Microarray analysis reveals similarity between $CD8^+CD28^-$ T cells from young and elderly persons, but not of $CD8^+CD28^+$ T cells

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Abstract

We isolated highly purified $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells populations from healthy young and elderly persons for gene expression profiling using Affymetrix oligonucleotide microarrays. We demonstrate that the gene expression profile of $CD8^+CD28^-$ T cells is very similar in young and elderly persons. In contrast, $CD8^+CD28^+$ in elderly differ from $CD8^+CD28^+$ in young persons. Hierarchical clustering revealed that $CD8^+CD28^+$ in elderly are located between $CD8^+CD28^+$ in young and $CD8^+CD28^-$ (young and old) T cells regarding their differentiation state. Our study demonstrates a dichotomy of gene expression levels between $CD8^+CD28^+$ T cells in young and elderly persons but a similarity between $CD8^+CD28^-$ T cells in young and elderly persons. As $CD8^+CD28^+$ T cells from elderly and young persons are distinct due to a different composition of the population, these results suggest that the gene expression profile does not depend on chronological age but depends on the differentiation state of the individual cell types.

Introduction

Aging is universal but the precise mechanisms linking the aging immune system to diseases in the elderly are poorly understood. Recent advances in the study of global patterns of gene expression with the use of microarray technology, coupled with extensive data analysis using bioinformatic tools, have provided new insights into the mechanism of the aging of the immune system. This method can provide a wealth of information at the level of gene expression and is a powerful method to identify genes and pathways involved in complex processes.

Aging has often been associated with a 'decline' in immune function. The immune system undergoes dramatic restructuring with age, leading to a decline in immune responses and an increased vulnerability of old individuals to infectious diseases. T cell immunosenescense has traditionally been associated with thymic involution, since a striking decline in the output of new thymus-derived T cells occurs with age [1]. However, absolute T cell numbers remain comparable between young and old individuals [2], requiring new T cells to be generated extra-thymically or by clonal expansion of pre-existing T cells. One intriguing change observed in the T cell pool with aging is the marked increase in the proportion of $CD8^+$ T cells lacking expression of the CD28 surface receptor [3], a major costimulatory molecule required for functional T cell activation [4]. The loss of CD28 expression on T cells is the most consistent biological indicator of aging in the human immune system, and the frequency of $CD28^-$ T cells is a key predictor of immunocompetence in the elderly. $CD8^+CD28^-$ T cells are often oligoclonal in nature and do not proliferate well in response to antigenic stimulation [5, 6]. These clonally expanded T cells can persist in humans for years [7]. $CD8^+CD28^-$ T cells occur in virtually all healthy elderly subjects [5], and an elevated number of this T cell subset is associated with numerous autoimmune conditions such as systemic lupus erythematosus [8] and rheumatoid arthritis [9].

Additionally, persistent antigenic stimulation such as CMV infection drives the accumulation of $CD8^+CD28^-$ T cells. It was therefore of interest to analyze the gene expression profile of $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets from young and elderly persons.

Material and methods

Purification of $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells

Peripheral blood was obtained from four young and four elderly healthy volunteers (Table 1). The health status of these persons was defined by the absence of severe diseases (diabetes, rheumatoid arthritis, dementia and cancer), acute infectious diseases and drugs which are known to interfere with the immune system. All participants had given their written informed consent and blood collection was approved by the Ethics Committee of Innsbruck Medical University. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech AB, Uppsala, Sweden). $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells were enriched from PBMCs in a series of separations using magnetic beads. To obtain sufficiently high subset numbers for analysis, purifications were only performed in CMV-seropositive persons who had high numbers of $CD8^+CD28^-$ cells. PBMCs were depleted from non T cells by application of a Pan T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD8^+$ T cells were then positively selected using CD8 Multisort Microbeads (Miltenyi Biotec). After enzymatic removal of the CD8 Multisort Microbeads, $CD8^+$ T cells were stained with an allophycocyanin-conjugated mAb recognizing CD28 (BD Pharmingen) and $CD28^+$ cells were obtained by positive selection using anti-allophycocyanin microbeads and a LS column (Miltenyi Biotec). The purity of the obtained $CD8^+CD28^+$ population was more than 90%. The $CD28^-$ fraction was depleted of residual $CD28^+$ cells applying an additional LD column (Miltenyi Biotec).

Affymetrix microarray experiment

Total RNA was extracted using TRI reagent (Sigma-Aldrich, Vienna, Austria) from freshly purified $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells. Glycogen (Roche) was added as a carrier for RNA precipitation at a concentration of 1 µg/ml. As a minimum amount of 1.5 µg RNA for microarray analysis was needed, a two-cycle target labeling protocol was used in our case to amplify total RNA (Affymetrix). Hybridization was done onto high-density oligonucleotide human genome array GeneChips U133 Plus 2.0 (Affymetrix). This chip comprises more than 54.000 probe sets and analyzes the expression level of over 47.000 transcripts and variants including 38.500 well-characterized human genes. The arrays were scanned at 3 µm resolution using a GeneChip® Instrument System. Affymetrix gene chip hybridization experiments were outsourced to the Microarray Facility Tuebingen, Germany, an authorized Affymetrix Service Provider. Both, experimental and data analysis workflow were fully

compliant with the MIAME 2.0 Standard. Gene Chip Annotation Data for U133 Plus 2.0 Array were supplied by Affymetrix Inc.

Microarray data analysis

CARMAweb (Comprehensive R-based Microarray Analysis web service) was used for data preprocessing (background correction, quality control and normalization) [10]. Affymetrix microarray suite version 5 (MAS5) algorithm was applied for probe-level normalization and background correction. The coefficient of variability of $CD8^+CD28^+$ T cells between the two young persons and between the two old persons was 11% and 14%, respectively, while the coefficient of variability of $CD8^+CD28^-$ T cells was 17% and 15%, respectively. To minimize the negative effects of random noise, filtering steps were applied. Genes that were consistently absent were excluded from analysis. Analyses were done using TM4 MeV software [11]. The resulting genes were then analyzed on the basis of their biological process, function or pathway from collectedly protein database by Panther classification system [12].

Validation of microarray results by using quantitative RT-PCR

cDNA first strand synthesis was reverse transcribed from 1 µg total RNA preparation using Reverse Transcription System (Promega) and oligo dT 15 primer. Several genes that indicated a remarkable difference between subsets were selected for validation by means of quantitative RT-PCR with the LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland). cDNA primers for amplification of target genes were designed using Primer3 software [13] (Table 2). PCR was performed in a total volume of 22µL with 60pM (6µL) primers. cDNA template of $2.5x10^{-2}$ µg total RNA (5µL) and 11µL 2x SYBR Green 1 Master (Roche Diagnostics) for 50 cycles. cDNA from 3 young and 3 elderly persons were checked to ensure consistency of the results. mRNA expression was normalized to GAPDH.

Results

We used highly purified $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells populations from healthy young and elderly persons for gene expression profiling using Affymetrix oligonucleotide microarrays. Hierarchical clustering analysis was used to explore the relationship between $CD8^+$ T cell subsets in different age groups. Firstly, we identified differentially expressed genes between $CD8^+CD28^+$ and $CD8^+CD28^-$ in young and elderly persons. For this purpose, one-way ANOVA test with $p > 0.05$ was used and samples were divided into four groups based on different subsets and age groups ($CD8^+CD28^+$ young, $CD8^+CD28^+$ old, $CD8^+CD28^-$ young, and $CD8^+CD28^-$ old). A set of 4964 probe IDs corresponding to 4115 genes were identified to be differently expressed between the groups. Unsupervised hierarchical clustering revealed three distinct groups, namely $CD8^+CD28^+$ young, $CD8^+CD28^+$ elderly and $CD8^+CD28^-$ young and elderly. The elderly $CD8^+CD28^+$ group is clustered between the $CD8^+CD28^+$ young and $CD8^+CD28^-$ young and elderly subsets. Our analysis thus reveals that $CD8^+CD28^-$ from young and elderly persons are clustered together (Figure 1).

The hierarchical clustering also revealed that genes were clustered in 21 different patterns. Genes in cluster 3, 5, 7, 17 and 19 appear to represent genes that are typically expressed at a high level in young $CD8^+CD28^+$ T cells. Genes which are highly expressed in elderly $CD8^+CD28^+$ are represented in cluster 13, while clusters 2, 4, 6, 8, 9, 11, 14, 20 and 21 are marked for effector signature (Figure 1A). This hierarchical clustering shows that the gene expression profile of $CD8^+CD28^+$ in elderly persons was closer to both $CD8^+CD28^-$ in young and elderly persons than to the $CD8^+CD28^+$ T cells from young persons (Figures 1A and B). This analysis also suggests that the $CD8^+CD28^-$ T cell subsets from young and elderly persons have a similar gene expression profile. We also confirmed that our Affymetrix data are in accordance with the result obtained from RT- PCR as shown in some randomly selected genes in Figure 2. For instance, granzymes (GZMB and GZMH) are typically higher expressed in $CD8^+CD28^-$ T cells whereas CD28, CCR7 and MAL expression is high in $CD8^+CD28^+$ T cells, which is in accordance with the literature [14].

Gene expression profile of $CD8^+CD28^+$ T cells from young persons

A list of 4115 genes which was found to be differentially expressed between $CD8^+$ T cell subsets was used for further analysis. Pavlidis template matching method (PTM) [15] with $p<0.01$ and 2-fold difference was performed to identify genes that are highly expressed in $CD8^+CD28^+$ cells from young persons compared to other subsets. 409 genes met these criteria. Later, we identified the biological processes in which the genes were involved, their

molecular function or related pathways using panther classification category. Genes responsible for biological processes such as cell proliferation and differentiation as well as cell adhesion mediated signaling are overrepresentated in $CD8^+CD28^+$ T cells from young persons. $CD8^+CD28^+$ T cells subsets from young persons express PIK3CD, MAL and IL6R which play a role in cell growth and differentiation. But also lymph node homing markers such as CD62L (SELL) and CCR7 as well as other adhesion molecules (e.g. PECAM1, DCHS1, F11R, FN1 and NRCAM) were highly expressed. A complete list of genes with a higher expression in $CD8^+CD28^+$ T cells from young persons compared to other groups is shown in Table 3.

Gene expression profile of $CD8^+CD28^+$ T cells from elderly persons

The same procedure was applied to analyze the gene expression of $CD8^+CD28^+$ T cells from elderly persons. 96 genes were found to be highly expressed in $CD8^+CD28^+$ T cells from elderly persons compared to $CD8^+CD28^+$ T cells young and $CD8^+CD28^-$ T cells (young and elderly). Genes with a typically high expression included GATA3, BIRC3, FAS, RGS1, and MAP3K1. Genes involved in inflammation mediated by cytokine and chemokine signaling pathway, induction of apoptosis, and signal transduction were IFNGR1, FAS and TNFRSF18 (Table 4).

Gene expression profile of $CD8^+CD28^-$ T cells

$CD8^+CD28^-$ T cells from young and elderly persons do not greatly differ in their gene expression profile. Genes encoding lytic granule proteins like granzyme B (GZMB), granzyme H (GZMH), perforin (PRF1), granulin (GRN) are highly expressed in $CD8^+CD28^-$ T cells. We could also confirm previous findings that killer cell lectin-like rectors and killer Ig-like receptor family members such as KLRD1, KLRF1, KLRC3, KIR2DL5A, KIR3DL2 and KIR2DS2 were highly expressed in $CD8^+CD28^-$ T cells. Additionally, we observed a high expression of genes playing a role in signal transduction in receptor-mediated cell-adhesion e.g. ITGAL and ITGAM, and in transcription e.g. GAS7 (Table 5).

Discussion

We demonstrate that the gene expression profile of $CD8^+CD28^-$ T cells is very similar between young and elderly persons, while $CD8^+CD28^+$ in elderly persons have a gene expression profile different compared to $CD8^+CD28^+$ T cells from young persons. Hierarchical clustering also showed that $CD8^+CD28^+$ T cells in the elderly are clustered

between $CD8^+CD28^+$ from young and $CD8^+CD28^-$ T cells from young and elderly persons. Thus, three different clusters were produced by this analysis i.e. young $CD8^+CD28^+$, elderly $CD8^+CD28^+$ and $CD8^+CD28^-$ from both young and elderly. This overall clustering classifies genes into 21 different sub-cluster based on expression level and pattern (Figure 1).

As about half of the $CD8^+CD28^+$ T cell population in young persons is considered antigen-inexperienced or naïve, it was interesting to have a closer look at the genes that are typically highly expressed in this subsets. Genes responsible for T cell activation, cell proliferation and differentiation as well as adhesion molecules are highly expressed in young persons. For example, PIK3CD a member of the phosphoinositide 3-kinases (PI3Ks) family regulates numerous biological processes including cell growth, differentiation, survival, proliferation, migration and metabolism. It has been shown that PI3K activation during the first nine hours of T cell stimulation is essential for T cell proliferation [16]. We also observed high expression of a hydrophobic protein called MAL in $CD8^+CD28^+$ T cells from young persons which is associated with human T cell differentiation [17].

Another gene that may play an important role is the interleukin 6 receptor complex (IL6R and IL6ST). The IL6 receptor is a protein complex consisting of an interleukin 6 signal transducer (IL6ST/GP130/IL6-beta), a receptor subunit shared by many other cytokines. Dysregulation of the production of IL6 (a potent pleiotropic cytokine that regulates cell growth and differentiation and plays an important role in the immune response) and the receptor are implicated in the pathogenesis of many diseases, such as multiple myeloma, autoimmune diseases and cancer [18, 19].

The $CD8^+CD28^+$ T cells in elderly persons over-express genes associated with the induction of apoptosis such as FAS and TNFRSF18. FAS is a member of the TNF-receptor superfamily and contains a death domain. FAS has been shown to play a central role in the physiological regulation of programmed cell death, and has been implicated in the pathogenesis of various malignancies and diseases of the immune system [20, 21].

It is widely accepted that cell-mediated immune functions decline with age, rendering an individual more susceptible to infection and possibly cancer, as well as to age-associated autoimmune diseases. The exact causes of the decline in T cell functions are not known. One possible cause could be the development of defects in the transduction of mitogenic signals following TCR stimulation. Signal transduction genes such as EPHA4, TIAM1 and RGS1 and cell structure and metabolism such as ANK3 are also upregulated in $CD8^+CD28^+$ cells from elderly persons. It has been shown that EPHA4 is highly expressed in lesional skin biopsy specimens in patients with cutaneous T cell lymphomas related to Sezary syndrome (Sz), a

malignancy of $CD4^+$ memory skin-homing T cells. The patients have erythroderma, lymphadenopathy, and peripheral blood involvement [22]. Another signaling protein TIAM1 contributes to the invasion and metastasis of the human giant-cell lung carcinoma cells [23].

In accordance with previous studies, $CD8^+CD28^-$ T cells express several different NK cell receptors (KIR) and produce high levels of granzyme, perforin and granulin, indicating an increased cytotoxic capacity [14]. The gain of NK receptor expression on $CD8^+CD28^-$ T cells may facilitate their effector functions as compensation for impaired proliferation [24].

In contrast to $CD8^+CD28^+$ T cells, $CD8^+CD28^-$ T cells are very similar between young and elderly persons. Only few genes were found to be differentially expressed such as TNFRSF1A, a gene that plays a role in the apoptosis signaling pathway and CD274, a ligand of the programmed death (PD)-1 which is associated with T cell exhaustion and disease progression in HIV-specific cells [25].

A critical question raised by these results is whether the observed differences between $CD8^+CD28^+$ in young and elderly persons reflect age-related differences in gene expression at the level of the single cell. It has been shown that $CD8^+CD28^+$ T cells are functionally heterogeneous and may contain different cell types. Using additional surface markers, this subset is for instance known to contain $CD28^+CD45RA^+$ and $CD28^+CD45RA^-$ T cells [26]. Furthermore, the $CD28^+CD45RA^-$ subset contains a subpopulation which expresses high levels of $CD62L^+$ and $CCR7^+$, and is referred to as central memory T cells (T_{CM}) [27, 28].

Recently we described that $CD45RA^+CD28^+$ T cells – a phenotype generally considered as naïve cells – from old persons differ from their young counterparts [29]. $CD45RA^+CD28^+$ T cells from elderly individuals have significant shorter telomeres and a restricted TCR repertoire in almost all 24 Vβ families. Cells from elderly persons also have a low expression of CD62L and CCR7. Additionally, they display an effector-like cytokine production and give rise to typical recall responses suggesting that this cell population is not exclusively naïve in the elderly [29]. All these facts imply that the $CD8^+CD28^+$ T cell subset is quite distinct between young and elderly persons and the difference in gene expression rather reflects the heterogeneous composition of this T cell population.

Taken together, our results confirm that a significant number of differentially expressed genes underlie the differentiation from naïve to effector and memory T cells. Many genes in each group fit well with known biological differences between these subsets, providing evidence that our data are valid. In conclusion, our study demonstrates a dichotomy of gene expression levels between $CD8^+CD28^+$ T cells in young and elderly persons and similarity between $CD8^+CD28^-$ T cells from young and elderly persons. Together with previous findings from

our group these results suggest that the gene expression profile does not depend on chronological age, but rather on the number of differently functioning cell types in a given population.

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Table 1. Characteristics of young and elderly persons

Donor number	Age	Gender	Anti-CMV IgG levels	Donor symbol	CD8 subset	Affymetrix	RT-PCR
Young donors							
1	27	male	2900	Y1_CD28P	$CD28^+$	x	
				Y1_CD28N	$CD28^-$	x	
2	34	male	11000	Y2_CD28P	$CD28^+$	x	x
				Y2_CD28N	$CD28^-$	x	x
3	30	female	5900	Y3_CD28P	$CD28^+$		x
				Y3_CD28N	$CD28^-$		x
4	29	male	8900	Y4_CD28P	$CD28^+$		x
				Y4_CD28N	$CD28^-$		x
Old donors							
1	72	male	8400	O1_CD28P	$CD28^+$	x	x
				O1_CD28N	$CD28^-$	x	x
2	81	female	4800	O2_CD28P	$CD28^+$	x	
				O2_CD28N	$CD28^-$	x	
3	75	female	2000	O3_CD28P	$CD28^+$		x
				O3_CD28N	$CD28^-$		x
4	81	male	21600	O4_CD28P	$CD28^+$		x
				O4_CD28N	$CD28^-$		x

All young and old persons are Cytomegalovirus (CMV) seropositive. Anti-CMV IgG levels were determined by ELISA (Enzygnost®, Dade Behring, Vienna, Austria).

Table 2. List of primers used for quantitative RT-PCR analysis

Gene symbol	Gene name	Primer sequence (5'-3' direction)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Fwd: GCATCCTGGGCTACACTGAG Rvs: CCCTGTTGCTGTAGCCAAAT
CD28	CD28 antigen	Fwd: TTTCCCGGACCTTCTAAGC Rvs: CGGGGAGTCATGTTCATGTAG
CCR7	Chemokine (C-C motif) receptor 7	Fwd: GTGGTGGCTCTCCTTGTCAT Rvs: ATAGGGAGGAACCAGGCTTT
GZMB	Granzyme B	Fwd: GACCCAGCAGTTTATCCCTGT Rvs: CTGGGCCTTGTTGCTAGGTA
GZMH	Granzyme H	Fwd: CCATTCCTCCTCCTGTTGG Rvs: ACTAGGATGCCGCCACAC
ITGAM	Integrin, alpha M (CD11b)	Fwd: GCAACCTCTCGTTTGACTGG Rvs: GAACGGCTCCACTTTGGTCT
MAL	Mal, T cell differentiation protein	Fwd: CTGGGTGATGTTCGTGTCTG Rvs: GACTGAGGCGCTGAGGTAAA

Table 3. List of genes with a 2-fold higher expression in $CD8^+CD28^+$ T cells (young) compared to $CD8^+CD28^+$ T cells (elderly) and $CD8^+CD28^-$ T cells (young and elderly) ($p<0.01$)

Gene name	Symbol	Biological process/function/pathway
BTB and CNC homology 1, basic leucine zipper transcription factor 2	BACH2	Transcription factor
complement component 1, q subcomponent binding protein	C1QBP	Complement mediated immunity
complement component (3d/Epstein Barr virus) receptor 2	CR2	Complement mediated immunity
cytochrome b-5	CYB5	
decay accelerating factor for complement (CD55, Cromer blood group system)	DAF	Complement mediated immunity
dachsous 1 (Drosophila)	DCHS1	Cell adhesion
F11 receptor	F11R	Cell adhesion
filamin B, beta (actin binding protein 278)	FLNB	Cell structure, integrin signaling pathway
fms-related tyrosine kinase 3 ligand	FLT3LG	Cytokine/chemokine mediated immunity
fibronectin 1	FN1	Cell adhesion
forkhead box P1	FOXP1	Cell cycle control, cell proliferation and differentiation
guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	GNA11	G-protein mediated signaling
growth factor receptor-bound protein 2	GRB2	Signal transduction
major histocompatibility complex, class II, DO alpha	HLA-DOA	Immunity and defense
homeo box A3	HOXA3	Transcription regulator
interleukin 16 (lymphocyte chemoattractant factor)	IL16	Cytokine/chemokine mediated immunity
interleukin 6 receptor	IL6R	Cytokine/chemokine mediated immunity
interleukin 6 signal transducer (gp130, oncostatin M receptor)	IL6ST	Cytokine/chemokine mediated immunity
integrin, alpha 6	ITGA6	Cell adhesion, inflammation mediated by chemokine & cytokine signaling
mal, T-cell differentiation protein	MAL	Cell proliferation and differentiation
Adenylate kinase 5	AK5	Signal transduction
insulin-like growth factor 1 receptor	IGF1R	Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade
neuronal cell adhesion molecule	NRCAM	Cell adhesion
pyruvate dehydrogenase kinase, isoenzyme 1	PDK1	Protein metabolism
platelet/endothelial cell adhesion molecule (CD31 antigen)	PECAM1 (CD31)	Cell adhesion
phosphoinositide-3-kinase, catalytic, delta polypeptide	PIK3CD	Signal transduction, T cell activation
protein kinase C, alpha	PRKCA	Signal transduction
protein tyrosine phosphatase, receptor type, S	PTPRS	Signal transduction
special AT-rich sequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's)	SATB1	Transcription factor
signal-regulatory protein beta 2	SIRPB2	Signal transduction, signaling molecule
transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	TCF3	Transcription factor
tumor necrosis factor receptor superfamily, member 7	TNFRSF7 (CD27)	Signal transduction, TNF receptor
leucine rich repeat neuronal 3	LRRN3	Signal transduction
nerve growth factor receptor (TNFRSF16) associated protein 1	NGFRAP1	Immune response
B-cell CLL/lymphoma 11B (zinc finger protein)	BCL11B	Transcription factor
chemokine (C-C motif) receptor 7	CCR7	Cytokine/chemokine mediated immunity
CD28 antigen (Tp44)	CD28	T cell activation
selectin L (lymphocyte adhesion molecule 1)	SELL (CD62L)	Cell adhesion

Table 4. List of genes with a 2-fold higher expression in $CD8^+CD28^+$ T cells (elderly) compared to $CD8^+CD28^+$ T cells (young) and $CD8^+CD28^-$ T cells (young and elderly) ($p<0.01$).

Gene name	Symbol	Biological process/function /pathway
ADP-ribosylation factor-like 1	ARL1	Intracellular protein traffic
baculoviral IAP repeat-containing 3	BIRC3	Inhibition of apoptosis
baculoviral IAP repeat-containing 4	BIRC4	Inhibition of apoptosis
dual specificity phosphatase 4	DUSP4	Oxidative stress response
ELK3, member of ETS oncogene family	ELK3	Transcription factor
Fas (TNF receptor superfamily, member 6)	FAS	Signal transduction, apoptosis
GATA binding protein 3	GATA3	Transcription factor
guanine nucleotide binding protein (G protein), q polypeptide	GNAQ	Signal transduction
mitogen-activated protein kinase kinase kinase 1	MAP3K1	Protein metabolism and modification
ankyrin 3, node of Ranvier (ankyrin G)	ANK3	Cell structure
Interferon gamma receptor 1	IFNGR1	Inflammation mediated by cytokine and chemokine signaling
T-cell lymphoma invasion and metastasis 1	TIAM1	Signal transduction
Tumor necrosis factor receptor superfamily, member 18	TNFRSF18	Induction of apoptosis
Anthrax toxin receptor 2	ANTXR2	Immunity and defense
Complement 1q tumor necrosis factor-related protein 3	C1QTNF3	Complement mediated immunity
Regulator of G-protein signaling 1	RGS1	Signal transduction
EPH receptor A4	EPHA4	Signal transduction

Table 5. List of genes with a 2-fold higher expression in $CD8^+CD28^-$ T cells (young and elderly) compared to $CD8^+CD28^+$ T cells (young) and $CD8^+CD28^+$ T cells (elderly) ($p<0.01$).

Gene name	Symbol	Biological process/function/pathway
actin related protein 2/3 complex, subunit 5-like	ARPC5L	Cell structure and motility
CD300A antigen	CD300A	Immunoglobulin receptor
cardiotrophin-like cytokine factor 1	CLCF1	Signal transduction
chemokine (C-X3-C motif) receptor 1	CX3CR1	Cytokine/chemokine mediated immunity
Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	FCGR3B (CD16)	Immunoglobulin receptor
Fc receptor-like and mucin-like 2	FCRLM2	Immunoglobulin receptor
growth arrest-specific 7	GAS7	Transcription factor
granulin	GRN	Signal transduction
granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	GZMB	Granulocyte mediated immunity, apoptosis signaling
granzyme H (cathepsin G-like 2, protein h-CCPX)	GZMH	Granulocyte mediated immunity, apoptosis signaling
major histocompatibility complex, class II, DP alpha 1	HLA-DPA1	MHC II-mediated immunity
major histocompatibility complex, class II, DR beta 1	HLA-DRB1	HLA-DRB1
insulin-like growth factor 2 receptor	IGF2R (CD222)	Cell adhesion, migration, apoptosis signaling
integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1, alpha polypeptide)	ITGAL (CD11A)	Cell adhesion
integrin, alpha M (complement component receptor 3, alpha	ITGAM (CD11B)	Cell adhesion
killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5A	KIR2DL5A	NK cell mediated immunity
killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 5	KIR2DS5	Ligand mediated signaling
killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	KIR3DL1	NK cell-mediated immunity
killer cell lectin-like receptor subfamily C, member 3	KLRC3	NK cell-mediated immunity
killer cell lectin-like receptor subfamily D, member 1	KLRD1	NK cell-mediated immunity
killer cell lectin-like receptor subfamily F, member 1	KLRF1	NK cell-mediated immunity
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1	LILRB1	Immunoglobulin receptor
lipopolysaccharide-induced TNF factor	LITAF	Transcription factor
lysozyme (renal amyloidosis)	LYZ	Immunity protein
mitogen-activated protein kinase 1	MAPK1	Signal transduction
megakaryoblastic leukemia (translocation) 1	MKL1	Transcription factor
2'-5'-oligoadenylate synthetase-like	OASL	Synthase and synthetase
perforin 1 (pore forming protein)	PRF1	Immunity and defense
protein kinase C, beta 1	PRKCB1	Signal transduction
protein kinase C, theta	PRKCQ	Signal transduction
protein tyrosine phosphatase, non-receptor type 12	PTPN12	Protein phosphatase
pyrin and HIN domain family, member 1	PYHIN1	Transcription factor
RAP2A, member of RAS oncogene family	RAP2A	Signal transduction
related RAS viral (r-ras) oncogene homolog 2	RRAS2	Signal transduction
runt-related transcription factor 3	RUNX3	Transcription factor
SATB family member 2	SATB2	Transcription factor
SLAM family member 7	SLAMF7	Immunoglobulin receptor
suppression of tumorigenicity 7	ST7	Tumor suppressor
transcription factor 4	TCF4	Transcription factor
transcription factor Dp-2 (E2F dimerization partner 2)	TFDP2	Transcription factor
transforming growth factor, beta receptor III (betaglycan, 300kDa)	TGFBR3	Signal transduction, cytokine receptor
tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	Signal transduction, TNF receptor, apoptosis signaling
vav 3 oncogene	VAV3	Signal transduction
Vinculin	VCL	Cell adhesion
killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2	KIR2DS2	NK cell-mediated immunity
killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2	KIR3DL2	NK cell-mediated immunity
killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 3	KIR3DL3	NK cell-mediated immunity

Legends

Figure 1. (A) Hierarchical clustering [30] of $CD8^+CD28^+$ (28P) and $CD8^+CD28^-$ T cells (28N) from two young (Y) and two elderly persons (O). A set of 4964 probe IDs corresponding to 4115 genes were identified as differentially expressed between $CD8^+$ T cell subsets and age groups using one-way ANOVA test. Each row in the heat map represents a gene and each column represents a $CD8^+$ T cell subset of a different donor. A red color indicates higher than median expression (up-regulation) and green indicates lower than median expression (down-regulation). The expression values are calculated in log2 scale. A hierarchical clustering with complete linkage clustering model using Euclidean distance was performed that results in 3 different groups based on T cell subsets and age. This clustering also revealed that genes were grouped into 21 clusters based on the similarity on pattern and expression value. **(B)** A higher magnification of linkage distance showing three distinct groups, $CD8^+CD28^+$ from young, $CD8^+CD28^+$ from elderly and $CD8^+CD28^-$ from both, young and elderly. **(C)** As an example, cluster 13 includes genes that are higher expressed in $CD8^+CD28^+$ T cells from elderly persons compared to all other T cell subsets.

Figure 2. Differences in the gene expression profile between $CD8^+$ T cell subsets from young and elderly persons analyzed by microarray **(A)** and quantitative RT-PCR **(B)**. Bars represent mean gene expression level as percentage ± SEM relative to the $CD8^+CD28^+$ T cell population from young which was considered basal level. $CD8^+CD28^+$ T cells from elderly persons (grey), $CD8^+CD28^-$ from young (black) and elderly persons (white). Abbreviations: GZMB, Granzyme B; GZMH, Granzyme H; ITGAM, integrin αM; CCR7, chemokine (C-C motif) receptor 7; MAL, T cell differentiation protein.

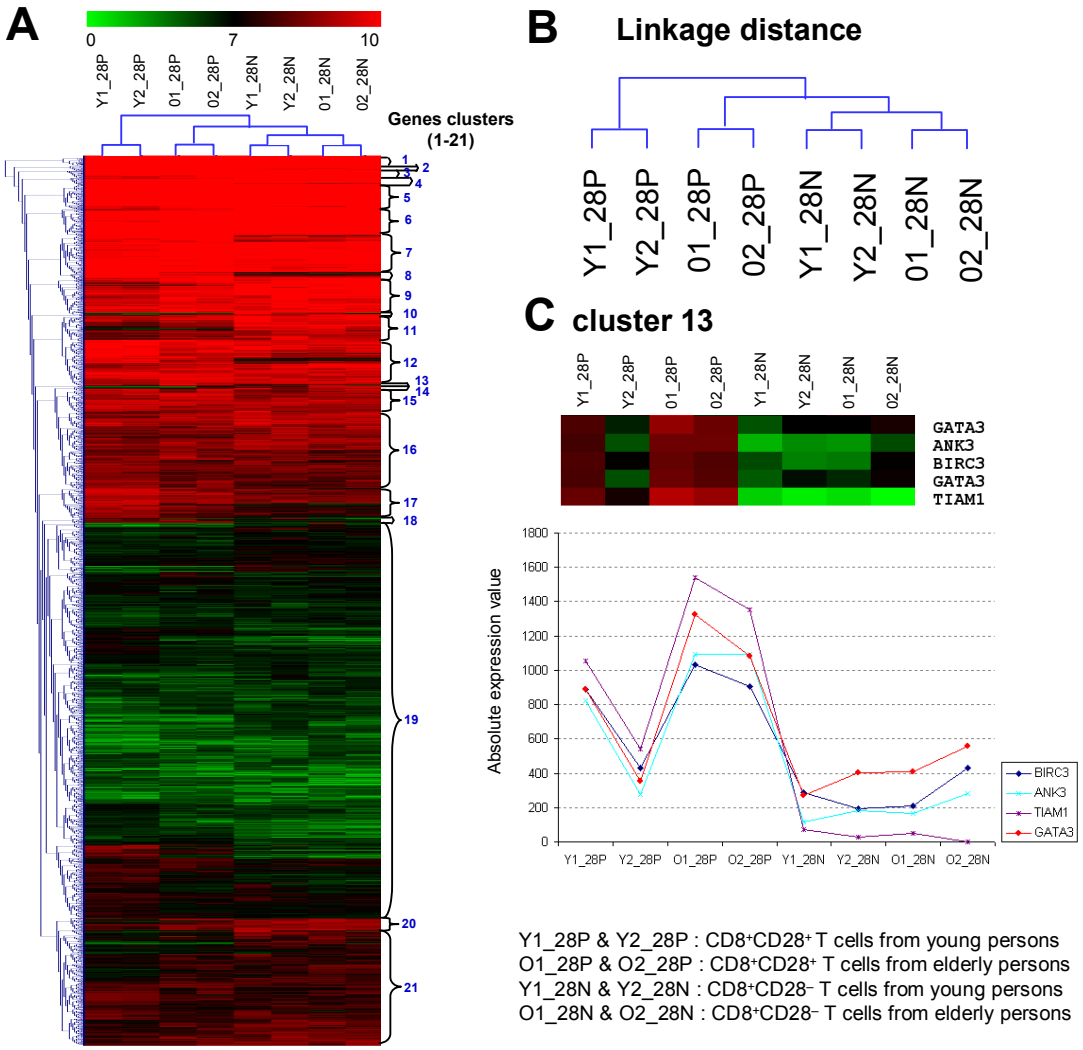
A
0
7
10
Y1_28P
Y2_28P
01_28P
02_28P
Y1_28N
Y2_28N
01_28N
02_28N
Genes clusters
(1-21)
1
2
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
B
Linkage distance
Y1_28P
Y2_28P
01_28P
02_28P
Y1_28N
Y2_28N
01_28N
02_28N
C
cluster 13
GATA3
ANK3
BIRC3
GATA3
TIAM1
Absolute expression value
1800
1600
1400
1200
1000
800
600
400
200
0
BIRC3
ANK3
TIAM1
GATA3
Y1_28P & Y2_28P : CD8+CD28+ T cells from young persons
O1_28P & O2_28P : CD8+CD28+ T cells from elderly persons
Y1_28N & Y2_28N : CD8+CD28- T cells from young persons
O1_28N & O2_28N : CD8+CD28- T cells from elderly persons

Figure 1

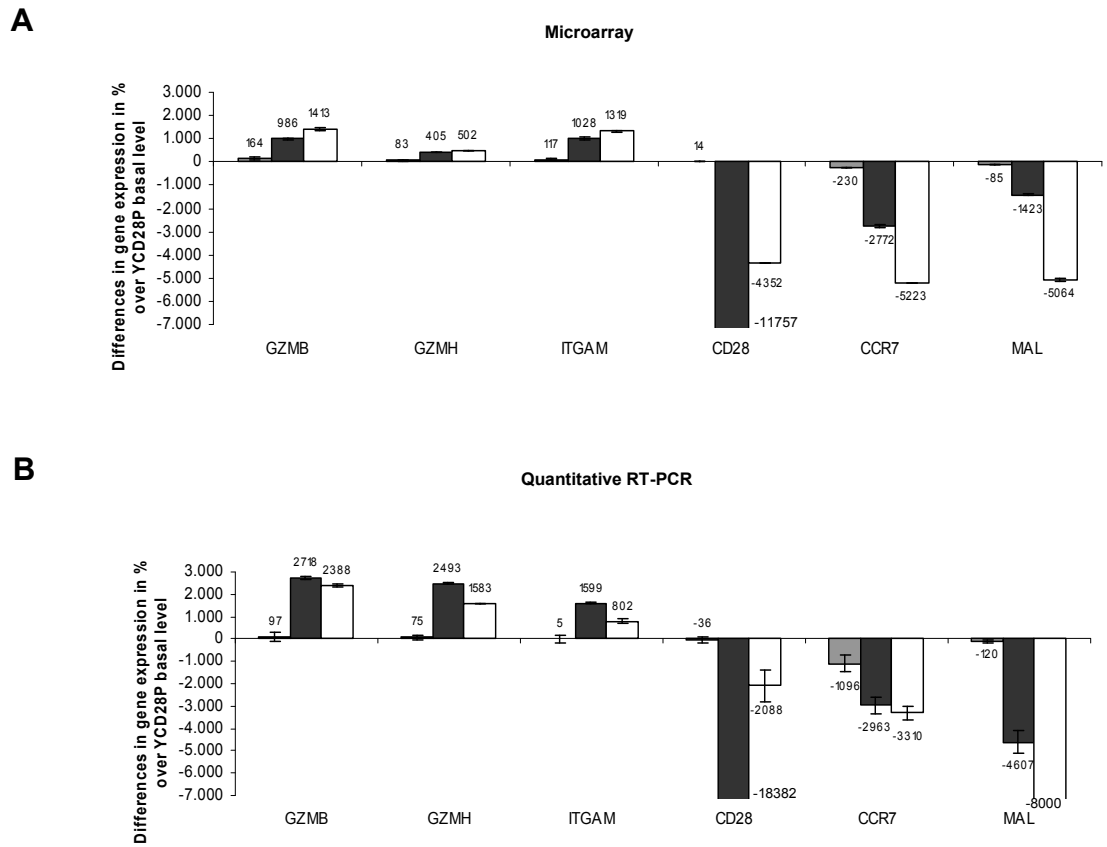
A
Microarray
Differences in gene expression in % over YCD28P basal level
3.000
2.000
1.000
0
-1.000
-2.000
-3.000
-4.000
-5.000
-6.000
-7.000
164
986
1413
83
405
502
117
1028
1319
14
-4352
-11757
-230
-2772
-5223
-85
-1423
-5064
GZMB
GZMH
ITGAM
CD28
CCR7
MAL
B
Quantitative RT-PCR
Differences in gene expression in % over YCD28P basal level
3.000
2.000
1.000
0
-1.000
-2.000
-3.000
-4.000
-5.000
-6.000
-7.000
97
2718
2388
75
2493
1583
5
1599
802
-36
-2088
-18382
-1096
-2963
-3310
-120
-4607
-8000
GZMB
GZMH
ITGAM
CD28
CCR7
MAL

Figure 2