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Transcriptional Profiles of Mart-1(27-35) Epitope Specific TCReng Human CD8+ and CD4+ T Cells upon Epitope Encounter as Elucidated by RNA-Seq

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Abstract

Adoptive cell therapy (ACT) with in vitro expanded populations of T cells engineered to express tumor epitope specific T cell receptors (TCR) is now undergoing clinical trials for various malignancies. In this context, ACT with the melanoma epitope, Mart-$1_{(27-35)}$, specific TCR engineered T cells has shown encouraging results in metastatic melanoma. A number of biological processes regulating T cell functions have, however, turned out to be impediments in this form of cancer therapy. As such, efforts are underway to gain a fuller understanding of the biology (functionality and constraints) of TCR-engineered (TCReng) T cells so as to extract more robust therapeutic effects from ACT. Traditional T cell-based assays are, however, somewhat inadequate for the purpose. Using Next Generation RNA-Seq (NGS) and qRT-PCR assays, we examined the transcriptome of melanoma epitope, Mart-$1_{(27-35)}$, specific TCReng human T cells. We found that the transcriptional profiles of the Mart-$1_{(27-35)}$, specific TCReng T cell (both CD8+ and CD4+) is remarkably similar when exposed to the cognate peptide. The genes responsible for T-cell activation, apoptosis, cellular proliferation, cytolytic response, and T-cell differentiation showed similar patterns of expression. Further, our analysis also revealed a number of alternate splice variants and novel isoforms related to immune response previously not associated with T cell activation, as well as expression of a number of recently discovered long non-coding RNAs (lncRNA).

Keywords: Mart-1; Engineered T-cells; Melanoma; Adoptive cell therapy; RNA-Seq; Trancriptome

Introduction

ACT with in vitro expanded populations of T cells, generated from tumor infiltrating lymphocytes, has shown remarkable results in human tumor immunotherapy [1-6]. These approaches are based on fundamental principles underlying T cell activation and effector function [7-9]. Unfortunately, while these approaches have proved therapeutically quite useful, various biological processes that regulate T cell functions and fate have turned out to be impediments in extracting a more uniform and sustained result with these approaches [7,8]. Accordingly, considerable efforts are underway to understand these regulatory mechanisms so that a more robust and long-lived anti-tumor response could be obtained by targeting these regulatory pathways.

Given that in vivo experiments that can be performed in animals are not feasible in humans, human tumor immunology has to rely on a variety of in vitro assays. Nonetheless, various in vitro bioassays and molecular analyses have generated important information on the function and fate of T cells [10,11]. Although these in vitro immune assays have been quite useful, they have proved to be inadequate for obtaining a comprehensive insight into the biology of anti-tumor T cells - especially for obtaining a better understanding of the regulatory elements that control the function and the fate of the anti-tumor T cells following activation and expansion. In this context, microarrays for some time [12] and NGS [13] more recently, have added considerable insights into the transcriptional signatures underlying T-cell activation in response to TCR stimulation by CD3/CD28 antibodies [14,15]. However, to our knowledge, the power of NGS has not been used to elucidate the biology of TCReng T cells responding to a relevant human tumor associated antigen.

Here we present the results of NGS with the Mart-$1_{(27-35)}$ epitope specific TCReng human $CD8^+$ as well as $CD4^+$ T cells showing remarkable similarities as well as important dissimilarities in the $CD8^+$ and $CD4^+$ T cells made to respond to a human melanoma associated epitope through a set of alpha/beta chain of receptors specific for the epitope. We also describe a large number of spliced variants as well as a number of large non-coding RNAs (lncRNA) expressed by both cell types in common, or expressed selectively by them.

Materials and Methods

Study subjects

The study population was drawn from melanoma patients of a cohort without frailty and other co-morbid health conditions. Blood samples from HLA-A2 positive patients were taken for the study with informed consent. Informed consent was obtained from each participant who volunteered to donate 50–100 milliliters peripheral blood for this research study approved by the Institutional Review Board of the University of Connecticut Health Center. Patients with active comorbidities and those with immune conditions and taking immunosuppressive agents were excluded from the study. The details of the collection of samples and cell preparation have been described [16,17].

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Tissue culture

Tissue cultures were performed in Iscove's medium (HyClone Laboratories Inc., USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO Inc., USA), henceforth described as complete medium (CM).

T cell purification: $CD4^+$ and $CD8^+$ T cells were purified using Dynal magnetic beads (Invitrogen, USA), as described previously [17]. Purity of the isolated cells was verified and >98% pure cells were used for the study.

DC culture

The method of generating myeloid dendritic cells (DC) from peripheral blood mononuclear cells (PBMC) used in this study has been described previously [16,17]. Briefly, monocytes/macrophages were isolated as adherent cells from Ficoll-Hypaque gradient derived mononuclear cell populations from blood. The adherent cells were then cultured in CM containing 1000 U/ml of GM-CSF (R&D Systems, Minneapolis, USA) and 1000 U/ml of IL-4 (R&D Systems, Minneapolis, USA), for 7 days. The non-adherent and loosely adherent dendritic cells were harvested by vigorous washing.

Retroviral vector construction

Mart-$1_{(27-35)}$ epitope specific DMF5 TCR was isolated from a high avidity tumor infiltrating lymphocyte (TIL) clone as has been previously described [10,11]. The PG13 packaging cell line to produce DMF5 retrovirus was cultured in DMEM (Hyclone Laboratories Inc., USA) supplemented with 10% FBS. The cultures were grown to 70% confluence. Fresh medium was added and the supernatant containing the virus was harvested 16 h later. Mart-1 TCR expressing cells were tested for their functional ability and then frozen in liquid nitrogen for later use. The viability of thawed cells was always in excess of 90%. No significant difference in functional profile was observed in these cells after thawing. This protocol has been previously described [11].

Generation of Mart-1 TCR transduced $CD4^+$ and $CD8^+$ T cells

PBL derived $CD4^+$ and $CD8^+$ T cells were activated by plate-bound anti-CD3 (5μg) and anti-CD28 (1μg/ml) antibodies in the presence of 100U/ml IL-2. After 48 hours, the cells were infected with DMF5 TCR retrovirus containing supernatant in the presence of Retronectin (Takara, Japan) as per manufacturer's protocol. After 48hr of infection, cells were stained with Mart-1 specific tetramer (Beckman Coulter, USA) and analyzed by flow cytometry using the FACSCalibur instrument (BD Biosciences, USA). The transduced cells were rested in culture for 7-10 days with regular medium changes, and subsequently used for downstream analyses.

Phenotypic analysis by flow cytometry

The immunofluorescence procedure for phenotypic analysis by flow cytometry has been previously described [16].

Cytokine ELISA: Cytokines; IFNγ, TNF-α, IL-2, IL-4 and IL-10 were quantified by ELISA (DuoSet ELISA Development System, R&D System, USA) as per manufacturer's protocol.

Tetramer assay

Analysis for Mart-$1_{(27-35)}$ antigen specific TCR bearing T cells has been described previously [16,17]. The T cells from the transduced population were washed twice in PBS and then incubated with 1μl of Mart-$1_{(27-35)}$ conjugated to Allophycocyanin HLA-A2 tetramer (Beckman Coulter Inc., Brea CA), and $CD4^+$/$CD8^+$ conjugated to FITC or PE (BD Biosciences, San Jose CA), at room temperature for 30 minutes. The stained cells were washed twice, and re-suspended in FACS buffer. Thereafter, the number of tetramer positive cells was determined using FACSCalibur (Becton Dickinson, USA), and the acquired flow cytometry data were analyzed using the FloJo software (Treestar Inc., San Jose CA).

Total RNA extraction and quantification

2×10^6 $CD8^+$ and $CD4^+$ cells were cultured for 0h and 4h with autologous plate bound DCs pulsed with Mart-1 peptide. Total RNA was extracted using the RNeasy mini kit (Life Technologies, San Diego CA) following the manufacturer's protocol. RNA quantification was done using the Nanodrop 1000 nucleic acid quantifier (Thermo Scientific, Wilmington DE) as well as Qubit 2.0 (Life Technologies, CA) in triplicates and averaged. RNA concentrations as measured by the Qubit instrument, were used for further analyses.

NGS

0.5 mg of total RNA was used for preparing 100 bp read median length paired-end libraries for sequencing using Illumina Truseq RNA Seq library kit (Illumina Corp., San Diego, CA) following the manufacturer's protocol. The prepared libraries from 4 samples were pooled together (each sample having a unique adaptor sequence). These pooled samples were loaded onto 2 lanes of an Illumina HiSeq2000 flowcell at the Genomic Services Core facility UConn Health Center. Individual reads from the pooled samples were detected using the Illumina CASAVA (version 1.82) and each sample produced approximately 40 million raw paired reads. Raw Fastq data also produced the quality metrics. Average Phred score of ≥ 40 per position were used for alignment.

NGS data analysis

Data was analyzed using the Tuxedo suite software tools (TopHat - version 2.0.11, Cufflinks - version 2.2.1, Cummerbund - version 2.7.2) available from the Center for Bioinformatics and Computational Biology, University of Maryland (http://cufflinks.cbcb.umd.edu) [18-20]. The tools were installed and run on a computational cluster with 18 nodes, 908 CPU, and 3TB RAM installed at the Cell Analysis and Modeling facility at the UConn Health Center, Farmington CT. Data analysis was done as described [19]. Briefly, samples were aligned to the Hg19 Human Genome (http://hgdownload.cse.ucsc.edu/downloads.html) using the TopHat software package [20]. Aligned reads were then used as input for the Cufflinks package in order to build transcripts from the exon junctions quantified by TopHat 19. Cuffmerge was used to pool the replicate samples, and statistically significant differences between the samples were obtained using Cuffdiff [19]. The R software and cummeRbund package were installed on a local machine and data analyzed (http://CRAN.R-roject.rg/package=cummeRbund).

Visualization of mapped reads in the samples

Local copies of Integrative Genome Viewer (IGV) and IGV Tools were installed on an Apple Macintosh computer [21,22]. TopHat aligned files were converted to IGV format using BEDTools and the "counts" function of IGV Tools [23]. Each sample was viewed in IGV as average alignment tracks. Log2 fold frequency of reads were plotted to better visualize individual genes and chromosomal coverage.

Assessment of reads quality

The RNA-SeQC package was installed on the UConn Health Center

Cell Analysis and Modelling computer cluster [24]. All TopHat aligned files were used as input for generating the quality metrics as explained by the RNA-SeQC software. Microsoft Excel was used for plotting graphs and tables to visualize the data obtained.

Results

Mart 1 TCR transduced CD4+ and CD8+ T cells and their specificity

MART-$1_{(27-35)}$ epitope specific TCReng CD8+ and CD4+ T cells from HLA-A2+ human donors were generated with freshly isolated CD8+ and CD4+ T cells as per published protocol [11] and were functionally characterized before sequencing. Figure 1A shows the number of the MART-$1_{(27-35)}$ epitope specific cells in the TCReng CD8+ as well as the CD4+ fractions. Figure 1B shows data obtained from ELISA of the two cell types and that they exhibit antigen specific functional fidelity. Figure 1C shows the NGS data of genes associated with Th1 and Th2 cytokines, and cytolytic effector function and shows the changes observed 4h after stimulation.

Quality analyses of the NGS data

NGS was performed using total RNA extracted from unstimulated, and Mart-1 epitope stimulated TCReng T cells at several time points (0 h, 1 h, 4 h, 24 h) from one donor, and at 0h and 4h from another

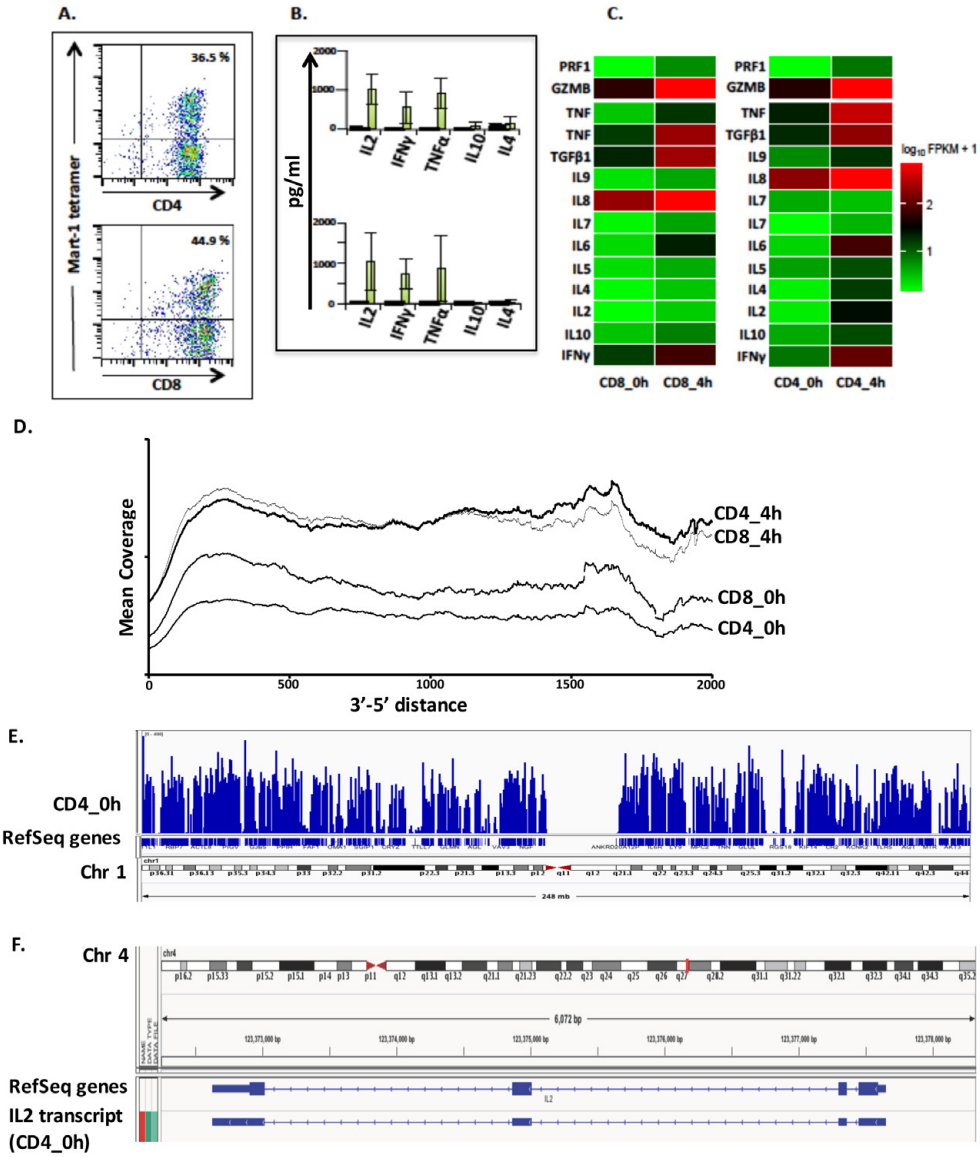

Figure 1: A. Flow cytometry from a representative donor showing the Mart-1 tetramer positive cells in both CD4+ and CD8+ cells. B. Antigen specific cytokine release assay of Mart-1 epitope specific TCR engineered CD4 and CD8 T cells. CD4 and CD8 T cells were transduced with the F5M1 virus carrying the Mart-1 TCR. CD4M1 and CD8M1 cells from four different biological replicates were stimulated with T2 alone or T2 pulsed with Mage3, control peptide, or T2 pulsed with Mart-1 peptide. Cytokines released 16 h after stimulation were measured by ELISA. C. Heatmap showing the expression of common Th1, Th2, and regulatory T cells following stimulation with cognate peptide presented on autologous DCs. D. Graph showing the cumulative 5'-3' alignment of the top 1000 transcripts from TopHat Hg19 aligned bam files of each sample using IGV tools and visualizing in IGV. E. Cufflinks output bam file of CD4_0h was aligned to all known RefSeq human chromosome 1 genes and to Chr. 1 using IGV. Genes of sample are displayed as logarithmic values. F. Data from Fig. 1B was used to examine the IL-2 gene and aligned against the available data from RefSeq, as well aligned to human chromosome 4 using IGV.

donor. The NGS data from 0 h and 4 h time point samples from the two donors were used as biological replicates for analyses. An overview of the data revealed considerable concordance in the transcriptional signatures although expected fluctuations in the levels of transcription of some of the immune function-associated cytokine genes, such as IL-2,γ,IFNTαF , etc, were observed.

Figures 1D and 1E show the analysis of NGS data quality. Since mRNA fragmentation may result from poor RNA extraction methods, and lead to 5'-3' sequencing bias [25-27], we examined sequencing bias using the RNA SeQC software package [24]. As shown in Fig. 1D, the mean coverage of reads from the top 1000 transcripts was found to be uniformly aligned to both the 5' and 3' ends, illustrating the fact that RNAs from our samples were not fragmented. Next, we examined the quality of our sequenced reads using IGV [21,22]. Genes assembled from sequenced reads of the human chromosome 1 (sample CD4_0h_1) were aligned against the known genomic regions of chromosome 1 compared to all the RefSeq genes available from the NCBI repository. Figure 1E shows that the sequenced reads did not map to the chromosomal centromere or to any intergenic regions. This was seen with all samples and chromosomes analyzed (collective data not shown).

The coverage of the IL-2 gene (4q26.32) was used as an example for the corroboration of sequenced reads assembled into exons against the Refseq human IL2 gene. Figure 1F shows that no read aligned to intronic regions, and all 4 IL-2 gene exons were mapped. Supplementary Table 1 lists data showing coverage of exonic and intronic alignment of all samples. As can be seen, a ratio of 9:1 exonic to intronic alignment was seen for all the samples, Based on the data shown, we concluded that the quality of our samples was quite satisfactory.

After establishing the quality of the NGS data, we examined the overall transcriptional profile of TCReng T cells upon encountering the cognate epitope. Table 1 shows a summary of the transcriptional profile of the TCReng CD8+ and CD4+ T cells stimulated by the cognate epitope at 4h. As shown, NGS revealed approximately 30,000 genes, of which a sizeable number showed significant modulation upon antigen exposure (i.e., both up- as well as down-regulation). Additionally, 72,000 known isoforms and about 20,000 previously unknown isoforms were detected.

We examined all the significantly expressed genes in both cell types and tabulated the top 20 most up-regulated and down-regulated genes in our data. As seen in Figure 2A, a majority of the up-regulated and down-regulated genes in each cell types are those associated with immune functions (cytokines IL27, IL1B, and IL18, and chemokines-CCL4, CXCL10, CXCL1, CXCL9, CCL20, CCL8, and CCL3). Additionally, Figure 2B heatmaps also show a distinct change in transcriptional profile in both cell types following stimulation with DCs pulsed with the Mart-1 peptide.

Thereafter, we examined the transcriptional signatures associated with different functional pathways of T cells -such as effector function, differentiation, proliferative activities, apoptosis, etc., in greater details. Additionally, Supplementary file 1 lists all results of gene expression as shown in heatmaps in the figures as Log2 -fold changes along with the p- values.

Status of T-cell activation associated genes

Figure 3 shows a heatmap representing the transcriptional profile of NF-κB, NFAT, and AP1 (Jun) genes and genes downstream these pathways, including key genes and transcription factors associated with a number of T cell activities, (such as, IL-2 and IFN γ secretion). As shown in Figure 3A, all the isoforms of NF-κB and NFAT were up-regulated while Jun was down-regulated at the 4h time point in both cell types. The transcription factors T-bet and GATA-3, responsible for the Th1 and Th2 differentiation of T cells, were up-regulated. Further, a number of genes such as STAT3, STAT1, STAT5, IKK, JAK, and most isoforms of REL and MYC genes were also up-regulated. JUN, FOS, and SYK were down-regulated, and no significant changes in the expression of KRAS, HRAS, MAPK genes, EGR1, and CREM were observed. Taken together, most of the genes and transcription factors known to be associated with IL-2-driven pathways showed modulation in both T cell types upon encountering the cognate epitope. Of note, the transcriptional profile was found to be essentially identical in both cell types. It should be however noted that since both cell types were previously activated for the purpose of TCR transduction, some of the genes in these pathways were already up-regulated at baseline (i.e., at 0hr point). Nonetheless, further modulations in relevant genes in both cell types took place following exposure of the cognate epitope, Mart-$1_{(27-35)}$.

Figure 3 further shows the expression of cytolytic molecules and cytokines involved in T-cell differentiation. Granzyme B and Perforin, known to be mobilized by cytolytic T cells in response to TCR stimulation, were up-regulated in both cell types. The cytokines IFNγ,IL -2, IL10,α,IL4,and IL6,9 IL7, IL8, TNF were also up-regulated in both the cell types.

Status of genes encoding for T cell associated cytokines, chemokines, and their receptors

Figure 4A shows a heatmap illustrating the transcriptional profile

	CD4_0h	CD4_4h	CD8_0h	CD8_4h
Total genes expressed	30,472	30,473	30,760	30,758
0h only	1,946		637	
4h only		463		1,147
Up-regulated (Log 2 fold or greater)		1,452		915
Down-regulated (Log 2 fold or greater)		759		858
Total known isoforms expressed	72,878	72,878	71548	71548
0h only	15,403		4003	
4h only		17,234		5308
Up-regulated (Log 2 fold or greater)		4,210		3,071
Down-regulated (Log 2 fold or greater)		614		3,020
Total novel isoforms	22,565	22,565	21006	21006
0h only	1,822		1525	
4h only		2,649		1943

Table 1: Cuffdiff data from all samples was examined and tabulated using Microsoft Excel.

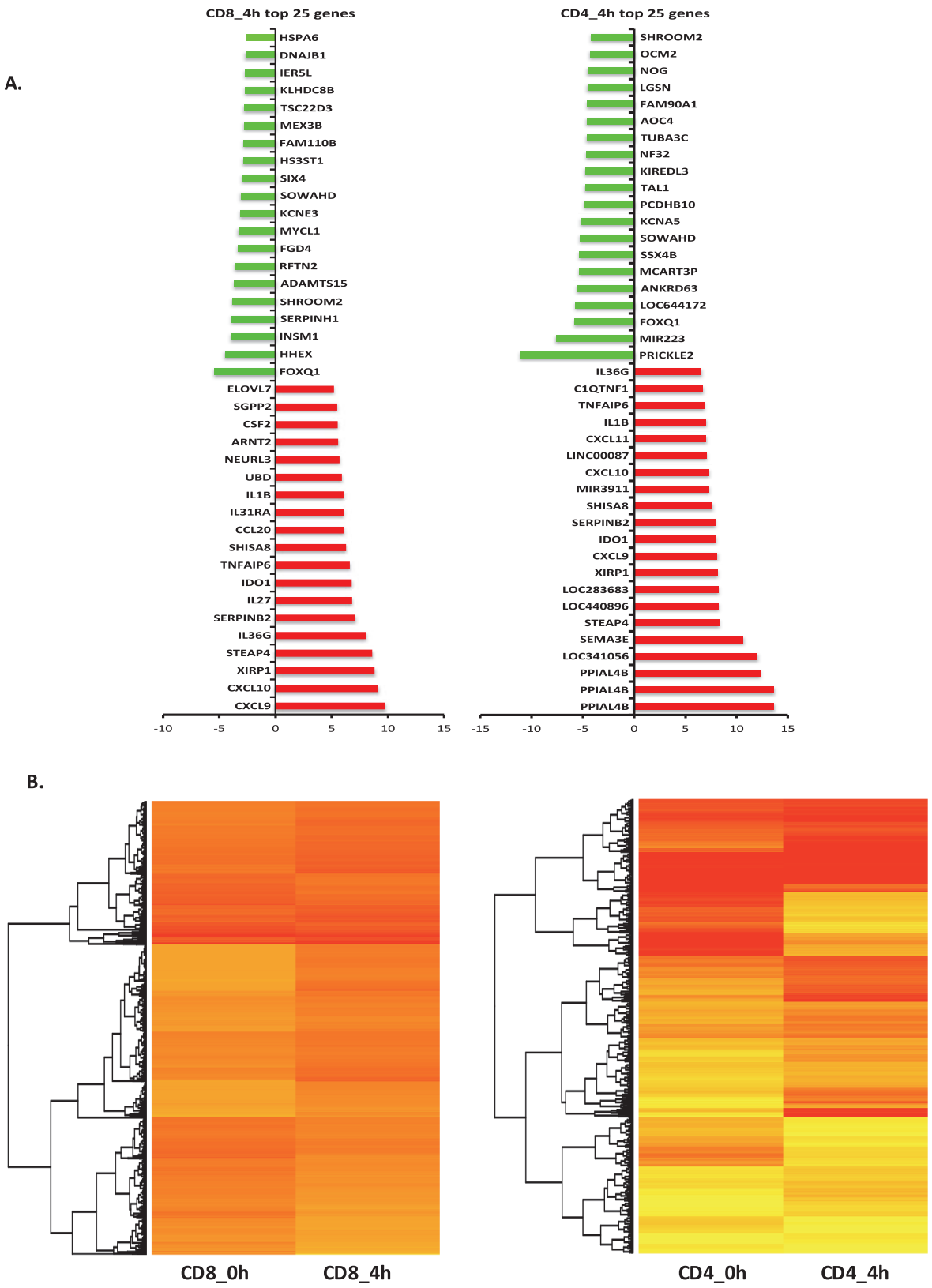

Figure 2: A. Bar Graph shows the 25 most up-regulated and down-regulated genes in both the CD4+ and CD8+ cell types following stimulation to Mart-1 for 4 h. B. Heatmaps show all significant genes ($p<0.05$).

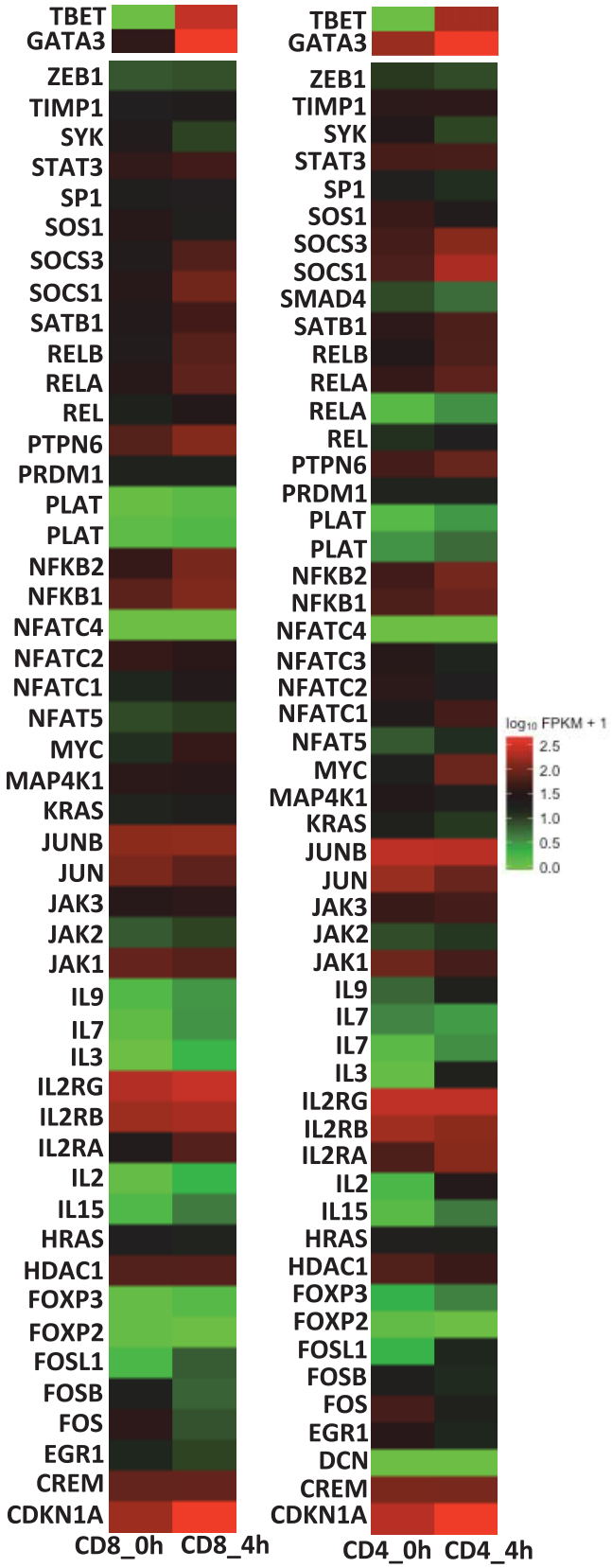

Figure 3: All genes associated with the NF-κB, NFAT and AP -1 pathways were tabulated and mined for in the NGS data. Heatmaps were generated using cummeRbund package installed in R software. FPKM normalized values of each gene were used and converted to their Log10 values to generate the graphs.

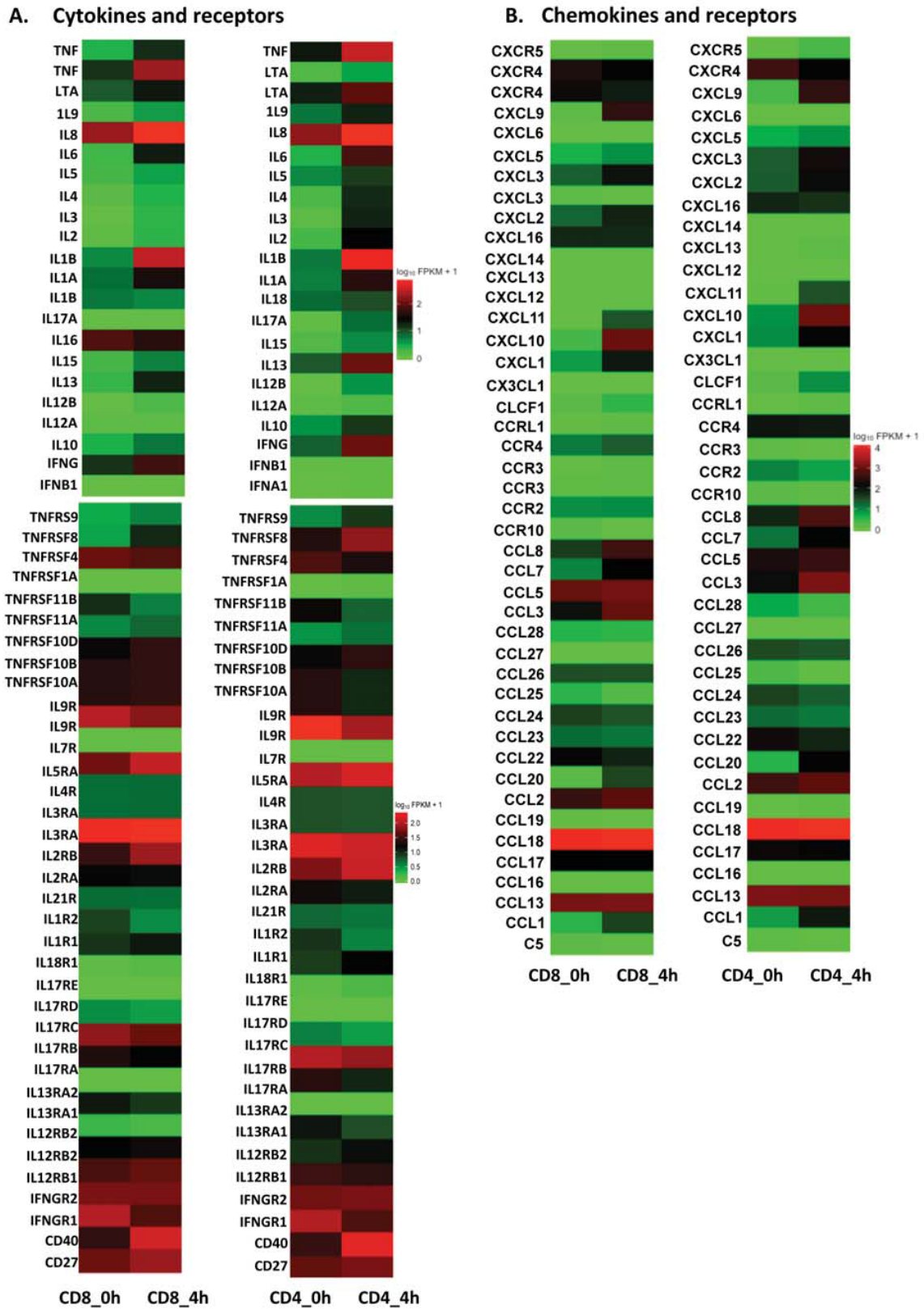

Figure 4: A. Heatmap displaying all known human cytokines and cytokine receptors expression in the two cell types. B. Heatmap showing the expression of known chemokines and chemokine receptors which showed a significant change in the two cell types. Heatmaps in both A. and B. were generated using the cuffdiff data and created using cummeRbund package installed on a local copy of R.

of inflammatory cytokines and their known receptors in TCReng CD8+ and CD4+ T cells following antigen exposure. As can be seen, both T cell types demonstrated considerable modulations (up- as well as down-regulation) in gene transcription upon antigen exposure. A similar pattern of modulation in gene transcriptions associated with inflammatory effector functions was observed with both cell types. Besides the cytokines mentioned in the previous section above, transcription for the genes for IL9, IL5, IL3, IL1B, IL1A, and IL13 clearly showed up-regulation 4 hours after antigen stimulation in both the CD8+ and CD4+ TCReng T cells. Additionally, many isoforms of the TNF receptors were up-regulated. Of interest, while the IL4RA, IL2RA, and IL18R1 transcripts also showed up-regulation, the transcripts for IL9R, IL5RA, IL3RA, IL1R, IL17R, IL13R and IL12R did not show any change in expression.

Since it is known that many inflammatory chemokines and their receptors undergo a change in expression in activated T cells in response to antigen stimulation, we examined their expression in these two cell types upon antigen exposure. Figure 4B shows that CCL1, CCL3, CXCR4, CCR4, CCL5, CXCL9, and CCL20 transcripts in both cell types were up-regulated with no noticeable change in the expression of CXCL16, CCL2, CCL27, and CXCR5.

Status of genes associated With T-cell co-stimulation and co-inhibition

In addition to TCR signaling in response to cognate peptide, signaling through co-stimulatory and co-inhibitory molecules present on the lymphocytic surface shapes their responses. Figure 5A shows the expression of some of the known molecules involved in co-stimulation and co-inhibition. As seen, the surface genes 4-1BB, OX40, CD80, and LAG-3 were found to be up-regulated in both cell types. Further, transcription of genes specifying some of the downstream molecules such as, FYN, CDC42, COT, and LYN showed up-regulated. Transcription of genes for PTEN, GADS, GRB2, CD28, and RAC1 showed down-regulation in both cell types.

Status of genes associated with cell proliferation and apoptosis

It has been well established that T cells undergo massive proliferation upon antigen-mediated stimulation, while a fraction undergo activation induced cell death. Hence, we looked for genes responsible for cellular proliferation, inhibition of cell proliferation, or cell death. Interestingly, we found only a few genes showing significant changes in expression. As shown in Figure 5B, TOP2A, PLK1 and CCNB1 were significantly up-regulated in the CD8+ T cells, while E2F1, PLK1, and MYBL2 were down-regulated in the CD4+ cells. Additionally, while the anti-proliferation gene, CDKN1A, was up-regulated in both cell types following antigen stimulation, p53 was up-regulated only in the CD8+ cells. The CDKN1B and CDKN2A genes were down-regulated in both cell types.

Figure 5C shows an example of modulations in transcripts that regulate apoptotic activities (both pro and anti) in both cell types. As can be seen, while the anti-apoptotic genes BCL2L1 and BCL2A1 were up-regulated, the pro-apoptotic genes did not show much change in either cell type.

Recently, the significance of lncRNAs has been evaluated in cancer, and a large number of these have been discovered in human tumor cells [26]. The lncRNA database was used to curate all known human lncRNAs [27]. Figure 5D shows all the lncRNA transcripts seen in our samples. As evident from the heatmap, the lncRNAs NEAT1, MIAT, MALAT1, JPX, HOTAIRM1, and GAS5, show an up-regulation in both the cell types.

Data mining also led to alternate splice variants in the samples. After correcting for variance, we cataloged splice variants common to both the cell types as shown in Table 2. As is seen, although the majority are not known to have a function associated with the immune response, MITF and CD160 were found to be differentially expressed in the two cell types.

Discussion

ACT with tumor antigen specific TCReng T cells [4,5,28-32] or with CAR engineered [33-36] T cells have generated considerable interest in translational tumor immunotherapy. While both approaches have shown substantial anti-tumor activities, a number of intrinsic regulatory processes within T cell biology (such as engagement of co-inhibitory pathways, emergence of exhaustion, AICD, elaboration of regulatory cytokines etc.) as well as extrinsic mechanisms (associated with tumor microenvironments) have turned out to stand in the way to extract more robust and sustained results. Accordingly, a great deal of effort is underway to better understand these processes. In this context, while various cell-based assays and microarrays have added valuable information, there is now a need for better tools and techniques to obtain better understanding of the biology of these powerful anti-

Alternate splice variants common to both CD8_4h and CD4_4h		
Splice Variant Name	**locus**	**Function (RefSeq)**
ADM2	chr22:50919927-50924866	This gene encodes a protein which is a member of the calcitonin-related hormones. The encoded protein is involved in maintaining homeostasis in many tissues, acting via CRLR/ RAMP receptor (calcitonin receptor like receptor/receptor activity modifying protein) complexes.
ARHGEF39	chr9:35658286-35665278	Rho guanine exchange factor 39
CCDC117	chr22:29156746-29185386	Coiled coil domain containing 117
CD160	chr1:145695566-145715673	CD8 marker. CD160 shows a broad specificity for binding to both classical and nonclassical MHC class I molecules.
CD86	chr3:121774208-121839988	Binding of this protein with CD28 antigen is a costimulatory signal for activation of the T-cell.
CIR1	chr2:175212877-175358497	Corepressor interacting with RBPJ
FAM20A	chr17:66531256-66597095	This locus encodes a protein that is likely secreted and may function in hematopoiesis
FBP1	chr9:97365420-97402531	Fructose-1,6-bisphosphatase 1, a gluconeogenesis regulatory enzyme, catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate.
GNA12	chr7:2767678-2883959	guanine nucleotide binding protein (G protein) alpha 12
MITF	chr3:69788585-70017488	It regulates the differentiation and development of melanocytes retinal pigment epithelium and is also responsible for pigment cell-specific transcription of the melanogenesis enzyme genes.

Table 2: The cuffdiff files splicing.diff of each set of samples were analyzed and the CD8+ samples compared against the CD4+ samples to generate the table. Data was tabulated using Microsoft Excel.

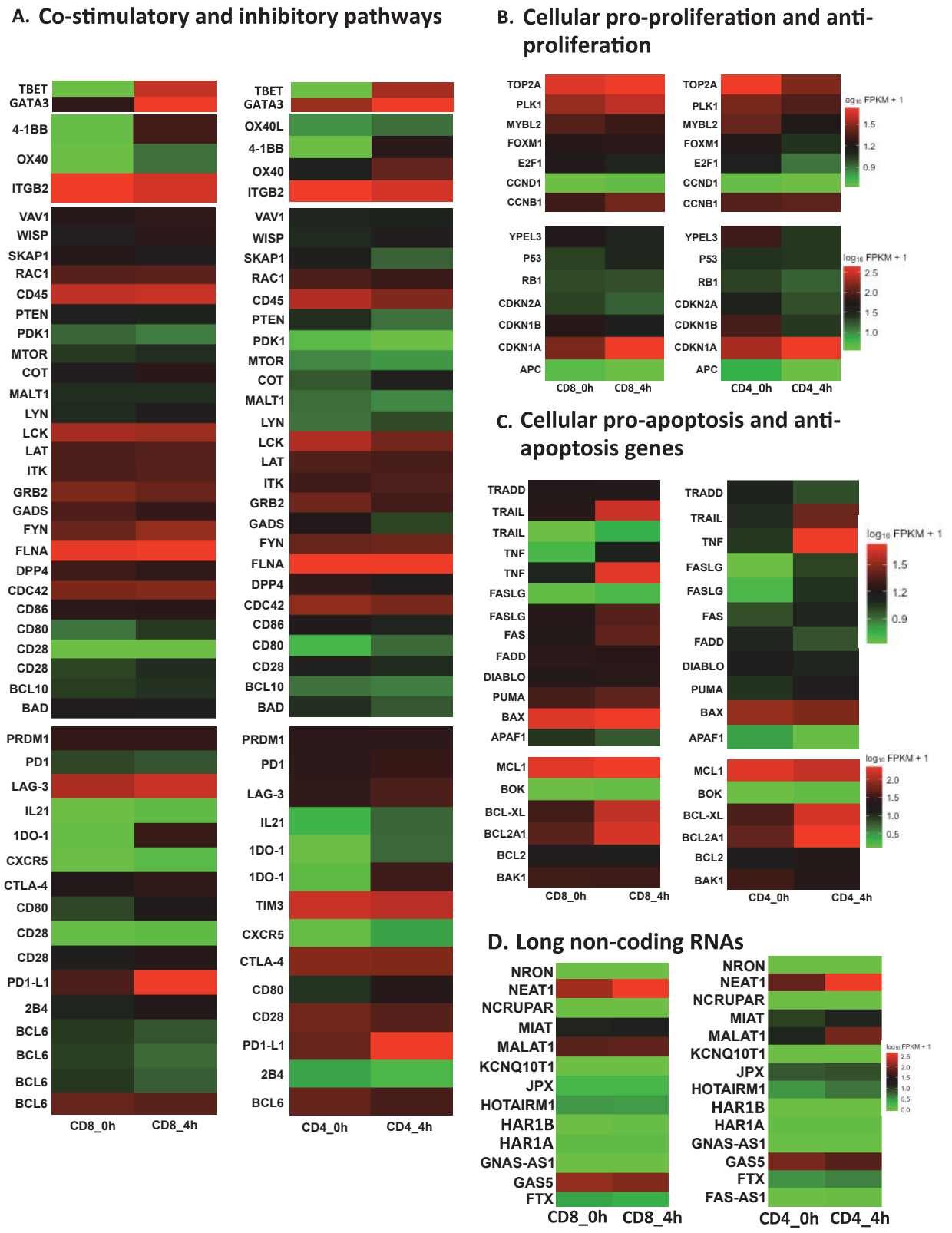

Figure 5: A, B, and C. List of known genes responsible for T-cell stimulation and inhibition in an antigen specific manner, positive and negative cellular proliferation, genes associated with the intrinsic and extrinsic apoptotic pathways were curated from NCBI and used to generate heatmaps using cummeRbund. Genes not expressed in the samples were discarded. D. NGS data was mined for a known set of human lncRNA curated from the lncRNA database (http://www.lncrnadb.org/). Log10 FPKM values of the genes found in the samples were used to generate heatmaps using cummeRbund. Heatmap was generated using cummeRbund.

tumor effector cells. This study was carried out to obtain an expanded understanding of the biology of TCReng T cells by examining their transcriptional profiles using NGS [13]. Since the human melanoma associated MART-1 epitope has turned out to be a good model for immunological studies in a relevant human system and as the MART-$1_{(27-35)}$ specific TCReng CD8+ and CD4+ T cells exhibit multifunctional effector functions, we elected to define the transcriptional profiles of the MART-$1_{(27-35)}$ TCReng T cells using RNA-Seq. Admittedly, the transcriptional profile of T cells responding to an epitope vary with time. Changes in expression can occur as early as 15 seconds for early genes and as late as 24 hour for late genes [37]. We chose to define the transcriptional signatures at 4h time point believing that this would be a reasonable time point for our present purposes. In this context, even though our study is somewhat limited, the results are noteworthy as they support most of our prior findings -- made through cell biological and molecular analytic techniques -- on the functional profiles of CD8+ as well as CD4+ T cells responding to a human tumor associated antigen. In addition, the data set also reveals a number of new aspects in T cell response to a tumor associated antigen.

For example, we have previously demonstrated that the human melanoma associated epitope, Mart-$1_{(27-35)}$, specific TCR engineered CD8+ and CD4+ exhibit potent anti-tumor effector functions in an epitope specific manner [10,11] and have shown that the TCR-engineered CD4+ T cells exhibit not only MHC class I restricted helper functions, they also exhibit epitope specific cytolytic effector functions of their own [11]. It should be pointed out that while the mechanisms underlying human CD8+ cell mediated effector functions have been studied extensively, the mechanisms underlying the generation of MHC class I restricted multifunctional effector responses by CD4+ T cells remain to be deciphered. In this context, as shown here, NGS provides a remarkable overview of the similarities in the transcriptional profiles of Mart-$1_{(27-35)}$ epitope specific TCReng CD8+ and CD4+ T cells responding to the Mart-$1_{(27-35)}$ peptide.

As summarized in Table 1, of the genes expressed in CD8+ and CD4+ T cells at 4h post epitope encounter, 166 and 103 genes were found to be unique to the respective cell populations. Of these genes, four genes (PI3, IL31, FRDM7, and CAMK2A) were found to be common to both the cell types. CAMK2A is a member of the serine/threonine protein kinase family and to the Ca^{2+}/calmodulin-dependent protein kinases subfamily. Since Ca2+ signaling is integral to T-cell activation, the expression of this transcript following antigen presentation may be an important finding. Interestingly, we found that IL27 was up-regulated in the engineered CD8+ T cells. IL27 is known to activate T-bet and suppress GATA3 in CD4+ cells, which may explain Th1 type phenotype [38]. Taken together these data sets support our published data on Th1 programming of TCR engineered CD4 T cells [10,11]. Besides IL31 and IL27, we also found the expression of PI3, GREM1, CD160, and CAM2KA up-regulated in both cell types. These genes are known to play an important part in T-cell activation [39-42], and this data set provides confidence in our NGS data.

We have previously shown that the Mart-$1_{(27-35)}$ engineered CD8+ and CD4+ cells, when stimulated with the Mart-1 peptide presented upon, APCs, secrete IL2 and IFNγ and no detectable amounts of IL10, IL4, or TGF - characteristic of Th1 type differentiation [10,11,43]. Interestingly, we have found that, 4h post-antigen stimulation; transcripts for all these cytokines are up-regulated. Since no IL-10, IL-4 or TGF-βprotein was found in ELISA at 24 hr. [10,11], our findings suggest that post-transcriptional regulation might play a significant role in functional programming of effector T cells.

Besides the well reported and characterized genes described above, we also found the up-regulation of some of the other noteworthy inflammatory response genes IL8, IL1B, IL6, and CXCL9, and CXCL10 (Figures 4A and 4B). It has been well established that IL8, CXCL9, and CXCL10 are known chemo-attractants responsible for T cells moving to an inflammatory site [44,45]. The fact that these genes are up-regulated in both engineered cells, provides new insights towards the functional profile of these cells and support the idea that CD4+ T cells are also capable of exhibiting multifunctional inflammatory activities potentially helpful in anti-tumor immune responses.

Lymphocyte homeostasis has been widely studied, and is known to be controlled by cytokines besides peptides, small molecules, hormones, regulatory cells, and antigen receptors among numerous other factors [46]. Besides looking at the major cytokines secreted during T-cell differentiation (Figure 3A), we also examined the expression of known inflammatory chemokines (Figure 3B). We found that both the CD8+ and CD4+ engineered cells exhibited the expression of these inflammatory cytokines.

Chemokines play a significant role in tumor biology and inflammation [46]. Our analysis revealed increased expression of the inflammatory chemokines and their receptors molecules (Figure 3B). This result was expected and some of the results have been previously reported [47]. In order to further understand and investigate other mechanisms of chemokine effects, we examined the expression of known chemo attractants expressed in hematopoietic cells. As seen in Figure 3B, the chemokines XCL1, CXCR4, CXCR3, CXCL9, CXCL10, CCL7, CCL3, CCL20, CCL2, and CCL1, and CXCL19 are up-regulated in both the stimulated cell types. Similar chemokine profiles in both the engineered CD4+ and CD8+ T cells supports our idea that Mart-1 TCR transduced CD4+ cells are behaving in a similar manner as the Mart-1 TCR transduced CD8+ cells.

Figure 4A shows up-regulation of T-bet and GATA-3 transcription factors, associated with differentiation of CD4+ T cells into Th1 and Th2 lineages [47,48]. This data set further emphasizes the post-transcriptional regulation of T cells effector function, highlighting the need for further studies.

Co-stimulatory and co-inhibitory molecules play critical roles in determining the functional profile of effector T cells. We carefully examined the status of co-stimulatory and co-inhibitory genes in TCR engineered CD4 and CD8 T cells, Interestingly, as shown in Figure 5A, the expression of both co-stimulatory (OX40 and 4-1BB) and co-inhibitory (PD1, PDL1, CTLA4, Tim3, and LAG3) transcripts are up-regulated. These findings are significant as they highlight the importance of post-transcriptional regulation of co-stimulatory and co-inhibitory factors, and emphasize the need for further investigation.

T-cell proliferation in response to TCR activation following cognate recognition has been previously reported [48]. A list of 4418 genes associated with cellular proliferation was compiled from the Gene Cards database (http://www.genecards.org) and matched against our data. After eliminating non-significant results and those with p-values<0.05, we found that a majority of the genes were those related to the immune response. In addition, the heat maps presented in Figure 4B are consistent with cells undergoing proliferation.

lncRNA is an emerging area of transcriptional control and only recently being recognized to play a role in tumor biology. A long non-coding RNA lincRNA-Cox2, has recently been shown to mediate the regulation of NF-κB – a gene associated with a number of immune response genes, in mice [49]. Our analysis of data has revealed a

CD4_4h unique novel isoforms			
Gene Name	**Log2 Fold**	***P* value**	**Function (RefSeq)**
TTLL10	1.97634	0.0425631	Inactive polyglycylase
EPB41	3.84993	0.0381522	Erythrocyte membrane protein band 4.1
CELSR2	3.21594	0.0262131	Member of the Flamingo subfamily, part of the cadherin superfamily. Specific function of this particular member has not been determined
HIPK1	-3.49575	0.0180169	Serine/threonine-protein kinase involved in transcription regulation and TNF-mediated cellular apoptosis.

Table 3: Cuffdiff data was examined for all genes, which were marked as novel isoforms using the coding as indexed by cufflinks. Gene Log2 fold differences as calculated by cuffdiff were used to generate the table using Microsoft Excel.

CD4_4h unique novel isoforms			
Gene Name	**Log2 Fold**	***P* value**	**Function (RefSeq)**
IL21	1.97634	0.0184314	Cytokine with immunoregulatory activity. In synergy with IL15 and IL18 stimulates interferon gamma production in T-cells
TNFSF10	2.77851	0.0278063	This protein preferentially induces apoptosis in transformed and tumor cells, but does not appear to kill normal cells although it is expressed at a significant level in most normal tissues. This protein binds to several members of TNF receptor superfamily.
TNFSF4	2.53106	0.0449031	The protein encoded by this gene is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family. This cytokine is a ligand for receptor TNFRSF4/OX4. It is found to be involved in T cell antigen-presenting cell (APC) interactions]
ODZ1	2.73746	0.00378027	The protein encoded by this gene belongs to the tenascin family and teneurin subfamily.

Table 4: The cuffdiff files promoter.diff of the samples were compared against each other using all promoters with p<0.05. Functions of the genes regulated by the promoters were obtained from RefSeq.

number of lncRNA (Figure 4D), although their significance in immune response is currently not understood. Our review of the current literature reveals that HOTAIRM1, NEAT1, and MALAT1, among others have been implicated in various solid tumors and hematological malignancies [49-52].

A major advantage of RNA-Seq over microarrays is its ability to identify sequences not previously reported and potentially considered novel genes or isoforms [13]. In this study we have identified a number of isoforms previously not known to be expressed in T cells. Supplementary File 2 shows a list of novel isoforms common to both cell types. Following a thorough examination of these isoforms, we found that some of these genes may be associated with currently known immune responses. These include the genes TNFRSF8, TNFSF13, BCORL1, CABP1, TLK2, IL11RA, MUM1, and SIPA1. Table 3 catalogs the novel isoforms of known genes unique to each cell type.

Among these, we found that HIPK1, a serine-threonine protein kinase known to be a regulator of TNF mediated apoptosis was up-regulated in the CD4+ engineered cells. In the CD8+ cells, IL21, TNFSF10, and TNFSF4, which are known to have immune response functions, were found to be up-regulated.

Our study has also yielded information about the regulation of promoters for a number of genes. Table 4 catalogs 7 promoters significantly up-regulated in both cell types and common to both. As can be seen the genes for which these promoters are up- regulated and are all involved in cellular proliferation and differentiation. This is a novel finding and has not been previously reported. Additionally, since these promoters are common to both the cell types, it leads us to believe that the two cell types may have significant functional similarities. This finding warrants further investigation and may explain some of the unique functions attributed to, and observed in, the TCR transduced cell types. Further, while characterizing all splice variants has been beyond our scope, the finding of alternate splice variant of CD160 in both cell types is of some interest. An alternate splice variants of CD160 is known in CD8+ cells. Its expression in CD4+ cells (Table 2) supports the idea that this variant of CD160 might also affect the MHC Class 1 restricted TCReng CD4+ cells in a similar fashion.

Finally, it is admitted that this is only an exploratory first attempt to obtain a better understanding of the transcriptional profile of human tumor epitope specific T cells in action. Clearly, further studies involving more cases, other TCRs, T cells expressing TCRs naturally, and against other epitopes, will be needed for a full appreciation of the transcriptional profiles of T cells responding to tumor associated epitopes. Besides confirming the power of NGS in this line of investigation, our studies clearly reveal many interesting leads and novel insights into the biology of CD8+ and CD4+ TCR-engineered human T cells, in general, and in anti-tumor immune responses, in particular.

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