Unbiased transcriptomes of resting human CD4+CD45RO+ T lymphocytes

Gene expression profiling of cells of the immune system isolated ex vivo is a unique tool to assess gene expression in vivo. Exemplified for CD4+CD45RO+ effector/memory T helper (TE/M) lymphocytes of human peripheral blood, we have analyzed the influence of different isolation procedures and storage conditions on the gene expression profile [1], and describe a method for the generation of unbiased transcriptomes.

TE/M lymphocytes were isolated from fresh human peripheral blood to >98% purity by FACS as CD3+ CD4+ CD45RO+ CD45RA− cells. Prior to this, cells were enriched either by MACS with whole-blood CD4 beads (CD4 MACS), density gradient centrifugation (FICOLL), alone or in combination with CD3 MACS, or by lysis of erythrocytes (LYSIS) followed by MACS depletion of CD15− cells. A total of 7 different protocols were compared among each other and to T cells stimulated for 3 h after isolation with PMA and ionomycin (Fig. 1A and Supporting Information File 1). Cell isolation procedures lasted for 45–120 min, and were carried out according to manufacturer’s instructions at 4°C, unless indicated otherwise. Preparation-induced de novo transcription was blocked in one of the protocols by the addition of 2 μg/mL Actinomycin D (ActD). The transcriptomes of TE/M cells from two unrelated healthy donors (D1, D2) were determined on Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix, Inc) that contain 54 675 probe sets representing approximately 47 400 transcripts and variants including 38 500 genes [2]. The data were evaluated using the High Performance Chip Data Analysis (HPCDA) [3] and Genes@Work software [4].

The nonstimulated samples clustered by donor, irrespective of the isolation protocol used, demonstrating that the interindividual differences exceeded those induced by the isolation procedures (Fig. 1B). Natural genome-wide variation in human gene expression has been shown to be able to account for significant fold changes between individuals in a population [5, 6] and should be taken into account during experimental planning. Genes encoding CD8, CD14, CD16, CD19, CD20, or CD56, indicating contamination of the CD4+ T cells with CD8+ T cells, monocytes, granulocytes, B lymphocytes, and NK cells confirmed the purity of the preparations (Fig. 1D). An exception was the contamination of CD4 T cells isolated by LYSIS with residual erythrocytes, as indicated by expression of the globin genes HBA and HBB (Fig. 1D).

Comparing the different protocols, up to 759 Affymetrix probe set IDs (AffyIDs) were differentially expressed, by a factor of 1.5 or more (Fig. 1C and Supporting Information File 2). Cells isolated by CD4 MACS in the presence of ActD showed the lowest expression of genes indicating activation or stress, such as JUN and FOS [7], very similar to cells isolated by CD4 MACS in the absence of ActD (Fig. 1D). Sixteen AffyIDs were differentially expressed between cells isolated by CD4 MACS in the presence versus absence of ActD. Of these, only the pseudogene POLR2J4 showed an absolute fold change of ≥ 1.5 (1.6). These subtle differences could also reflect technical variation between the same cells, RNA prepared, amplified, and hybridized in parallel [8]. Thus, in cells isolated by CD4 MACS and FACS neither the degradation of RNA, nor the de novo transcription ex vivo skewed the in vivo transcriptomes. In general terms, ActD might qualify as a tool to prevent de novo transcription ex vivo in any kind of isolation protocol, but it cannot prevent degradation of transcripts, and thus is no alternative to isolation technologies preserving the in vivo transcriptome.

Compared with cells isolated by the CD4 MACS protocol, cells isolated by the FICOLL or LYSIS procedures showed differential expression of more than 100 AffyIDs at an absolute fold change of ≥ 1.5 (Fig. 1C). Among them were immediate response genes like JUN and FOS, the Eif1 gene encoding a translation factor [9], and the genes encoding the anti- and proapoptotic proteins Bel-2 and Bel10 (Fig. 1D) [10]. In the LYSIS samples the differences in expression were most pronounced.

The TE/M cells that were stimulated after isolation cluster on a completely different branch clearly separated from the branches of the nonstimulated data of the two donors (Fig. 1B). They are set apart from each of the two donors by over 11 000 differentially expressed AffyIDs (absolute fold change ≥1.5) (Fig. 1C), revealing that changes induced by stimulation outweigh technical and biological variability.

Decisive factors for the conservation of in vivo transcriptomes are the time...
Figure 1. (A) Schematic overview of the isolation procedures compared. The three main procedures for enrichment were direct magnetic isolation of CD4+ cells (CD4 MACS), with (w/) or without (w/o) 2 μg/mL Actinomycin D (ActD), Ficoll-density gradient centrifugation (FICOLL) using *LSM1077 or **PM400, alone or in combination with magnetic isolation of CD3+ cells (CD3 MACS) and lysis of erythrocytes (LYSIS), either at RT or at 4°C, followed by magnetic depletion of CD15+ cells. Cells were then stained and CD3+CD4+CD45RO+CD45RA− cells were isolated by FACS. Ten milliliters blood were collected for each test and approximately 10^6 cells were ultimately collected for transcriptome analysis. (B) Cluster analysis of transcriptomes of human peripheral effector/memory T helper (T\text{E/M}) cells of two donors (D1, D2). The heat map visualizes differences in expression of Affymetrix probe set IDs with an absolute fold change (absFC) /2.265/1.5 (red/upregulated, green/downregulated). (C) Summary of modulation of gene expression induced by the preparation protocol of the T\text{E/M} cells. Numbers indicate the numbers of differentially expressed Affymetrix probe set IDs between the differently isolated cells, of the two donors analyzed; light gray = numbers of regulated IDs with absFC /2.265/1.5; dark gray = numbers of regulated IDs with absFC ≥2. (D) Expression levels of genes exemplifying the impact of the isolation procedure on purity, cellular activation, stress response, and programmed cell death. The color gradient is based on the lowest (yellow) and highest (green) expression value of each individual AffyID.
Figure 2. (A) Schematic overview of the storage conditions compared. Ten milliliters samples of freshly drawn peripheral human blood were either processed immediately or stored in the sample tube for the time indicated, either at 4°C or at RT. (B) Cluster analysis of transcriptomes of TEmily cells, stored under conditions, as indicated, and isolated via FICOLL as described in Fig. 1A. The heat map visualizes differences in expression of Affymetrix IDs with an absolute fold change (absFC) ≥1.5 (red/upregulated, green/downregulated). (C) Summary of modulation of gene expression induced by different storage conditions, before preparation of TEmily cells. Numbers indicate the numbers of differentially expressed Affymetrix probe set IDs between the differently stored cells, of the two donors analyzed; light gray = numbers of regulated IDs with absFC ≥1.5; dark gray = numbers of regulated IDs with absFC ≥2. (D) Expression levels of genes exemplifying the impact of storage conditions on cellular activation, the stress response, and programmed cell death. The color gradient is based on the lowest (yellow) and highest (green) expression value of each individual AffyID.
between blood sampling and preparation of RNA, and the storage conditions of the blood before cell isolation. Blood samples were drawn and either processed directly or kept at 4°C or room temperature (RT) for 2 or 6 h before T_{E/M} cells were isolated. Gene expression was compared among the various cell preparations and with that of T_{E/M} cells isolated from 1-day-old buffy coat fractions obtained from the German Red Cross, stored at RT (Fig. 2A). CD3^+CD4^+CD45RO^+CD45RA^- cells were isolated by density gradient centrifugation and FACs, as described above in Fig. 1A (FICOLL^®). T_{E/M} cells immediately isolated from fresh blood differed from cells isolated from buffy coats by expression of more than 10,000 AffyIDs, with an absolute fold change of ≥1.5 (Fig. 2B, 2C), suggesting that T_{E/M} cells isolated from buffy coats no longer express the transcriptomes they used to express in vivo. Instead, they express genes indicating activation, i.e. CD69, JUN, FOS, and ELF1, and stress response, i.e. HIF1A, ATF4, and CXCR4 [11–13] (Fig. 2C and D). Already 2 h after blood draw, T_{E/M} cells stored at 4°C differentially express 677 AffyIDs with an absolute fold change of ≥1.5, and 192 AffyIDs with an absolute fold change of ≥2 (Fig. 2C), as compared with cells isolated immediately after blood draw. In samples stored for the same duration at RT these numbers were elevated to 2048 and 646 differentially expressed AffyIDs, respectively. These differences exceeded those observed between cells isolated by the various methods compared here (Fig. 1C, 2C and Supporting Information Fig. 3) and grew progressively more pronounced with prolonged storage (Fig. 2C and D).

In summary, we communicate here a method for the generation of transcriptomes of human T_{E/M} cells isolated ex vivo, reflecting their transcriptome in vivo. With blood processed directly after sampling, a fast direct enrichment of CD4^+ T cells from the blood by CD4 MACS and the final purification by FACS of CD3^+CD4^+CD45RO^+CD45RA^- cells, transcriptomes are obtained that are neither biased by de novo transcription nor by RNA degradation ex vivo, and thus reflect the situation in vivo.

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The array data discussed in this publication has been deposited in NCBI’s Gene Expression Omnibus and is accessible through GEO Series accession number GSE54247 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54247).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: ActD: Actinomycin D · AffyID: Affymetrix probe set ID · FICOLL: density gradient centrifugation protocol · HPCDA: high performance chip data analysis · LYSIS: erythrocyte lysis · RT: room temperature · T_{E/M}: effector/memory T helper lymphocyte

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The detailed Materials and methods for Technical comments are available online in the Supporting information