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Comparative kinetic analyses of gene profiles of naïve CD4$^+$ and CD8$^+$ T cells from young and old animals reveal novel age-related alterations

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Summary

It is well established that immune responses are diminished in the old. However, we still do not have a clear understanding of what dictates the dysfunction of old T cells at the molecular level. Although microarray analysis has been used to compare young and old T cells, identifying hundreds of genes that are differentially expressed among these populations, it has been difficult to utilize this information to pinpoint which biological pathways truly affect the function of aged T cells. To better define differences between young and old naïve CD4$^+$ and CD8$^+$ T cells, microarray analysis was performed pre- and post-TCR stimulation for 4, 12, 24 and 72 h. Our data indicate that many genes are differentially expressed in the old compared to the young at all five time points. These genes encode proteins involved in multiple cellular functions such as cell growth, cell cycle, cell death, inflammatory response, cell trafficking, etc. Additionally, the information from this microarray analysis allowed us to underline both intrinsic deficiencies and defects in signaling only seen after activation, such as pathways involving T-cell signaling, cytokine production, and Th2 differentiation in old T cells. With the knowledge gained, we can proceed to design strategies to restore the function of old T cells. Therefore, this microarray analysis approach is a powerful and sensitive tool that reveals the extensive changes seen between young and old CD4$^+$ and CD8$^+$ naïve T cells. Evaluation of these differences provides in-depth insight into potential functional and phenotypical differences among these populations.

Key words: CD4 T cells; CD8 T cells; gene expression; kinetic analyses; microarray analysis; Naïve.

Introduction

T cell-mediated immunity is a critical component of the immune response to prevent infectious diseases and cancer. It is well established that T-cell responses are compromised in old mice, rats and humans (Linton *et al.*, 2005; Jiang *et al.*, 2007; Yager *et al.*, 2008). It is known that the activation of an effective immune response in aged hosts is lowered because of the number of available naïve T cells capable of reacting to new antigens (Fagnoni *et al.*, 2000; Gupta *et al.*, 2004). In the aged host, a decrease in the naïve T-cell population and an increase in memory T cells (Zanni *et al.*, 2003) creates an imbalance in memory/naïve T-cell populations, which may, in part, account for the hyporesponsive state of the aged (Fulop *et al.*, 2005). Additionally, the function of naïve T cells decreases in the old as a result of the accumulation of age-related defects, limiting the activation of immune responses to new antigens (Sambhara & McElhaney, 2009; Weiskopf *et al.*, 2009). Because of naïve T-cell deficiency, the elderly are at a higher risk to new infections (Ely *et al.*, 2007) and cancers (Fulop *et al.*, 2010) and respond poorly to vaccination (Kumar & Burns, 2008).

Many causative defects have been identified in old naïve T cells that limit the optimal activation of an immune response such as deficiency in T-cell receptor (TCR) signaling, lower reactivity to antigenic stimulation resulting in decreased proliferation (Canonica *et al.*, 1985), modifications in production and secretion of cytokines (Nagel *et al.*, 1988), altered apoptosis (Aggarwal & Gupta, 1998), and reduced cytotoxic activity of CD8$^+$ T cells (Perillo *et al.*, 1993). The underlying cause or mechanism of these defects at the molecular level is not well understood. However, without understanding what dictates the dysfunction of old naïve CD4$^+$ and CD8$^+$ T cells at the molecular level, it will not be possible to optimize immune responses in the old. Transcriptome analysis provides valuable insight into the molecular pathways and processes that could be differentially activated in discrete populations. Furthermore, gene expression analysis provides critical information to understand the biological functions of different cellular subpopulations. Several groups have performed microarray analysis comparing mouse or human CD4$^+$ and CD8$^+$ T-cell populations (Han *et al.*, 2006; Lazuardi *et al.*, 2009; Cao *et al.*, 2010). A critical issue lacking in these reports is that in these analyses naïve and memory T-cell populations were not discriminated, making the interpretation of the results more difficult. Although these studies reveal major transcriptional differences between young and old T cells, it has been difficult to elucidate which molecule, signal transduction pathway or network, is pivotal in leading to the dysfunction of old T cells, nor which element should be targeted to enhance the old CD4$^+$ or CD8$^+$ naive T-cell responses. We took an alternative approach to discriminate between the intrinsic and functional differences between young and old T cells, by using an activation time course. Young and old CD4$^+$ and CD8$^+$ naïve (CD44^{low}/CD62L^{high}) T cells were isolated and either not-stimulated or stimulated with anti-CD3 plus anti-CD28 for 4, 12, 24 and 72 h: At these time points, cells were collected, RNA was isolated, labeled, and hybridized to a whole mouse genome chip for microarray analysis. Data analysis was approached from two perspectives: (i) to reveal the innate differences between young and old naïve CD4$^+$ and CD8$^+$ T cells; and (ii) to discover the changes in T-cell function in old T cells as defined by altered responses after TCR stimulation. The combination of both analyses resulted in insights into the fundamental differences that exist in the transcriptome of matched old and young T-cell populations in contrast to the acquired functional differences in aged cells. Selected signaling pathways relevant to T-cell activation are presented and discussed. In conclusion, we present a detailed transcriptional analysis of the differences between young and old naïve T-cell populations, which may lead to a better understanding of the dysfunction of aged T cells and to the identifica-

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tion of potential molecular targets that might enhance the aged T-cell responses.

Results and discussion

Global genomic analysis of young and old naïve CD4$^+$ and CD8$^+$ T cells pre- and post-TCR stimulation

T cells are a critical component of the immune system to fight infectious diseases and cancer. Cumulative data over more than 20 years indicate that naïve and memory old CD4$^+$ and CD8$^+$ T cells are dysfunctional or altered when compared to young T cells (Engwerda *et al.*, 1994). It seems that the old naïve T cells have a more profound defect than old memory T cells (Haynes *et al.*, 2003; Cicin-Sain *et al.*, 2007), which could be a reason why T-cell responses against new antigens are severely compromised in the aged. Additionally, the transition of naïve phenotype to memory is compromised in old T cells (Dominguez & Lustgarten, 2008). The dysfunction of old T cells are reflected in processes like TCR activation, proliferation, type and quantities of cytokine secreted, differentiation, migration, and others. Transcriptome analysis provides valuable insight into the molecular pathways and biological processes underpinning changes in cell behavior. Previous studies have analyzed gene profiles of young and old CD4$^+$ or CD8$^+$ T-cell populations (Han *et al.*, 2006; Lazuardi *et al.*, 2009; Cao *et al.*, 2010), revealing hundreds of genes involved in processes – such as T-cell activation, cell cycle, cytokine production, signaling pathways, and others – are differentially expressed between young and old. However, as these studies were performed with mixed populations of naïve and memory CD4$^+$ and CD8$^+$ T cells making the interpretation of the results more difficult, we hypothesize that the comparison of the transcriptome of young and old naïve T cells pre- and post-TCR stimulation will provide critical information to (i) identify inherent differences among these populations and (ii) discover functional deficiencies present in old naïve T cells. For these experiments, CD44^{low}/CD62L^{high} CD4$^+$ and CD8$^+$ T cells were sorted from naïve young (3 months old) and old (20 months old) Balb/c mice. RNA was isolated prior to stimulation or 4, 12, 24, and 72 h post-stimulation with anti-CD3 plus anti-CD28. Isolated RNA was fluorescently labeled using the One-Color Low RNA Input Linear Amplification Kit PLUS (Agilent, Santa Clara, CA, USA) and hybridized to the 4X44K whole mouse genome chip (Agilent). Feature extracted microarray data were normalized and analyzed using GeneSpring (Agilent) software. Figure 1A shows the schematic representation of the experimental approach.

Two analytical approaches were taken to distinguish genes that are differentially expressed pre- and post-stimulation. Analysis of the data indicate that 2116 genes were differentially expressed by at least 2-fold at time 0 in old vs. young CD8$^+$ T cells (Fig. 1B), (Table S1 for complete gene list). After stimulation, 5860 genes were differentially expressed in at least 2 of 4 time points after stimulation (Table S2 for complete gene list), and 1050 genes are differentially expressed at time 0 and at least in 2 of 4 time points (Table S3 for complete gene list). We also analyzed the genes with differential expression in at least 3 of the 4 time points post-TCR stimulation (Fig. 1C). We observed 385 genes that were differentially regulated at time 0 and in at least 3 of 4 time points (Table S4 for complete gene list) and 1272 genes at least in 3 of 4 time points but not at time 0 (Table S5 for complete gene list). With regard to CD4$^+$ T cells (Fig. 1D), 2596 genes are differentially expressed at least by 2-fold at time 0 in old vs. young (Table S6 for complete gene list), 5447 genes at least in 2 of 4 time points (Table S7 for complete gene list), and 1209 genes at time 0 and in at least 2 of 4 time points (Table S8 for complete gene list). Analysis of genes regulated at time 0 and at least in 3 of 4 times post-stimulation

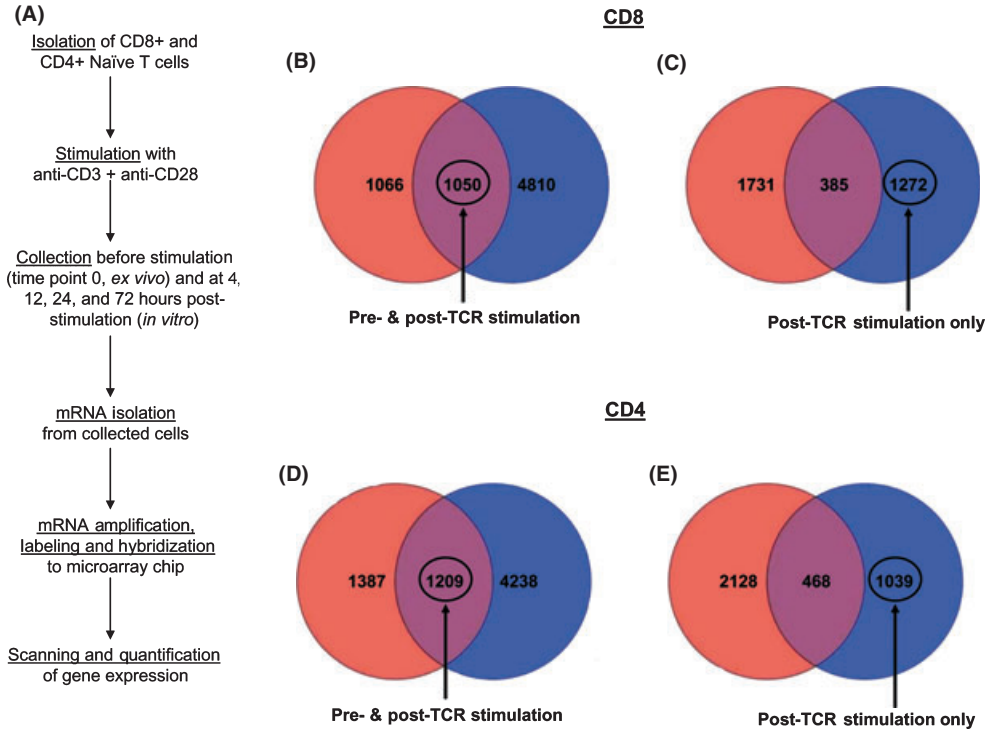


Fig. 1 Gene selection algorithm of a microarray-based kinetic analysis of naïve CD4$^+$ and CD8$^+$ young and old T cells. (A) Schematic representation of experimental design. Venn diagrams overlaying genes that are differentially regulated between old and young naïve T cells. (B) Pre- and post-stimulation in CD8$^+$ naïve T cells. (C) Post-stimulation only in CD8$^+$ naïve T cells. (D) Pre- and post-stimulation in CD4$^+$ naïve T cells. (E) Post-stimulation only in CD4$^+$ naïve T cells.

(Fig. 1E) revealed that 468 genes were differentially regulated (Table S9 for complete gene list) and 1039 genes at least in 3 of 4 time points but not at time 0 (Table S10 for complete gene list). Tables S1 to S18 discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO series accession number GSE28165 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28165). These results show major transcriptional changes both at time 0 and following TCR stimulation in old naïve $CD4^+$ and $CD8^+$ T cells when compared to young naïve $CD4^+$ and $CD8^+$ T cells. The outcome of these transcriptional differences may modify certain pathways and/or signal networks, resulting in altered effector functions in aged T cells as is discussed later.

Differential gene expression of cellular processes between young and old naïve $CD4^+$ and $CD8^+$ T cells

To obtain a detailed look at inherent and acquired differences between old and young naïve $CD4^+$ and $CD8^+$ T cells, we designed two separate analytical approaches. First, we selected genes that were differentially regulated both at time 0 (pre-activation) and upon TCR activation in 2 of 4 time points (see scheme in figure 1) between old and young animals. The overlap between these lists represent genes that are (i) inherently different in old and young populations prior to stimulation but following TCR activation show no alterations in gene expression, and (ii) genes that are inherently different in old and young populations but change significantly upon TCR activation (Tables 1A and 2A represent the genes in the overlap) (see Table S3 for CD8 T cells and Table S8 for CD4 T cells). Secondly, we were interested in genes that are differentially regulated between old and young T cells only after TCR activation. To derive this list, we selected genes that were 2-fold up- or down-regulated in old vs. young in 3 of the 4 time points during TCR activation alone (Tables 1B and 2B) (see Table S5 for CD8 T cells and Table S10 for CD4 T cells). Gene lists from both these analyses were imported into Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc., Redwood City, CA, USA) for pathway enrichment analysis. This type of analysis segregates genes into categories pertaining to defined biological processes or signaling pathways and calculates the probability that the genes found in a given biological function group are not enriched by chance (see *P*-value columns in Tables 1 and 2). Not surprisingly, the most significant enrichments occurred in pathways associated with immune response, inflammatory response, and T-cell signaling (Tables 1 and 2), providing information on which cellular processes are dysregulated in old T cells. Although we identified genes that are commonly differentially expressed in both old $CD4^+$ and $CD8^+$ T cells such as ANXA1, CCL1, CCL5, CCR2, IL-4, HAVCR2, LTB4R1, and other genes that are involved in several cellular processes, the majority of genes that are differentially expressed between young and old T cells are specific to either old $CD4^+$ or old $CD8^+$ T cells. This indicates that functional dysregulation (e.g., activation, migration, immune response, etc.) in old $CD4^+$ and $CD8^+$ T cells is likely to be cell specific and brought about by changes in distinct signaling pathways. This implies that if, for example, we would like to restore the activation and proliferation activity of old $CD4^+$ T cells, genes such as Jak3, SOCS1, and PI3KCD should be targeted, while in old $CD8^+$ T cells genes such as PENK, NFC2, and IRAK3 should be targeted.

It is known that the old immune system favors Th2 responses (Gardner & Murasko, 2002) and that the transcription factor GATA3 promotes Th2 differentiation while blocking differentiation of the Th1 program (Zhu *et al.*, 2006). Our data indicate that the expression of GATA3 is up-regulated post-stimulation in naïve old $CD4^+$ T cells, providing clues as to why old T-cell responses have a predisposition toward a Th2 response. Another transcription factor that promotes Th2 responses is c-Maf (MAF) (Ho *et al.*, 1998). Old $CD4^+$ T cells overexpressed c-Maf when compared to young T cells, further contributing toward a predisposition to a Th2 response. It is not clear why GATA3 is predominantly up-regulated in old $CD4^+$ T cells after TCR activation. For this, it is important to understand how GATA3 expression is regulated. Zhou *et al.* (2001) demonstrated that GATA-1 represses GATA-3 autoactivation. As shown in Table 2, GATA1 is highly expressed in young $CD4^+$ T cells and down-regulated in old $CD4^+$ T cells. Kurata *et al.* (2002) show that down-regulation of GATA1 induces Th2 cell differentiation by releasing GATA-3 from its repression. Therefore, the low expression of GATA1 in old T cells allows the high expression of GATA3 resulting in a predominant Th2 response. GATA1 interacts with a variety of proteins, either cofactors or transcription factors, which lead to transcriptional activation or repression of GATA1 target genes such as GATA3 (Ferreira *et al.*, 2005). Perhaps, the interactions of these cofactors are dysregulated in old T cells, preventing the expression of GATA1 in these cells. That could provide an explanation at the molecular level why old $CD4^+$ T cells have a tendency toward a Th2 type response.

Tables 1 and 2 list many genes that are up- or down-regulated pre- and postactivation (Tables 1A and 2A) or only postactivation (Tables 1B and 2B). Proteins encoded by these genes could influence many biological functions in old T cells. We observed that LAG-3 (Lymphocyte activation gene 3) gene expression is down-regulated in old $CD8^+$ T cells. LAG-3 negatively regulates the expansion of activated T cells and T-cell homeostasis, helping to maintain a homeostatic balance of T-cell populations (Workman & Vignali, 2005). It is well established that the old immune system is characterized by clonal expansion of T-cell clones (Ku *et al.*, 2001). Based on these results, it is possible to speculate that T-cell clonal expansions occur in the old because of dysregulation of mechanisms that prevent the expansion of these cells. We have recently demonstrated that old T cells expressed less T-cell receptors on the cell surface than young T cells (Mirza *et al.*, 2010). Furthermore, we demonstrated that the interactions of the TCR complex subunits such as zeta/epsilon associations is lower in old T cells when compared to young T cells, which correlates with, and is predicted to cause lower surface TCR expression in old T cells (Mirza *et al.*, 2010). However, it is not clear why old T cells pre- and post-stimulation express lower TCR. NCK2 (NCK adaptor protein 2) belongs to a family of adaptor protein containing Src homology-2 (SH2) and -3 (SH3) domains. These proteins function by coupling tyrosine phosphorylation via SH2 domains to downstream effectors through SH3 domains. Recently, Takeuchi *et al.* (2008) demonstrated that NCK2 interacts with CD3ε limiting the ability of CD3ε to interact with other subunits of the TCR, resulting in the reduction of TCR cell surface expression. Our data indicate that NCK2 is highly up-regulated pre- and post-TCR stimulation in old $CD4^+$ T cells, which could explain why there is less interaction among the TCR subunits and cell surface expression in aged T cells. Analysis of the genes that are up- or down-regulated in old T cell compared to young T cells is critical to better understand the overall behavior of old naïve T cells.

Patterns of gene expression pre- and post-stimulation between young and old naïve $CD4^+$ and $CD8^+$ T cells

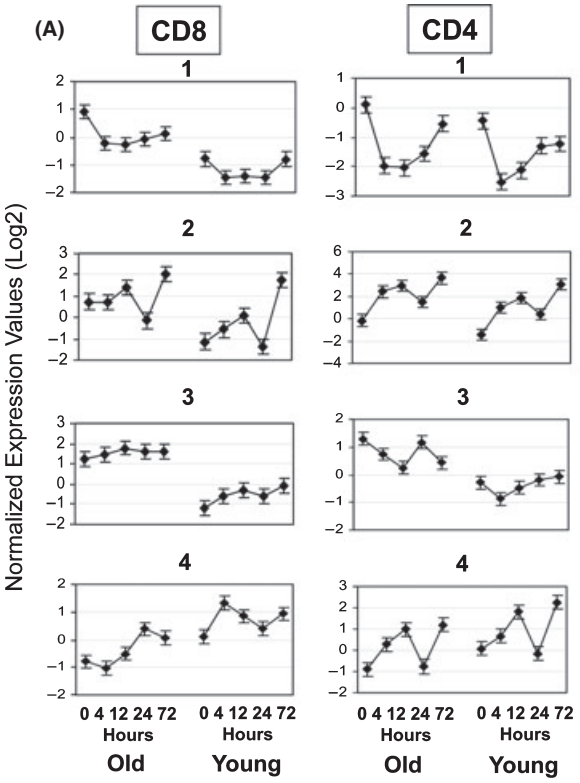
To assess the kinetic dynamics of TCR stimulation-dependent gene regulation, the K-means (Genespring, Agilent) cluster algorithm analysis was used. This type of analysis allows us to visualize changes in gene expression patterns over time and identify specific gene expression patterns that differentiate young from old naïve $CD4^+$ and $CD8^+$ T cells. Twelve patterns were identified (four are shown in Fig. 2, and complete gene lists

Table 1 Significant biological functions of the genes differentially expressed between old and young CD8+ T cells (A) pre- and post-TCR stimulation and (B) post-TCR stimulation only

CD8	(A) Pre- and post-TCR stimulation			(B) Post-TCR stimulation only		
Biological function	*P*-value	No. genes	Genes	*P*-value	No. genes	Genes
Cell movement of T lymphocytes	2.27E-11	22 Up	BLR1, C3, CAMP, CCL5, CCL8, CCL21C, CCR2, CCR5, CHI3L3/CHI3L4, CXCL9, FASLG, FUT7, IFNG, IL10, IL1B,IL21, IL4, ITGB1, S100A4, SPP1, TLR4	1.25E-03	7 Up	APP, CCL1, CCR4, COL4A3, IL2, IL5, VIP
		9 Down	CCL22, CCR9, CD86, COL4A1, CTSL, CXCL10, CXCL12, ITGAE, PLP1		4 Down	CCL24, IL6, IL17A, TNFRSF21
Infiltration by T lymphocytes	1.21E-06	10 Up	C3, CCL21C, CCR2, CCR5, FASLG, IFNG, IL10, IL1B,IL4, ITGB1	1.05E-03	4 Up	APP, COL4A3, IL2, IL5
		3 Down	CCL22, CD86, CXCL10		2 Down	IL6, TNFRSF21
Inflammatory response	3.52E-11	57 Up	AIF1, ANXA1, BLNK, C3, CAMP, CASP1, CASP4, CCL5, CCL8, CCL21C, CCR2, CCR5, CD38, CHI3L3/CHI3L4, CLEC5A, CSF2, CSF2RA, CXCL2, CXCL9, CXCR3, CYPF16, FASLG, FPR-RS2. HAVCR2, HCK, HP,IFNG, IL10, IL18RAP, IL1B, IL21, IL4, IRAK3, ITGB1, KLRG1, LCN2, LTB4R, LTF, LYN, LYZ, MAF, NOX1, NT5E, PENK, PROS1, PTGER3, RORA, S100A4, S100A8, S100A7A, SAA3, SERPINF1, SLPI, SPP1, TLR4, TNFSF4	3.04E-09	27 Up	AFAP1L2, APP, CCL1, CCR4, CD38, CPB2, CSF3R, F11R, F2R, FABP4, GNAS, IL2, IL3, IL5, IL9, IL20RA, IL22, IRAK3, LTF, LYST, MDS1, NOD1, NT5E, S100A14, SLC7A2, ULBP1, VIP
		12 Down	CCL22, CXCL10, CXCL12, EDN1, IFNA1/IFNA13, ITGA9, ITGB6, PDE4D, PLAUR, PTAFR, TGM2, ZFP36		20 Down	ACP5, ADORA3, APCS, CCL24, CCND1, CD200, EPHX2, FLT1, GHRL, HNF4A, IL6, IL17A, KL, PLA2G4A, PLAUR, TLR5, TLR7, UACA, UTS2D, XCR1
Immune response	3.92E-14	68 Up	ANXA1, ANXA3, AQP9, BLNK, BMPR1A, C1R, C3, CAMP, CASP1, CCL5, CCL8, CCL21C, CCR2, CCR5, CD36, CD38, CENPF, CLEC5A, CLEC7A, CSF2, CXCL9, DUSP3, FASLG, FCGR2B, FOXJ1, FPR-RS2, HAVCR2, HCK, HLA-A, HLA-DMB, HLA-DQA1, HLA-DRA, IER3, IFNG, IGH, IL1R2, IL10, IL18RAP, IL1B, IL21,IL4, ITGAX, KLRC1, KLRE1, LAIR1, LCN2, LTB4R1, LTBR, LTF, LYN, MAF, MPO, P2RY14, PENK, PGLYRP1, PLD2, PRDM1, PROS1, PTGER3, RORA, SLPI, SPP1, TLR4, TNFRSF17, TNFSF4, TNFSF13B, UBE2L6, ZBTB7B	1.47E-06	30 Up	APP, AQP9, CCR4, CD22, CD38, CPB2, DOCK1, EBI2, GATA3, ICOSLG, IGSF6, IL2, IL3, IL5, IL9, IL22, IL18R1, IL1RL1, IL20RA, IL23A, IL23R, IL7R, LAIR1, LTF, LYST, MGST1, NOD1, SLC7A2, ULBP1, VIP
		21 Down	CCL22, CCR9, CD86, CTSL2, CXCL10, CXCL12, DAF2, DNTT, EDN1, GATA2, IFNA1/IFNA13, ITGB6, KLRA17, LAG3, LRRC23, NPPA, PLP1, PLXNB1, PTAFR, PVRL2, TNFRSF11A		21 Down	APCS, APLN, CD200, DDX58, EPHA2, GBP2, HNF4A, IL6, IL12RB2, IL17A, IRF7, KIT, MX2, NCF2, NDRG1, POU2AF1, SERPINB1B, TLR5, TLR7, TPO, VCAN

Table 2 Significant biological functions of the genes differentially expressed between old and young CD4+ T cells (A) pre- and post-TCR stimulation and (B) post-TCR stimulation only

CD4	(A) Pre- and post-TCR stimulation			(B) Post-TCR stimulation only		
Biological function	*P*-value	No. Genes	Genes	*P*-value	No. Genes	Genes
Activation of T lymphocytes	1.74E-05	19 Up	BCL2, CCL5, CCR2, CD81, CD86, CENPF, CLEC7A, EFNB2, HLA-DRA, HLA-DRB1, ICOSLG, IFNG, IL4, IL10, IL13, IL1RL1, TBX21, TLR4, TNFSF4	7.30E-05	17 Up	CCL22, CD74, CD274, CSF2, DUSP3, EGR2, FCER1G, GATA3, GZMA, HAVCR1, HYOU1, IL9, IL21, IL24, IL1RL1, IL2RA, SOCS1
		7 Down	CEACAM2, DCT, DLL4, IFNAB, KLRA8, MBP, PIK3CD		9 Down	CD8A, FGF1, IFNA1, IL12B, ITGA3, JAK3, KLRD1, MGST1, TACR1
Differentiation of T lymphocytes	2.66E-05	22 Up	BCL2, BCL11A, CD86, CEBPB, FCGR2B, HLA-DQA1, ICOSLG, IFNG, IGF2, IL4, IL10, IL13, IL12RB1, IL1RL1, LMO1, MAF, NCK2, PRDM1, RNF128, TBX21, TNFSF4, TNFSF13B	2.52E-07	21 Up	APC, BMI1, CCL1, CCL17, CCL22, CD74, CDKN2A, CLEC4E, CSF2, EBI3, FCGR2B, FZD7, GATA3, ID1, IL5, IL9, IL21, IL1RL1, IL1RN, IL2RA, PICALM
		9 Down	AP3D1, C5AR1, DLL4, IFNAB, MBP, NOG, PIK3CD, RUNX1, SOX4		11 Down	ANXA1, CCL19, CD8A, GATA1, ID3, IFNA1, IGF1, IL12B, JAK3, MYB, RORC
T-cell development	4.51E-09	35 Up	BCL2, BCL11A, BIRC5, CASP1, CCL5, CCR5, CD86, CEBPB, CHEK1, CIITA, EPHA3, FCGR2B, HLA-DQA1, ICOSLG, IER3, IFNG, IGF2, IGH, IL4, IL5RA, IL10, IL13, IL12RB1, IL1RL1, LMO1, MAF, PENK1, PERP, PRDM1, RNF128, RUNX2, TBX21, TLR4, TNFSF4, TNFSF13B	1.14E-08	38 Up	ADCY7, APC, BMI1, CALCA, CASP7, CCL1, CCL17, CCL22, CCNB1, CD74, CD274, CDKN2A, CLEC4E, CNR2, CSF2, DKK1, EBI3, EGR2, FCER1G, FCGR2B, FCGR3, FZD7, GATA3, GZMA, ID1, IL5, IL9, IL21, IL1RL1, IL1RN, IL2RA, INHBA, PICALM, PMAIP1, SOCS1, SPHK1, TGM2, TOP2A
		19 Down	ABL1, AP3D1, C5AR1, CCR9, DLL4, EOMES, GNAS, IFNAB, IL6RA, MBP, NOG, NOTCH3, NR4A1, PIK3CD, RELA, RUNX1, SIRPA, SOX4, TERT		17 Down	ANXA1, ARRB1, CCL19, CD8A, ELF4, GATA1, ID3, IFNA1, IGF1, IL12B, JAK3, KIT, KLRD1, MYB, PIM2, RORC, TRP73
Immune response	5.80E-14	57 Up	BCL2, BLNK, BMPR1A, CASP1, CCL3, CCL5, CCR2, CCR4, CCR5, CD22, CD79B, CD81, CD86, CEBPB, CENPF, CIITA, CLEC7A, CSF1, CXCL10, DDR1, EFNB2, EIF4EBP1, FCGR2B, FOXJ1, GEM, HAVCR2, HDAC9, HLA-DMB, HLA-DQA1, HLA-DRA, HLA-DRB1, ICOSLG, IER3, IFNG, IGH, IL4, IL10, IL13, IL12RB1, IL1RL1, IL1R2, LTB4R1, LTBR, MAF, MS4A1, MX1, PENK1, PLSCR1, PRDM1, PROS1, TBX21, TLR4, TNFRSF13C, TNFRSF17, TNFSF4, TNFSF13B, TUB	2.20E-12	48 Up	CADM1, CCL17, CCL22, CD63, CD74, CD274, CLEC4E, CNR2, CSF2, CXCL9, CXCL11, DNTT, DUSP3, EBI2, EBI3, EDN1, EGR2, FCER1G, FCGR2B, FCGR3, GATA3, GCNT1, GZMA, H28, HAVCR1, HC, HYOU1, IL3, IL5, IL9, IL19, IL21, IL24, IL1R2, IL1RL1, IL1RN, IL2RA, INPP5D, ITGA1, LAIR1, NFIL3, NOD2, PELI1, PLA2G10, PRG2, PTGES, PTPRE, SOCS1
		31 Down	ABL1, AP3D1, C5AR1, CAMP, CCR9, CEACAM2, CERK, CLEC5A, CXCL4, DCT, DLL4, EDN2, IFNAB, IL6RA, IRF7, KLRA8, KLRB1A, LBP, LCN2, LTB4R2, LTF, MBP, MMP7, PIK3CD, PLXNA1, RARRES2, RELA, S100A9, SIRPA, TRPC2, UBE2L6		23 Down	ANXA1, CCL19, CD8A, CD226, CEACAM10, EDG1, FGF1, HNF4A, IFNA1, IGF1, IL12B, ITGA3, JAK3, KIT, KLRD1, KLRK1, LTF, MGST1, NDRG1, PLG, PSG19, SECTM1A, TACR1
Inflammatory response	9.04E-12	32 Up	BLNK, CASP1, CASP4, CCL3, CCL5, CCR2, CCR4, CCR5, CEBPB, CSF1, CXCL10, CXCR3, CYSLTR1, F11R, HAVCR2, HDAC9, IFNG, IL4, IL10, IL13, IL12RB1, KLRG1, LTB4R1, MAF, MEFV, MGLL, NT5E, PENK1, PROS1, S100A4, TLR4, TNFSF4	5.45E-09	40 Up	AIF1, CALCA, CCL1, CCL17, CCL20, CCL22, CCL24, CD63, CLEC4E, CSF2, CXCL9, CXCL11, CYP4F16, DARC, EDN1, F2R, FCER1G, FCGR3, GNAZ, HC, IL3, IL5, IL9, IL19, IL21, IL24, IL1RN, IL2RA, INHBA, INPP5D, ITGA1, NOD2, PLA2G10, PLAA, PRG2, PTGES, RAP1GAP, SOCS1, SPHK1, TGM2
		34 Down	ADORA3, ALOX5, AOAH, C5AR1, CAMP, CHI3L3, CLEC5A, CXCL1, CXCL4, EDN2, EDN3, GHRL, GNA12, GNAS, HRH3, IFNAB, IL6RA, LBP, LCN2, LSP1, LTB4R2, LTF, LYZ, NFRKB, ORM1, PIK3CD, PNOC, PPBP, RARRES2, RELA, S100A8, S100A9, SAA3, XCR1		18 Down	ANXA1, BDKRB1, CCL19, CEACAM10, EDG1, FGR, HNF4A, IFNA1, IGF1, IL12B, ITGA3, LTF, NFATC4, PDGFB, PLG, SERPINA1D, TACR1, TRP73



(B)

Pattern 1		Pattern 2		Pattern 3		Pattern 4	
CD8	*CD4*	*CD8*	*CD4*	*CD8*	*CD4*	*CD8*	*CD4*
Abcb1a	Abcb1a	Adam8	Capsl	C1r	Bcl11a	Acads	Adam15
Acox1	Adam19	Aifm2	Casp4	Ccr5	Blnk	Adam28	Arid5a
Adam15	Arid5b	Anxa3	Ccr4	Cdkn2a	Bmpr1a	Ccr1l1	Armcx1
Alox8	Bcl2	Bcl2l2	Cd81	Cdkn2b	Casp1	Cd24a	Armcx6
Bex2	Ccl5	C1qtnf6	Creb3l2	Ctla2a	Cd300lf	Cdc2l5	C1ql3
Casp1	Ccr9	Cd36	Csf1	Cysltr2	Cd86	Cmtm1	Hes1
Casp4	Cd22	Cd38	F11r	Dusp3	Cebpb	Cmtm8	Lsr
Cd300lf	Cd79b	Creb3l2	Il10	Entpd1	Csprs	Ctsl	Lss
Cebpd	Cdk5r2	Ctla4	Il12rb1	Fasl	Ctbp2	Cxcl10	Lypd6
Casp1	Ciita	Dkkl1	Il1r2	Il10	Ctsf	Cxxc5	Ptprf
Casp4	Cxcr3	Gng2	Il4	Il18rap	Ctsh	Defb15	Rab7
Cd300lf	Cxcr4	H1f0	Itga7	Il4	Efna2	H2afy	Slamf9
Cebpd	Cxcr6	Havcr2	Mgll	Klre1	Efnb2	Igsf3	Tceal3
Csf2ra	Eomes	Itga8	Myo1d	Lilrb4	Entpd1	Mapk13	Tceal6
Dock6	Foxq1	Klra16	Myo6	Pard3	H2-DMb2	Mid1	Trib3
H2-DMb2	H2-T3	Kremen2	Pros1	Pde4a	Igf2	Serpina3m	
Hoxa1	Icosl	Lag3	Rhod	Pde8a	Igh	Serpinb5	
Il1r2	Klra8	Lsr	Serpina3g	Pros1	Il5ra	Sox4	
Irak3	Ly6c	Mdm1		Ryk	Klrg1	Sstr1	
Itgb1	Maf	Mid2		Sept10	Lmo1	Sstr4	
Lair1	Myo1f	Plekhg3		Serpina3f	Lmo2	Sult2b1	
Maf	Runx2	Plekhh3		Serpina3g	Lrp4	Sult5a1	
Mapk12	S100a6	Rhoc		Tcrg	Ly6f		
Myo6	Sfn	Rhod		Tnfsf4	Naip2		
Nlrp10	Sntb2	S100a6			Naip5		
Oas1d		Tlr4			Nlrp10		
Oas1f					Serpina9		
Rhou					Tlr4		
Rora					Tnfrsf13c		
S100a4					Tnfrsf17		
Sp100					Tnfsf13b		
Tnfsf13b							
Wnt3							

Fig. 2 Cluster analysis of differentially regulated genes between young and old CD4$^+$ and CD8$^+$ T cells. Gene lists created in Fig 1 containing genes that are differentially regulated at time point 0 and at least 2 of the 4 other time points. (A) Four prominent and biologically relevant expression pattern profile plots with ranges of expression values within each pattern. (B) Gene lists for the patterns in A.

and patterns for CD8 T cells are available in Table S11 and for CD4 T cells, Table S12), in which a 2-fold difference in gene expression was observed between young and old at time 0 and at least in 2 of 4 time points after stimulation. We observed patterns such as: pattern (1), genes with high expression levels at time 0, in which gene expression decreases after stimulation; pattern (2), gene expression levels increases after stimulation; pattern (3), genes with higher expression levels in the old than in the young at time 0 and post-stimulation; and pattern (4), genes with lower expression levels in the old than in the young at time 0 and post-stimulation (Fig. 2A). We also found patterns such as genes that decrease in first hours post-stimulation but then gene expression augments, or genes that increase in the first hours post-stimulation but then gene expression decreases (Tables S11 and S12). In addition, in this approach, the biological behavior of genes over time can be tracked and provides insights into why the T-cell responses of the old are different than the young. For example, in pattern 1 we observed that Maf is highly expressed in old CD4$^+$ and CD8$^+$ T cells. As described earlier, high expression of Maf is associated with Th2 responses that are a characteristic of the aged. Among the most interesting patterns are 3 and 4 where genes are expressed at high or low levels in old T cells regardless of TCR stimulation. This information could generate new hypotheses that were not previously considered in aging T-cell biology. In pattern 3, we observed that Entpd1 (CD39) is also highly expressed in old CD4$^+$ and CD8$^+$ T cells. Expression of this receptor on T effector cells has been associated with immuosuppressive loops (Deaglio *et al.*, 2007). We also observed that phosphodiesterases (Pde8a and Pde4a) are highly expressed in old T cells. These molecules regulate the intracellular levels of cAMP and cGMP cyclic nucleotides, which play an important role as second messengers in multiple physiologic processes. These include the regulation of immune response such as inflammation (Smith & Spina, 2005), which could provide an explanation why the old immune system is characterized by a chronic inflammatory status (Chung *et al.*, 2009). In pattern 4, we observed that many genes associated with microtubules, cell cycle replication, migration, and other functions are expressed at low levels in old CD4$^+$ and CD8$^+$ T cells. Not much is known about the role of these genes in T-cell biology. Perhaps, the lack of these proteins affects the cell cycle of T cells, diminishing cell activation, replication, migration, and other functions. Furthermore, such analyses enable the identification of genes that are dysregulated in old T cells, genes that were not previously considered biologically relevant in altering the function of these cells. We identified many other genes of interest that were differentially expressed between young and old T cells. For example, the data show that Ctsl (cathepsin L) (pattern 4, CD8 T cells) is high in the young. High expression of this gene correlates with a Th1 response suppressing Th2 responses (Onishi *et al.*, 2004), supporting our previous observation of a switch to Th2 responses as T cells age. In CD4$^+$ T cells, we observed that Hes1 (Hairy/Enhancer of Split) (pattern 4) is highly expressed in the young but not in the old. Hes1 proteins are targets of the Notch signaling pathway in which this program is critical for T-cell development (Chari & Winandy, 2008). This might suggest that defects in the Notch pathways could affect the T-cell developmental process in the old.

Also of great interest are genes which were differentially regulated both at time 0 and after activation (e.g., patterns 3 and 4, Fig. 2). These genes might be critical as they represent both innate and functional differences affecting essential biological processes. It is well established that aging is characterized by a pro-inflammatory status with an increase in

the level of specific cytokines, chemokines, and other factors. This state of subclinical, chronic inflammation has been called 'inflamm-aging' (Franceschi *et al.*, 2000). However, it is not yet completely understood what triggers or how inflamm-aging is maintained. Many reports have indicated that IL-1β, IL-6, TNF-α, CCL2, and CCL5 are elevated in the old (Gerli *et al.*, 2000; Ferrucci *et al.*, 2005). Why these molecules are high in the old is not known. IL-1β is produced by macrophages and T cells, and the production of this cytokine is controlled by multi-protein complexes termed inflammasomes (Martinon *et al.*, 2009). Many of the inflammasome-related genes are elevated in old T cells (Tables S3 and S8). IL-1β can induce the production of other pro-inflammatory cytokines such as IL-6, TNF-α, IL-8, IL-17, and IL-1 itself (Rausch-Fan *et al.*, 2005; Cahill & Rogers, 2008; Kramer *et al.*, 2008) potentially leading to a pro-inflammatory positive feedback loop. We also observed that the receptors for CCL2 (CCR2 and CCR4), CCL5 (CCR5), and other chemokines such as CXCL10 and its receptor CXCR3 are elevated in old T cells, which could further enhance the pro-inflammatory status of the old. This is in agreement with the studies of Chen *et al.* (2003) where they demonstrated by microarray analysis and protein levels that old $CD4^+$ and $CD8^+$ T cells are associated with increased expression and secretion of CCL3, CCL4, and CCL5 in C57BL/6 mice. Additionally, many other genes involved in inflammation such as ANXA1, Pde4a, Pla2g, PENK1, S100a6, and MAPK12 are elevated in old T cells. Taken together, this information begins to provide an explanation why we observe a chronic inflammatory status in the old. Previously, we provide examples of pathways that were not previously identified as important in the aging immune systems, suggesting possible explanations as to why some of the functions of old T cells are dysregulated or altered. There are many other pathways that can be analyzed such as migration, adhesion, and numerous other signal transduction pathways, which will not be discussed in detail at this time.

Differential gene expression at each time point

We next tabulated the number of genes that were up- or down-regulated by at least 2-fold in old $CD4^+$ or $CD8^+$ T cells when compared to young T cells at each individual time point before and after TCR stimulation. This type of analyses allows us to have a general picture about the behavior of the transcriptome. Furthermore, we grouped differentially regulated genes into gene ontologies to examine which cellular processes were enriched with particular genes at each time point. As an example, we analyzed the genes that were up- or down-regulated in immune system processes in $CD8^+$ (Fig. 3A) and $CD4^+$ T cells (Fig. 3B) (Complete gene lists for $CD8^+$ T cells Table S13 and for $CD4^+$ T cells Table S14). The analysis revealed that in old $CD8^+$ T cells at all five time points, CCL5 and TLR4 are up-regulated (discussed genes are labeled with a symbol), while CCL1, CCL9, IL7R, and other genes are only up-regulated after TCR stimulation (Fig. 3A). Genes such as Ddx58 (or RIGI) and Gbp2, which are involved in immune regulation (Imaizumi *et al.*, 2008; Kylaniemi *et al.*, 2009), are up-regulated in young but not in old $CD8^+$ T cells after 12 h following stimulation maintaining high expression after 24 and 72 post-TCR activation. Genes such as TNFSF14 (LIGHT) and S100a9 are up-regulated in old $CD8^+$ T cells at time 0 and only during the first 12 h post-TCR activation. Analysis of $CD4^+$ T cells (Fig. 3B) show that CCL5 and TLR4 are also up-regulated in the old. RORC (retinoic acid receptor-related orphan receptor) is not differentially expressed at time 0; however, after TCR stimulation, the expression of this gene is higher in young $CD4^+$ T cell than old $CD4^+$ T cells at all times. Expression of RORC in naive T cells reduces levels of Foxp3 and knockdown of RORC promotes Foxp3 expression (Burgler *et al.*, 2010). This is in agreement with our data, indicating that in the old there is double the amount of Tregs when compared to the young (Sharma *et al.*, 2006). Perhaps, the lack of expression of these regulatory elements alters the balance of the different T-cell populations. Immunoglobulin-like receptors (LILRB or ILT) are involved in functional shaping of T-cell responses inducing tolerance (Thomas *et al.*, 2010). Although this receptor is selectively expressed on APCs such as monocytes, macrophages, and dendritic cells, we observed that LILRB4 (ILT3) gene starts to be expressed in old $CD4^+$ T cells after 24 h post-stimulation and its expression significantly augments after 72 h. We have recently reported that the negative regulatory molecule B7-H1 is highly expressed in old naïve $CD8^+$ T cells impairing the proper activation of aged $CD8^+$ T cells (Mirza *et al.*, 2010). It is possible to speculate that in the old, molecules that negatively regulate the T-cell responses are up-regulated. This is supported also from the microarray data (Tables S3 and S8) which show that Tim-3 (HAVCR2), a negative regulator of T-cell responses (Sabatos *et al.*, 2003), is also up-regulated in naïve old $CD4^+$ and $CD8^+$ T cells. It is not clear to us why molecules that inhibit T-cell responses are up-regulated in the old, but their high expression could be driven by the pro-inflammatory status in the old, which is also indicative of exhausted T cells following viral infections (Blackburn *et al.*, 2009).

Data mining of assorted genes of interest linked to age-related deficiencies or pathologies

Another usage of this kinetic microarray data is as a functional tool to query relationships between genes (or their encoded proteins) involved in previously reported pathologies or suspected immune deficiencies. Figure 4 shows multiple examples of genes encoding proteins involved in T-cell biology. We observed that NFATC1 (nuclear factor of activated T cells) at time 0 has the same level of expression in young and old $CD8^+$ T cells. However, after TCR activation, this gene is highly up-regulated in the young while it substantially decreases in the old. NFATC1 is critical for the activation of certain cytokines such as IL-2. Although the expression of the IL-2 gene is higher in old $CD8^+$ T cells, it is known that old $CD8^+$ T cells secrete less IL-2 than young $CD8^+$ T cells. NFATC1 is critical for the activation of the IL-2 promoter and induces the production of IL-2 (Northrop *et al.*, 1994). This could explain why old $CD8^+$ T cells produce less IL-2 than young $CD8^+$ T cells. Additionally, low NFATC1 nuclear translocation is associated with calcium flux and has been implicated in the induction of anergy (Srinivasan & Frauwirth, 2007). Perhaps, the low expression levels of NFATC1 in old T cells after activation might affect the calcium flux impairing the proper activation of these cells. The levels of S100A6 (S100 calcium binding protein) gene expression is higher in old $CD4^+$ and $CD8^+$ T cells. The role of S100A6 in T-cell biology in not defined; however, S100 proteins regulate a diverse group of cellular functions including cell–cell communication, cell growth, cell structure, energy metabolism, contraction, and intracellular signal transduction, and they play a role as pro-inflammatory mediator in acute and chronic inflammation (Perera *et al.*, 2010). It is possible that overexpression of S100A6 might alter the function of old T cells and/or induce inflammatory processes in the aged. This type of analysis provides the opportunity to identify specific genes that have not been implicated in aging T-cell biology which could influence specific cellular processes and function or be involved in disease generation.

Analysis of signaling pathways through the evaluation of specific genes pre- and post-stimulation

It is critical to understand the contextual significance of differential gene expression and how these changes relate to the modulation of specific signaling pathways. Recently, several reports have indicated

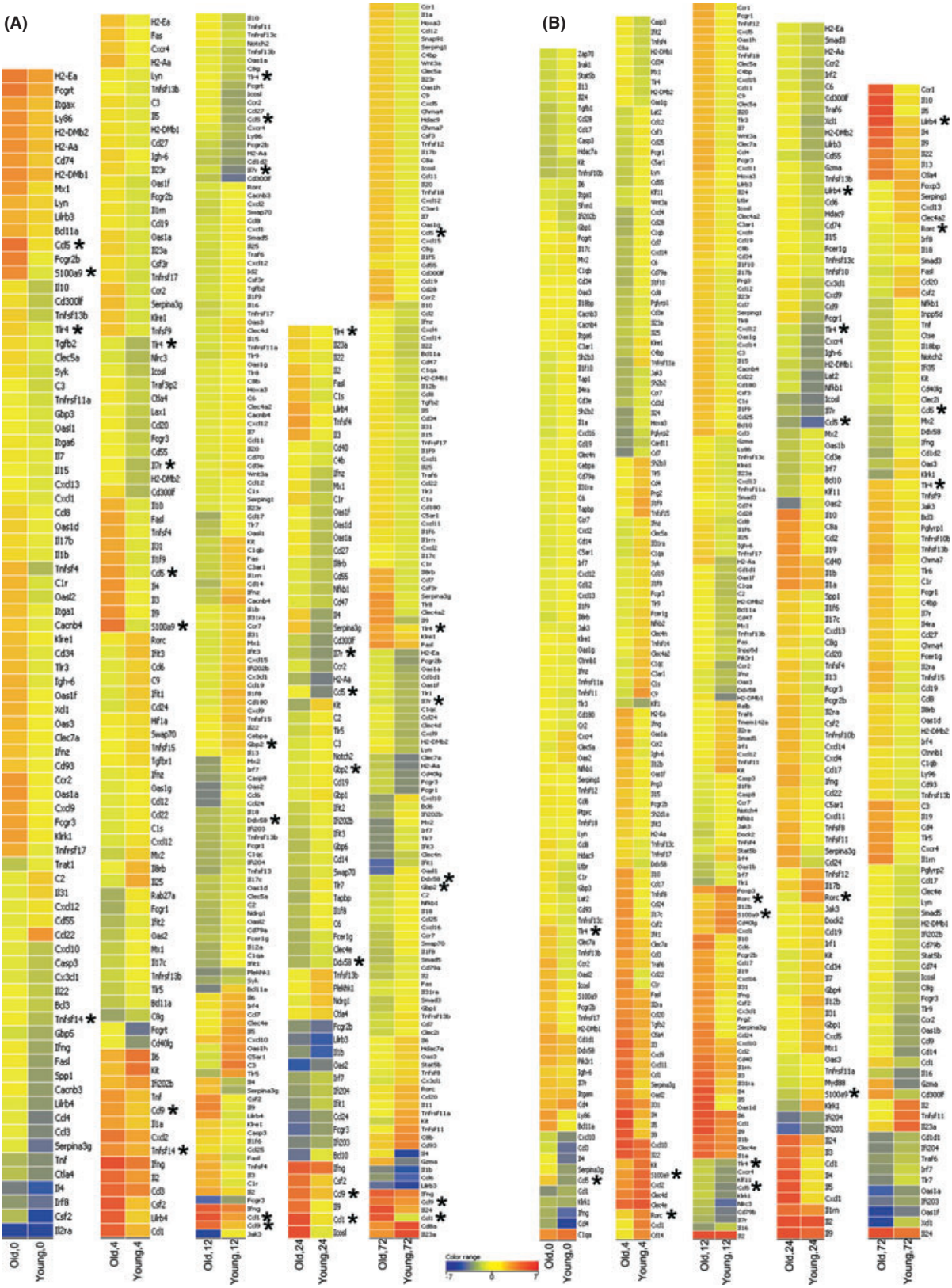


Fig. 3 Heat maps of expression levels of genes contained in the immune processes gene ontology. Differentially regulated genes by 2-fold or more for each time point. Shown in hierarchical clustered heat maps for $CD8^+$ (A) and $CD4^+$ (B) T cells. Color bar depicting the range of differential gene expression, in log 2. Asterisks denote mentioned in the text.

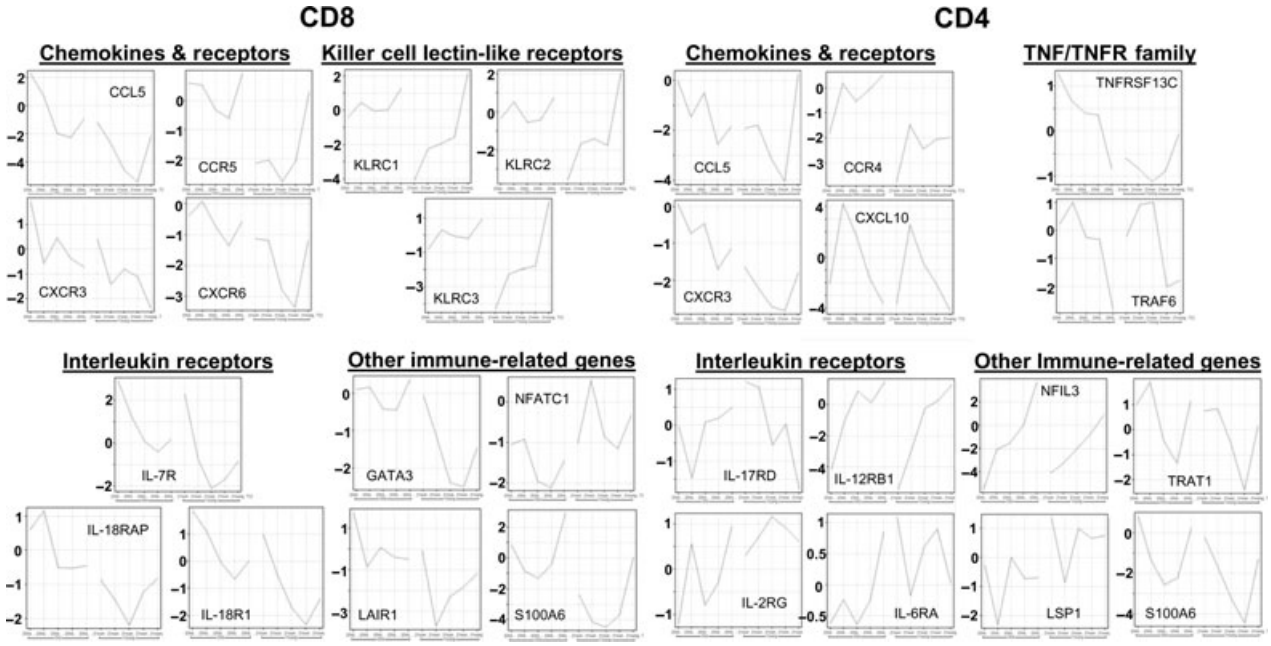


Fig. 4 Expression profile plots of differentially regulated genes. The gene expression profile of selected gene families pre- and post-stimulation was analyzed in old and young $CD8^+$ and $CD4^+$ T cells. Axis labels are the same as in Fig. 2.

the importance of the Wnt-β-catenin pathway in the generation of memory T-cell responses (Wu *et al.*, 2007; Zhao *et al.*, 2010). We depict the Wnt-β-catenin pathway and populate it with gene expression levels found in $CD8^+$ T cells (Fig. 5). Adjacent to every step of the pathway, we included the profile plot of the gene, allowing the visualization of the differential gene expression between young and old. Wnt is a family of 19 secreted glycoproteins involved in signal transduction pathways that regulate the transcriptional activity of hundreds of genes that impact cell differentiation, communication, apoptosis/survival and proliferation (Petersen & Reddien, 2009). The Wnt/β-catenin pathway is highly regulated at various levels of the signaling cascade (Petersen & Reddien, 2009), as well as positively or negatively regulated by feed back loops. Wnt/β-catenin signaling is a key regulator of T-cell development at various stages of thymocyte differentiation (Ioannidis *et al.*, 2001). Although it was thought that Wnt/β-catenin pathway was terminated post-thymocyte maturation, recent data indicate that Wnt/β-catenin is an active and important pathway in regulating different functions in mature T cells (Wu *et al.*, 2007) and is critical for regulating memory T-cell responses (Gattinoni *et al.*, 2009). A major problem found in old T-cell responses is the improper transition from naïve T-cell phenotype into a memory T-cell phenotype (Dominguez & Lustgarten, 2008). We have demonstrated that old mice could not develop memory responses against tumor antigens while young animals did (Dominguez & Lustgarten, 2008), indicating that the transition from a naïve phenotype to a memory phenotype is compromised in the old. The canonical Wnt pathway is activated when Wnt ligands, which are secreted glycoproteins, initiate signaling by interacting with cell surface receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). As a result of this interaction, the cytosolic amount of β-catenin increases and thus translocates to the nucleus where it associates with transcription factors of the TCF1/LEF1 family. Because of its transactivating ability, the β-catenin–transcription factor complex binds to DNA and activates Wnt target genes. If β-catenin is not released from the complex, this pathway cannot be activated. Regarding the Wnt/β-catenin pathway, our data indicate that the expression of (i) LRP5/6 is lower in the old, which could restrict the binding of Wnt Fz-LRP5/6; (ii) Dkkl1 expression is higher in the old. Currently, there is no information in the literature about the role of Dkkl1 in T-cell biology. Dkkl1 encodes for a protein that antagonizes Wnt/β-catenin preventing the binding of Wnt to the Fz/LRP receptor, inhibiting the activation of this pathway (Krupnik *et al.*, 1999); (iii) Other key molecules in the Wnt/β-catenin pathway such as CK1/2 (casein kinase 1/2), Gsk3b (glycogen synthase kinase 3-beta), Src (oncogne Src), and APC, (APC gene), which are critical for the disruption of the 'β-catenin complex', accumulation and for nuclear translocation of β-catenin, are differentially expressed in old $CD8^+$ T cells when compared to young $CD8^+$ T cells (Fig. 5). Similar gene pattern expression was found in old $CD4^+$ T cells (data not shown). Based on the analysis of the Wnt-β-catenin pathway, we hypothesized that there is a deficiency in the disruption of the 'β-catenin complex' resulting in the cytosolic accumulation and lack of nuclear translocation of β-catenin; consequently, TCF1 is not activated preventing old naïve $CD4^+$ and $CD8^+$ T cells from becoming memory cells. Inhibitors of Gsk-3β, which mimic the Wnt-pathway, have shown to enhance the formation of memory T-cell responses (Wu *et al.*, 2007). To enhance the generation of memory responses it might be possible to treat old mice with inhibitors of Gsk-3β and possibly normalize the generation of memory responses in the aged. Thymic involution is high in old age; despite that, this organ still generates T cells at reduced levels (Lynch *et al.*, 2009). As Wnt/β-catenin pathway is a key regulator of T-cell development, alterations within this pathway might also affect the differentiation of t cells in thymus at old age. The information from this microarray analysis allowed us to evaluate pathways and/or networks that were not previously considered and to gain insight into the behavior of the old immune system which might provide possible therapeutic approaches to restore the T cell function in the aged.

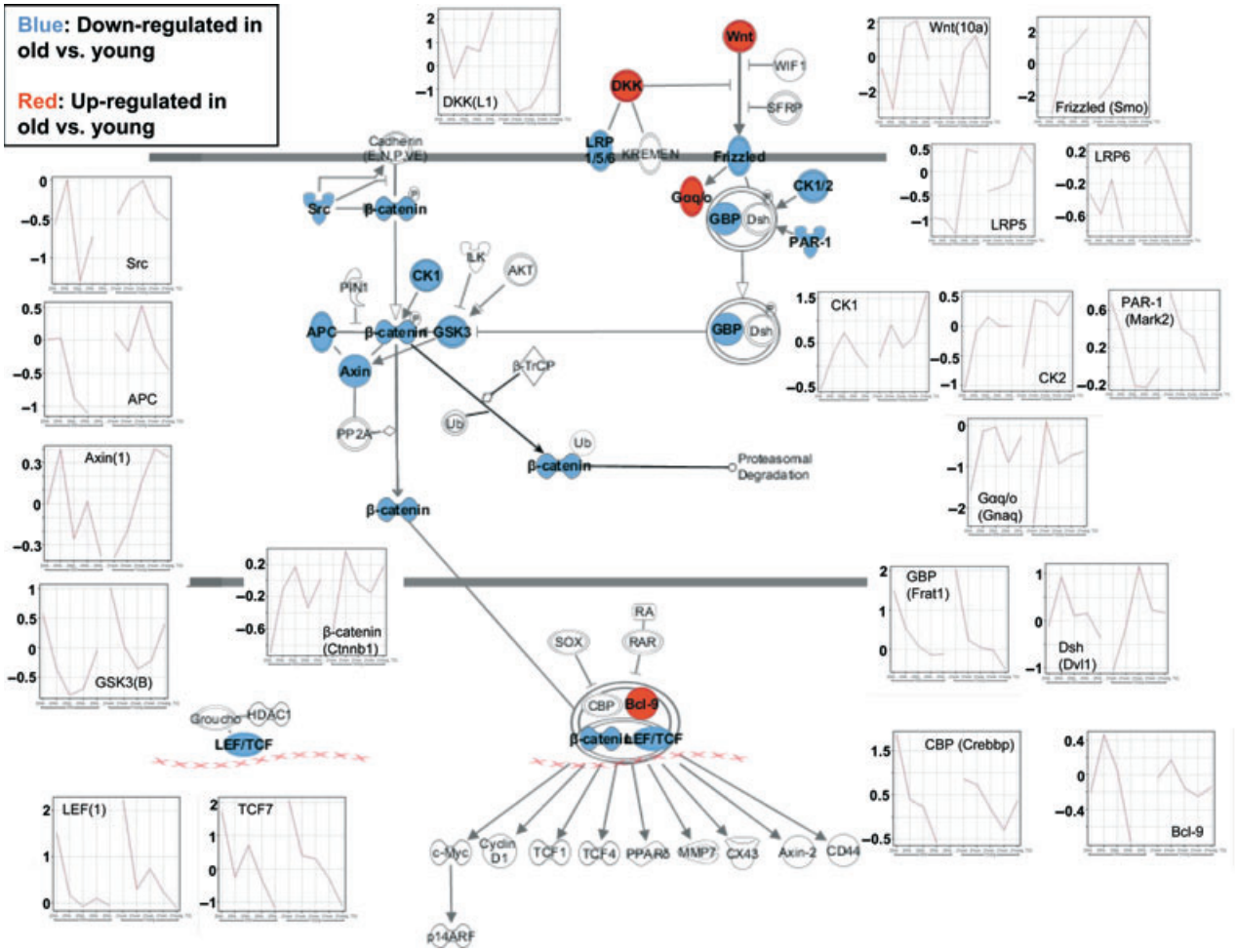


Fig. 5 Kinetic changes in gene expression of selected components of the Wnt/β-catenin signaling pathway in aging CD8$^+$ T cells. Wnt/β-catenin pathway was generated by Ingenuity Pathway analysis. Adjacent to each step of the pathway are the gene profile plots of old and young CD8$^+$ T cells. Axis labels are the same as in Fig. 2. Red indicates up-regulated genes, blue down-regulated genes.

We built pathways to visualize and understand why old CD4$^+$ T cells have a propensity toward a Th2 response and high secretion of IL-4 and IL-10 (Fig. 6). Analysis of genes that induce and regulate Th2 differentiation indicate genes that are critical in these pathways such as IL4, GATA3, and c-Maf are significantly expressed at higher levels in old CD4$^+$ T cells pre- and post-stimulation (Fig. 6A). GATA1 – which is a negative regulator of the Th2 master regulator, GATA3 – is expressed at high levels in young CD4$^+$ T cells, which could provide an explanation as to why young and old differ in Th1 vs Th2 differentiation. Analysis of the IL-10 signaling pathway reveals a robust induction of IL-10 transcription by old CD4$^+$ T cells, which correlates with higher induction of STAT3 gene expression over time resulting in elevated expression of IL-4R (Fig. 6B). We also evaluated the IL-4 signaling and production pathway (Fig. 6C). The results indicate that intermediate steps such as Jak3 expression are elevated in activated old cells and critical molecules such as GATA-3 and c-MAF are highly expressed in the old resulting in higher expression of IL-4 and IL-10. Although the expression of STAT6 is similar between young and old T cells, however, GATA3 is capable of inducing its own expression via a STAT6-independent positive autoregulatory loop (Ouyang *et al.*, 2000), explaining the high expression of this gene in aged T cells. In summary, GATA-1 – the negative regulator of GATA3 – is highly expressed in young T cells whereas genes such as IL-4, IL-4R, GATA3, and c-MAF, which are critical in driving Th2 responses, are significantly elevated in old CD4$^+$ T cells. This pattern of gene expression pre- and post-stimulation provides an explanation about the molecular mechanism supporting or favoring elevated Th2 responses in the old. The Th2 responses are maintained in the old by the high production of IL-4 and IL-10. The data presented in Fig. 6 suggest that there is an auto-feedback mechanism driving the Th2 differentiation in old CD4$^+$ T cells. While we still do not understand the molecular basis of suppressed GATA1 expression in old T cells, perhaps it might be possible to augment the expression of GATA1 and/or diminish the expression of GATA3 to balance the Th1 and Th2 responses in the old.

This type of analysis is also critical because we found genes such as Camk4, Otub1 (in CD4$^+$ and CD8$^+$ cells), Socs3, Gzmb1 (in CD8$^+$ cells), Tgfb1, Cdk5 (in CD4$^+$ cells), and many others (Table S15 for CD8s and Table S16 for CD4s) that were differentially expressed at time 0 between young and old CD4$^+$ and CD8$^+$ T cells, but after stimulation these genes were equally expressed between young and old. We also found genes such as IL-7r, GATA3 (in CD8$^+$ cells) Ndgr1, IL-10, Cebpd (in CD4$^+$ cells), and others (Table S17 for CD8s and Table S18 for CD4s) that were expressed at the same level at time 0, but after TCR stimulation gene expression was markedly different. Only a T-cell activation time course, such as the one presented here, can reveal important changes in gene

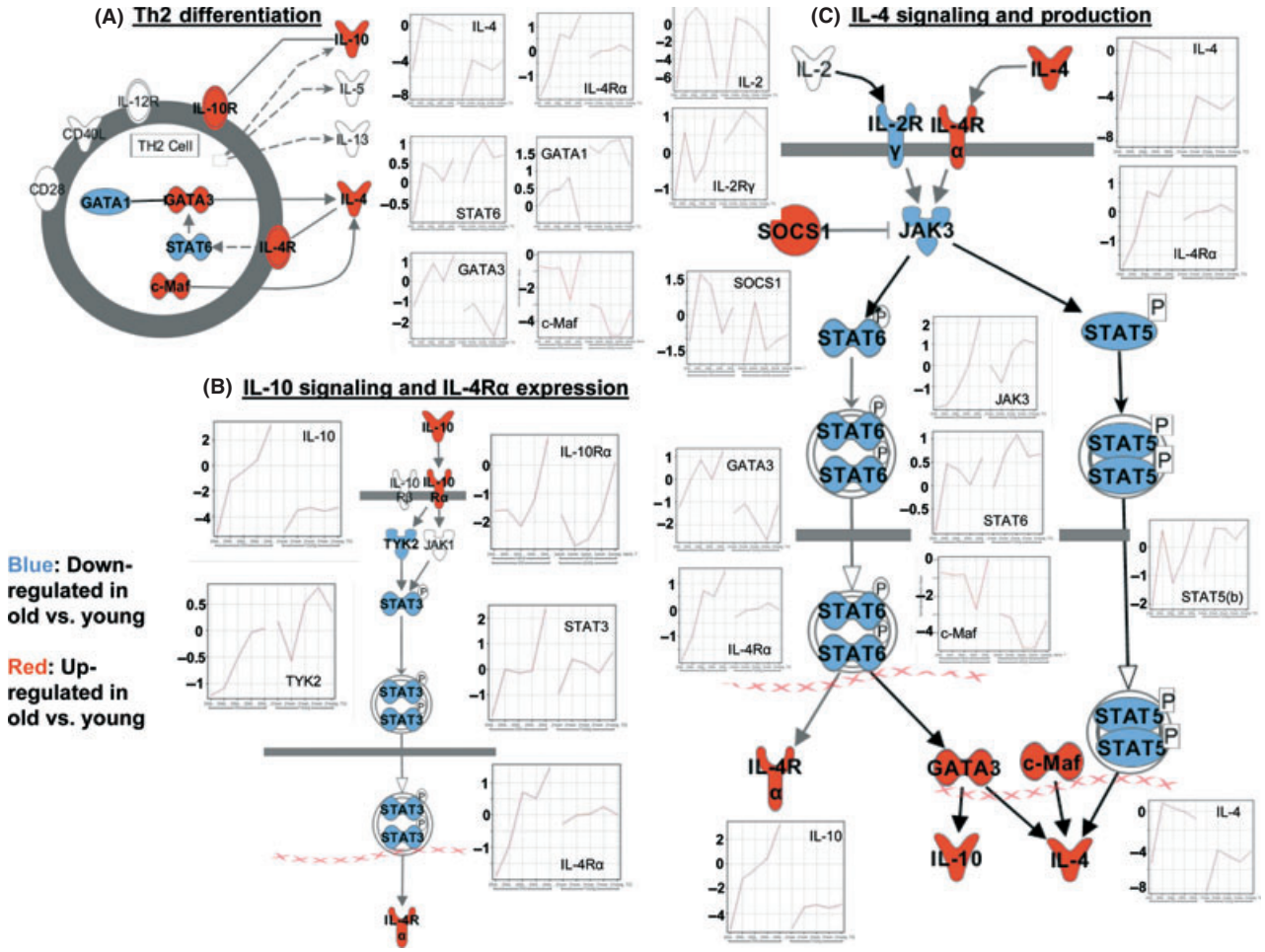


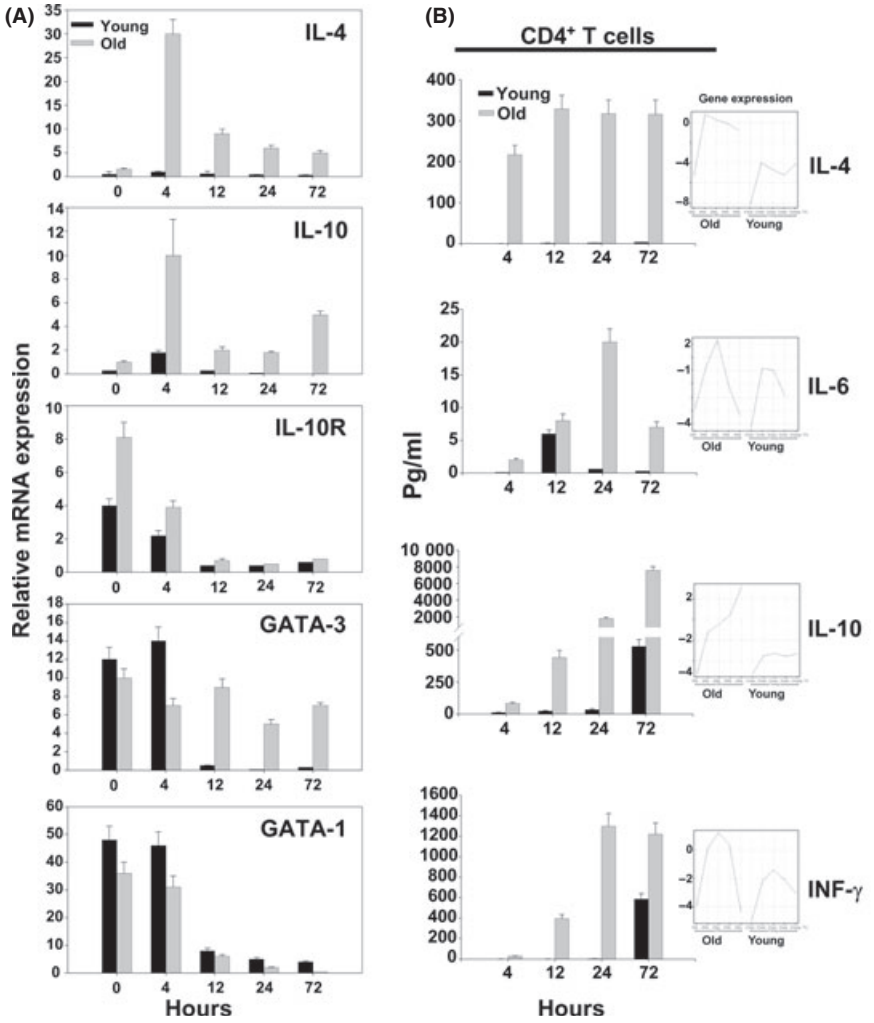
Fig. 6 Kinetic changes in gene expression of selected components of Th2 responses. Pathways were generated by Ingenuity Pathway analysis. (A) Pathway analysis of Th2 differentiation. (B) IL-10 Signaling and IL-4Rα expression, and (C) IL-4 signaling and production. Adjacent to each step of the pathway are the gene profile plots of old and young CD4$^+$ T cells. Axis labels the same as in Fig. 2.

expression kinetics leading into new insights of why some processes are deficient or altered in the old.

Validation of gene expression

To validate the microarray analysis, we performed real-time quantitative RT-PCR (QRT-PCR) analysis. We selected specific genes for CD4 T cells. Data shown in Fig. 7A indicate the relative gene expression from young and old CD4$^+$ T cells in relation to gene expression of GAPDH. We observed that genes such as IL-4, IL-10, IL10R, and GATA-3 are highly expressed in old CD4$^+$ T cells while GATA-1 is expressed at low levels. These results from the QRT-PCR analysis confirmed the data from the microarray analysis. To confirm that changes in cytokine gene expression observed between young vs. old results in differential cytokine secretion, we determined the production of IL-4, IL-6, IL-10, and INF-γ after 4, 12, 24, and 72 post-stimulation in CD4$^+$ T cells. The data indicate that old CD4$^+$ T cells rapidly start secreting these cytokines compared to young CD4$^+$ T cells, and the levels of cytokines produced are significantly higher in old CD4$^+$ T cells than in young CD4$^+$ T cells (Fig. 7B). We also analyzed the secretion of these cytokines by CD8$^+$ T cells, although old CD8$^+$ T cells produce more of these cytokines than young CD8$^+$ T cells (Fig. S1); the levels are much lower than those of CD4$^+$ T cells. As we observed that many other cytokines and chemokines were differentially expressed between young and old T cells and to extend these results, we used a Mouse Cytokine Array Profiler able to evaluate a panel of cytokines and chemokines. Protein analysis indicate that cytokines and chemokines such as IL-3, IL-4, IL-10, IL-13, M-CSF, CCL1, CCL3, and CCL5 are produced in higher levels in old CD4 T cells while TNF-α is secreted in higher levels by young cells (Fig. 7C). A similar pattern was observed with old CD8 T cells in which they produced higher levels of IL-3, IL-4, IL-13, M-CSF, CCL1, CCL3, and CCL5, and young CD8 T cells secrete more TNF-α. Although this method does not indicate the absolute quantities of protein produced by the cells, it allows for the rapid evaluation of several secreted factors. The positive correlation between the production of these factors and their expression levels in the microarray further validate the results of the microarray analysis.

In summary, for the first time, this study compares the gene profiles of naïve young and old CD4$^+$ and CD8$^+$ T cells pre- and post-TCR stimulation. Based on the kinetic analysis, we could determine whether differential expression was maintained post-stimulation and therefore identify gene expression modifications. The data indicate that alterations in immune functions in old CD4$^+$ and CD8$^+$ T cells is attributed to modifications in various cellular process and signaling pathways, and this information is critical to optimize the CD4$^+$ and CD8$^+$ T-cell responses in the



(C) CD4 T cells[a]

Factors[b]	Young	Old
IL-3	4095	17625
IL-4	615	1840
IL-10	910	2010
IL-13	1409	8950
TNF-α	2305	1880
M-CSF	515	1250
CCL1	1330	12830
CCL3	1455	2606

(D) CD8 T cells[a]

Factors[b]	Young	Old
IL-3	3705	5805
IL-4	875	545
IL-13	810	1815
TNF-α	3765	2660
M-CSF	475	680
CCL1	7095	16945
CCL3	41345	69654
CCL5	225	4285

[a] 72 h supernatant

[b] relative integrated intensity

Fig. 7 Validation of gene expression. (A) expression of IL-4, IL-10, IL-10R, GATA-3, and GATA-1 for CD4 T cells pre- and post-stimulation was evaluated by QRT-PCR. Data indicate the relative gene expression from old CD4$^+$ T cells in relation to gene expression from young T cells. (B) Secretion of IL-4, IL-6, IL-10, and INF-γ was evaluated in young and old CD4$^+$ T cells post-stimulation by ELISA. Each value represents the mean of triplicate wells ±SE. (C) Analysis of protein profiler for the detection of cytokines and chemokines productions. Supernatants of 72-h stimulated young and old CD4 and CD8 T cells were used for this analysis. Each value represents the mean of duplicate wells. Values indicate relative integrated intensity.

old. We certainly do not understand the ramification of all the genes that are differentially expressed between young and old T cells. However, this type of analysis reveals the transcriptional modulation of specific genes which might reflect functional changes in the aged immune system. Such information allows us to postulate new hypotheses addressing why old T cells are dysregulated compared to young T cells. This study improves our understanding of the aged T-cell biology and the underlying mechanism for T-cell dysregulation in the old and might provide the basis to perhaps reverse age-associated T-cell deficiencies.

Experimental procedures

Harvesting splenocytes

For these experiments, spleens were harvested from Balb/c mice aged 3–4 months (young) and 20 months (old). Single-cell suspensions were generated by passing splenocytes through 40-μm nylon mesh strainers after mechanical disruption of spleens. Red cell contaminants were removed by incubating splenocytes in red cell lysis buffer at 37 °C according to instructions' manual. Cell pellets were washed thoroughly in RPMI1640 containing 10% FBS and then resuspended in a buffer of cold PBS (Dulbeccos'; Invitrogen, Carlsbad, CA, USA) containing 1% BSA (Sigma, St. Louis, MO, USA) and 4 mM EDTA recommended for enrichment and cell sorting purposes. Negative isolation was used to enrich CD4$^+$ and CD8$^+$ T cells from total splenocytes. Briefly, biotin-conjugated antibodies to CD45R and CD4 were incubated with splenocytes on a rotating shaker for 10 min at 4 °C for CD8 T-cell isolation. CD4 T-cell enrichment was performed with biotin-conjugated antibodies to CD45R (eBioscience, San Diego, CA, USA) and CD8 (eBioscience). Excess unbound antibodies were washed by centrifugation using the buffer recommended. Washed cells were incubated with biotin binder beads (Invitrogen) on rotating platform at 4 °C for 30 min. Bead bound cells were depleted on magnets and the supernatants enriched with CD4 or CD8 cells for collection.

Cell staining and sorting

CD4$^+$ T cells were surface stained with fluorescent antibodies to CD4, CD44 and CD62L and CD8$^+$ T cells with fluorescent antibodies to CD8,

CD44, and CD62L. Naïve $CD4^+$ and $CD8^+$ T-cell populations from young and old animals were sorted based on $CD44^{low}/CD62L^{hi}$ expression on the BD FACS Aria.

Cell culture

Sorted naïve T-cell subpopulations were washed in RPMI1640, and cell viability more than 99.99% was assessed. Cells (1×10^6) from naïve subpopulations were taken prior to plating and kept in RLT buffer (Qiagen, Valencia, CA, USA) at −80 °C. This was denoted as the time point $t = 0$ sample. The remaining cells were plated at 1.5×10^6 mL^{-1} per well into 24-well plates precoated with antibodies to CD3 and CD28 at 2 μg mL^{-1}. $CD4^+$ and $CD8^+$ T cells from young and old animals were collected after 4, 12, 24, and 72 h of stimulation. Cell pellets were resuspended in RLT buffer and kept at −80 °C. T lymphocytes are small cells and the amount of RNA extracted is limited; therefore, multiple independent sorts were carried out to generate RNA sufficient for microarray investment.

RNA extraction and microarray processing

RNA was isolated using the RNeasy micro kit (Qiagen) according to instructions enclosed. Cells from each independent sort contributed toward all the five time points. For each time point, the RNA was pooled together from cells isolated from four independent sortings using young and old animals. RNA quantity and integrity were verified with the Nanodrop (Thermo Scientific, Austin, TX, USA) and Bioanalyzer using the Pico chips (Agilent). Using the Quick Amp Labeling kit, one color (Agilent), 200 ng RNA per sample was labeled. Dye incorporation and amplified RNA amounts were verified with the Nanodrop. Then, 1.65 μg of each sample was hybridized to a 4X44K mouse whole genome slide, washed, and feature extracted according to manufacturer's (Agilent) instructions.

Microarray data analysis

Gene expression imaging, quality control, fold change, GO (Gene Ontology), and clustering analysis was performed with GeneSpring GX 11.0 (Agilent Technologies Inc.). To obtain a detailed look at inherent and acquired differences between old and young naïve $CD4^+$ and $CD8^+$ T cells, we designed two separate analytical approaches. First, we selected genes that were differentially regulated between old and young in both time point 0 h (unstimulated) and upon TCR activation in 2 of 4 of the time points taken (see scheme in figure 1). The overlap between these lists represents genes that are a) inherently different in old and young populations (derived from time point 0 h) and b) change significantly upon TCR activation (Fig. 1B,D). Secondly, we were interested in genes that are differentially regulated between old and young T-cells only after TCR activation. To derive this list, we selected genes that were 2-fold up- or down-regulated in old vs. young in 3 of the 4 time points during TCR activation and not at time point 0 h (Fig. 1C,E).

Gene lists from both these analyses were imported into Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc.) for pathway enrichment analysis. This type of analysis segregates genes into categories pertaining to defined biological processes or signaling pathways and calculates the probability that the genes are truly associated with a given functional group (see *P*-value columns in Tables 1 and 2). Differentially expressed genes were then classified as either up, down, or indeterminately regulated in old vs. young naïve T cells upon visual inspection of their respective expression profile plots.

Clustering analysis was performed using the gene lists containing genes that are differentially regulated between old and young naïve T cells pre- and post-TCR stimulation (Fig. 1B,D) to discover groups of seemingly unrelated genes that exhibit similar expression patterns over time. The GeneSpring K-means clustering algorithm was used to define 12 different expression profile types for each cell type. Normalized data values were then averaged for each pattern group and graphed along with the range of expression values (Fig. 2).

Fold change analysis was performed on all the tested entities to determine genes differentially expressed at each time point independently. These were classified as either up- or down-regulated in old vs. young naïve T cells and graphed (Fig. 3A,B 'All Genes'). GO Analysis was then performed on the 2-fold change gene lists at each time point, and those genes categorized under immune system process were selected to undergo hierarchical clustering to generate heat maps for each time point (Fig. 3A,B 'Immune System Process' and heat maps).

Pathway analysis from Ingenuity was used to generate specific signaling pathways.

Quantitative RT-PCR (QRT-PCR)

Total RNA isolated for the microarray analysis was reverse transcribed with oligo dT primers using SuperScriptIII (Invitrogen). The resulting cDNA was amplified by PCR using the LightCycler and FastStart DNA Master^{PLUS} SYBR GreenI reagent (Roche, Tucson, AZ, USA) and the following gene-specific primers (Operon, Huntsville, AL, USA): GAPDH-F 5′-accacagtccatgccatcac-3′, GAPDH-R 5′-tccaccaccctgttgctgt-3′, IL10-F 5′-catgggtcttgggaagagaa-3′, IL10-R 5′-cattcccagaggaattgcat-3′, IL10RA-F 5′-gcaaggatgctgagagaagc-3′, IL10RA-R 5′-ctcaaagagcttgcccagt-3′, IL4-F 5′-tctgtagggcttccaaggtg-3′, IL4-R 5′-tggactcattcatggtgcag-3′, GATA1-F 5′-ttcccattctcctgcctcta-3′, GATA1-R 5′-cactcaggaactgaggcaca-3′, GATA3-F 5′-gctaggcctacatgctctgtg-3′, GATA3-R 5′-ccctccagagtacatccacct-3′. The cDNA amount in each sample was normalized to the crossing point of the housekeeping gene GAPDH. Relative mRNA fold change for each gene between each time point in old and young naïve T cells was calculated using the respective crossing points applied in the following formula: $F = 2^{-(G - H)}$, where F = fold difference, G = gene of interest, and H = housekeeping gene.

Profiling Cytokines from supernatants

A Mouse Cytokine Array Panel A kit (R&D Systems, Minneapolis, MN, USA) was used to simultaneously detect the relative levels of multiple cytokines present within the same sample of supernatant. Instructions outlined in the array protocol were followed. Briefly, supernatants harvested at the indicated time points were centrifuged to remove cellular particulate and stored at −80 °C. Supernatants were thawed, assayed for total protein with BCA protein assay kit (Pierce, Rockford, IL, USA). In the case of young and old CD4 naïve T-cell supernatants, 3000 μg of protein from each time point was used in each array, and 2500 μg of protein was used for young and old naïve CD8 T-cell fraction. A secondary antibody IRDye 800CW Streptavidin (LI-COR,) diluted to 1:2000 was used to fluorescently detect the cytokines and chemokines hybridized to the array. Array images were collected and analyzed using LI-COR Odyssey Infra-red Imaging System. A template was created to analyze the intensity of each spot in the array and an averaged background signal was subtracted for each spot. The cytokine level was determined by averaging the integrated intensity signal of duplicate spots. Corresponding integrated signal intensities on different arrays were compared to determine the relative change in cytokine levels at the specified time points between supernatant samples from young and old.

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Author contributions

N.M., K.P., and D.B.H. designed experiments, performed microarray analysis and evaluation of data. A.L.D. helped in obtaining cells and culture assays. N.M., K.P., D.B.H., and J.L. planned the study, interpreted the data, and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. ELISA analysis of IL-4, IL-6, IL-10 and INF-γ secreted by young and old CD8 T cells 4, 12, 24 and 72 post-stimulation.

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