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Technical comment

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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 ($T_{E/M}$) 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.

$T_{E/M}$ 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 $T_{E/M}$ 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].

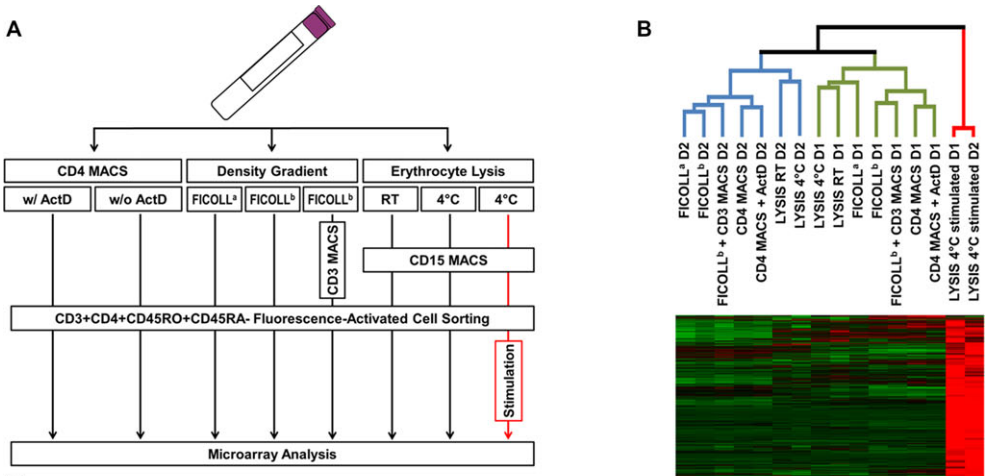
The nonstimulated samples clustered by donor, irrespective of the isolation protocol used, demonstrating that the interindividual differences exceeded those induced by the isolation procedures (Fig. 1B). Natural genome-wide variation in human gene expression has been shown to be able to account for significant 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 Bcl-2 and Bcl10 (Fig. 1D) [10]. In the LYSIS samples the differences in expression were most pronounced.

The $T_{E/M}$ 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



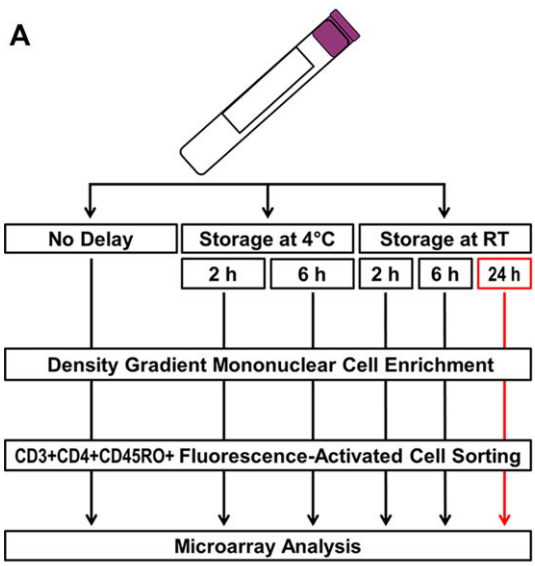
C

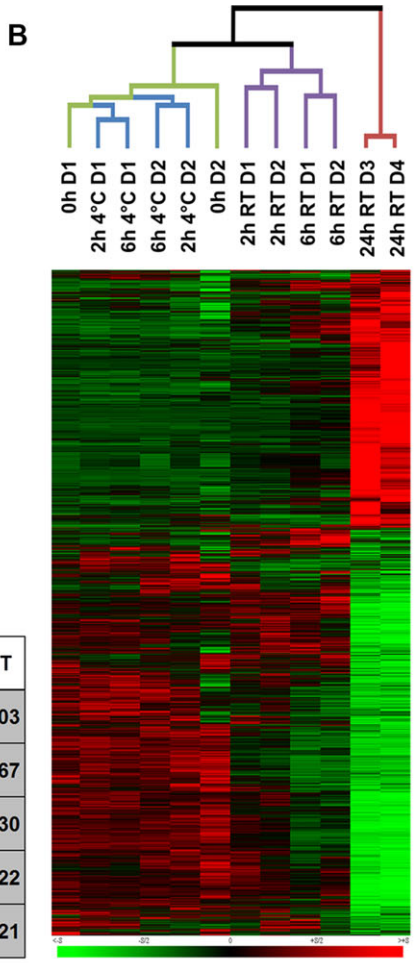
	CD4 MACS w/o ActD		FICOLL[a]		FICOLL[b]		FICOLL[b] w/ CD3 MACS		LYSIS RT		LYSIS 4°C		LYSIS 4°C stimulated	
CD4 MACS w/ ActD	1	0	194	65	208	52	131	39	759	246	311	66	11663	8893
CD4 MACS w/o ActD			246	70	140	45	115	34	625	209	206	46	11671	8936
FICOLL[a]					11	1	29	9	337	127	268	96	12284	9201
FICOLL[b]							7	2	595	176	345	92	12086	9149
FICOLL[b] w/ CD3 MACS									563	156	339	78	11651	8946
LYSIS RT											321	86	11597	8699
LYSIS 4°C													11745	8809

D

			CD4 MACS w/ ActD		CD4 MACS w/o ActD		FICOLL[a]		FICOLL[b]		FICOLL[b] w/ CD3		LYSIS 4°C		LYSIS 20°C		LYSIS 4°C stimulated	
			D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2
Lineage Marker	205456_at	CD3E	113	125	153	105	104	114	121	102	129	98	117	92	88	102	57	74
	203547_at	CD4	226	179	212	149	172	201	201	175	249	142	175	140	161	187	65	64
	230037_at	CD8	8	1	1	1	2	3	2	2	3	1	9	2	8	3	1	2
	201743_at	CD14	64	2	45	5	3	7	1	1	4	1	2	2	7	12	1	2
	204006_s_at	CD16	18	2	17	2	7	2	11	2	9	0	16	11	14	13	1	9
	206398_s_at	CD19	35	14	9	6	19	14	38	6	9	2	5	23	1	2	13	7
	228599_at	CD20	22	22	17	23	28	32	23	38	34	43	3	46	41	22	2	2
	209968_s_at	CD56	3	4	6	3	2	2	9	2	7	5	2	3	9	4	2	7
	214414_x_at	HBA	209	35	140	64	204	412	224	317	5	17	1447	1060	1646	2732	1288	733
	209116_x_at	HBB	192	47	131	88	217	554	247	353	8	25	1175	951	1227	2262	1075	684
Activation Marker	209795_at	CD69	1047	1425	1178	1363	2522	1803	1441	1433	1841	1551	1536	1366	1336	2315	12855	10325
	227404_s_at	EGR1	6	4	3	4	6	2	6	2	6	2	5	2	24	35	933	1122
	212227_x_at	EIF1	4173	4510	4540	4199	5306	5293	6208	4738	5521	4306	4859	5091	7409	7289	10340	8709
	209189_at	FOS	19	6	12	2	108	40	49	14	14	7	373	13	398	1989	375	800
	201465_s_at	JUN	26	34	47	23	79	23	44	23	53	29	439	143	412	946	484	480
Stress Response	202672_s_at	ATF3	13	1	5	18	18	18	14	11	20	18	11	17	19	21	767	2071
	200989_at	HIF1A	2215	3103	2301	2920	2809	3093	2425	2825	2612	2884	2532	2855	2414	2766	8778	7193
	200806_s_at	HSP60	1431	1417	1354	1152	1319	764	1016	884	1253	645	1474	698	1357	1184	5404	4398
	200692_s_at	HSP70	904	1100	831	948	884	800	954	987	854	831	898	756	879	917	2722	3254
	200599_s_at	HSP90	3026	3653	2594	3762	3179	3846	2666	3963	2901	4071	2879	3551	2901	3970	7586	6048
Apoptosis	203685_at	BCL2	1671	2153	1802	2123	1749	2096	1734	2035	1743	1925	1754	2027	1839	1948	867	829
	1557257_at	BCL10	39	75	83	84	185	117	145	129	91	88	128	89	281	278	400	429
	208905_at	CYCS	2214	2569	1994	2482	2031	2412	2045	2478	2055	2673	2143	2037	1919	2003	7242	7504
	211333_s_at	FASLG	65	61	75	37	46	72	46	73	62	59	56	68	42	73	1074	837
	202239_at	PARP4	1098	1547	1136	1672	1054	1631	953	1517	1227	1613	1107	1208	1075	1068	220	273

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 [a]LSM1077 or [b]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 ($T_{E/M}$) cells of two donors (D1, D2). The heat map visualizes differences in expression of Affymetrix probe set IDs with an absolute fold change (absFC) ≥1.5 (red/upregulated, green/downregulated). (C) Summary of modulation of gene expression induced by the preparation protocol of the $T_{E/M}$ 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 ≥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.





C

	2h at 4°C		2h at RT		6h at 4°C		6h at RT		24h at RT	
No Delay	677	192	2048	646	922	404	4326	1867	10008	8003
2h at 4°C			1805	488	238	49	4531	1839	10118	7767
2h at RT					1531	333	1395	232	9093	6730
6h at 4°C							3976	1560	9958	7722
6h at RT									7789	5321

D

			No Delay		2h at 4°C		2h at RT		6h at 4°C		6h at RT		24h at RT	
			D1	*D2*	*D1*	*D2*	*D1*	*D2*	*D1*	*D2*	*D1*	*D2*	*D3*	*D4*
Activation Marker	209795_at	CD69	2522	1803	3382	2445	6035	6652	4193	3434	7382	6865	17391	8468
	212227_x_at	EIF1	5306	5293	5960	6066	8067	8373	6483	5863	10250	8924	23218	18246
	209189_at	FOS	108	40	129	175	380	429	271	276	1528	651	14762	1354
	242903_at	IFNGR1	113	52	123	105	202	142	162	144	259	329	592	291
	213281_at	JUN	34	17	59	51	129	75	83	54	152	135	509	352
Stress Response	200779_at	ATF4	2530	2767	2465	2428	3113	2593	2648	2764	4449	3713	7802	7740
	1558678_s_at	CXCR4	5949	4816	6616	6404	10222	10535	6528	5538	11046	9542	21630	13593
	200989_at	HIF1A	2809	3093	2497	2534	2605	2597	2383	2625	4059	4596	6992	5470
	200692_s_at	HSP70	884	800	792	800	783	854	843	691	1061	1225	3019	3074
	200064_at	HSP90	1949	2048	1699	1691	1496	2120	1763	1965	2069	2303	4602	4192
Apoptosis	203685_at	BCL2	1749	2096	1664	1890	2055	1853	1678	1994	1554	1899	429	262
	1557257_at	BCL10	185	117	201	126	378	368	249	245	437	514	1131	746
	1553096_s_at	BIM	198	182	171	245	254	178	199	190	266	211	385	392
	202239_at	PARP4	1054	1631	1177	1516	1020	969	1094	1327	823	887	354	524
	205641_s_at	TRADD	665	656	744	829	747	910	725	660	743	613	388	379

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 $T_{E/M}$ cells, stored under conditions, as indicated, and isolated via FICOLL[a] 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 $T_{E/M}$ 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[a]). $T_{E/M}$ 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 *EIF1*, 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

Keywords: $CD4^+$ T cells • Cellular activation • Gene expression • Microarray • Transcription

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