shown, indicating comparable purities of manually and automatically isolated CD14+ monocytes. Finally, in figure 1D, RNA integrity numbers (RIN) of all CD14+ monocyte-derived RNAs are displayed. RIN values close to 10 indicated high-quality RNA. Average RIN values of PAXgene or Ficoll-derived RNAs ranged between 9.7 and 8.9, respectively (data not shown).

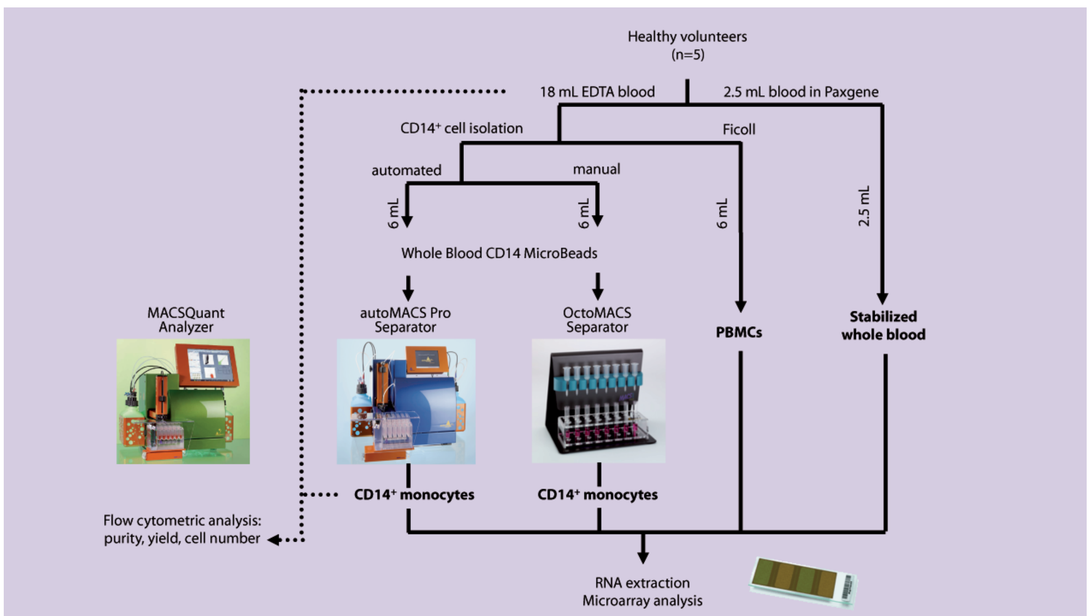


Figure 1A

Scheme of the experimental setup. Samples from five donors were used for whole blood and PBMC preparation as well as manual and automated CD14+ monocyte separation (20 experiments in total).

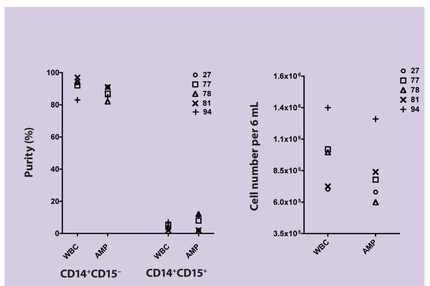


Figure 1B

Purity and recovery of manually or automatically isolated CD14+ monocytes from whole blood. Enrichment of monocytes was performed following magnetic labeling, using Whole Blood Columns (WBC) for manual isolation and the autoMACS Pro Separator (AMP) for automated isolation. Purities and recoveries of samples from five donors are shown.

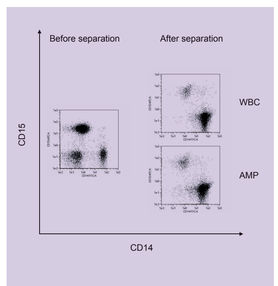


Figure 1C

Exemplary flow cytometric analysis of CD14+ monocytes before and after manual (WBC) or automated (AMP) separation (donor 77). The main contaminating cell fraction represented CD14+CD15+ granulocytes.

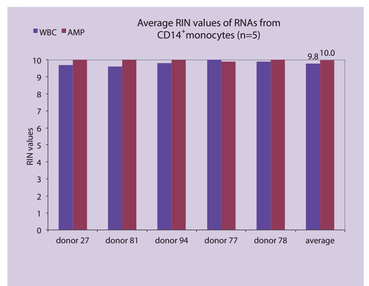


Figure 1D

Overview of RIN values of RNAs from CD14+ monocytes isolated manually (WBC) or automatically (AMP).

## 2 High concordance between manual and automated separation methods

RNAs were subjected to whole genome microarray analysis and the resulting data were analyzed by Principal Component Analysis (PCA). Figure 2A displays three distinct populations; clustering is based on the sample source (whole blood, PBMCs or CD14+ monocytes). The comparison of automated versus manual CD14+ monocyte isolation revealed a clear separation of the five different donors into five doublets, illustrating the fact that donor differences were more significant than cell separation protocol differences. Pearson correlation coefficients between manually and automatically separated CD14+ monocyte samples showed a very high intra-donor concordance in all five cases, with values of >0.992 (fig. 2B and data not shown). Further analysis showed no systematic variation in the gene expression profiles between the two separation methods.

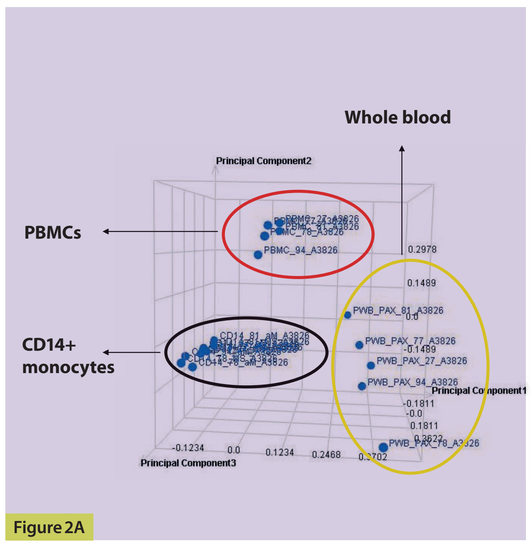


Figure 2A

PCA of all 20 samples (input: median-centered intensity profiles; RESOLVER analysis).

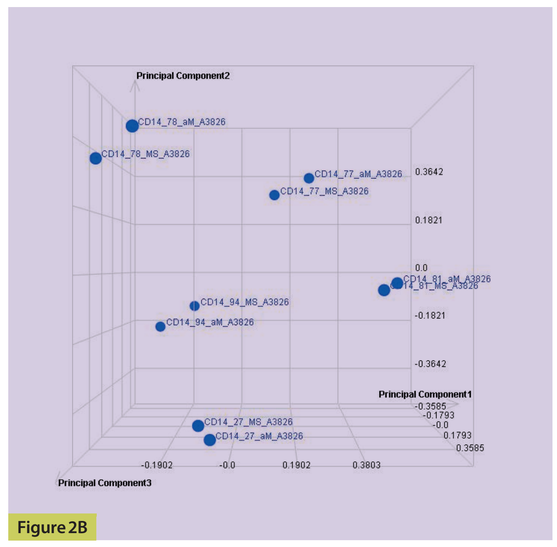


Figure 2B

"Zoom-in PCA" of CD14+ monocytes (ten intensity profiles from five donors; manual (MS) vs. automated (aM) isolation; input: median-centered intensity profiles, RESOLVER analysis).

## 3 Analysis of isolated CD14+ monocytes revealed superior sensitivity

In order to prove the enrichment of monocyte-specific genes in isolated CD14+ monocytes versus whole blood/PBMCs, a "between-subject" t-test was performed. More than 3,700 gene probes showed an enrichment factor of 2 to 4, and >380 probes were expressed at levels that were 4–16-fold higher in monocytes, among them, e.g., the CD14 gene as expected (figs. 3A and 3B). Accordingly, numerous genes were found to be strongly overexpressed in whole blood and/or PBMCs. In particular "contaminating" genes, such as i) hemoglobin, which is mainly derived from erythrocytes, with more than 10,000-fold higher median signal intensities in whole blood versus purified CD14+ monocytes, or ii) platelet factor-4, which is mainly derived from platelets, with more than 100-fold higher median signal intensities in whole blood or PBMCs versus CD14+ monocytes. These genes were negligible in purified CD14+ monocytes, leading to a higher overall sensitivity and detection of monocyte-specific genes (fig. 3B)[5].

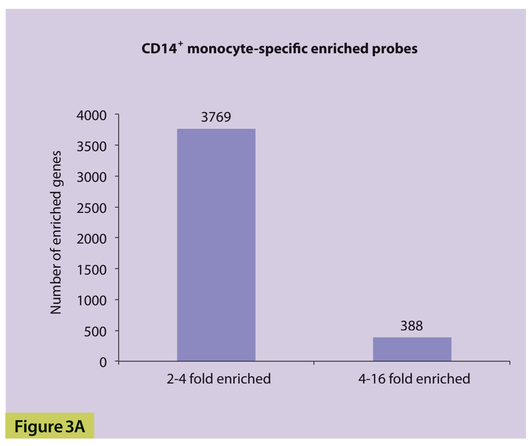


Figure 3A

Summary of CD14+ monocyte–specific probes compared to whole blood or PBMCs. The median signal intensities of CD14+ cells versus whole blood and PBMC signal intensities were compared in a "between-subject" t-test (TIGR MeV). Probes with >2-fold differential expression and a p-value of 0.01 were selected.

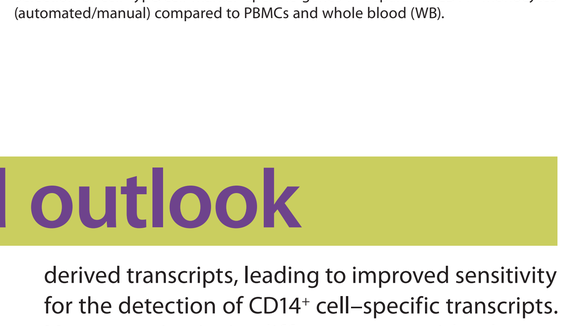


Figure 3B

Selection of cell type/cell marker–specific genes in separated CD14+ monocytes (automated/manual) compared to PBMCs and whole blood (WB).

# Conclusion and outlook

- SOPs were established for automated as well as manual magnetic isolation of CD14+ monocytes by MACS Technology directly from whole blood.
- The protocols required minimal handling steps, took less than 30 min, and resulted in sufficient yields and excellent RNA quality for gene expression profiling experiments.
- Expression profiles demonstrated high concordance between manual and automated separation strategies.
- Compared to whole blood or PBMCs, the highly enriched monocyte preparations showed negligible signs of contaminating erythrocyte- or platelet-derived transcripts, leading to improved sensitivity for the detection of CD14+ cell–specific transcripts.
- Monocyte-intrinsic differences provide deeper insights into the pathology of immune-related diseases and offer a greater chance to detect clinically relevant biomarkers.
- Our approach, also referred to as "MACS-4-omics", can easily be expanded towards direct isolation of other immune cell subsets, such as granulocytes, B cells, T cells, or NK cells.
- "MACS-4-omics" is not restricted to genomics but may be applied to all areas of a systems biology approach.

**References**

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