INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 18: 1127-1139, 2006

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Mitogen stimulation activates different signaling pathways in early- and late-divided T cells as revealed by cDNA microarray analysis

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Received June 20, 2006; Accepted August 17, 2006

Abstract. Mobilization of tumor-reactive CD8$^+$ T cells remains the major challenge of cancer immunotherapy. Knowing how and when the T cell response expands and differentiates after antigen stimulation would make a significant contribution to the development of tumor vaccines. In the current study, we used CFSE-based cell sorting and cDNA microarray to identify the gene expression profile of adjacent generations of T cells after PHA stimulation. Early-divided generations of T cells responded to stimulation by activating cell cycle and surviving gene pathways, while late generations of T cells had more dramatic changes in transcription of cytokine genes. Reconstruction of biochemical pathways, activated in both early and late generations of T cells, also confirmed the impact of division in focal-adhesion kinases. Because most tumors are infiltrated by lymphocytes, our studies indicate a novel approach to identify 'systemic biological responses' of T cells, which could determine the design, and optimization of effective tumor vaccines.

Introduction

Studies in previous years have focused on the identification and characterization of tumor antigens recognized by T cells. Among these tumor antigens, some consist of tumor proteins, which are present at low levels in healthy tissue, such as HER-2 and Folate receptor α (1,2). The processes of malignant transformation and metastasis coincide with increased amounts of these proteins. The increased amount of these proteins apparently coincides with the presence of CD8$^+$ cells, which recognize peptides from these proteins. The CD8$^+$ cells, present in malignant effusions, become activated by culture in the presence of IL-2, and mediate lysis of autologous tumors (3-5). Such cells are also present in healthy individuals (4,5). These results raise the novel hypothesis that immunity to cancer is induced *in vivo* by self-vaccination. Administration of a peptide HER-2 vaccine to cancer patients at high risk of metastasis resulted in at least 9- to 11-month delays in occurrence of metastasis in the vaccinated group compared with the non-vaccinated patients (6). In contrast, in patients with progressive disease dominated by an increased level of the prostate specific antigen, the same vaccine was insufficient to delay disease recurrence (7).

These findings together raise the need for characterization of the immune response potential of the T cells in an individual, before and after cancer vaccination. The potential of T cells to respond to antigen will indicate whether a cancer vaccine is expected to function as intended or not. Therefore it is important to elucidate which responses should be considered as indicators of activation.

The response of a T cell to a stimulus is characterized by cell division followed by differentiation (8,9). Cell division results in several successive generations of daughter cells, while differentiation is characterized by activation of transcription of cytokine genes (8). Cell division precedes expression of cytokine genes. Transcription products of cytokine genes are in general detectable after 2-3 divisions (10). The presence of cytokines in the activation environment polarizes the T cell response towards a type 1, 2, T regulatory phenotype or T-inflammatory phenotype (11-13). A large amount of work has been recently performed in characterization of the transcriptional profile of T cells from various sources after stimulation with antigen and cytokines (14-16).

To identify the activation potential and the early-activated genes in T cells, in this study, we re-examined this question using microarray technologies. We also hypothesized that the

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Abbreviations: HER-2, HER-2 protooncogene; IFN-γ, interferon γ; CFSE, carboxy-fluorescein acetate; PHA, phytohaemagglutinin; TIL, tumor infiltrating lymphocytes; MFI, mean fluorescence intensity; FA, focal adhesion; MAPK, mitogen activated protein kinase; PKC, protein kinase C; ERK, extracellular signal regulated kinase

Key words: gene transcription profile, T lymphocytes, cancer vaccine, focal adhesion kinase, cytokine-cytokine receptor

gene-pathways of activated T cells interact, and the responses are 'global' rather than associated with restricted specific pathways of differentiation. Expression of the transcripts of the genes encoding effector cytokine molecules (IFN-γ, perforin, granzyme) in lymphocytes associated with tumor cells only indicate that a response to the tumor exists. It does not however indicate that this response can expand to proliferation of T cells at re-activation with antigen/tumor Ag. Less differentiated T cells will expand and complete their differentiation program while more differentiated cells will die by T cell receptor-induced apoptosis.

If this hypothesis is correct then the transcriptional profile of T cells should change with the cell division. In this case genes other than the ones encoding for cytokines and effector-proteases will be activated first. Furthermore T cells expressing such functional signatures will die at re-stimulation. The response to vaccine will recapitulate division and differentiation of new generations of naïve T cells.

Materials and methods

Reagents and technical support. RNA extraction kits were obtained from Qiagen (Valencia, CA). Carboxy-fluorescein acetate (CFSE) was obtained from Invitrogen (Carlsbad, CA) and stored in aliquots at 4˚C. Phytohaemagglutinin (PHA) obtained from Gibco (Carlsbad, CA), was reconstituted at 1 mg/ml and stored frozen in aliquots. The RNA samples were separately analyzed by the Genomics Core Resource in MD Anderson Cancer Center and Agilent Technologies, Inc. (Palo Alto, CA). The Onto analysis software programs for microarrays were available online at http://vortex.cs.wayne.edu/ontoexpress/ (17).

CFSE labeling and PHA stimulation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-centrifugation and resuspended in PBS. PBMC were suspended in CFSE solution of 5 μM. After 10-min incubation, unincorporated CFSE was washed out by dilution in PBS, followed by centrifugation (18). CFSE-labeled lymphocytes were suspended into RPMI culture media containing 10% FCS. PHA (5 μg/ml) was added per $1x10^5$ CFSE-labeled lymphocytes. Lymphocytes were analyzed by flow cytometry for 4 consecutive days to verify their division (Fig. 1). Sterile flow-cytometry sorting was performed for separation of different generations of T cells, at Baylor College of Medicine, based on the dilution of CFSE (Fig. 2).

RNA isolation and gene chip hybridization. We used TRIzol reagent for isolation of total RNA from cultured cells (1 ml of TRIzol per $5\text{-}10x10^6$ cells), followed by chloroform/isopropyl alcohol precipitation of RNA. All the above procedures were performed in an RNase-free environment. Total RNA isolated using TRIzol was further purified using the Qiagen RNeasy cleanup procedure. The A260/A280 ratio was at least 1.9 for purified RNA. The quality of RNA was also assessed by agarose gel electrophoresis, in which the 28S band was 2-fold more intense than the 18S ribosomal RNA band. The RNA preparation was free from genomic DNA contamination.

RNAs from activated generation 1 and undivided T cells were analyzed using chips available from the Genomics Core Resource in MD Anderson Cancer Center (19). RNAs from activated generation 4 and generation 3 T cells were handled as follows: starting from total RNA, the cDNA synthesis was conducted with Cy3 and Cy5 fluorescence modified nucleotides being introduced inside, according to the manufacturer's (Agilent Technologies, Inc.) specifications with corresponding dye swaps. Both overnight hybridization and wash protocols were strictly followed for acquiring useful data.

Analysis of microarray results. The variability of the measurements was estimated using replicate spotting for each gene transcript on the array. When comparing the two channels, The smooth curves that estimate the within-channel standard deviation of the values of the two channels, were pooled to produce a single curve estimating the standard deviation (Σ) across the entire array, The smoothed t-statistic was computed as t-score = [log (b) - log (a)] divided by standard deviation (Σ), for each gene transcript. We selected the differentially expressed genes using a cut-off of t-score of 3.0. The smoothed t-score value is an estimate of the log ratio of gene expression levels between samples that have been re-scaled to account for the observed variability between the two determinations for each transcript.

To refine the gene expression profiles of divided T lymphocytes we used the Agilent Human 1 cDNA microarrays (G4100A) (20). These chips contain 12,814 unique clones. After hybridization, the Agilent gene chips were scanned by an Agilent DNA microarray scanner into the TIF (Tag Image File) format, analyzed with Feature Extraction Software version 5.1.1 (Agilent Technologies, Inc.), which uses the LOWESS (locally weighed linear regression curve fit) normalization method. The background signals were subtracted and the expression values of individual genes were obtained as the log ratio of the dye-normalized red (Cy 5) and dye-normalized green (Cy 3) channel signals. By comparing plots showing the results of designed array versus its dye-swap hybridization array, statistically significant ($p<0.01$) data points in both hybridizations were identified based on their individual fold increase between the two generations. Transcripts that indicated unchanged gene expression ($p>0.01$) and discordant results in two dye-swapped chips (having a positive slope in plot comparison) were not taken into consideration.

To better characterize the functionally-related genes which showed different expression levels between the two pairs of generations of T cells (1 versus 0, and 4 versus 3), we used the Onto-Express program (21,22) to research the following functional categories: biological processes, cellular roles, and molecular functions. Data were inputted with the Binomial distribution and Bonferonni correction. To construct gene pathways as graphical representations of gene interactions, the software performed searches based on the inputted information in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. The Onto-Pathway Express program (17) was also utilized to identify the biologically meaningful increase/decrease in gene transcription. The data were analyzed with hyper-geometric distribution, the probability model best suited to calculate the significance values (23) and a p value threshold of 0.05. The basic rank criterion considered was the impact factor. The impact factor considers the number of significantly different expressed genes, normalized fold change

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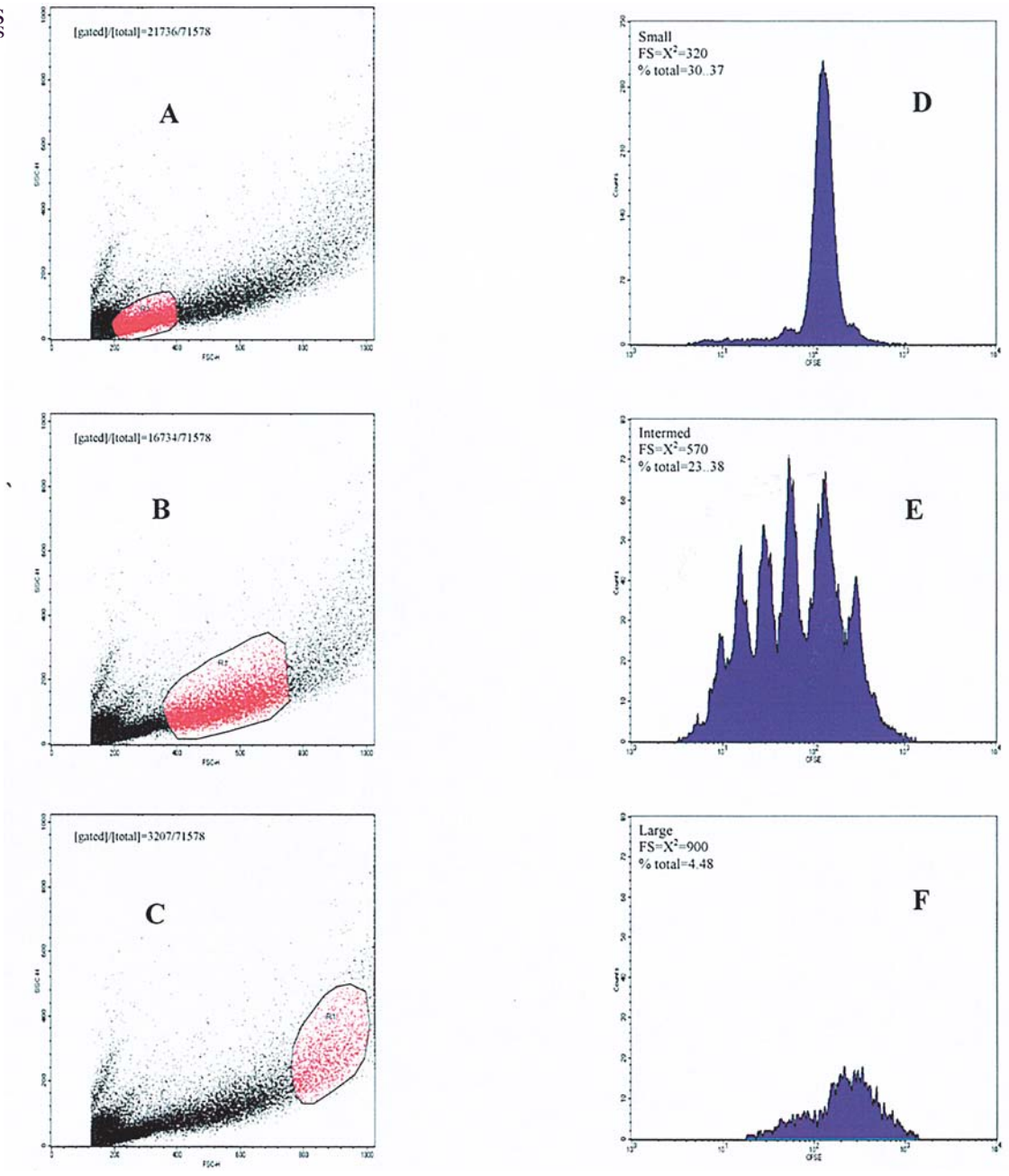

Figure 1. (A-C) Histogram of distribution of lymphocyte population according to their size four days after PHA stimulation. Patterns of cell division in T lymphocytes of small (D), intermediate (E) and large (F) size. Of note, the small, intermediate and large size T cells represented 30.36%, 23.37% and 4.48% of the total gated cells respectively.

of the genes and their perturbation factors on the pathway, which 'reflect the relative importance of each differentially regulated gene'.

Results

Mitogen stimulation induced different rates of division of cells of different sizes. CFSE as a fluorescent dye binds to DNA and is diluted through cell divisions. Fig. 1 shows that the majority of cells of small size divided few times. The majority of cells (>90%) accumulated in one large peak corresponding to a strong CFSE signal corresponding to cells, which did not divide or divided only once. The small population of large size cells divided less than cells of intermediate size. It is possible that most of the cells were in interphase. In contrast, the intermediate size cells formed six clearly distinguished peaks of fluorescence. This indicates that one parental population divided at least five times during four days. Therefore cells for microarray analysis were isolated from the intermediate size population. PHA is a T cell only mitogen. The small size cells are B-cells, which did not divide.

Following cell division, each daughter cell retains one-half of the fluorescence of the mother cell. This is illustrated by the well-defined peaks of fluorescence. Each well-defined peak of fluorescence with the mean fluorescence intensity (MFI) of 1/2, 1/4, 1/8, 1/16 etc. of the MFI of undivided cells corresponded to T cells which divided 1, 2, 3 and 4 times respectively. Cells which divided 0, 1, 2, 3 and 4 times were

Table I. Genes of increased expression level between generation 1 divided and undivided T cells.[a]

Fold increase	Accession	Symbol	Gene name
25.17	NM_053056	CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1)
18.40	AB067516	KIAA1929	KIAA1929 protein
15.58	NM_002090	GRO3	GRO3 oncogene
14.83	NM_003118	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
14.05	NM_002019	FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
13.46	NM_005093	CBFA2T2	core-binding factor, runt domain, α subunit 2; translocated to, 2
12.98	NM_015841	ADAR	adenosine deaminase, RNA-specific
12.79	NM_001191	BCL2L1	BCL2-like 1
12.06	NM_005435	ARHGEF5	Rho guanine nucleotide exchange factor (GEF) 5
11.94	NM_002460	IRF4	interferon regulatory factor 4
11.23	X65551	MKI67	antigen identified by monoclonal antibody Ki-67
10.70	NM_001826	CKS1	CDC28 protein kinase 1
10.66	NM_001566	INPP4A	inositol polyphosphate-4-phosphatase, type I, 107 kD
10.66	NM_000213	ITGB4	integrin, ß 4
10.50	NM_002593	PCOLCE	procollagen C-endopeptidase enhancer
10.40	NM_003726	SCAP1	src family associated phosphoprotein 1
10.09	NM_002734	PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, α (tissue specific extinguisher 1)
9.70	NM_002886	RAP2B	RAP2B, member of RAS oncogene family
9.56	L12146	PPP2R3	protein phosphatase 2 (formerly 2A), regulatory subunit B" (PR 72), α isoform and (PR 130), ß isoform
9.44	AL022069	RPS6KA2	ribosomal protein S6 kinase, 90 kD, polypeptide 2
9.37	NM_005197	CHES1	checkpoint suppressor 1
9.23	NM_005236	ERCC4	excision repair cross-complementing rodent repair deficiency, complementation group 4
8.96	NM_002131	HMGIY	high-mobility group (nonhistone chromosomal) protein isoforms I and Y
8.87	X59869	TCF7	transcription factor 7 (T-cell specific, HMG-box)
8.74	NM_000136	FANCC	Fanconi anemia, complementation group C
8.72	NM_002732	PRKACG	protein kinase, cAMP-dependent, catalytic, γ
8.52	NM_003392	WNT5A	wingless-type MMTV integration site family, member 5A
8.27	NM_000875	IGF1R	insulin-like growth factor 1 receptor
8.13	NM_005207	CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like
8.11	NM_001530	HIF1A	hypoxia-inducible factor 1, α subunit (basic helix-loop-helix transcription factor)
8.09	NM_000323	RET	ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
8.01	NM_002253	KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
7.72	NM_000597	IGFBP2	insulin-like growth factor binding protein 2 (36 kD)
7.71	NM_002311	LIG3	ligase III, DNA, ATP-dependent
7.57	NM_005225	E2F1	E2F transcription factor 1
7.56	NM_000633	BCL2	B-cell CLL/lymphoma 2
7.55	NM_001973	ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)
7.47	NM_001423	EMP1	epithelial membrane protein 1
7.29	J04102	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)
7.26	NM_000214	JAG1	jagged 1 (Alagille syndrome)
7.22	NM_004375	COX11	COX11 homolog, cytochrome c oxidase assembly protein (yeast)
7.20	NM_003929	RAB7L1	RAB7, member RAS oncogene family-like 1
7.19	M12783	PDGFB	platelet-derived growth factor ß polypeptide [simian sarcoma viral (v-sis) oncogene homolog]
7.17	NM_003455	ZNF202	zinc finger protein 202
7.13	NM_001224	CASP2	caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2)
7.06	NM_005180	BMI1	B lymphoma Mo-MLV insertion region (mouse)
7.06	D11327	PTPN7	protein tyrosine phosphatase, non-receptor type 7
7.02	NM_005582	LY64	lymphocyte antigen 64 homolog, radioprotective 105 kD (mouse)
6.93	NM_003426	ZNF74	zinc finger protein 74 (Cos52)
6.74	NM_003443	ZNF151	zinc finger protein 151 (pHZ-67)

[a]We first determined the Cy3 to Cy5 ratio for each microarray spot. Ratios >6.7 were considered significant. Details in Materials and methods.

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Genes of decreased expression level between generation 1 divided and undivided T cells.[a]

Fold increase	Accession	Symbol	Gene name
0.05	X07109	PRKCB1	protein kinase C, ß 1
0.06	NM_002488	NDUFA2	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 2 (8 kD, B8)
0.06	NM_005252	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
0.06	NM_004276	CABP1	calcium binding protein 1 (calbrain)
0.07	NM_002716	PPP2R1B	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), ß isoform
0.07	NM_021574	BCR	breakpoint cluster region
0.07	NM_001732	BTN1A1	butyrophilin, subfamily 1, member A1
0.08	NM_002110	HCK	hemopoietic cell kinase
0.09	NM_001188	BAK1	BCL2-antagonist/killer 1
0.09	NM_009588	LTB	lymphotoxin ß (TNF superfamily, member 3)
0.09	NM_006259	PRKG2	protein kinase, cGMP-dependent, type II
0.09	NM_005252	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
0.10	D10202	PTAFR	platelet-activating factor receptor
0.10	NM_005433	YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
0.10	NM_000022	ADA	adenosine deaminase
0.10	M68520	CDK2	cyclin-dependent kinase 2
0.11	NM_000887	ITGAX	integrin, α X (antigen CD11C (p150), α polypeptide)
0.11	NM_002359	MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)
0.11	NM_005694	COX17	COX17 homolog, cytochrome c oxidase assembly protein (yeast)
0.11	NM_005171	ATF1	activating transcription factor 1
0.11	NM_080549	PTPN6	protein tyrosine phosphatase, non-receptor type 6
0.12	NM_002422	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase)
0.12	NM_003032	SIAT1	sialyltransferase 1 (ß-galactoside α-2,6-sialytransferase)
0.13	Z11695	MAPK1	mitogen-activated protein kinase 1
0.13	AF047182	NDUFA6	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 6 (14 kD, B14)
0.13	M31523	TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
0.13	NM_000655	SELL	selectin L (lymphocyte adhesion molecule 1)
0.13	NM_005175	ATP5G1	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1
0.13	NM_003441	ZNF141	zinc finger protein 141 (clone pHZ-44)
0.14	NM_004757	SCYE1	small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)
0.14	NM_002228	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)
0.14	NM_001436	FBL	fibrillarin
0.14	NM_005394	PMS2L8	postmeiotic segregation increased 2-like 8
0.15	U47077	PRKDC	protein kinase, DNA-activated, catalytic polypeptide
0.15	NM_006243	PPP2R5A	protein phosphatase 2, regulatory subunit B (B56), α isoform
0.15	NM_000038	APC	adenomatosis polyposis coli
0.16	NM_013387	HSPC051	ubiquinol-cytochrome c reductase complex (7.2 kD)
0.16	NM_003248	THBS4	thrombospondin 4
0.16	NM_003326	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34 kD)
0.16	NM_001618	ADPRT	ADP-ribosyltransferase [NAD^+; poly (ADP-ribose) polymerase]
0.16	NM_006435	IFITM2	interferon induced transmembrane protein 2 (1-8D)
0.16	NM_000448	RAG1	recombination activating gene 1
0.16	NM_000655	SELL	selectin L (lymphocyte adhesion molecule 1)
0.17	NM_032996	CASP9	caspase 9, apoptosis-related cysteine protease
0.17	NM_003869	CES2	carboxylesterase 2 (intestine, liver)
0.17	M19961	COX5B	cytochrome c oxidase subunit Vb
0.17	NM_001987	ETV6	ets variant gene 6 (TEL oncogene)
0.17	NM_001350	DAXX	death-associated protein 6
0.17	NM_005003	NDUFAB1	NADH dehydrogenase (ubiquinone) 1, α/ß subcomplex, 1 (8 kD, SDAP)
0.17	NM_006286	TFDP2	transcription factor Dp-2 (E2F dimerization partner 2)

[a]We first determined the Cy3 to Cy5 ratio for each microarray spot. Ratios <0.17 were considered significant. Details in Materials and methods.

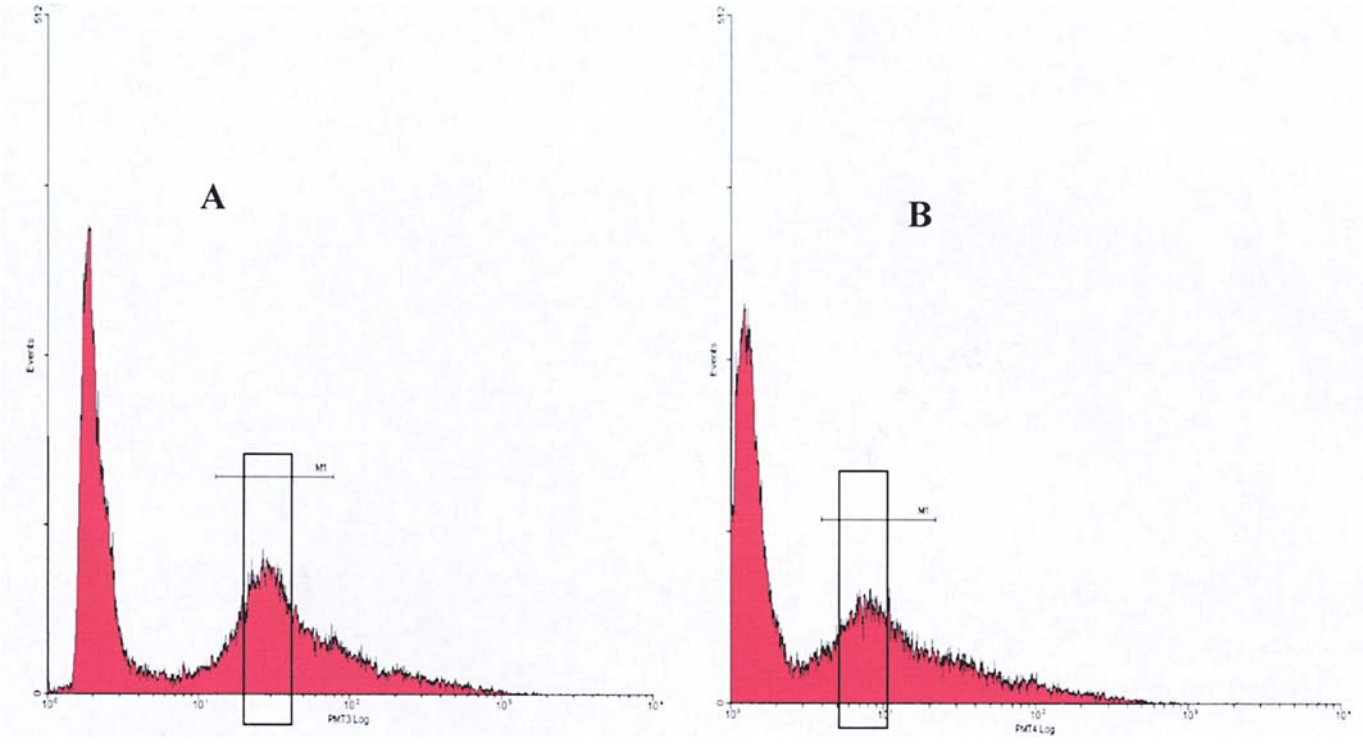

Figure 2. Isolated T lymphocytes were sorted based on the intensity of fluorescence CFSE-label. The gate indicates that cells were isolated only from the peak area corresponding to division 3 (A) and division 4 (B).

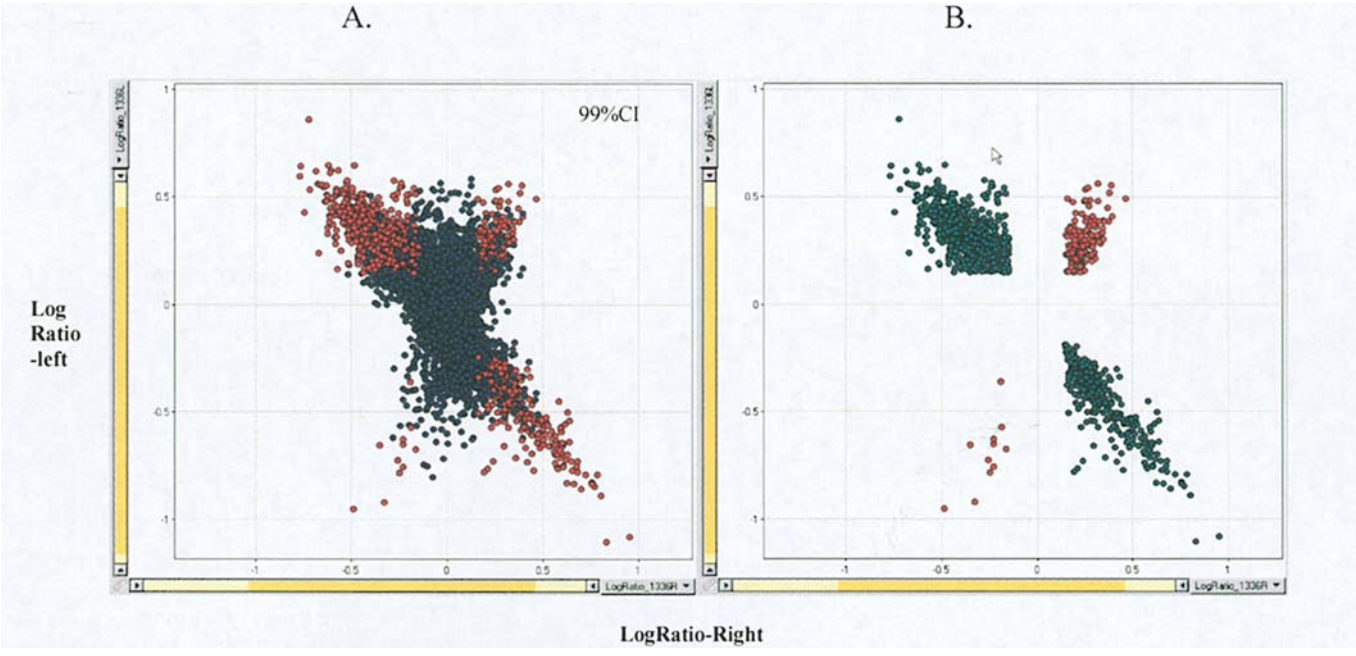

Figure 3. Differently expressed genes between generation 4 and 3 T cells were identified by microarray. (A) Comparisons of gene expression levels between two generations of T cells are depicted based on dye-swap hybridization assays. (B) Differently expressed genes (green in B) were identified by removing unchanged genes (blue in A) and discordant genes (red in B).

gated according to the position of the peaks and separated by sorting in cells in division 0, 1, 3 and 4, respectively. Cells in division 2 were not selected to avoid contamination of cells in division 1 and 3. For cell sorting gates were placed in the middle of the peak to avoid contamination (Fig. 2). The sorted cells were collected for RNA extraction and microarray analysis. This approach allowed the analysis of cells of similar size and metabolism, distinguished between divided cells and avoided contamination by mRNA from other cells (B-cells). The intermediate size cells represented 23.88% of the total; the small size cells represented 30.37% of the total, while the large-size cells represented 4.5% of total cells.

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Genes of increased expression between T cells divided 4 times and 3 times.

unigene_code	Gene name	Fold increase	p value
Hs.182825	ribosomal protein L35	4.44	5.01E-17
Hs.914	major histocompatibility complex, class II, DP α 1	4.41	1.71E-10
Hs.76038	isopentenyl-diphosphate δ isomerase	4.20	3.91E-07
Hs.181125	immunoglobulin λ locus	4.15	2.86E-11
Hs.100431	small inducible cytokine B subfamily (Cys-X-Cys motif), member 13 (B-cell chemoattractant)	4.08	2.45E-14
Hs.165590	ribosomal protein S13	3.76	3.29E-15
Hs.166468	programmed cell death 5	3.69	8.75E-15
Hs.179779	ribosomal protein L37	3.66	7.08E-15
Hs.9963	TYRO protein tyrosine kinase binding protein	3.64	2.40E-13
Hs.180255	major histocompatibility complex, class II, DR ß 1	3.64	4.59E-11
Hs.227751	lectin, galactoside-binding, soluble, 1 (galectin 1)	3.62	3.35E-09
Hs.181366	major histocompatibility complex, class II, DR ß 5	3.61	2.34E-14
Hs.239176	insulin-like growth factor 1 receptor	3.53	3.57E-14
Hs.119324	kinesin-like 4	3.48	1.70E-10
Hs.1051	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	3.46	5.80E-11
Hs.8372	ubiquinol-cytochrome c reductase (6.4 kD) subunit	3.41	7.87E-10
Hs.156110	immunoglobulin κ constant	3.40	1.63E-11
Hs.1499	heat shock transcription factor 1	3.35	6.94E-12
Hs.3709	low molecular mass ubiquinone-binding protein (9.5 kD)	3.34	1.56E-13
Hs.75538	ribosomal protein S7	3.28	1.58E-13
Hs.3066	granzyme K (serine protease, granzyme 3; tryptase II)	3.27	1.44E-09
Hs.82202	ribosomal protein L17	3.27	2.13E-13
Hs.83753	small nuclear ribonucleoprotein polypeptides B and B1	3.26	9.09E-13
Hs.174131	ribosomal protein L6	3.25	2.44E-13
Hs.112058	CD27-binding (Siva) protein	3.25	3.61E-11
Hs.44532	diubiquitin	3.24	4.72E-06
Hs.74407	nucleolar protein p40; homolog of yeast EBNA1-binding protein	3.24	3.77E-13
Hs.75659	MpV17 transgene, murine homolog, glomerulosclerosis	3.21	1.50E-03
Hs.23044	RAD51 (S. cerevisiae) homolog (E coli RecA homolog)	3.21	1.73E-11
Hs.184693	transcription elongation factor B (SIII), polypeptide 1 (15 kD, elongin C)	3.19	4.34E-13
Hs.278613	interferon, α-inducible protein 27	3.17	6.73E-07
Hs.89529	aldo-keto reductase family 1, member A1 (aldehyde reductase)	3.16	7.74E-13
Hs.97681	DNA (cytosine-5-)-methyltransferase 2	3.13	8.50E-13
Hs.50842	interferon-induced protein 35	3.12	5.07E-11
Hs.695	cystatin B (stefin B)	3.09	1.19E-12
Hs.226307	phorbolin (similar to apolipoprotein B mRNA editing protein)	3.08	9.19E-05
Hs.288986	survival of motor neuron 1, telomeric	3.08	1.16E-12
Hs.107476	ATP synthase, H^+ transporting, mitochondrial F1F0, subunit g	3.07	2.22E-12
Hs.182241	interferon induced transmembrane protein 3 (1-8U)	3.07	1.67E-12
Hs.21635	tubulin, γ 1	3.05	5.73E-09
Hs.75596	interleukin 2 receptor, ß	3.04	6.96E-12
Hs.146360	interferon induced transmembrane protein 1 (9-27)	3.04	1.62E-12
Hs.90443	NADH dehydrogenase (ubiquinone) Fe-S protein 8 (23 kD) (NADH-coenzyme Q reductase)	3.01	5.71E-05
Hs.1741	integrin, ß 7	3.01	2.80E-11
Hs.282997	glucosidase, ß; acid (includes glucosylceramidase)	3.00	1.46E-03

Differentially expressed genes between pairs of generations of activated T lymphocytes. We found that a number of genes were up- and down-regulated. The magnitude of the transcriptional response was different between activated generation 1 and undivided T cells by PHA (Tables I and II). The significantly different levels of expression of activated genes were defined based on fold increase in the expression levels of the corresponding mRNAs. Because the MDACC chips showed higher

Table IV. Genes of decreased expression between T cells divided 4 times and 3 times.

unigene_code	Gene name	Fold increase	p value
Hs.180016	semenogelin II	0.12	2.46E-19
Hs.1513	interferon (α, ß and ω) receptor 1	0.13	1.47E-20
Hs.128856	CSR1 protein	0.14	7.73E-21
Hs.21951	*Homo sapiens* Xq pseudoautosomal region; segment 1/2	0.15	6.35E-13
Hs.86161	GPI anchored molecule like protein	0.15	2.10E-17
Hs.113262	5-hydroxytryptamine (serotonin) receptor 4	0.15	2.02E-10
Hs.72879	melanoma antigen, family A, 1 (directs expression of antigen MZ2-E)	0.15	1.00E-18
Hs.200598	KIAA0537 gene product	0.16	2.18E-14
Hs.263395	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	0.16	9.21E-13
Hs.170027	mouse double minute 2, human homolog of; p53-binding protein	0.17	1.14E-19
Hs.66744	twist (*Drosophila*) homolog (acrocephalosyndactyly 3; Saethre-Chotzen syndrome)	0.17	3.95E-19
Hs.194679	WNT1 inducible signaling pathway protein 2	0.17	6.18E-19
Hs.169854	hypothetical protein SP192	0.17	1.27E-17
Hs.1827	nerve growth factor receptor (TNFR superfamily, member 16)	0.18	1.03E-17
Hs.12056	asialoglycoprotein receptor 1	0.19	2.11E-16
Hs.2820	oxytocin receptor	0.19	1.13E-18
Hs.146688	prostaglandin E synthase	0.19	7.48E-19
Hs.103124	ATPase, Ca^{++} transporting, plasma membrane 3	0.19	6.00E-12
Hs.79090	exportin 1 (CRM1, yeast, homolog)	0.19	1.58E-18
Hs.1985	spectrin, α, erythrocytic 1 (elliptocytosis 2)	0.19	1.41E-18
Hs.301174	glyceraldehyde-3-phosphate dehydrogenase pseudogene 14	0.20	3.96E-18
Hs.199179	RAN binding protein 2	0.20	1.73E-13
Hs.2913	EphB3	0.20	1.35E-17
Hs.24979	hypothetical protein DKFZp761P1010	0.21	2.72E-17
Hs.6975	PRO1073 protein	0.21	1.20E-17
Hs.38481	cyclin-dependent kinase 6	0.22	2.72E-17
Hs.256278	tumor necrosis factor receptor superfamily, member 1B	0.22	4.71E-17
Hs.2958	RNA binding motif protein, Y chromosome, family 1, member A1	0.22	8.20E-04
Hs.115617	corticotropin releasing hormone-binding protein	0.22	1.04E-11
Hs.159651	death receptor 6	0.22	1.55E-08
Hs.104133	GABAA receptor γ 3 subunit [human, fetal brain, mRNA Partial, 1536 nt]	0.22	1.51E-14
Hs.73923	pancreatic lipase-related protein 1	0.22	6.31E-14
Hs.41749	protein kinase, cGMP-dependent, type II	0.23	5.20E-05
Hs.171763	CD22 antigen	0.23	7.30E-11
Hs.288618	potassium channel, subfamily K, member 10	0.23	1.68E-15
Hs.42824	hypothetical protein FLJ10718	0.23	1.08E-11
Hs.28505	ubiquitin-conjugating enzyme E2H (homologous to yeast UBC8)	0.23	3.54E-11
Hs.47860	neurotrophic tyrosine kinase, receptor, type 2	0.23	8.31E-05
Hs.227850	KIAA0953 protein	0.24	1.88E-16
Hs.106511	protocadherin 17	0.24	5.91E-06

activation of transcription, we used the 6.74-fold increase and 0.05-fold increase as cut-off values. We found 100 activated genes from a total of 1824 genes. Comparison of the transcript levels from cells in the first division and undivided T cells revealed that genes related with cell cycle and survival were dramatically up-regulated, such as Cyclin D1 (fold increase: 25.17), BCL2L1 (12.79), CDK2 (0.10), BAK1 (0.09), TNFSF4 (0.16). This illustrated that T cells activate early more genes associated with cell division and survival.

The Agilent chips identified 1325 genes differently expressed in generation 4 compared with generation 3 (Fig. 3). To select significantly activated genes, we used a 3-fold increase and a 20-fold decrease in the amount of transcribed products as cut-off values (Tables III and IV). Up-regulation and down-regulation of gene expression following division indicated that daughter cells expressed higher levels of the genes associated with translation, such as ribosomal proteins. Significant up-regulation of expression of genes involved in

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Identified gene clusters among differentially expressed genes between dividing T cells.

Gene cluster	Gene no.		p values	
Generations	1 vs. 0	4 vs. 3	1 vs. 0	4 vs. 3
Molecular functions				
Signal transducer activity	23	51	0.27697	0.00071[a]
Receptor activity	10	28	0.17826	0.00724[a]
Receptor binding	8	15	0.39455	0.00667[a]
Binding	78	183	0.17857	0.00038[a]
Nucleotide binding	16	46	0.04962[a]	0.33669
Protein binding	41	72	0.04428[a]	0.00753[a]
Transcription regulator activity	19	26	0.17176	0.06631
Transcription factor activity	17	19	0.06937	0.09705
Catalytic activity	40	114	0.38994	0.27099
Biological processes				
Physiological process	85	246	0.1954	0.00369[a]
Metabolism	63	166	0.4458	0.06596
Nucleotide	-	64	-	0.08620
Intracellular macromolecular	32	75	0.20565	0.21987
Cellular process	88	244	0.38518	0.00026[a]
Transport	-	49	-	0.12220
Regulator of cellular metabolism	-	34	-	0.00481[a]
Cell communication	36	69	0.24474	0.00025[a]
Regulation of biological process	42	60	0.30792	0.00013[a]
Cellular components				
Cell	70	227	0.17423	0.00304[a]
Plasma membrane	27	45	0.46883	0.00780[a]
Cytoplasm	53	88	0.37383	0.15286
Organelle	47	138	0.49119	0.25581
Nucleus	35	77	0.38520	0.16987
Endoplasmic reticulum	2	20	0.10683	0.09258
Mitochondria	11	21	0.06662	0.03276[a]
Protein complex	14	60	0.14193	0.01617[a]
Significant p values			2/23	14/23

programmed cell death (PCD5) and cytolysis (granzyme) was also observed.

Mitogen activation induced distinct gene ontology profiles in divided T cells. To ascertain whether the different levels of expression of the gene transcripts associate with the activation of functional genes in specific processes we performed clustering analysis. Table V shows the gene transcripts expressed in generation 4 versus generation 3: 275 genes were important in molecular function, 183 genes were involved in binding/cell adhesion, and 114 genes were involved in catalytic processes, while 267 genes were involved in biological processes. The results confirmed the activation of translation and of cellular metabolism of later generations of T cells.

Less activated genes were identified by clustering analysis, when generation 1 of divided was compared with undivided T cells (Table V). Significant differences ($p<0.05$) were observed only in genes involved in protein binding and nucleotide synthesis and transport. It is possible that differences resulted from different designs of the chips, i.e. from the different probes in each chip. However it is also possible that stronger specific gene activation occurs in later divisions. Cells which divided once activated a large number of genes although there was less specificity in the activated pathways.

Mitogen stimulation activated different signaling pathways in early- and late-divided cells. To identify the impact of mitogen stimulation in biological processes, the impact factors were determined for each activated pathway.

Between generation 1 and undivided T cells, mitogen stimulation had the strongest impact on the focal adhesion pathway (impact factor 327.39) and the least impact on the cytokine-cytokine receptor interaction pathway (impact factor 68.71). Similar results were obtained with generation 4 and 3 stimulated T cells (focal adhesion impact factor 34.77). The least activated pathway was the Notch signaling (impact factor

Table VI. Impacted pathways in T lymphocytes by mitogen stimulation identified by the Onto-Pathway Express program.

Pathways	Impact factor		p-value		Input/pathway	
Generation	1 vs. 0	4 vs. 3	1 vs. 0	4 vs. 3	1 vs. 0	4 vs. 3
Focal adhesion	327.39	34.77	6.5052E-2	1.2891E-1	10/163	5/163
Regulation of actin-cytoskeleton	166.01	34.11	3.0062E-1	3.5965E-1	6/209	5/209
Cytokine-cytokine receptor interaction	68.71	27.06	2.5202E-1	4.9285E-1	6/258	10/258
TGF-ß signaling	-	23.76	-	2.1907E-1	-	3/83
Jak-STAT signaling	-	14.44	-	1.8960E-1	-	5/159
MAPK signaling	127.20	12.37	8.7642E-2	9.2687E-2	12/247	3/247
Cell cycle	-	11.22	-	3.1436E-1	-	3/98
Phosphatidylinositol signaling	184.49	-	3.4935E-1	-	2/69	-
Wnt signaling	123.39	-	3.3275E-3	-	8/146	-
Apoptosis	70.09	-	1.4254E-1	-	5/98	-

Table VII. Impacted focal adherent pathway components in T lymphocytes by mitogen stimulation

Generation	Component	Fold increase
4 vs. 3	Integrin ß-chain (ITGB)	3.01
	VAV	2.01
	Integrin-linked kinases (ILK)	1.93
	Caveolin (CAV)	0.45
	Filamin	0.58
1 vs. 0	Cyclin D1 (CycD)	25.17
	FMS-like tyrosine kinase 1 (FLT1)	14.05
	Integrin ß-chain (ITGB)	10.66
	Insulin-like growth factor type 1 receptor (IGF1R)	8.27
	Kinase insert domain receptor (KDR)	8.01
	B-cell CLL/lymphoma 2 (Bcl-2)	7.56
	Platelet-derived growth factor ß (PDGFB)	7.19
	Protein kinase C (PKC)	0.05
	Mitogen-activated protein kinase (MAPK)	0.13
	c-JUN	0.14

3.15) (Table VI). Therefore although we used different chips, the increase in the impact factor was similar between the least affected and the most affected pathway (5.22- vs. 10.9-fold.)

The focal adhesion (FA) pathway is an example of precise and efficient coordination of signaling events (24), which connects the extracellular environment with intracellular signaling and protein function. Connection is made through transmembrane integrin-receptors and cytoskeletal components. Different conditions of ECM, of type of integrin, and of cellular environment result in different responses of the FA. The focal adhesion kinase (FAK) and paxillin are the main components of focal adhesion complex, induced by Rho (Ras homolog)-family members Fac and CDC42. Integrin signaling uses as a second intracellular messenger system tyrosine and serine/threonine kinases, and corresponding phosphatases. This pathway signals bi-directionally (25,26).

Between generation 1 and undivided T cells, the MAPK signaling pathway had the most activated genes (12 genes compared with 10 genes of the focal adhesion pathway). Ten genes were found in the focal adhesion pathway as differentially expressed, either up- or down-regulated (Table VII). They included CyclinD1, ITGB, Bcl-2, PKC, MAPK, and c-JUN. Cyclin D is the cell cycle protein. Bcl-2 and Bcl-xL are the main pro-survival molecules, which are activated after mitogen, antigen and/or cytokine activation of lymphcytes. c-Jun can bind DNA and regulate transcription. It is also notable that the anti-apoptotic pathway is strongly activated, showing that early generations of divided T cells have better survival ability than the later generations.

It is interesting that JAK-STAT and TGF-ß signaling pathway are found to be activated when comparing generations 4 and 3. The comparison also indicated that the cytokine-cytokine receptor interaction pathway had the most affected genes. Therefore the least activated pathway in division 1 had the most activated genes in division 4. There were 10 genes activated in this pathway compared with 5 genes activated in the focal adhesion pathway. Activated genes of the focal adhesion pathway consisted of integrin ß-chain (ITGB), filamin, integrin-linked kinases (ILK), VAV, and Caveolin.

Filamin belongs to the transmembrane integrin family, involved in ECM signaling (27). ITGB mediates various interactions. Localization of ITGB isoforms affects myotubule morphology and myogenesis. Tyrosine-phosphorylated caveolin can interact with FA proteins by FAK, affecting cell motility and directional control (28). Intracellular serine/threonine integrin-linked kinases (ILK) are one of the convergence points of integrin- and growth factor-signaling pathways. The small adaptor protein, vav, couples tyrosine kinase signals with the activation of the Rho/rac GTPases, thus leading to cell differentiation and/or proliferation.

Therefore the activated genes were different in the cells which divided once compared with cells which divided 4 times. The results indicate that the identification of the affected pathway is not sufficient for definition of a T cell response, but all factors should be considered in defining a global response to activation (Table VII).

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fore responses at the first division consisted of ı of the genes controlling adhesion, cell division, and responses to extracellular signals, while responses at the fourth division consisted of activation of cytokine receptor signaling as we expected.

Discussion

We identified novel differences in gene expression profiles from divided generation 1 compared with undivided and from generation 4 compared with generation 3, of T lymphocytes. The transcription profiles of generation 0 and 1 T cells have more genes activated in surviving and proliferation, while generation 4 and 3 T cells have more in cytokine signaling and cell function. We identified activated gene pathways, which are affected quantitatively and qualitatively when T cells are activated by mitogen. In both generations of T cells, the FA pathway is the most activated, while early generations of T cells have activated survival pathways and later generations of T cells have activated JAK-STAT and TGF-ß signaling pathway. Our results together define functional 'signatures' of responses to PHA and indicate that a cancer immunogen should activate T cells to divide at least 4 times to expect any effector cytokine secretion.

Mitogen activated a number of genes related to signal transduction and gene transcription (Table V). These genes, especially the pathway related genes (Table VII), connected membrane binding, signal transduction, and metabolism. Modulation of transcription of gene expression of T cells during differentiation of effector cells was reported. Of these, transcripts encoding for CD62L, GPI-linked proteins, chemokines CCR2 and CCR5, perforin, granzyme, TCR signaling, and cytokine signaling proteins had increased expression in effector T cells. However, the conclusions of this study were obtained by comparing murine $CD8^+$ T cells 8 days after stimulation of naïve and unstimulated T cells (29). Hess *et al* (14) recently reported the kinetic profiles of gene expression of naïve $CD4^+$ T cells after activation. Upon anti-CD3 and anti-CD28 antibody stimulation, human naïve $CD4^+$ T cells had 196 genes with changed expression levels during the first 24 h. The genes were functionally involved in maintenance of rest status, adhesion/migration, cell cycle progression, and cytokine production. Chtanova (15) reported genes preferentially transcribed by T cells. These genes provided 'signatures' for T cell responses. For example, they differentiated γδ T cells from αß T cells by a number of genes down-regulated during activation, including genes involved in the pre-mRNA splicing process, such as transformer 2ß, suppressor of white apricot homologous 2, and pre-mRNA splicing factor 16. Mao *et al* (30) used PHA-stimulated lymphocytes in the presence of IL-2 to identify unknown genes and to associate them to biological processes. To identify genes specific for T cell activation, the authors examined genes co-regulated with IL-2. They found some ESTs (expressed sequence tags) that encoded previously unknown proteins involved in GTP metabolism, while others were linked to newly published cDNAs and cytokine receptor genes. In support of our conclusions, the authors found that genome scale analysis of gene transcription during T cell activation can facilitate the identification of genetic functional networks. Adarichev *et al* created the portrait of experimental autoimmune arthritis (31) using the same algorithm for the functional classification of genes. The authors found similar gene clusters to those in the current study. The main gene clusters were related to chemokine, cytokine, activation, inflammation and cell adhesion.

Compared with the previous studies our contribution was to connect the differentially expressed genes into a logical biological scenario. Our results refine the definition of activated T cells specifically to adjacent generation (pair generations), revealing that at the level of mRNA transcription, the metabolism of cellular components and special characteristics were changed.

The significant change between adjacent generations of T lymphocytes in our study (Table VII) showed that early generation activated T cells are resistant to apoptosis, sensitive to growth stimulation and proliferate well, as shown by the expression levels of growth factor related, Cyclin-D1 and Bcl-2. The large number of activated genes of MAPK pathway, 12 in early generations versus 3 in later generations (Table VI), indicate the rapid and dramatic response of T cells to external stimuli. After the quick expansion, the later generations of T cells (generation 4) decrease their responses to ECM signals while increasing the motility and proliferation ability of daughter cells.

This phenomenon is interpreted as daughter T lymphocytes being well prepared to respond by effector-function when they meet a target, while rejecting other activators, e.g. Notch-ligands, through decreased sensitivity.

Our conclusions are supported by the activation of naïve T cells. Stimulation of TCR and of co-stimulatory molecules regulates serial transcription of genes such as those involved in the cell cycle, cytokine production and survival (29,32-34).

Global gene transcription profiling based on the relationship between effector molecule-expression and the number of divisions has important implications for development of cancer vaccines and prediction of the effectiveness of vaccine-activated lymphocytes associated with tumors. T cells isolated from freshly-resected tumors can proliferate in the presence of small amounts of cytokines. Cytokines induce proliferation of antigen-activated T cells *ex vivo*. Analysis of the transcriptional profile of cytokine, e.g. IL-2, IL-15 or IL-7-propagated T cells, undergoing several divisions can be compared with the transcriptional profile of T cells to a standard mitogen, and to several antigens which can prime or re-activate $CD8^+$ cell proliferation *in vitro*. Based on this scenario, an increase in the level of granzyme B or perforin, for example, after activation with IL-2 will indicate that *ex vivo* activated cells are differentiated and are unlikely to expand more or they are incompletely differentiated and are likely to expand at recall with the same Ag. If the first scenario is found to be correct then it will be necessary to replace the Ag used for vaccination to select a different population of effectors.

Furthermore, the MAPK and PKC pathways are the main effector pathways of TCR-signaling. A number of transcriptional regulators, e.g. Foxp-3, are activated by ERK-signals of insufficient strength. Expression of ERK-targeted genes and of transcriptional regulators by stimulation with a tumor antigen in relation to the 'normal' stimulation levels will predict whether the vaccine will activate anti-tumor effector cells or regulatory cells (T_{reg}) which inhibit the inflammatory responses.

Global lymphocyte gene transcription profiling should become useful for prediction of cancer progression due to escape from immunosurveillance or establishment of tolerance. In the study of colorectal cancer metastasis, increased mRNA levels of T cell activation markers in TIL, such as IFN-γ, granzyme B, and CD45RO, were found in tumor samples and correlated with absence of signs of early metastasis invasion and increased survival (35). Another study of gene profiling in melanoma (36) found 33 genes in the pretreatment samples to be predictive of clinical response. Of these 16 out of 33 genes were related to T cell activation, including CTL-mediated cytolysis, TGF-ß/IFN/TNF regulation, JAK/STAT pathway, as is described in the current study. For example, the expression level of INF-γ regulatory factor 2 (IRF2) was enhanced and IFN-α inducible protein 27 (IFI27) suppressed in pretreated samples that regressed completely compared with those of nonresponding patients. However, in the current study, IRF2 was up-regulated 1.2-fold and IFI27 3.2-fold in generation 4 T cells.

Our findings define a 'systemic biology approach' for identification of the responsiveness or unresponsiveness to tumor and of the immunogenicity of cancer vaccines. Prior to the easy observation of dramatic changes in surface molecules and effector cytokines, T cells respond with changes in transcription, translation and adhesion which target specific genes. Such early changes are linked temporally with changes in effector molecules. The detection of early changes in apparently unrelated genes would allow the development of better vaccines for human cancer.

Acknowledgements

This work was supported in part by an NIH Core grant (no. 16662), DOD grant (no. 01-1-299), and funds from the Graduate School of Biomedical Science and Agilent Technologies, Inc. We thank Dr Scott Vacha (Agilent Technologies, Inc.) for assistance and guidance in microarray analysis.

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