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Sensitive Gene Expression Profiling of Human T Cell Subsets Reveals Parallel Post-Thymic Differentiation for CD4$^+$ and CD8$^+$ Lineages[1]

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The differentiation of CD4$^+$ or CD8$^+$ T cells following priming of naive cells is central in the establishment of the immune response against pathogens or tumors. However, our understanding of this complex process and the significance of the multiple subsets of differentiation remains controversial. Gene expression profiling has opened new directions of investigation in immunobiology. Nonetheless, the need for substantial amount of biological material often limits its application range. In this study, we have developed procedures to perform microarray analysis on amplified cDNA from low numbers of cells, including primary T lymphocytes, and applied this technology to the study of CD4 and CD8 lineage differentiation. Gene expression profiling was performed on samples of 1000 cells from 10 different subpopulations, defining the major stages of post-thymic CD4$^+$ or CD8$^+$ T cell differentiation. Surprisingly, our data revealed that while CD4$^+$ and CD8$^+$ T cell gene expression programs diverge at early stages of differentiation, they become increasingly similar as cells reach a late differentiation stage. This suggests that functional heterogeneity between Ag experienced CD4$^+$ and CD8$^+$ T cells is more likely to be located early during post-thymic differentiation, and that late stages of differentiation may represent a common end in the development of T-lymphocytes. ***The Journal of Immunology,*** **2007, 179: 7406–7414.**

CD4$^+$ and CD8$^+$ T cells are major players of our defenses against pathogens and eventually embody the memory of cellular immunity, but their post-thymic differentiation (i.e., following encounters with foreign Ags) is complex. Despite thorough studies, it remains the subject of controversies, for instance, regarding the pathway of differentiation itself (i.e., distinct models have been derived from either mouse model or human studies), or regarding the nature of the cells that should confer optimal immune protection (i.e., either early or highly differentiated T cells) (1). CD4$^+$ and CD8$^+$ T cells are thought to undergo unique developmental programs after activation to evolve along distinct pathways of differentiation (2). However, recent studies in humans have reported intriguing similarities regarding the phenotype of CD4$^+$ or CD8$^+$ T cell subsets of differentiation (3–5). Altogether, this emphasizes the need for a clarification of both CD4$^+$ and CD8$^+$ T cell post-thymic development, which is central for our comprehension of an effective immune response and development of T cell based therapies.

The development of DNA microarray analysis has been a major technological advance in the recent years. The possibility to analyze simultaneously the expression of several thousand genes from biological samples represents a key step toward a better understanding of biological mechanisms of diseases and has opened new directions of investigation in various fields. However, a major limitation of this approach lies in its need for a substantial amount of material (i.e., mRNA), which can restrict significantly its application. In this study, we describe methodologies to perform gene profiling analysis on a limited number of immunomagnetic and flow cytometry sorted cells (including primary cells like human T lymphocytes), taking advantage of global PCR techniques that enable 1–10 million fold mRNA amplification (6–8). The DNA microarrays used include 1070 genes selected for their relevance to the field of immunology. We present this technology and its application to the study of T lymphocyte differentiation.

For this purpose, we have performed microarray analysis on 10 different subsets of CD4$^+$ or CD8$^+$ T cells. These subsets, sorted by MACS and five-color FACS (based on the expression of CD8/CD4, CD27, CD28, CD45RA, and CCR7), defined the major steps of a well established pathway of T cell differentiation in humans, i.e., from naive to highly differentiated cells (1, 9). The methodology developed by our group to reliably assess gene expression from a small number of cells was particularly suited to this study because analysis had to be performed on multiple T cell subsets obtained from an unique blood sample, which contain relatively small numbers of cells per subsets. Bioinformatic analysis was conducted to compare

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the expression profiles of the different T cell subsets and to assess potential similarity between $CD4^+$ and $CD8^+$ T cell differentiation.

Materials and Methods

Cell lines and blood samples

The human B cell like Raji and T cell like Jurkat cell lines were cultured in RPMI 1640 medium, 2 mM glutamine, 10% (v/v) FCS at 37°C in 95%/5% air/CO_2. Blood samples were obtained from healthy volunteers by venepuncture. PBMCs were purified from heparin-anticoagulated blood by Ficoll-Hypaque (Sigma-Aldrich) separation. The study was approved by the Local Research Ethics Committee.

Flow cytometry and T cell subset sorting

For cell surface staining, a panel of titrated anti-human Abs was added to freshly isolated PBMC for 15 min at room temperature. For intracellular staining, cells were washed, permeabilized with FACS Permeabilization buffer (Becton Dickinson), and incubated for 15 min at room temperature in the dark with Abs for intracellular molecules (Perforin, Granzymes A and B). Cells were then washed and stored at 4°C until flow cytometry analysis or sorting was performed. Samples were analyzed on a Becton Dickinson LSRII. For cell sorting, PBMC were obtained by Ficoll separation from 20 ml healthy donors' heparinized blood samples. $CD4^+$ or $CD8^+$ T cells were enriched using magnetic bead (Miltenyi Biotec) before cell surface staining (for CD4/CD8, CD45RA, CCR7, CD28, and CD27) and then sorted according to their phenotype using a FACSVantage with a high abort rate. Ten to ten thousand cells were sorted into 96-well plates (conic bottom), directly into 6.4 μl lysis buffer (including detergent, tRNA, and Protease). Cell purity was between 95 and 98% for each sorted population. However, owing to the preliminary step of CD4/CD8 magnetic bead enrichment, sorted populations were at least 99% pure in terms of CD4 or CD8 expression, assuring minimal contamination between CD4 and CD8 lineages. Anti-CD8 (APC-Cy7), Anti-CD4 (APC-Cy7), anti-CD27 (FITC), anti-CD28 (PE), anti-CCR7 (PE-Cy7 or APC), anti-perforin (PE), anti-granzyme A (FITC), and anti-granzyme B (Alexa 647) Abs were purchased from BD Pharmingen. Anti-CD45RA (PE-TexasRed) and anti-CD28 (PE-TexasRed) was purchased from Beckman Coulter. Anti-CD27 (Alexa 700) was purchased from Biolegend.

Microarray experiments

Unamplified labeled samples were generated using the μMACS one step cDNA Labeling Kit (Miltenyi Biotec). In brief, cells were centrifuged at $300 \times g$ and resuspended in 1 ml Lysis/Binding Buffer. Lysate was sheared through a 21G needle and cleared using a LysateClear Column. Fifty microliter Oligo(dT) MicroBeads were added to the lysate and mRNA fixed and purified on a micro column. mRNA was transcribed to labeled cDNA using the thermoMACS Separator (Miltenyi Biotec) in a 60 min, 42°C in-column incubation with cDNA labeling mix and 1 μl Cy3-dCTP or Cy5-dCTP (1 mM, GE Healthcare). Labeled cDNA/mRNA hybrids were washed and RNase H digested in a 5 min, 42°C in-column incubation. cDNA was eluted with 50 μl cDNA Elution Buffer.

For the generation of amplified cDNA (SuperAmp Service, Miltenyi Biotec), the mRNA was extracted from the 1–10000 cell samples using magnetic beads and transcribed into cDNA using tagged random and oligo(dT) primer. First strand cDNA was 5′ tagged using 21.5 U terminal deoxynucleotidyl transferase (GE Healthcare) and incubating for 60 min at 37°C before heat inactivating at 70°C for 5 min. Tagged cDNA was globally amplified (Expand Long Template PCR System DNA Pol Mix, Roche) using primer complementary to the tag sequence and incubating at 78°C for 30 s, 20 cycles of 94°C for 15 s, 65°C for 30 s, and 68°C for 2 min followed by 21 cycles of 94°C for 15 s, 65°C for 30 s, and 68°C for 2.5 min with an extension of 10 s/cycle and a final step of 68°C for 10 min. PCR product was purified and cDNA yield measured. Two hundred nanograms of purified PCR product (High Pure PCR Product Purification Kit, Roche) was labeled with Cy5- or Cy3-dCTP (GE Healthcare) in a Klenow Fragment (20 U) reaction for 2 h at 37°C before heat inactivating at 70°C for 5 min. A 2.5 μg combined Cy3- and Cy5 labeled and purified (CyScribe GFX Purification Kit, GE Healthcare) cDNAs were used for each microarray analysis.

PIQOR Microarray Immunology, human, sense (Miltenyi Biotec) hybridization was performed according to the manufacturer's instructions using an automized hybridization machine (a-Hyb, Miltenyi Biotec). In brief, a volume of labeled sample was adjusted to 100 μl with nuclease-free water and 100 μl 2× Hybridization Solution prewarmed at 42°C was added. After mixing, the solution was preheated to 65°C for 2 min before application to the a-Hyb. Microarrays were incubated in distilled water at 95°C for 2 min, dipped in ethanol (p.a.), blow-dried, and inserted into the a-Hyb. Microarray processing in the a-Hyb was as follows: incubation in Pre-Hyb Solution for 5 min at 63°C, hybridization with the labeled cDNAs for 960 min at 63°C, wash with Washbuffer I for 1 min at 50°C (two cycles), and second wash with Washbuffer II for 1 min at 35°C (two cycles). Pump speed at all incubations was set to 1 ml/min. Microarrays were blow-dried and scanned with the ScanArray Lite (GSI Lumonics) and the Agilent DNA-Microarray Scanner. Signal processing and quantification was conducted with ImaGene software version 5.0 (BioDiscovery). For each spot, the local signal was measured inside a fixed circle of 230–280 μm diameter, and background was measured outside the circle within specified rings 30 μm distant to the signal and 100 μm wide. Signal and background was taken to be the average of pixels between defined low and high percentages of maximum intensity with percentage parameter settings for low/high being 2/97% for signal and 0/97% for background. Local background was subtracted from the signal to obtain the net signal intensity and the ratio of Cy5/Cy3. Subsequently, the mean of the ratios of four corresponding spots representing the same cDNA was computed. The mean ratios were normalized to the median of all mean ratios by using only those spots which were unflagged (excluding empty spots, poor spots, negative spots) and for which the fluorescent intensity in one of the two channels was 2-fold the mean background value (2 bkg dataset). The whole data set is accessible in the GEO databases (www.ncbi.nlm.nih.gov/geo/) with the accession number GSE6732. Agilent whole human genome arrays (44k) where hybridized and analyzed according to the manufacturer's instructions. In brief, 2.5 μg of combined Cy3- and Cy5-labeled and purified cDNAs were adjusted to a volume of 200 μl and denatured 5 min at 95°C. After adding of 50 μl control targets (Agilent) and 250 μl 2× hybridization buffer (Agilent), samples were incubated on the microarrays at 65°C for >16 h. Afterward microarrays were washed with Washbuffer I for 1 min at 37°C, with Washbuffer II for 1 min at 25°C, and dried after 30 s incubation in Acetonitrile. Scanning was performed using the Agilent DNA-Microarray Scanner. Scanned images were analyzed using the Agilent Feature Extraction software (Version 9.1) by which the local background was subtracted and a rank consistency based probe selection for Lowess normalization was done. After filtering the data with respect to signal significance a two-tailed *t* test was used to determine the signal vs background significance. Spots with a *p*-value of >0.01 were omitted. Exported raw data were further processed by the Luminator software (Agilent) yielding expression values.

Real-time PCR experiments

Transcript levels were measured by real-time quantitative PCR using PerkinElmer Applied Biosystems prism model 7000 sequence detection system (PE ABI 7000 SDS). Forward and reverse primer sequences were as follows (5′-3′): FosB1, ACAAAGTGGGTGTGTGGCCT and AGG CAGGACAAAACACAAGGA; IFNG, ATAATGCAGAGCCAAATTG TCTCC and ATGTCTTCCTTGATGGTCTCCAC; JunB, CGGCAGCTA CTTTTCTGGTCA and AATCAGGCGTTCCAGCTCC; KLRD1, TGAC TGCTGTTCTTGCCAAGA and GAGAAGCACAGAGATGCCGAC; GAPDH, GACCTGACCTGCCGTCTAGAA and TCAGTGTAGCCC AGGATGCC. GAPDH was used to normalize TaqMan data. Amplification efficiencies of target and reference gene were measured to be identical ($E_{target} = E_{ref} = 2$). Six ng of globally amplified cDNA libraries were used as template for each PCR analysis, all assays were performed in triplicate. Threshold cycle, Ct, was measured as the cycle number at which the SYBR green emission increases above threshold level. The following cycle conditions are used: 95°C 10 min followed by 50 cycles of 95°C for 15 s, 60°C for 1 min. For each amplified product, melting curves were determined according to the supplier's guidelines ensuring specific amplification. For each run, negative controls were performed by omitting the template. If no amplification product was detectable, the gene was considered "not expressed" in the respective sample.

Bioinformatic analysis

Pearson correlation coefficient analysis was performed with the unfiltered ratio 2bkg dataset logarithmized to the basis of two (see above). One and two dimensional average linkage hierarchical clustering (10) using Euclidean distance as well as statistical analysis of microarrays (SAM)[4] (11) was performed with TIGR MeV version TM4 (12) setting the percentage cutoff filter to 30%. For two class unpaired SAM, level 5 $CD4^+$ and $CD8^+$ T cells were assigned to Group B while level 2–4 T cells were assigned to Group A.

[4] Abbreviations used in this paper: SAM, statistical analysis of microarrays.

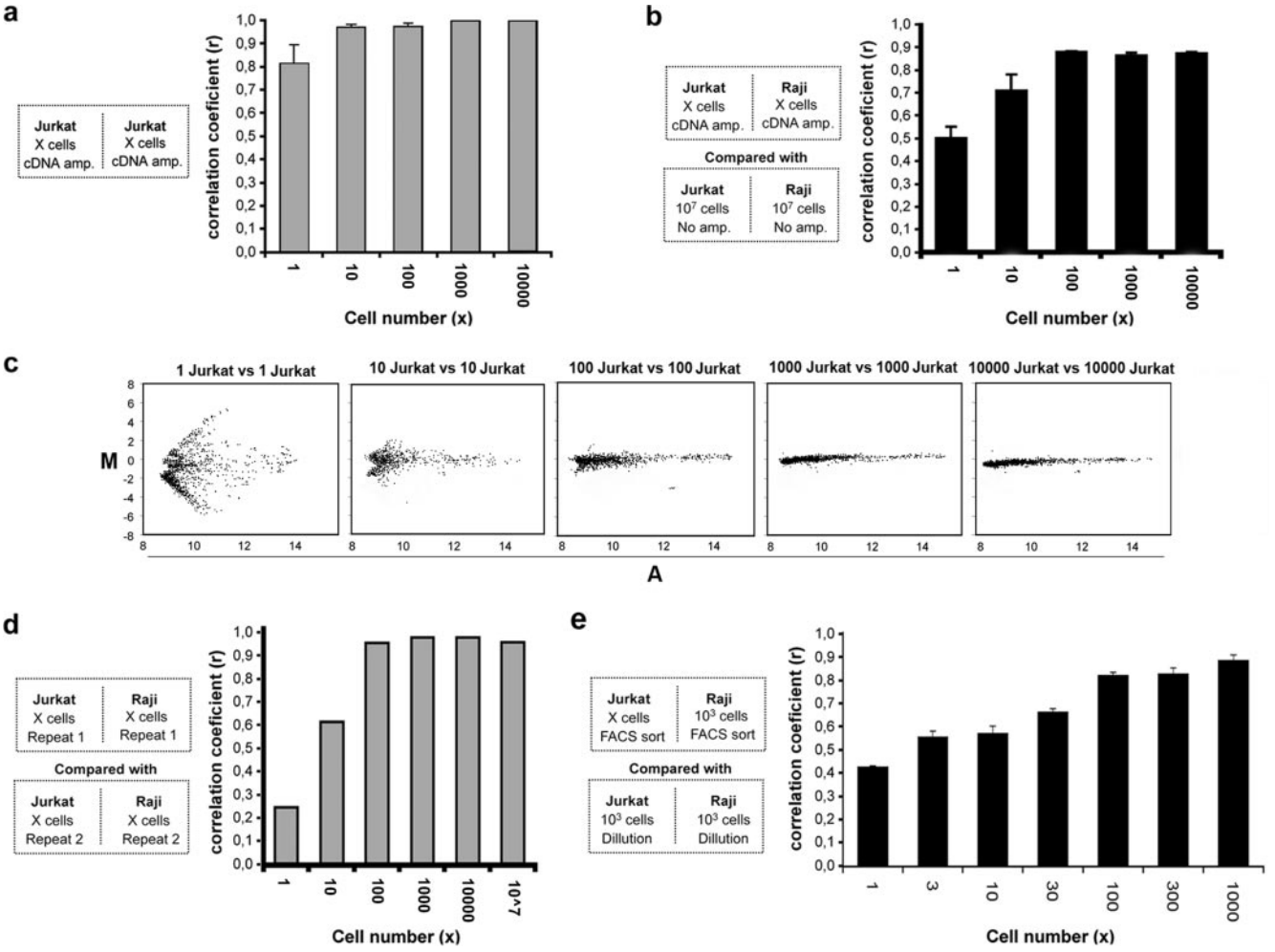


FIGURE 1. Similarity between gene expression profiles from various cell numbers of Jurkat T cells and Raji B-cells. RNA of Jurkat and Raji cells for various cell numbers was extracted and amplified in independent duplicates, and the resulting gene expression profiles were compared using Pearson correlation coefficients (r). These coefficients take into consideration both the number of expressed genes and their ratio of expression (both in the quadruplicate setting) and illustrate the degree of similarity between gene expression profiles. *a*, r of signal intensities for given Jurkat vs Jurkat cell number gene expression profiles (amplified cDNA). *b*, r of gene expression ratios (Cy5/Cy3) for given Jurkat (Cy5) vs Raji (Cy3) cell number (amplified cDNA) compared with 1×10^7 Jurkat vs Raji cell gene expression profile (unamplified cDNA). Bars show SD between repeats. *c*, Representative MvA plots (variability (M) as a function of mean (A), raw signal intensities, log2) for given number of Jurkat vs Jurkat cells. *d*, r for independent repeats ($n = 2$) of a given number of Jurkat and Raji cells. *e*, r for gene expression profiles from given (1–1000) FACS sorted Jurkat vs Raji cell numbers compared with the Jurkat vs Raji gene expression profile of diluted (i.e., no FACS sorting) 1000 cells.

Results

Validation of the methodology using immortalized cells

To obtain the amount of materials (i.e., mRNA) necessary to perform the experiments, previous studies of primary human lymphocytes by microarray analysis used at least 10^5 cell samples (13–16). In an effort to reduce the required amount of sample, we have worked on a method that could enable us to apply microarray technology to the analysis of low cell numbers. This methodology implies the sorting of pure cell population directly into cell lysing buffer, followed by bead-based mRNA extraction, cDNA synthesis, global PCR amplification, and Klenow Fragment labeling. In addition, hybridization of fluorescently labeled probes was done on DNA arrays with individually selected cDNA fragments of 200–400 bp, set in quadruplicate to improve on reliability of the results.

Experiments to test the efficacy of our method applied to low cell numbers of human lymphocytes were performed using the immortalized Jurkat T cell and Raji (Human Burkitt's lymphoma) B cell lines. First, we undertook "self-on-self" experiments comparing the expression profiles of 1, 10, 100, 1000, and 10000 Jurkat T cells to a respective second set of independently diluted and amplified Jurkat T cells (duplicates representing a total of 10 microarrays). To assess the reproducibility in gene expression profiles between the amplified materials from the different cell number samples, Pearson correlation coefficients (r) were calculated based on the expression of the 1070 genes present on the DNA arrays (Fig. 1*a*). The mean correlation coefficients are 0.815, 0.969, 0.972, 0.997, and 0.996 for 1, 10, 100, 1000, and 10000 cells, respectively. Next, we repeated the same experiments as described above using whole genome oligonucleotide arrays which resulted in comparable correlation coefficients (0.733, 0.8, 0.97, and 0.945 for 1, 10, 100 and 1000 cells). However self-on-self experiments do not allow addressing the reproducibility of differential gene expression. Therefore, following a more rigorous approach, a set of microarrays (duplicates, 12 microarrays) was obtained with amplified material from 1, 10, 100, 1000, or 10000 Jurkat T cells hybridized against the same numbers of Raji B-cells (all titrated by dilution) and compared with profiles obtained with nonamplified material from 10^7 Jurkat cells vs Raji cells. Correlation coefficients

close to 0.9 were obtained between the nonamplified material and the amplified material down to 100 Jurkat vs 100 Raji cell samples (Fig. 1*b*), and are indicative of a good uniformity between nonamplified and amplified sample profiles. Even 10 Jurkat vs 10 Raji cell samples yielded satisfactory uniformity (with a correlation coefficient beyond 0.7). MvA plots of all arrays are shown in Fig. 1*c*. Reproductibility between arrays was satisfactory from 100 cell samples onwards as shown with the comparison of repeat experiments performed with same cell number (Fig. 1*d*). Correlation coefficients remained in general slightly below those obtained with conventional methods using non amplified materials (with high cell number samples), which likely corresponds to the cost of using amplification protocols from small cell numbers. In a second set of experiments (duplicates, 18 microarrays), the influence of the FACS sorting procedure on gene expression profiling was tested. An increasing number of Jurkat cells (1, 3, 10, 30, 100, 300, 1000) were sorted by FACS and their amplified cDNA hybridized against amplified Raji cDNA (1000 cells). Gene expression profiles from these experiments were compared with the profile from 1000 Jurkat vs Raji cells obtained by dilution. Pearson correlation coefficients indicated that the FACS sorting procedure had almost no influence on the gene expression profiles (with r values beyond 0.8 for 100 to 1000 cell gene expression profiles) (Fig. 1*e*). Because amplified cDNA can sometimes be generated when 0 cells are applied (e.g., from resulting DNA contaminations of the enzymes used) due to the high degree of amplification, negative controls with 0 cell amplifications were also labeled and hybridized vs a Raji cDNA pool. The signals of the Raji cells were then the only detectable ones and correlation coefficients of those arrays to arrays hybridized with increasing numbers of Jurkat cells were insignificant (in the range of 0.1; data not shown).

Microarray analysis on low numbers of primary cells

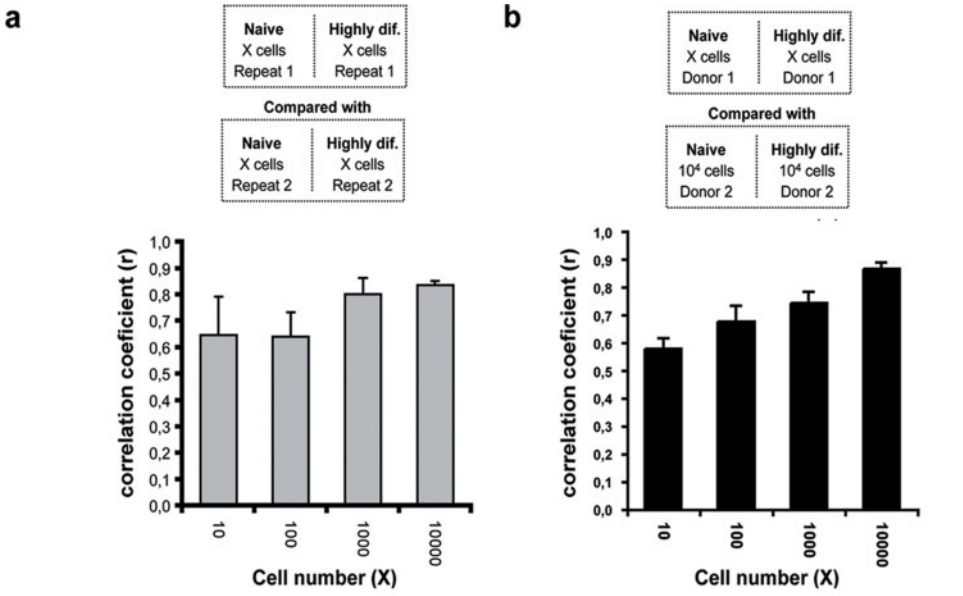
The results of these experiments validated the potential of our methodology; nonetheless Jurkat and Raji cells are immortalized cells, known to show strong levels of gene expression so that these observations may not apply to the study of primary cells. Another set of experiments (20 microarrays) was therefore performed using primary $CD8^+$ T lymphocytes obtained from peripheral blood from two donors. For this purpose, naive ($CD27^+$, $CD28^+$, $CD45RA^+$, and $CCR7^+$) as well as highly differentiated ($CD27^-$, $CD28^-$, and $CCR7^-$) $CD8^+$ T cells were sorted immunomagnetically and by flow cytometry into 10, 100, 1000, and 10000 cell samples. Naive and highly differentiated cell samples were hybridized in a competitive manner on the microarrays (in duplicate) to provide differential gene expression profiles between these two subsets. Pearson correlation coefficients of independent repeats (Fig. 2*a*) and of various cell numbers for the two donors (Fig. 2*b*) revealed that uniformity was satisfactory with 1000 cell samples (correlation coefficient close to 0.8). Gene expression ratios derived from our data were in strict agreement with the phenotype and known characteristics of naive or highly differentiated $CD8^+$ T cells (Fig. 2*c*). Naive $CD8^+$ T cells presented strong expression of genes encoding for CCR7, CD27, and CD28. Another example was the high expression level of CD31 whose expression is lost during post-thymic peripheral expansion of naive T cells in relation to TCR triggering events or due to homeostasy (17). In addition, the gene coding for the IL-7 receptor α-chain (important for the homeostatic proliferation and survival of naive cells, Ref. 18) was strongly expressed. In contrast, highly differentiated $CD8^+$ T cells showed high levels of mRNA encoding for the integrins CD11a and CD11c, as well as LAMP-3, a lysosomal glycoprotein (also known as CD63), abundantly presented on the lytic granule-membrane (19). Genes encoding for effector molecules like Perforin and Granzyme B were also highly expressed. The observation of less satisfactory data with 10 cell samples may be related to the low gene expression levels in resting primary T cells, as well as to the intercell variability, more perceptible at this level. Altogether, these data demonstrate though the applicability of our microarray methodology to the study of low cell number samples and its consistency for the analysis on primary lymphocytes. Because 100 cell samples appeared to be at the limit of satisfactory analysis, we decided to work with 1000 cell samples for subsequent studies.

Comparative study of multiple subsets of $CD4^+$ or $CD8^+$ T cell differentiation

The possibility to do gene expression profiling on as low as 1000 MACS and FACS sorted primary cells enabled us to perform a comparative study of 10 different subpopulations, defining major steps of both $CD4^+$ or $CD8^+$ T cell differentiation processes (Fig. 3, *a* and *b*), FACS sorted from a single blood sample of 20 ml. Because previous studies showed a good uniformity of microarray data on lymphocyte subsets between different donors (13, 14), experiments were performed only on two donor blood samples (still representing a total of 20 microarrays). For both $CD4^+$ or $CD8^+$ lineages, the probes obtained for each subset were hybridized in a competitive manner against the probes from respective naive cell pool standards (composed of three independent FACS isolated and amplified 1000 $CD4^+$ or $CD8^+$ cell samples). This analysis provides therefore information on gene expression differential between the subsets of Ag experienced cells and the naive cells. The expression of 350 genes on average (of the 1070 present on the arrays) could be detected (i.e., with fluorescent intensity being two fold the background value) for each subset. Differences in gene expression (i.e., percentages of genes that were down-regulated or up-regulated) between naive pool standards and each T cell subset (Fig. 3*c*) were calculated. The lack of difference for pool samples hybridized against themselves indicated that there was no technical error with the arrays. The small percentages observed between the naive subsets and the naive pool standards represent technical bias associated with independent FACS isolations and cDNA amplifications, but could be considered as inconsequential for the rest of the analysis. As expected, overall gene expression profiles of Ag experienced T cell subsets differed significantly (40–60%) from the profiles of naive cell standards, in particular when reaching highly differentiated cell subsets. This is consistent with the establishment of a biological process and changes in gene expression following T cell priming with Ags and differentiation. Interestingly, the gene profile analysis of highly differentiated $CD4^+$ T cells showed the acquisition of a cytolytic program by these cells (with expression of genes encoding for the lytic granule membrane protein LAMP-3, and the cytotoxic factors granzyme B and perforin), similar to what is observed with $CD8^+$ T cells (Fig. 4*a*). These observations are in line with recent ex vivo characterization of cytotoxic $CD4^+$ T cells (20–22) and support their existence in vivo. The establishment of this program appeared to be linked with differentiation of $CD4^+$ T cells in a two step process: a limited expression of cytolytic factors at the $CD27^-/CD28^+$ stage followed by a collective expression at the $CD27^-/CD28^-$ stage. Flow cytometry analysis confirmed the microarray data (Fig. 4*b*).

Parallel between both $CD4^+$ and $CD8^+$ T cell differentiation processes

The intriguing correspondence between the markers used to define the $CD4^+$ or $CD8^+$ lineage differentiation pathways, together with the acquisition of cytolytic capacities by $CD4^+$ T cells



c

	10 cells		100 cells		1000 cells		10000 cells	
	Donor 1	Donor 2	Donor 1	Donor 2	Donor 1	Donor 2	Donor 1	Donor 2
CCR7	0.78	0.10	0.01	0.07	0.06	0.14	0.04	0.08
CD27	0.22	ND	0.06	0.02	0.09	0.04	0.04	0.05
CD28	2.19	ND	0.05	0.55	0.07	0.83	0.05	0.28
IL-7R	1.45	0.46	0.08	0.17	0.02	0.35	0.14	0.08
CD31	1.08	0.26	0.17	0.42	0.12	0.22	0.11	0.35
CD11a	14.20	0.79	3.50	2.00	3.85	1.74	3.57	3.67
CD11c	0.39	1.27	4.13	4.17	2.51	2.09	2.00	2.98
PERFORIN	19.56	5.34	146.14	6.71	49.97	4.29	58.35	13.45
GRANZYME B	207.50	56.63	531.40	65.98	308.92	48.19	374.23	53.28
LAMP-3	1.27	0.38	2.18	2.40	4.60	2.18	5.00	2.61

FIGURE 2. Similarity between gene expression profiles from various cell numbers of primary T lymphocytes. Cy5 labeled cDNA of FACS sorted highly differentiated $CD8^+$ T lymphocytes ($CCR7^-/CD28^-/CD27^-$) were hybridized against Cy3 labeled cDNA of FACS sorted naive $CD8^+$ T lymphocytes ($CD45RA^+/CCR7^+/CD28^+/CD27^+$) for various cell numbers (10 to 10000) in duplicates from two donors, and the resulting gene expression profiles were compared using Pearson correlation coefficients (r). *a*, r for independent repeats ($n = 2$) of a given number of naive vs highly differentiated $CD8^+$ T lymphocytes. Bars show SD between donors. *b*, r for gene expression profiles of given numbers of naive vs highly differentiated $CD8^+$ T lymphocytes from one donor compared with the gene expression profile for 10000 naive vs highly differentiated $CD8^+$ T lymphocytes from another donor. *c*, Expression ratio of genes usually associated with naive (*top*) or highly differentiated $CD8^+$ T cell (*bottom*) phenotypes are shown for experiments with naive vs highly differentiated $CD8^+$ T lymphocytes from the two donors at various cell numbers. Down-regulated genes (associated with naive cells) are highlighted in green, and up-regulated genes (associated with highly differentiated cells) are highlighted in red.

like for $CD8^+$ T cells, prompted us to investigate further the potential similarity between the differentiation of these two lineages at the molecular level. The design of the study (i.e., competitive hybridization of cDNA probes from the different subsets of Ag experienced cells against naive cell cDNA probes) enables us to assess the similarity between both CD4 or CD8 lineage differentiation in terms of gene expression evolution from their respective naive stages. Interestingly, we observed a consistent increase of correlation coefficients comparing $CD4^+$ and $CD8^+$ T cells at equivalent stage of differentiation (e.g., CD4 stage 2 compared with CD8 stage 2) along differentiation stages (Fig. 5*a*): while correlation coefficients showed little correspondence between the two lineages for early stages of differentiation, an increasing similarity between $CD4^+$ and $CD8^+$ cell data sets could be observed with late differentiation (i.e., stage 5). This suggests that the differentiation program of $CD8^+$ and $CD4^+$ cells initially involves different deviations (qualitatively and/or quantitatively) from the naive cells, but that with late differentiation, $CD8^+$ and $CD4^+$ cells evolve toward much more similitude as observed with their gene expression profiles. The highest heterogeneity appears to be located early during post-thymic differentiation, when $CD4^+$ and $CD8^+$ T cells are likely to exert their lineage specific role in the immune response. The similarity in gene expression profile between highly differentiated $CD4^+$ and $CD8^+$ T cells could even be more pronounced than the similarity between two subsets of the same lineage (e.g., early differentiated $CD8^+$ T cells compared with late differentiated $CD8^+$ T cells), as seen with the whole set of correlation coefficients (Fig. 5*b*). To further understand the basis of the parallel between both lineage differentiation pathways when reaching late stages, we next looked for gene clusters. A SAM (11) was computed using a two class unpaired study design and assigning the gene expression profiles of $CD4^+$ and $CD8^+$ T cells at the $CD27^-/CD28^-$ stage to a first group and the earlier stages of differentiation to a second group. One dimensional clustering of SAM results identified a series of genes differentially expressed (down-regulated or up-regulated) at late stages of differentiation and common to both $CD4^+$ and $CD8^+$ T cells (Fig. 5*c*). Our results are in line with initial microarray analysis of $CD8^+$ T cell subsets, which, for instance, also showed increased expression of gene like CCR7, CD28, MCAM, LEF1, PRKCA, CD31, SELL, and IL-7R in $CD8^+$ T cells with a $CD45RA^+$ and $CCR7^+$ (or $CD27^+$) phenotype (i.e., overlapping with stage 1 $CD8^+$ T cell subset to a large extent), and increased expression of genes like CCL5, GZMB, GZMA, CD63, PRF1, IFNG,

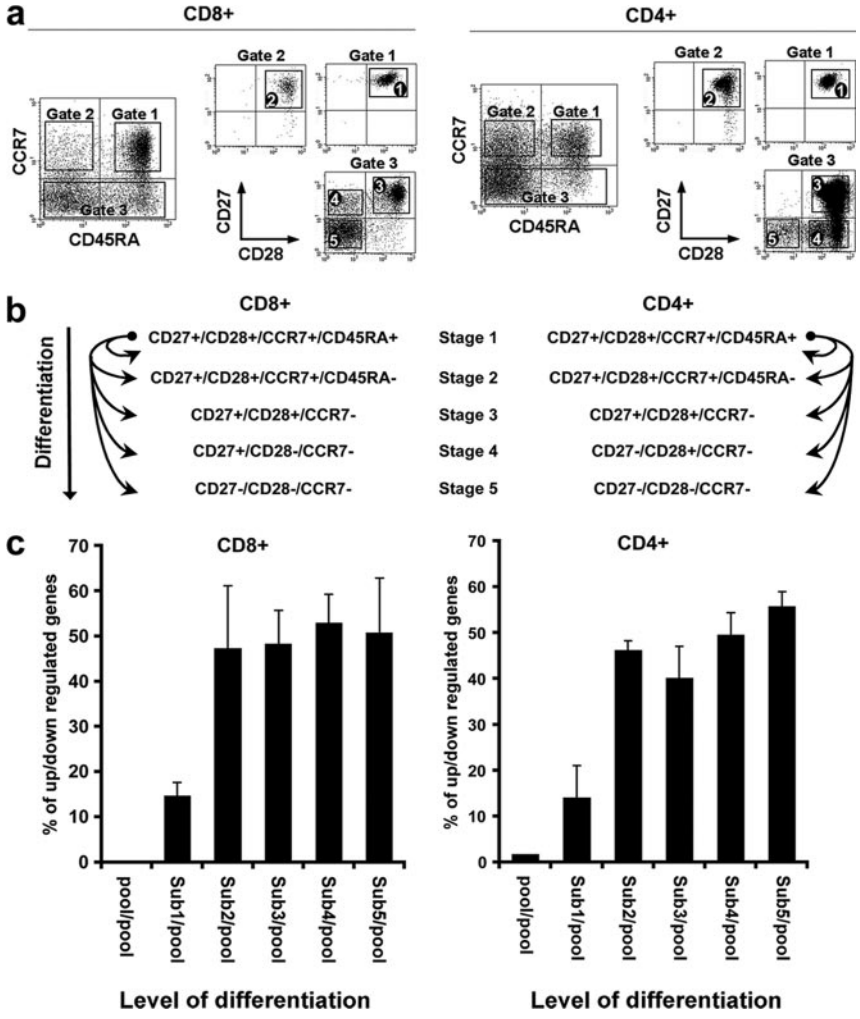


FIGURE 3. $CD8^+$ or $CD4^+$ T cell subpopulations defining distinct stages of differentiation based on the cell surface expression of CD45RA, CCR7, CD28, CD27. *a*, Definition of $CD8^+$ or $CD4^+$ T cell subsets of differentiation and FACS sorting. $CD8^+$ or $CD4^+$ T cell populations from two donors were first gated according to CD45RA and CCR7 expression and then sorted based on the expression of CD28 and CD27 into five distinct subsets defining five stages of differentiation (stage 1 = naive cells, and stages 2 to 5 = increasingly differentiated cells). *b*, Comparative microarray analysis of the different subsets. Subset 1 represents naive cells, and subsets 2 to 5 represent increasingly differentiated Ag experienced cells. cDNA probes from each $CD8^+$ or $CD4^+$ subset (1000 cells) were hybridized against a standard pool of naive $CD8^+$ or $CD4^+$ cells (three independently FACS isolated and amplified samples of 1000 cells) respectively, as illustrated with the arrows. *c*, Differences in gene expression profiles between naive and Ag experienced cell subsets. Percentage of genes that are up or down-regulated (2-fold) between the different subsets of $CD8^+$ or $CD4^+$ cell differentiation (stages 1 to 5) and the standard pools are presented. Bars show SD between donors.

KLRD1, IFIT1, FGR, TNFR1, and ITGB2 in $CD8^+$ T cells with a $CD45RA^+$ and $CCR7^-$ (or $CD27^-$) phenotype (i.e., overlapping with stage 5 $CD8^+$ T cell subset to a large extent) (13, 14).

Of note, we observed the down-regulation of genes encoding for fosB, junB, and RASA1, three proteins involved in cell cycle entry and/or normal cell proliferation, suggestive of a state of quiescence

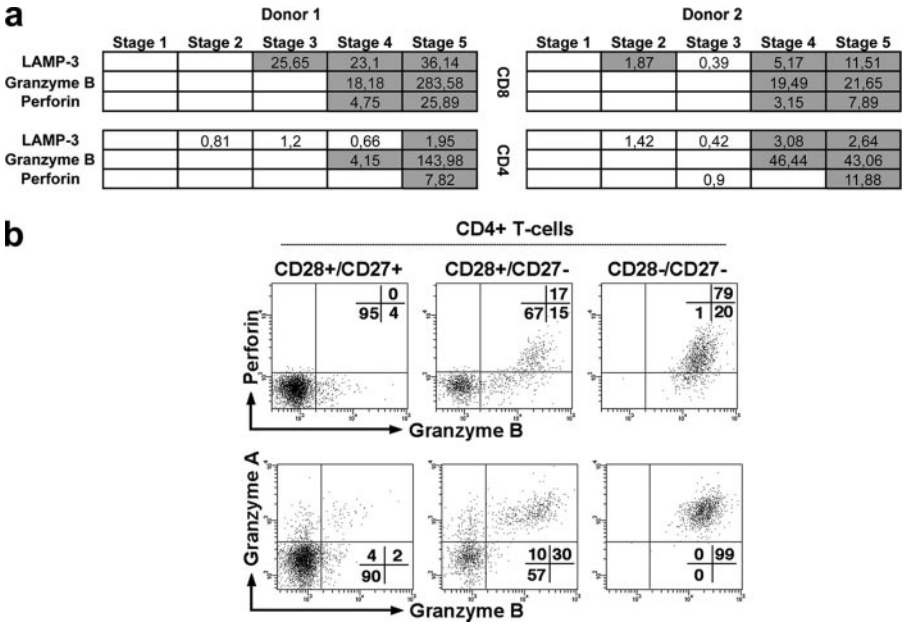


FIGURE 4. $CD4^+$ T cell differentiation and acquisition of cytotoxic potential. *a*, Expression of genes encoding for cytotoxicity related molecules in $CD8^+$ or $CD4^+$ T cells at distinct stages of differentiation. Gene expression ratios are shown, up-regulation is highlighted in gray. *b*, Representative example of cytotoxic factor expression in $CD4^+$ T cell subsets as assessed by FACS staining. Percentages of cells present in quadrants are shown.

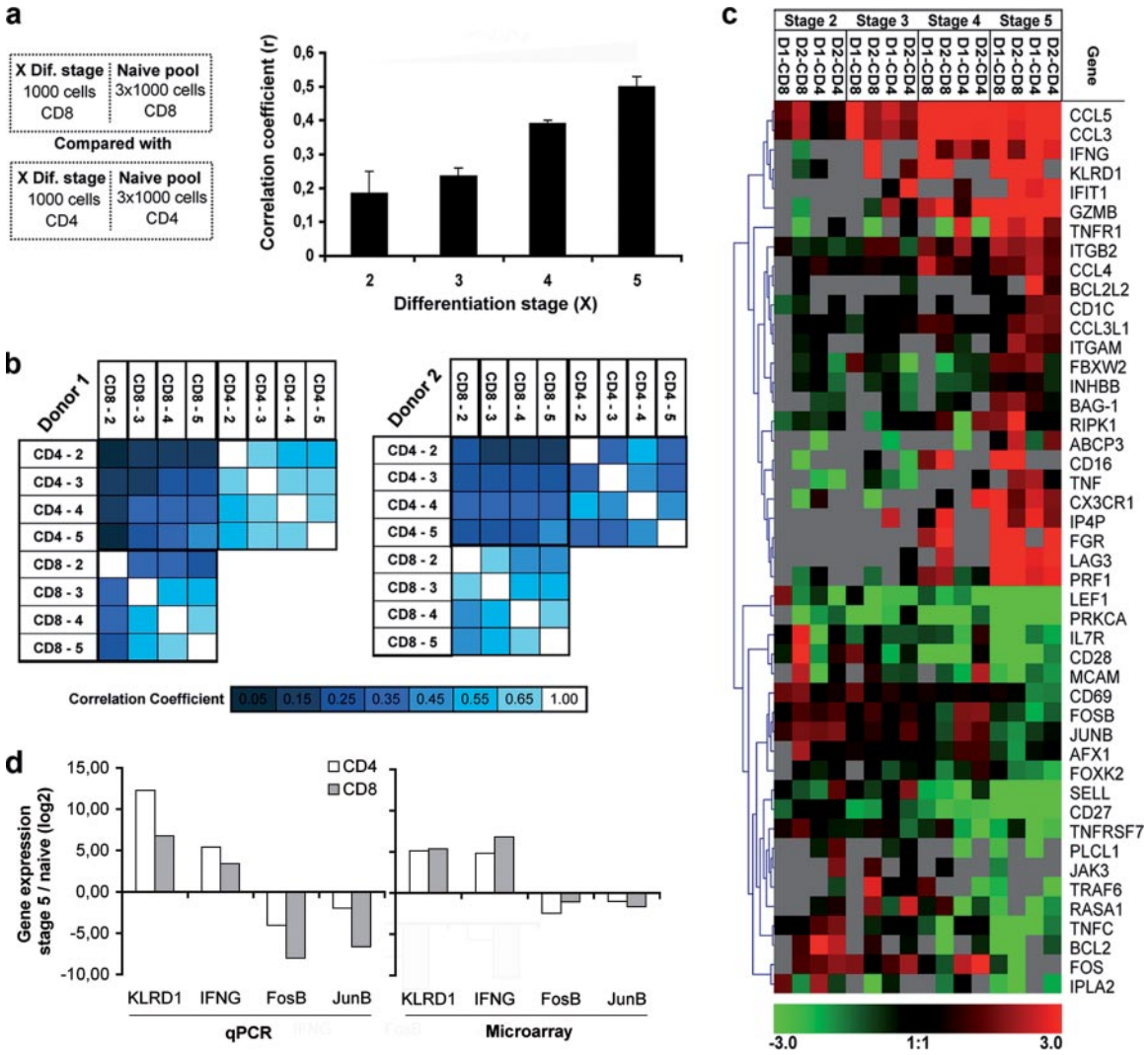


FIGURE 5. Similar evolution of CD8 or CD4 T cells with late differentiation. *a*, Similarity in gene expression profiles between $CD4^+$ and $CD8^+$ T cells at equivalent stage of differentiation. Mean Pearson correlation coefficients (r) between Ag experienced CD4 or CD8 subsets are shown for the two donors as well as S.D. between donors. *b*, Overall comparison of gene expression profiles for $CD8^+$ or $CD4^+$ T cell subsets of differentiation. Pearson correlation coefficients (r) are shown between all different Ag experienced CD4 or CD8 subsets for each donor. *c*, One dimensional hierarchical gene cluster derived from a two class unpaired statistical analysis of microarrays (SAM). Results are shown for two donors (D1 and D2), up or down-regulated genes are in red or green respectively. The color range bar indicates the log 2 gene expression ratio for each subset of Ag experienced T cells vs the naive pool standard. *d*, Validation of differential expression by real time PCR. Transcript level of KLRD1, IFNG, FosB, and JunB was measured by real time PCR in respective stage 5 and naive $CD4^+$ T cells (□) and $CD8^+$ (▨). Gene expression is shown as $\log_2$ x-fold differential expression for stage 5 vs naive T cells; comparing real time quantitative PCR and microarray.

for the T cells (23). Highly differentiated $CD4^+$ or $CD8^+$ T cells presented also reduced levels of the anti-apoptotic factor Bcl-2 gene (24), suggesting that T cells may have a limited survival capacity, under stress or upon activation. Similarly, the gene encoding for IPLA2, which was proposed to have a role in mitochondrial membrane repair and protection from oxidative stress (25) was also down modulated. Inversely, genes (e.g., KLRD1, CX3CR1, and CCL4), which have been associated with aged $CD8^+$ T cells (26), were clearly up-regulated in both highly differentiated $CD4^+$ or $CD8^+$ T cells. Overall, this suggests that late stages of differentiation may represent a common end in the development of both CD4 and CD8 T cell lineages. Validation of the microarray data was performed using real time quantitative PCR on a selection of genes. Real time PCR presented usually a higher dynamic range than the microarray for measuring the transcript level of KLRD1, INFG, FosB, and JunB, and it confirmed the differential expression of these genes between naive and highly differentiated cells (Fig. 5*d*).

Discussion

The coupling of global cDNA amplification to microarray analysis heralds a new era in gene expression profiling application. Direct reverse transcription and fluorescent labeling of mRNA to cDNA uses ~5–10 μg of total RNA. Efforts to reduce the amount of starting material have lead to a nowadays widely accepted T7 RNA polymerase based mRNA amplification protocol (27). Although a single round of this protocol yields ~1000-fold amplification, consecutive rounds of the original or modified protocols have been used to generate gene expression profiles from small tissue samples (27–29). We have recently established a magnetic bead based in-column T7 amplification procedure for starting material down to 50,000 cells (μMACS One-step T7 Template Kit,

our unpublished data). In this study, mRNA is captured using oligo(dT) magnetic beads directly from lysed cells, immobilized, and washed on magnetized columns and converted by several enzymatic steps which are completely performed within the column to amplified aRNA. We noted that besides intrinsic limitations like selective amplification of 3′ ends due to initial priming at the poly(A) RNA tail, in particular multiple rounds of T7 in vitro transcription, as needed when starting with low cell numbers, tend to be less robust and successful due to the high number of enzymatic reactions and purification steps. In contrast, PCR based amplification methods have been reported to skew original quantitative mRNA relationships. Thus, we have combined and modified several PCR based protocols published by e.g., Brady and Iscove (30), Hartmann (8), and Smith (7) to establish a new protocol with a particular emphasis on reproducibility and conservation of differential expression but also on ease of use, shortness of protocol and robustness. The protocol used in the present study circumvents shortcomings of PCR protocols yielding a sensitive and robust 2-day experimental procedure: i) magnetic bead based mRNA capture and first strand synthesis enhances efficacy of RNA isolation, ii) combined oligo d(T) and random primed cDNA synthesis leads to short overlapping fragments of comparable length avoiding PCR bias due to different transcript length, iii) uniform primer PCR avoids bias due to different primer annealing conditions, and iv) Klenow fragment labeling avoids additional PCR cycles.

The conditions to test the reproducibility of our protocol were chosen to be as comparable as possible to real applications. To this end, we have always used individual cells rather than "cell number equivalents" or "split pool" samples despite the fact that we have to deal with a certain biological bias at least when analyzing single cells. The biological bias is particularly high when the differential expression profiles generated from two different cell lines are independently repeated and correlated to each other. That is for example the RNA from 10 Jurkat and 10 Raji cells is amplified and hybridized against each other and the resulting ratios are correlated to arrays where independent 10 Jurkat and 10 Raji cells have again been hybridized against each other.

The reason for this rigorous test system is based on two experiences made in the course of establishing the protocol. First, using cell number equivalents normally means to extract the RNA from a large number of cells, to quality control the RNA and to take a certain amount of good quality RNA to perform the experiments. However, thereby one of the most critical steps when performing low cell number analysis is omitted, i.e., the isolation of high quality RNA from low cell numbers without having the chance of doing any in process quality control. Second, individual cells display individual characteristics despite the fact that they are, e.g., of a same line. This fact is not addressed when using split pools. The correlation coefficients yielded from self-on-self experiments as well as on repeated experiments of different cell types and using different microarray platforms document the applicability of our procedure for low cell analysis down to a single cell. Although using a very rigorous test protocol, we gain comparable or better results as reported by other amplification protocols (6–8, 28, 29, 31, 32).

This new technology enables us to perform microarray analysis on low number cell samples and in the case on FACS isolated primary lymphocytes to assess the gene expression profile from as low as 100 to 1000 cells. This represents a reduction of 2 to 3 log in required material, compared with previous studies using microarrays on primary lymphocytes (13–16, 33). It opens new possibilities of using microarray analysis, for instance to study gene expression profiles in rare cell subsets and/or in case of limited access to samples (e.g., small blood samples, biopsies). For instance, this includes the study of Ag specific T cells purified by multiparametric FACS sorting, to comprehend the difference between circulating tumor specific T cells and their tumor infiltrated counterpart, or to define precisely the characteristics of protective vs nonprotective T cells in HIV infection, issues that remain unanswered. In this study, we have applied this technology to provide the first study comparing CD4 and CD8 post-thymic lineage differentiation. Five $CD4^+$ and five $CD8^+$ T cell subsets defining a well established pathway of differentiation could be obtained from small single blood samples, and comparative analysis of the gene expression evolution along differentiation was performed. Gene expression profiles between subsets confirmed the establishment of a cytotoxic program by $CD4^+$ T cells as they differentiate in vivo. Interestingly, our data revealed a parallel between CD4 and CD8 lineage differentiation pathways, with similarities in the evolution of gene expression pattern occurring with late differentiation. Common sense or preconceived idea would argue in favor of unique developmental programs undergone by $CD4^+$ and $CD8^+$ T cells after activation to evolve along divergent pathways of differentiation and become increasingly distinct from each other as they differentiate further. In contrast, it appears that their respective program of differentiation may bring CD4 and CD8 lineages toward a common end, as suggested by shared alterations in expression for genes involved in cell cycling (like fosB, junB, and RASA1) or apoptosis (like Bcl-2, IPLA2, and TNFR1). On the same line, similar findings were reported in a recent study of $CD8^+$ $CD57^+$ (i.e., marker of senescence) cells by microarray, with the modulation of several factors that influence proliferation potency (e.g., fos and junB) or apoptosis (e.g., TNFR1) in these cells (33). In addition, the authors reported that these cells are characterized by the up-regulation of CCL5, PRF1, GZMB, KLRD1, CD11a, and CX3CR1 and the down-regulation of CCR7, IL7R, genes also highlighted in our cluster analysis of highly differentiated $CD8^+$ or $CD4^+$ T cells. Interestingly, recent efforts to examine gene expression modulation associated with cellular senescence point in the same direction (34). Microarray analysis of $CD4^+$ T cell clones that reached an apparent state of senescence in vitro (i.e., ceased to proliferate after extensive population doublings in culture) revealed alterations of a series of genes involved in fos regulatory pathway. Furthermore, the authors described the up-regulation of genes encoding for various proinflammatory molecules in these cells, which goes along with our analysis of the highly differentiated subsets (i.e., up-regulation of CCL3, CCL4, CCL5, CCL3L1, and IFNG). In keeping with data showing reduced proliferative capacity and telomere length in $CD4^+$ and $CD8^+$ T cells along differentiation (35, 36), late differentiation may therefore represent a common state of aged cells and probably the natural end of a T lymphocyte life.

It remains to be understood whether these cells (in particular cytotoxic $CD4^+$ cells) really own a particular role in the immune response or simply are no more than worn out T cells. Evidence in both virus infection or cancer settings support the idea that immune efficacy is held by early differentiated T cells, i.e., $CD4^+$ or $CD8^+$ subsets which present the highest proliferative capacity and functional heterogeneity (37, 38). However, recent data by Oxenius and colleagues (39) using mouse models of virus infection suggests that protective capacity of distinct subpopulations of memory T cells may vary in different conditions, related to the nature and the route of the challenge infection. In line with this possibility, we had previously shown that Ag specific $CD8^+$ T cells differ in differentiation phenotype in distinct virus infections (3). Depending on the pathogen and its site of replication, a particular T cell subset (i.e., either early or highly differentiated) may therefore be required to halt viral replication.

In summary, we have developed a procedure to perform gene expression profiling on low numbers of primary cells, which extends significantly the range of possibilities to use microarray technology. Applying this procedure to the study of T cell differentiation revealed an unexpected evolution of CD4 and CD8 lineages converging as cells reach late differentiation, which may correspond to a common end in T cell development.

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Disclosures

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