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Genomic-Scale Transcriptional Analysis of T-Cell Activation Reveals Novel Genes and Signaling Programs

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ABSTRACT

Genomic-Scale Transcriptional Analysis of T-Cell Activation Reveals Novel Genes and Signaling Programs

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T-cell activation is an essential step of the immune response, yet the cellular and molecular events underlying this complex process are not fully understood. Significantly, a comparative genome-scale transcriptional analysis of two T-cell subsets and the natural-mixed CD3+ population remains unexplored. Using microarrays, we investigated the temporal global transcriptional profile of human T-cell activation in CD3+, CD4+ and CD8+ T cells. Comparison of the microarray-based gene expression patterns between the three populations revealed largely conserved transcriptional patterns. Transcription patterns of selected genes were confirmed by Q-RT-PCR. We employed a Gene-Ontology-driven transcriptional analysis coupled with protein-abundance/activity assays to identify novel genes and cell-type-specific genes involved in the activation process.

Focusing on immune response, we identified potential genes involved in the communication between the two subsets, would-be effector-function-specific genes and novel chemokines in T cells. Increased expression of unexpected cytokines (GPI, OSM and MIF) suggests their involvement in T-cell activation. Differential expression of many receptors, novel in T-cell activation, including CCR5, TNFRSF25 and TNFRSF1A, suggests their role in this process.

We identified significantly regulated apoptotic genes in several protein families and detailed their transcriptional kinetics during the T-cell activation process. Transcription patterns of some selected genes were validated at the protein level. The simultaneous upregulation of NF-κB and IκB family genes at 48-96 hours, supported by the increase of phosphorylated p65 at 48-96 hours, suggests the NF-κB involvement. Examination of significant regulated genes revealed an increase of p38 and ERK1 signalings during T-cell proliferation (48-96 hours), explored using phosphorylation assays for p38 and ERK1.

Gene-Ontology-driven transcriptional analysis of T-cell response to H_2O_2 stress revealed transcription events, such as response to stimulus, and significant genes, such as BBC3, involved in this stress response process in the context of T-cell activation.

Analysis of T-cell activation, which integrates dynamic gene-expression data with protein-abundance and activity assays, has identified numerous novel genes and pathways that may be important players in T-cell activation. This has significantly broadened our understanding of the molecular orchestration of T-cell activation process, and provides a basis for further studies for understanding T-cell activation in health but also in malignancies and autoimmunity.

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I would also like to thank Maria Papoutsakis and Ellie Papoutsakis for their genuine hospitality, making me feel like home.

Finally, I simply can not have accomplished what I have without the unconditional support, encouragement and love of my parents.

Dedicated to my parents

Wang ShenPing and Zhang XiaoWei

For their unconditional love, encouragement and inspirations.

LIST OF ABBREVIATIONS

ATP	adenosine 5’ triphosphate
AICD	activation induced cell death
AIM-V	cell culture media
AML	acute myeloid leukemia
ALL	acute lymphoblastic leukemia
AP-1	activator protein 1
APC	antigen-presenting cell
BCL2	B-cell CLL/lymphoma 2
Ca^{2+}	calcium ion
CCL	chemokine (C-C motif) ligand
CCN	cyclin
CD2	adhesion molecule
CD3	subunit T-cell receptor
CD4	co-receptor T helper cells
CD8	co-receptor cytotoxic T cells
CD25	interleukin-2 alpha chain
CD28	co-stimulatory molecule
CD40	co-stimulatory molecule
CD69	surface protein, activation marker
CDC	cell division cycle proteins
CDK	cyclin-dependent kinase

cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CMV	Cytomegalovirus
COX	cytochrome c oxidase
CSF	colony-stimulating factor
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
CTL/T_C	cytotoxic T cells
CXCL	chemokine (C-X-C motif) ligand 1
Da	Dalton
DNA	deoxyribonucleic acid
EASE	Ontology software
EDTA	ethylene diaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzym-linked immunosorbent assay
ESTs	expression sequence tags
e.g.	for example
ERK	extracellular signal-regulated protein kinase
EtOH	ethanol
Fas/FasL	surface receptor/ligand that triggers cell death
FC	Fc fragment of immunoglobulin
GO	Gene Ontology
GZM	granzyme

HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IAP	inhibitor of apoptosis protein
IFN	interferon
IG	immunoglobulin
IL	interleukin
iROS	intracellular reactive oxygen species
ITG	integrin
JNK	c-jun NH2-terminal kinase
KLR	killer cell lectin-like receptor
LCK	leukocyte-specific protein tyrosine kinase
Mabs	monoclonal antibodies
MAP	mitogen-activated protein
MHC	major histocompatibility complex
min	minute
MLR	Mixed leukocyte reaction
mRNA	messenger ribonucleic acid
MRP	mitochondrial ribosomal protein
NADH	nicotinamide adenine dinucleotide (reduced form)
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-kappa B
nt	nucleotides

oligo	oligonucleotide
ORF	open reading frame
p38	mitogen-activated protein kinase 14
p65	p65 subunit of NF-κB complex
PBMCs	peripheral blood mononuclear cells
PDCD	programmed cell death
PI	Propidium Iodide
PSM	proteasome
Q-RT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
ROS	reactive oxygen species
s	second
SD	standard deviation
SEM	standard error of the mean
SIV	simian immunodeficiency virus
SNN-LERM	segmental-nearest-neighbor-logarithmic-expression-ratio method
SOMs	self-organizing maps
SOTA	self-organizing tree algorithm

TCR	T-cell receptor
Th/T_H	T helper cells
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
Tris	trihydroxymethylaminomethane
tRNA	transfer ribonucleic acid
VDAC	voltage-dependent anion channel
XCL	chemokine (C motif) ligand
ZAP-70	Zeta-chain-associated protein kinase 70

PREFIXES FOR MULTIPLES AND SUBMULTIPLES

k	kilo	10^{3}
c	centi	10^{-2}
m	milli	10^{-3}
μ	micro	10^{-6}
n	nano	10^{-9}

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CHAPTER 1: INTRODUCTION

1.1 IMMUNE RESPONSE

The immune system is a remarkable defense system, protecting the host against invading foreign pathogenic organisms. Even minor infections can be fatal without a working immune system. The immune system consists of a dynamic network of an enormous variety of cells and molecules to specifically recognize and eliminate a large variety of antigens through cooperation. The vertebrate immune system adapts over time to recognize particular pathogens more efficiently. Subsequent exposure to the same pathogen induces a memory response, characterized by a more rapid and heightened immune reaction.

The ability of a host to resist infection is called immunity, which includes both nonspecific innate immunity and specific adaptive immunity (1). The mechanisms of innate immunity fighting against infection are short-term, not specific to any particular pathogen. In contrast, the adaptive immunity is long-lived and able to recognize and remember specific pathogens, displaying a high degree of specificity as well as the remarkable property of memory. However, it takes time to select and mass-produce the most useful ones among the wide variety of recognition proteins of the adaptive immunity to fight against the particular pathogen. The innate immunity is always present and quick to respond. It provides an immediate and rapid defense for the host right after the exposure to a pathogen when the more powerful adaptive immune response is in preparation. Innate immunity and adaptive immunity work together to bring forth effective immune responses against infections as well as abnormal cells of the body that can develop into cancer. For example, phagocytes of innate immunity can generate a range

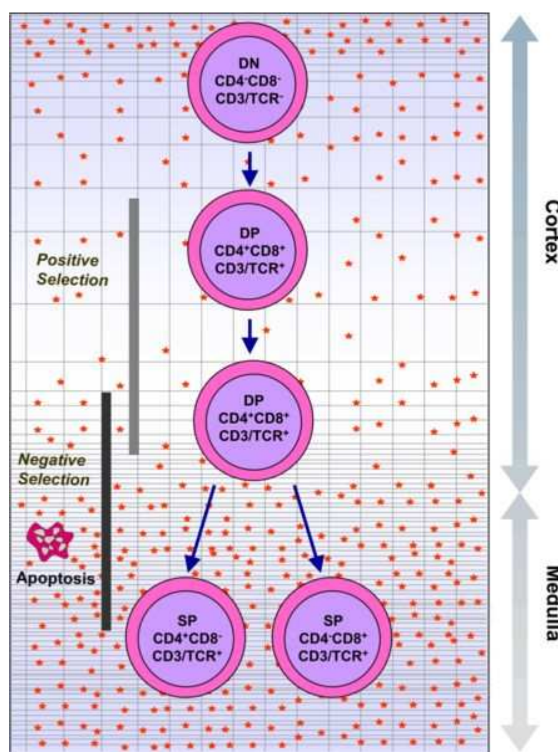
of cytokines that help to stimulate the adaptive immune responses, and present the phagocytosed antigen to its specific T cells (1). On the other hand, after activated by appropriately presented antigen, some T cells synthesize and secrete cytokines that may activate macrophages and/or neutrophils, increasing their ability to kill invaded microorganisms, a function of the innate immune response (1).

Adaptive immunity can be further categorized into humoral immunity and cellular immunity (1). Humoral immunity is mediated by secreted antibodies, produced by B cells. Secreted antibodies bind to antigens on the surfaces of invading microbes, which flags them for destruction. Cellular immunity does not involve antibodies but rather involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen.

The immune system can be compromised by immunodeficiency caused by inherited gene mutations or certain pathogens, such as human immunodeficiency virus (HIV), which can lead to destructive chronic infections or death. Diseases can also be caused by unwanted immune responses, such as allergy, autoimmune disease and transplantation rejection. Allergy is the consequence of antibody production against a harmless substance; autoimmune disease is caused by immune response directed toward the body's normal healthy components; transplantation rejection will be triggered by the differences in MHC type between donor and recipient if unchecked (1).

1.2 T-CELL DEVELOPMENT IN THYMUS

T cells are among the most versatile cells in the body. Precursor T cells from the bone marrow enter the thymus, where they undergo a stepwise maturation process (2) (Figure 1.1). In the first phase of T-cell development, double-negative progenitor lymphocytes make gene rearrangement and express T-cell receptor (TCR) protein and other cell-surface glycoproteins essential for the receptor's full function, such as CD4 and CD8. In the second phase, the double-positive (CD4+ and CD8+) T cells go through two types of screening. In the first screening, positive selection selects T cells that can recognize peptides presented by a self-MHC molecule and determines the commitment of the double-positive T cells to either CD4+ or CD8+ single-positive T cells. Cells that do not receive the peptide: MHC complex positive signal at this stage will undergo apoptosis. In the second screening, the negative selection eliminates potentially auto reactive cells that could be activated by the peptides normally presented by MHC molecules on the surface of healthy cells. Only a small subpopulation of the double-positive T cells survives the obstacle course of positive and negative selections and leaves the thymus and circulates in the secondary lymphoid organs. This population of mature circulating T cells is able to tolerate self-antigens, but is responsive to foreign antigens presented by self-MHC molecules. T cells remain naive until they encounter with antigens, which provoke the final phases of T-cell development and differentiation: the mature T cells get activated, divide and differentiate into various types of effector T cells and gain the ability to enter inflammations sites.



Source: Savino W (2006) The Thymus Is a Common Target Organ in Infectious Diseases. PLoS Pathog 2(6): e6

Figure 1.1. T-cell development in the thymus can be considered as a series of phases.

In the first phase, double-negative thymocyte progenitors enter the thymus, proliferate, and begin to rearrange T-cell receptor genes, which lead to production of CD4+ and CD8+ double-positive cells. In the second phase of the development, the double-positive thymocytes undergo positive selection, matching between the receptor specificity for MHC and the co-receptor molecules, which eventually leads to single-positive CD4+ or CD8+ T cells. In the third phase of development, negative selection eliminates cells with self-reactivity. Thymocytes that survive both positive and negative selection leave the thymus as mature single-positive CD4+ or CD8+ T cells and enter the circulation.

1.3 T-CELL ACTIVATION

T-cell activation is the process in which naive T cells encounter their specific antigen for the first time and are stimulated to differentiate into effector cells. T-cell activation is the first stage of a primary adaptive immune response. Naive T cells constantly circulate through out the body in search of stimulation. Initially, it is absolutely necessary for T cells to recognize their antigen on professional antigen-presenting cells (APC). Since APCs only reside in lymphoid tissues, naive T cells can only be activated in lymphoid tissues, such as the lymph nodes, spleen, and also the peyer's patches of the intestinal mucosa. Once activated, T cells may activate other naive T cells that also have specific receptors for the same antigen. Alternatively, the activated T cells can leave the lymph node, travel to peripheral sites infection and of inflammation and activate other cells locally. The selective homing of naive T cells to lymphoid tissues and activated T cells to peripheral tissues is due to differential expression of adhesion molecules on the surface of these cells. For example, naive T cells do not contain cell adhesion molecules that allows them to bind inflamed endothelial cells and enter those sites.

1.3.1 Requirements for T-cell receptor (TCR) ligation and co-stimulatory signal

T-cell activation requires the binding of T-cell receptor (TCR) to an antigen: MHC complex. However, binding of the T-cell receptor alone is insufficient to trigger naive T-cell activation, proliferation and differentiation of the progeny into effector cells (1). A second, co-stimulatory, signal is required. This signal is provided by B7 molecules expressed on the surface

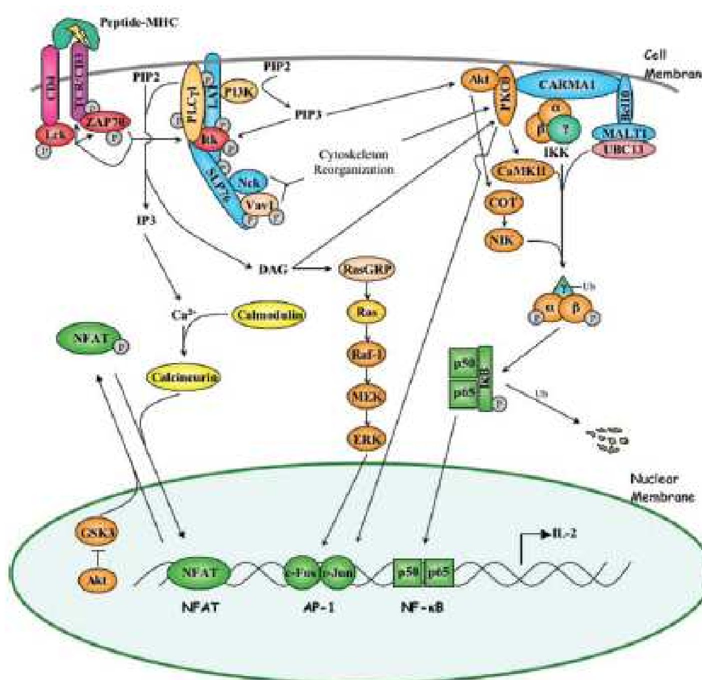
of antigen-presenting cells exclusively. B7 molecules interact with their appropriate ligands on T cells (CD28 on naive T cells, CD28 and CTLA4 on activated T cells). Binding of B7 to CD28 provides the critical second signal for T cells to become activated. Upon activation, T cells also express CTLA4. B7-CTLA4 interaction provides an inhibitory signal to T cells. This serves to competitively inhibit, and regulate excessive T-cell activation. The co-stimulatory signal can only be delivered by antigen-presenting cells. Therefore, the most critical point in the activation of T cells is the presence of appropriate antigen-presenting cells. This absolute requirement of T-cell activation for a co-stimulatory signal, and the fact that this signal can only be provided by antigen-presenting cells, serve as an important regulatory point to limit inappropriate T-cell activation by other host cells.

If any one of the above signals is absent (e.g. TCR interaction in the absence of co-stimulation from antigen-presenting cells), T cells will become non-responsive, a state described as anergy. In this state, T cells can not be activated by any subsequent stimulation it might receive in the future, even with appropriate co-stimulation.

1.3.2 T-cell activation induces the gene transcription regulation

Upon T-cell receptor ligation and co-receptor ligation, the signal is transmitted to the interior of T cells by the cytoplasmic tails of the CD3 proteins, containing immunoreceptor-tyrosine activation motifs (ITAMs), which associate with cytoplasmic protein tyrosine kinases. These kinases are activated by receptor clustering and then phosphorylate tyrosine residues in ITAMs (1). On formation of the T-cell receptor: MHC: co-receptor complex, the protein tyrosine kinase Lck activates the cytoplasmic protein tyrosine kinase ZAP-70, which is prominent in

initiating the intracellular pathway. Once activated, ZAP-70 triggers three major signaling pathways, DAG and Ca^{2+} mediated protein kinase C pathway, Ca^{2+} mediated phosphatase calcineurin pathway, and G protein RAS and RAC mediated MAP kinase pathway, which eventually regulate the activity of transcription factors such as NFAT, AP-1, and NF-κB (Figure 1.2). The orchestrated action of NFAT, AP-1, and NF-κB turn on the transcription of genes that direct T-cell proliferation and the development of effector functions (3).



Source: The Journal Of Biological Chemistry Vol. 279, No. 28, July 9, pp. 28827–28830, 2004

Figure 1.2. T cell receptor signaling events leading to activation of transcription factors.

This figure presents an overview of some of the key signaling events linking the binding of peptide-MHC to the T cell antigen receptor (TCR/CD3) and the CD4 costimulatory receptor. The existence of key phosphorylation events is indicated on some of the signaling proteins by small

gray circles labeled P; however, not all proteins undergoing TCR-stimulated phosphorylation are labeled. Ub designates ubiquitination. The nature of the different interactions is described in the accompanying text.

1.4 T-CELL SUBSETS AND EFFECTOR FUNCTIONS

There are two major types of effector T cells: cytotoxic CD8+ T cells, which kill a variety of target cells, CD4+ T helper cells (Th1 cells for cellular immune responses and Th2 cells for humoral immune responses). Additionally, in a small population of the total T cells, regulatory T cells suppress the immune response.

1.4.1 Helper T cells

The general function of effector CD4+ T helper cells is to secrete cytokines that activate other cells of the immune system. These cells have no cytotoxic activity and do not kill infected cells or clear pathogens directly. Instead, they control immune response by directing other cells to perform these tasks including cytotoxic CD8+ cells, B cells, macrophages, and natural killer cells. CD4+ T helper cells can be further categorized according to the cytokines they secrete and the cells they assist. Helper cells that secret cytokines that mainly activate macrophages are called Th1 cells, whereas helper cells that mainly help B cells to make antibodies are called Th2 cells.

The two helper T cell populations (Th1 and Th2) are commonly distinguished by the profiles of cytokines they secrete (1). Th1 cells direct cell-mediated functions such as cytotoxic

CD8+ T-cell and macrophage activation by secreting IL2, IFNG, TNF-beta, GM-CSF and IL3, which leads to inflammation and a cell-mediated immune response. Th2 cells mediate allergic or hummoral responses by providing cytokines that help activate B cells (IL4, IL5, IL6, IL13), eosinophils (IL5), and mast cells (IL3, IL4, IL10). The Th1 and Th2 responses are exclusive. A Th1 or a Th2 response, rather than a mixture of the two, can be elicited by certain infectious agents. The choice can have profound consequences for the human host. Leprosy, an infection caused by the Mycobacterium leprae, a bacterium that grows within the vesicular system of macrophages, is a case in point(1). Th1 cells give a more effective response to this bacterium, secreting cytokines that activate macrophages, which then destroy the bacteria they contain. When the host's response consists mainly of Th1 cells, the bacterial population is kept low, the disease progresses slowly, and the patient usually survive. On the other hand, if the CD4+ T-cell response consists mainly of Th2 cells, the antibodies made by the host can not reach the bacteria inside the macrophages, then the bacterial population expands. Unchecked bacterial growth within macrophages cause gross tissue destruction, which is eventually fatal.

Although we know about the types of cytokine patterns helper T cells tend to produce (1), we understand less about how the decision between Th1 and Th2 responses are made and how the patterns themselves are decided. The major influencing factors involve the affinity of the binding of the antigen to the T cells (including the type of the presentation, the concentration of antigen presented to the T cell) and the presence of cytokines during primary activation (such as the ones mentioned above). For instance, when IL4 is present during activation, CD4+ T cells develop into Th2 cells, while activation in the presence of IL12 results in development of Th1 cells. The cytokines produced by the Th1 and Th2 cells can suppress the differentiation of the

other population to bias the development of its own. For example, IFNG, secreted by Th1 cells, inhibits Th2 cell proliferation (4). The balance of the two helper cell subsets is of paramount importance and deregulation might contribute to the development and progression of certain diseases, such as cancer (5, 6).

1.4.2 Cytotoxic T cells

Cytotoxic CD8+ T cells can selectively eliminate infected or abnormal cells by three distinct but not mutually exclusive mechanisms: cell death induced by the perforin (direct), cell death induced by Fas pathway (direct), and cell death induced by cytokines (indirect).

Both the perforin and Fas pathways trigger apoptotic cascades in the target cells (7). Cytotoxic CD8+ T cells contain stored lytic granules, which are modified lysosomes containing a mixture of specialized proteins called cytotoxins. Cytotoxic CD8+ T cells start to synthesize cytotoxins in inactive forms and to pack them into lytic granules upon stimulation. Activated cytotoxic CD8+ T cells undergo a reorganization of their cytoskeleton, which leads to polarization and a directional release of lytic granules towards the target cell. Cytotoxic granules contain the pore-forming protein, perforin, lysosomal proteins and proteases known as granzymes. The perforin monomers insert into the target cell membrane, and polymerize in a Ca^{2+} dependent reaction resulting in pore formation. The pores contribute to access of granzymes and other lytic enzymes into the target cell (7). Granzyme A (GZMA) is the most abundant granzyme, which activates the apoptosis cascade through a caspase-independent mechanism, and overexpression of BCL2 does not protect against GZMA-mediated cell death (8). Cleavage assays in isolated nuclei demonstrated that GZMA cleaves lamins A, B, and C, causing

disruption of the nuclear lamina (9). GZMA also leads to degradation of histone H1, proteolysis of the tails of core histones, and single stranded DNA nicking (10). Granzyme B (GZMB) initiates apoptosis by cleaving caspases 3, 7 and 10 (11).

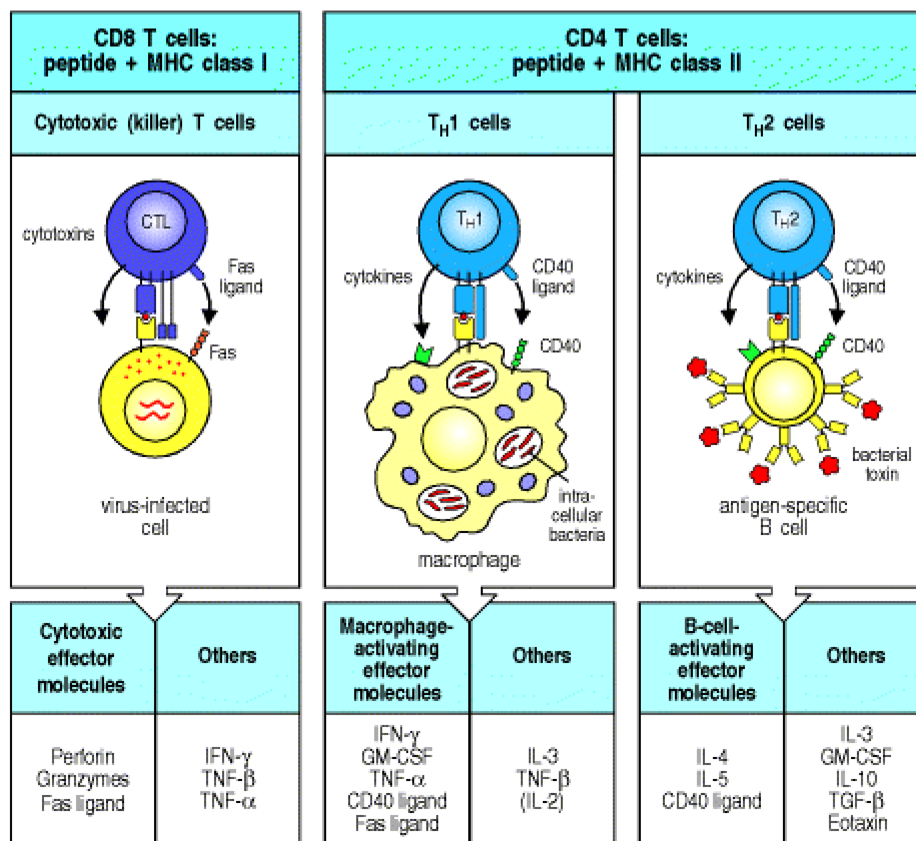
Activated cytotoxic CD8+ T cells also express membrane bound Fas Ligand (FasL). Once the effector cytotoxic CD8+ T cells recognize specific antigen on the target cell through TCR recognition, FasL will come into contact with Fas on the surface of the host target cell. The ligation of Fas and FasL will induce an intracellular signal to induce apoptosis in the target cell (12). In summary, the granule release mechanism is important in virus clearance, tumor rejection and cytotoxic function, while the Fas-mediated pathway is involved in homeostasis of the lymphocyte population following an immune response (13).

Cytotoxic CD8+ T cells can also induce cell death in target cell by cytokine secretion. Activated cytotoxic CD8+ T cells secrete cytokines including INFG, TNF and TNF-beta, which are important in the activation of macrophages. INFG also induces the expression of MHC I on host cells, which will increase antigen presentation to cytotoxic CD8+ T cells. Thus infected cells are more readily destroyed.

Figure 1.3 summarizes effector functions and specific secretion patterns for both CD4+ and CD8+ T cells.

Figure 1.3. Three main types of armed effector T cells produce distinct sets of effector molecules.

CD8 T cells are predominant killer cells, which recognize pathogen-derived peptides bound to MCH class I molecules. They release perforin, granzymes and IFNG. A membrane-bound effector molecule expressed on CD8 T cells is the ligand for Fas, a receptor whose activation induces apoptosis. CD4 T cells recognize peptides bound to MHC class II molecules and are of two functional types: Th1 cells and Th2 cells. Th1 cells are specialized for activation of of macrophages that are infected by or have ingested pathogens; they secrete IFNG as well as other effector molecules, and express membrane-bound CD40 ligand, and/or Fas ligand. These are both members of the TNF family but CD40 ligand triggers activation, whereas Fas ligand triggers death, so their pattern of expression has a strong influence on function. Th2 cells are specialized for B-cell activation; they secrete the B-cell growth factors IL4 and IL5. Th2 cells express mainly the membrane-bound effector molecule CD40 ligand, which binds to CD40 on the B cell and induces B-cell proliferation.



Source: Immunobiology, 3rd edition, Janeway C.A. and Travers, P., Current Biology Ltd./ Garland Publishing Inc., New York, 1997.

1.5 T-CELL RESPONSE TO H_2O_2 STRESS

1.5.1 Generation of ROS, especially in T cells

There are three main cellular ROS: the superoxide anion ($\bullet O_2^-$), the hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2). ROS generation reactions always start with the production of the superoxide anion ($\bullet O_2^-$) by an electron transfer to molecular oxygen. Electron transfer to O_2 can occur as a "leakage" of the respiratory chain reactions in mitochondria, where some electrons can escape to generate $\bullet O_2^-$ (14). Another source of ROS is the endoplasmic reticulum where $\bullet O_2^-$ is generated by NADPH cytochrome p450 and b_5 families of enzymes that can oxidize unsaturated fatty acid and xenobiotics and reduce O_2 to $\bullet O_2^-$ (15). This radical can be converted to H_2O_2 and O_2 spontaneously in the presence of protons from water ($H_2O \leftrightarrows H^+ + OH^-$). This process can also be catalyzed by enzymes such as superoxide dismutases (SODs). Three SODs have been discovered: SOD1 is found in the cytosol and nucleus, while SOD3 is extracellular and SOD2 is localized to the inner mitochondria membrane (16).

Furthermore, the plasma membrane-associated NADPH oxidase complex in phagocytic cells has been established as ROS producing enzymes. The activation of the complex causes the oxidative burst in macrophages and neutrophils in response to phagocytosis (17), catalyzing the one-electron reduction of oxygen by NADPH. This NADPH oxidase complex comprises the membrane-bound cytochrome b558 (a heterodimer of the subunits $gp91^{phox}$ and $p22^{phox}$) and at least three cytosolic proteins: $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ (phox: phagocyte oxidase). The small G protein Rac is required for the activation of the NADPH oxidase complex, which will help the cytosolic proteins translocate to the plasma membrane and associate with cytochrome b558 to

form the active superoxide-generating enzyme. The NADPH oxidase complex can be activated by the phosphorylation of a few cytosolic proteins: (1) phosphorylation of $p67^{phox}$ by the GTP-bound Rac recruits the $p67^{phox}$-$p47^{phox}$ complex to cytochrome b558 (18); (2) phosphorylation of $p47^{phox}$ by PKC and other serine-threonine protein kinases results in its binding to cytochrome b558 (19); (3) phosphorylation of $p40^{phox}$ releases its inhibition of forming the active $p67^{phox}$-$p47^{phox}$ complex and helps to activate the NADPH oxidase (20). The existence of functional components of this multisubunit enzyme complex has been demonstrated in non-phagocytic cells, suggesting the ROS producing role of the NADPH oxidase homologous in non-phagocytic cells. Moreover, it has also been hypothesized that diverse receptors stimulate ROS generation via activation of intracellular NADPH oxidase homologous to that of inflammatory cells (21).

In T cells, it has been reported that TCR stimulation leads to ROS generation, although only the $p40^{phox}$ is expressed among the known NADPH oxidase subunits (22). It has also been reported that T-cell mitogens (23), lectins (24), superantigens (mouse mammary tumor virus) (25), staphylococcal enterotoxin B, and staphylococcal enterotoxin A (26) can stimulate ROS generation in T cells.

External stimuli will cause excess ROS production by several possible mechanisms: (1) cellular injury leading to Ca^{2+} accumulation accelerates the electron transfer process and increases ROS formation; (2) chemicals that alter coupling of the respiratory chain, such as antimycin, or change the redox state of the cell, including alcohol and its metabolite acetaldehyde, cause excess ROS; (3) diseases or inflammations, such as HIV, are an important source of extra ROS production (as reviewed by Perl et al. (27)).

1.5.2 Redox control of signal transduction

Because of ubiquitous presence of ROS in biological systems, it is not surprising that a large number of signaling pathways are regulated by ROS. However, the underlying mechanisms of cellular response to ROS are not well understood. There is growing evidence that redox regulation might occur at multiple levels and involve several signal-transduction pathways. (1) ROS have been reported to activate protein tyrosine kinases (PTKs) and inactivate protein tyrosine phosphatases (PTPs). Thus, ROS lead to an apparent enhancement of tyrosine phosphorylation. Studies have shown that H_2O_2 readily inactivates a number of PTP family members while having no effect on serine/threonine phosphatases (28). (2) H_2O_2 can induce rapid increase in intracellular concentration of Ca^{2+} (29). (3) ROS are involved in regulation of activities of MAP kinases (30). (4) It has been reported that ROS induce the activity of transcription factors, such as NF-κB, AP-1, STAT3 and SP1 (31).

1.6 DNA MICROARRAYS: TECHNOLOGY, ANALYSIS AND APPLICATIONS

1.6.1 Principle of the method

Microarray technology is based on the principle that complementary nucleic acids will hybridize, which is also the basis for traditional gene expression analyses, such as Southern and Northern blotting. Hybridization provides high sensitivity and specificity of detection as a consequence of exquisite, mutual selectivity between complementary strands of nucleic acids. However, it is impossible to research on a large number of genes using the traditional techniques. In microarray-based technologies, the arrays, with the solid surface base, are spotted with thousands of target paring sequences, which can be simultaneously hybridized (32). Therefore, the microarrays enable the simultaneous detection of gene expression levels of thousands of genes due to a vast number of gene-specific targets (oligonucleotides or DNA) that are individually arrayed on a single matrix. The adaptable nature of the fabrication and hybridization methods allows the technique to be applied widely.

1.6.2 Applications of microarrays

Microarray technology has been classically used for investigating the effect of a given biotic or a biotic perturbation on the transcriptional output of a system (32). Underlying these experiments is the notion that analyzing the response of a system to a given perturbation can uncover the mechanism of signaling or the biological response to the perturbation, or both. One

example of a systematic genome-wide study was carried out in examining the response of human fibroblasts to serum (33). It was demonstrated that serum could cause large differential phenotypes that can be used to elucidate intracellular mechanisms and cellular function.

Microarray experiments based on temporal gene expression profiling have advanced the understanding of biological processes such as development, differentiation, the cell cycle and biological rhythms (34, 35). Hundreds of transcripts have been found to show rhythmic expression patterns in their steady-state message levels, with a periodicity very close to that of the cell cycle (36). These transcripts have been classified into separate clusters on the basis of the stage of the cell cycle corresponding to the peak phase of expression. DNA microarrays have also been used to study transcriptional changes following T-cell activation. The gene expression patterns of T-cell activation with or without co-stimulation by anti-CD28 antibody were compared (37, 38).

Microarray technology is also becoming an increasingly valuable tool in diagnosis, classification, and outcome prediction of various cancers. For example, two types of acute leukemia, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), are treatable by traditional chemotherapy; however, successful treatment is largely dependent on correct diagnosis. To find a distinct molecular signature for these two diseases, oligonucleotide microarrays were used to identify a set of 50 genes that can differentiate between AML and ALL with great accuracy (39). These methods have also been extended to the analysis of several classes of tumor. Su et al. have established a list of about 110 genes that are highly characteristic and therefore diagnostic of colon, bladder, kidney, liver, pancreas, ovary, prostate, lung, gastric and breast cancers (40). In addition to diagnostic purposes, gene expression signatures of tumor-

subgroups have been correlated with patient survival (41) and histopathological data to predict poor prognosis or to identify carriers of the BRCA1 gene in breast cancer (42).

Recently, arrays have also been used to construct transcriptional networks by monitoring the binding of transcription factors to genomic DNA (43). This method uses chromatin immunoprecipitation (ChIP) to detect physical interactions between known proteins and their DNA target sites. Instead of sequencing immunoprecipitated targets genes, DNA arrays were used to identify the target identities (‘ChIP-chip’). Using this methodology, it has been shown that transcription factors that are activated during a specific phase in the yeast cell cycle control the expression of transcription factors that regulate the respective subsequent phase, which revealed novel mechanistic insights into the continuous transcriptional coordination of the cell cycle (44).

CHAPTER 2: COMPARATIVE ANALYSIS OF TRANSCRIPTIONAL PROFILING OF CD3+, CD4+ AND CD8+ T CELLS IDENTIFIES NOVEL IMMUNE RESPONSE PLAYERS IN T-CELL ACTIVATION

2.1 INTRODUCTION

T cells are among the most versatile cells in the body and play a central role in adaptive immunity. T-cell maturation in thymus is a stepwise process, undergoing positive and negative selection to produce CD4+ and CD8+ T cells (2). When mature, T lymphocytes leave the thymus and are considered naive cells until they encounter activating signals in peripheral lymphoid organs, thus become activated, start to proliferate, differentiate into effector cells (helper and cytotoxic) and gain the ability to enter inflammations sites (45). Activation of the naive T cells in the peripheral immune system is the first step of the adaptive immune response.

Successful T-cell activation requires two major stimulatory signals to produce an effective immune response. First, the T-cell receptor complex (TCR) recognizes the cognate ligands presented by the major histocompatibility complex (MHC) on antigen-presenting cells (APCs) (46). Second, a co-stimulation signal is presented to T cells through the engagement of a co-receptor such as CD28 (47). In the absence of CD28 co-stimulation, TCR signaling alone results in anergy. T-cell activation, which begins with TCR activation with CD28 co-stimulation, triggers multiple signaling pathways and cellular events. Signaling downstream of TCR

engagement has been widely studied (3, 48, 49). Key events include activation of protein kinases such as LCK and ZAP-70, intracellular Ca^{2+} regulation, activation of MAP-kinase cascades, and activation and nuclear localization of crucial transcription factors including AP-1, NFAT, and NF-κB. However, our understanding of the activation process including subsequent proliferation and differentiation events is far from complete. A temporal genome-scale transcription profiling of T-cell activation process would provide a comprehensive understanding and insights into the molecular mechanisms underlying the process.

Gene expression analysis of T-cell activation at a single timepoint has been reported (50, 51), and using a single donor sample, the gene expression patterns of T-cell activation with or without co-stimulation by anti-CD28 antibody were compared (37, 38). However, to the best of our knowledge, the genome-scale donor-independent temporal gene expression analysis of primary, human T-cell activation has not been reported, and this is the goal of this study. Significantly, a comparative analysis of the programs of two T-cell subsets (CD4+ and CD8+) against each other and against the natural CD3+ population remains unexplored, and would likely yield significant new information. Comparison of the transcriptional patterns among the three populations should lead to the identification of the common transcriptional events shared by CD4+ and CD8+ T cells, and of subset-specific genes and genes potentially involved in the communication between CD4+ and CD8+ T cells. Among the differentially expressed genes, we focused on 'immune response' genes based on Gene Ontology (GO) classification in order to provide new insights into the expression of chemokines and cytokines, the orchestrated regulation of receptors, the interactions between the two subsets, and the homeostasis of resting

T cells. Such understanding would be helpful for enhancing, re-directing or modifying the activities of T cells under physiological and pathophysiological circumstances.

2.2 **MATERIALS AND METHODS**

2.2.1 Cells and culture system

Healthy-donor peripheral blood mononuclear cells (PBMCs) (AllCells, Berkeley, CA) were used to negatively-select CD3+ T cells, CD4+ and CD8+ T cells (Pan T Cell Isolation Kit II, CD4+ T Cell Isolation Kit II, and CD8+ T Cell Isolation Kit II, Miltenyi Biotech, Sunburn, CA). Cells were activated polyclonally with anti-CD3/anti-CD28 Mab (1:1)-coated magnetic beads (500 fmol/bead) (Dynabeads M-450 Epoxy, Dynal Biotech, Lake Success, NY) (52). The ratio of beads to cells was 3:1. CD3+ cell cultures from three individual donor samples were seeded at $1x10^6$ cells/mL in T-flasks and cultivated for 96 hours in serum-free AIM-V medium with 100 U/mL IL2 (Chiron, Emeryville, CA) and 2% human serum (Sigma-Aldrich St. Louis, MO) as described (53). CD4+ cells and CD8+ cells from another three individual donors were cultured for 72 hours in the same manner. Cell counting and sampling for flow cytometry and microarray analysis were carried out at 0, 4, 10, 48 and 96 hours in CD3+ T-cell experiments, E1-E5, and at 0, 6, 12, 24, 48 and 72 hours in CD4+ T-cell and CD8+ T-cell experiments, E7-E11. This study was approved by the Northwestern University IRB.

2.2.2 Flow cytometry

The following monoclonal antibodies (Mabs) for flow cytometry were purchased from BD Biosciences (San Jose, CA) unless otherwise stated and included CD3 (FITC+PE), CD4 PE, CD8 PE, CD25 PE, CD69 PE, TNFSF4 PE, CD40LG (TNFSF5) PE, TNFRSF9 PE, KLRD1 PE,

CD48 PE, GZMB PE (Invitrogen, Carlsbad, CA). Flow cytometry was carried out as described (54, 55). Briefly, all samples were gated on forward scatter and on propidium iodide negative (PI-) to eliminate debris and dead cells. For intracellular detection of GZMB, cells were first stained with anti-CD4-FITC (or anti-CD8-FITC) and then fixed, permeabilized, and stained as previously described (56). 10,000 gated events from each tube were acquired using a FACscan (BD Biosciences) or LSRII flow cytometer (Becton Dickinson). Quantibrite beads (BD Biosciences Immunocytometry Systems) labeled with different amounts of PE molecules were used to quantify surface or intracellular protein levels and normalize measurements between timepoints.

2.2.3 RNA extraction and quality control

Total RNA was extracted from frozen cells using the Total RNA Isolation Mini Kit (Agilent, Wilmington, DE). RNA samples were re-suspended in RNase-free water and stored at –80°C. RNA yield and purity were assessed spectrophotometrically at 260 and 280 nm (Biomate 3, Thermo Spectronic, Marietta, OH). RNA integrity was evaluated using a Bioanalyzer 2100 (Agilent).

2.2.4 DNA-microarray experiments and data analysis

Microarray-based transcriptional analysis was carried out for samples at each timepoint, using the ‘reference’ design (55), with Human Thymus Total RNA (Ambion, Austin, TX) as the reference RNA. Approximately half of the individual microarrays were replicated and the

correlation coefficient between these technical replicates was above 0.90. Detailed experimental procedures and the use of the SNNLERM-algorithm (57) for data normalization were described (55). Further analysis (significant genes identification, hierarchical clustering and gene ontology assignment) was carried out using 'MultiExperiment Viewer (MeV)' from The Institute for Genomic Research (TIGR) (58). Raw and normalized data were deposited in the Gene Expression Omnibus (GSE6607 (CD3+ T-cell experiment), GSE7571 (CD4+ T-cell experiment) and GSE7572 (CD8+ T-cell experiment)) (59). Within each population (three biological replicates using cells from different donors), multi-class SAM (Significance Analysis of Microarrays) with a false discovery rate <1% was used to select genes that show statistically different expression between groups. A group is defined as all the samples belonging to the same timepoint regardless of the donors. Briefly, there were 5 groups (0 hour, 4, 10, 48 and 96 hours) in the set of CD3+ experiments, E1-E3, and 6 groups (0 hour, 6, 12, 24, 48 and 72 hours) in the set of CD4+ experiments and CD8+ experiments, E7-E9. The three samples from biological experiments in each group were treated as replicates. To focus on the expression change, gene expression at each time point was compared to that of 0 hour in each experiment. Gene Ontology annotations, as curated by European Bioinformatics Institute, were retrieved from the Gene Ontology Consortium website (60). Hierarchical clustering was performed with the Euclidian distance metric.

2.2.5 Quantitative RT-PCR (Q-RT-PCR)

cDNA was obtained from total RNA samples using the High-Capacity cDNA Archive Kit and Q-RT-PCR was performed with Assays-on-Demand kits (Applied Biosystems; Foster City, CA) as described(54). The amount of mRNA for each sample was normalized using the average of two housekeeping genes (Glucuronidase-β and 18S). The use of GUSB (Hs99999908_m1) and 18S (Hs99999901_s1) genes as housekeeping genes has been previously tested in our lab (54, 55). Primers (Applied Biosystems, Foster City, CA) for the following functionally diverse set of genes were used: FOS (Hs01119267_g1), MYB (Hs00920564_m1), JUN (Hs99999141_s1), CAT (Hs00156308_m1), MAPK6 (Hs00957318_g1), SOD2 (Hs00167309_m1), SORD (Hs00973148_m1), STAT1 (Hs01014001_m1) in CD3+ T-cell experiments, E1-E3; and GZMA (Hs00196206_m1), GZMB (Hs00188051_m1), MYB (Hs00920564_m1), FASLG (Hs00899442_m1), EGR1 (Hs00152928_m1), EGR2 (Hs00166165_m1), and EGR3 (Hs00231780_m1) in CD4+ and CD8+ T-cell experiments, E8 and E9. Genes were chosen to reflect differentially expressed genes of a wide range of microarray signal intensities.

2.2.6 Supernatant ELISA assay of CCL20 and IFNG

Culture supernatants were collected at 4, 10, 48 and 96 hours in three CD3+ T-cell experiments, E3-E5, and analyzed for CCL20 and IFNG concentrations by ELISA (R&D Systems, Minneapolis) following the manufacturer's instructions.

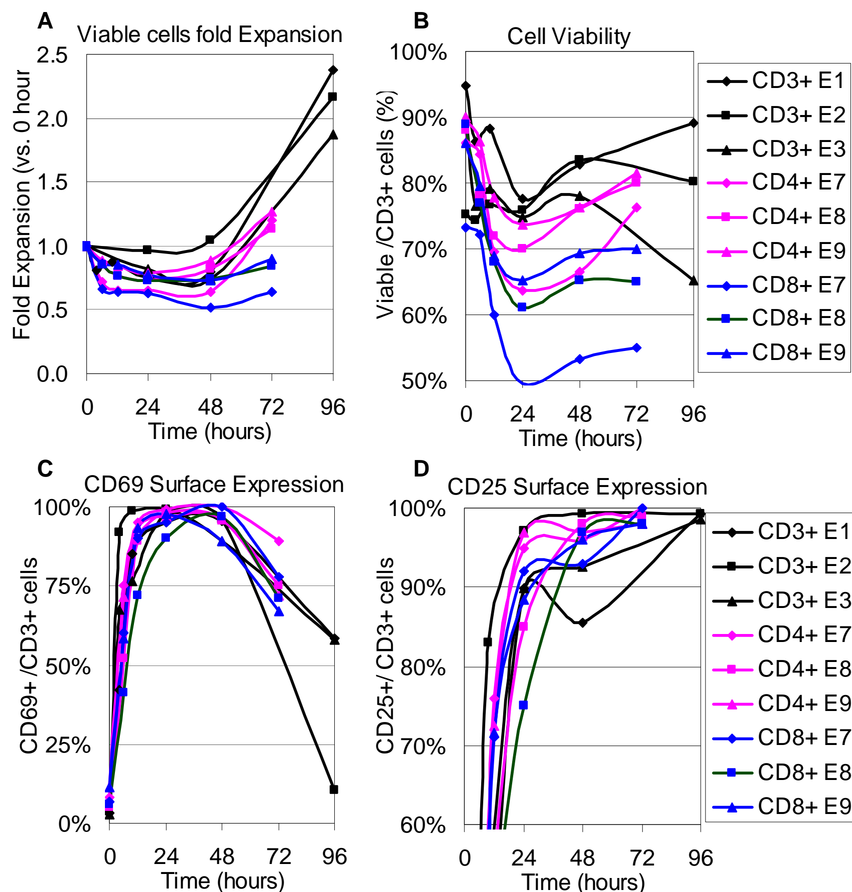
2.3 RESULTS

2.3.1 Primary human T-cell activation is donor independent

We aimed to capture important, donor-independent transcriptional events of T-cell activation. Three biological experiments, E1-E3, using CD3+ T cells, which contain both the CD4+ and CD8+ subsets, from three different healthy donors demonstrated similar phenotypic characteristics. T-cell proliferation, as measured by cell expansion, started at 48 hours after stimulation, and cell numbers doubled by 96 hours (Figure 2.1A). Cell viability remained around 80% throughout the 96 hours (Figure 2.1B). Surface expression of the early T-cell activation marker CD69 was rapidly upregulated within 10 hours, and then downregulated after 48 hours (Figure 2.1C). Expression of the other important surface marker CD25 (IL2RA) rapidly increased within 24 hours and stayed high (above 80%) from 24 hours to 96 hours (Figure 2.1D). We also examined the CD4+/CD8+ subset ratio but we found no significant changes during the 96 hours of the experiments (data not shown). CD4+ cells were ca. 60%, and CD8+ cells ca. 40% of the total T-cell population.

A separate set of experiments, E7-E9, was carried out using separately CD4+ and CD8+ T cells isolated from another three healthy donors. The time course analysis of this set of experiments was setup somewhat differently than in the CD3+ T-cell study (0, 6, 12, 24, 48 and 72 hours in the CD4+ and CD8+ subsets compared to 0, 4, 10, 48, and 96 hours in CD3+ T cells) in order to cover earlier timepoints. As we demonstrate below, the different time points in the two sets of experiments do not affect our ability to compare the data from the two studies, and in fact enhance and broaden the validity of the conclusions. Within each population, T cells

exhibited overall similar phenotypic characteristics (Figure 2.1). T-cell proliferation as



assessed

Figure 2.1. Phenotypic analysis of T-cell ex vivo activation upon anti-CD3/anti-CD28 stimulation of three populations.

Three independent biological experiments were carried out for each population. CD3+, CD4+ and CD8+ T cells were negatively selected from PBMCs of healthy donors and activated with anti-CD3/anti-CD28 antibodies. **(A)** T-cell expansion as assessed by cell numbers; **(B)** The percentage of the viable T cells as determined by flow cytometry; **(C)** The percentage of the viable cells expressing CD69; **(D)** The percentage of the viable cells expressing CD25. Data from 6 independent experiments (CD3+ T-cell experiments, E1-E3, and CD4+ and CD8+ T-cell

experiments, E7-E9) using cells from 6 different healthy donors are shown.

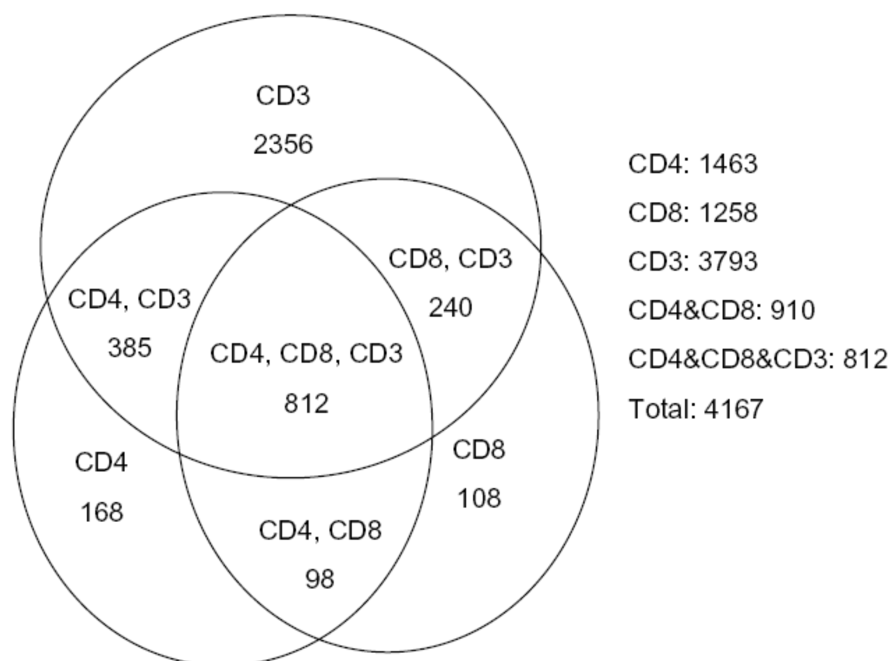


Figure 2.2. Venn diagram comparison of the significant genes identified as differentially expressed in the three populations.

SAM analysis (false discovery rate of <1%) identified 3793 significant genes in CD3+ population, 1463 significant genes in the CD4+ population and 1258 significant genes in the CD8+ population.

by cell numbers did not start until 48 hours. Expansion reached about 1.2 fold in CD4+ T cells and about 0.8 fold in CD8+ T cells by 72 hours. Cell viability remained around 75% in CD4+ T cells vs. around 60% in CD8+ T cells; the lower viability of CD8+ T cells was likely caused by the absence of the help from CD4+ T cells. Expression of the T-cell activation surface markers CD25 and CD69 in the two subsets was similar to that of CD3+ T cells.

Agilent microarrays that target 18,403 human genes were used to generate the transcriptional profile of activation for the combined CD3+ T-cell population, and CD4+ and CD8+ T-cell subsets. Comparing samples across all time points, multi-class SAM (false discovery rate of <1%) identified 3793 genes with statistically significant expression change in the CD3+ population, 1463 significant genes in the CD4+ population and 1258 significant genes in the CD8+ population in these temporal profiling studies. Hierarchical clustering of these significant genes (Appendix A1-A3) demonstrated that the common transcriptional patterns among the replicate biological experiments were reproducible. Thus, for simplicity and ease of presentation, gene expression data from their three biological experiments for each population were averaged for discussion and analysis below. In order to compare the transcriptional patterns of the three populations, the significant genes from all experiments were combined to a total of 4167 unique, significant genes distributed among the three cell populations as shown in the Venn diagram of Figure 2.2. Far more significant genes were identified in the CD3+ T-cell activation experiments than in the subset experiments, possibly reflecting a larger repertoire of genes during activation in the natural, mixed population of CD3+ cells, and thus the synergy and interplay of the two subsets in producing a more complex and multifaceted response. Nevertheless, a large number of the genes were shared by CD4+ and CD8+ T cells (910 out of

1463 and 1258 respectively), reflecting the common cellular events shared by CD4+ and CD8+ T cells during the activation process. Hierarchical clustering of these pooled significant genes (Appendix A4) demonstrated that the 3 populations shared largely similar transcriptional profiles regardless of the difference of the sampled timepoints, which, in perspective, broadens the significance of identified genes.

Q-RT-PCR was used to validate select microarray results. Fifteen significant genes with different expression intensities were selected. As previously reported (54), in our laboratory, data from these Agilent microarrays correlated strongly with the Q-RT-PCR results, although Q-RT-PCR data generally show larger fold changes compared to microarray data (Appendix A5). We thus conclude that the T-cell activation process under our experimental conditions is largely donor invariant, as assessed by both phenotypic data and transcriptional profiles.

2.3.2 Regulation of 'immune response' genes in T-cell activation

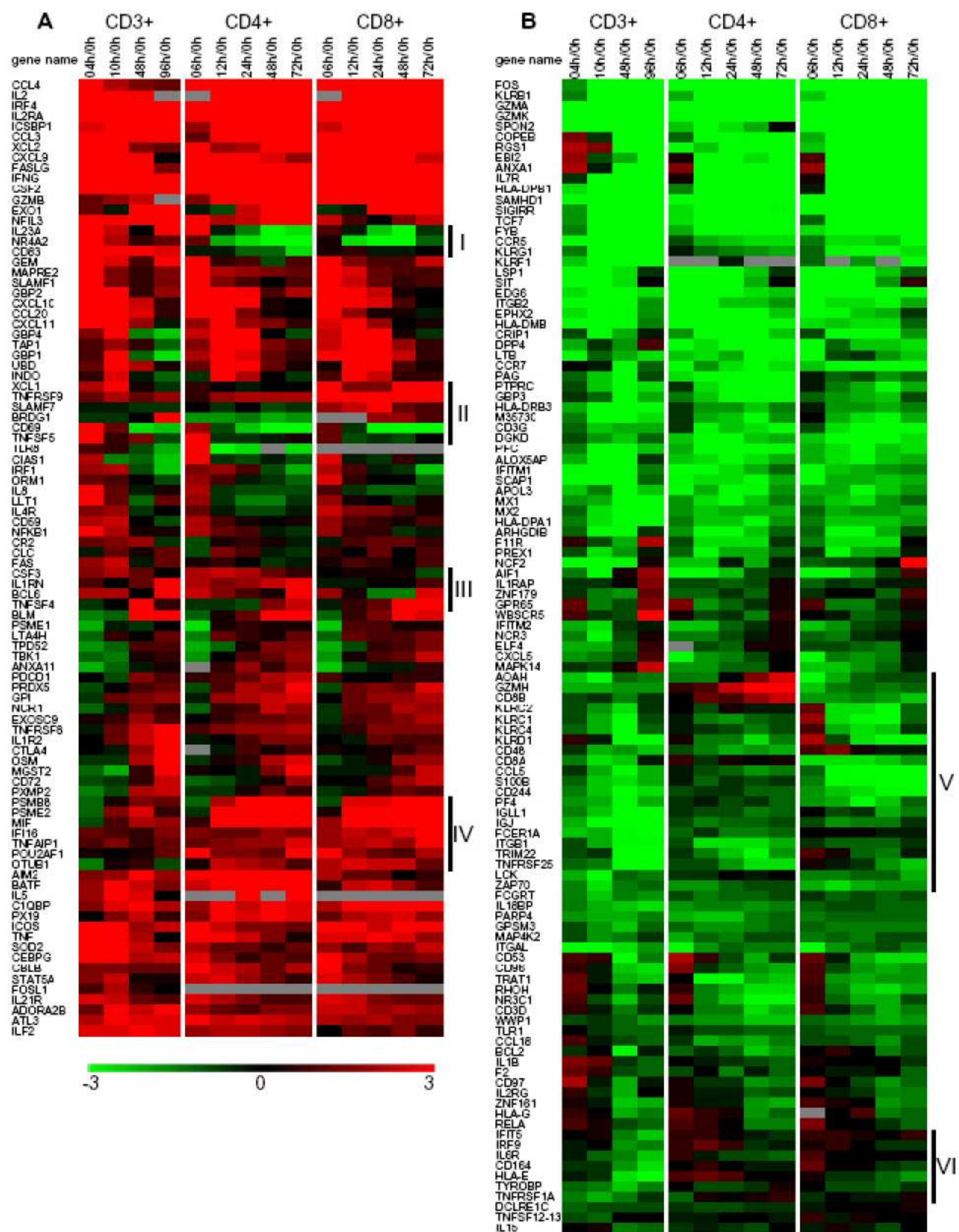
Ontological analysis using the MeV EASE module identified 203 genes associated with the term 'immune response' among the 4167 significant genes, consistent with the essential roles of T cells in the adaptive immune response. Hierarchical clustering revealed distinct expression patterns for these 203 genes and allowed us to divide them into two clusters: (A) Expression is mainly upregulated compared to resting T cells (0 hour) (Figure 2.3A); (B) Expression is mainly downregulated compared to resting T cells (0 hour) (Figure 2.3B).

Although we expected differences in gene expression patterns because of the different biology and functions of CD4+ and CD8+ T cells, the aforementioned 203 genes show, overall,

similar expression patterns among the 3 populations but with several notable exceptions. Within the upregulated cluster A, notable differences among the three populations include: (1) genes in

Figure 2.3. Expression profiles of significant genes associated with the Gene Ontology term 'immune response'.

Genes that were differentially expressed temporally in T-cell activation of the three (CD3+, CD4+ and CD8+) populations were divided into two groups (A with mostly upregulated genes and B with mostly downregulated genes) according to their distinct expression patterns based on hierarchical clustering using the Euclidian distance metric. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Clusters (I-VI) of genes with different expression patterns among the three populations were noted on the side. Expression data shown are averages from three independent biological experiments for each T-cell population.



clusters I (IL23A, NR4A2, CD83) and IV (PSMB8, PSME2, MIF, IFI16, TNFAIP1, POU2AF1, and OTUB1), which shared similar gene expression patterns between CD4+ and CD8+ T cells, but different than those of CD3+ T cells; (2) genes in cluster II (XCL1, SLAMF7, BRDG1, CD69, TNFRSF9 and CD40LG (TNFSF5)) with different gene expression patterns among the 3 populations, and cluster III (CSF3, IL1RN, BCL6 and TNFSF4) with different gene expression patterns between the CD4+ and CD8+ populations. Within the downregulated cluster B, there are a few genes with different expression patterns in cluster V (AOAH, CD8A, -B, KLRC1, -2, -4, KLRD1, CD48, CCL5, S100B, CD244, PF4, IGLL1, IGJ, FCER1A, ITGB1, TRIM22, TNFRSF25, LCK and ZAP-70), mainly between CD4+ and CD8+ T cells, and cluster VI (IFIT5, ISGF3G, IL6R, CD164, HLA-E, TYROBP and TNFRSF1A), mainly between CD3+ T cells and CD4+, CD8+ subsets.

Genes sharing similar expression patterns between CD4+ and CD8+ T cells, but different than CD3+ T cells, are likely important players in the communication between CD4+ and CD8+ compartments. Although not previously associated with T-cell activation, the decreased upregulation of NR4A2 (coactivator of general gene transcription) (61) and increased upregulation of IFI16 (transcriptional repressor) (62) and PSMB8, PSME2, and OTUB1(proteases) (63-65) are possibly involved in the delayed T-cell activation and proliferation of the CD4+ and CD8+ subsets compared to the CD3+ T cells. The significant downregulation (at 48-96 hours) of IGLL1 and IGJ (Immunoglobulins), FCER1A (receptor), CD164 (negative regulator proliferation) and IRF9 (transcription factor) in CD3+ T cells, but not in CD4+ or CD8+ subset suggests that these proteins are affected by the co-presence or and/or communication between the two subsets. Genes with different expression patterns between

CD4+ and CD8+ subsets are possibly involved in cell-type-specific development of characteristics and functions. For instance, expression of TNFSF5 (CD40LG) has been mainly reported in CD4+ T cells, facilitating the activation of CD8+ T cells (66). Indeed, preferential transcriptional upregulation of TNFSF5 was observed in CD4+ T cells, supported by a protein abundance assay (Figure 2.4). Interestingly, TNFSF5 was also upregulated in CD8+ T cells (although not as strongly as in CD4+ T cells), supporting the recently reported expression of TNFSF5 in CD8+ T cells in the absence of CD4+ T cells (67). This is an example demonstrating that, with the comparative analysis of the expression patterns among the 3 populations, our data capture significant differential transcriptional events. Transcriptional differences between CD4+ and CD8+ subsets were validated and supported by protein abundance assays of selected genes (TNFSF4, -5, -RSF9, KLRD1, CD48 and CD69) (Figure 2.4). Some of these genes (encoding cytokines and receptors) are discussed in detail below.

Cytokines act as messengers between cells, regulating their functions and activity. The production of cytokines is precisely controlled temporally in the immune response, and so are cytokine receptors. Thus, the significantly regulated cytokines and cytokine receptors were sorted based on their functions listed by NCBI website (http://www.ncbi.nlm.nih.gov/) (Figure 2.5 and Figure 2.6) and are discussed below.

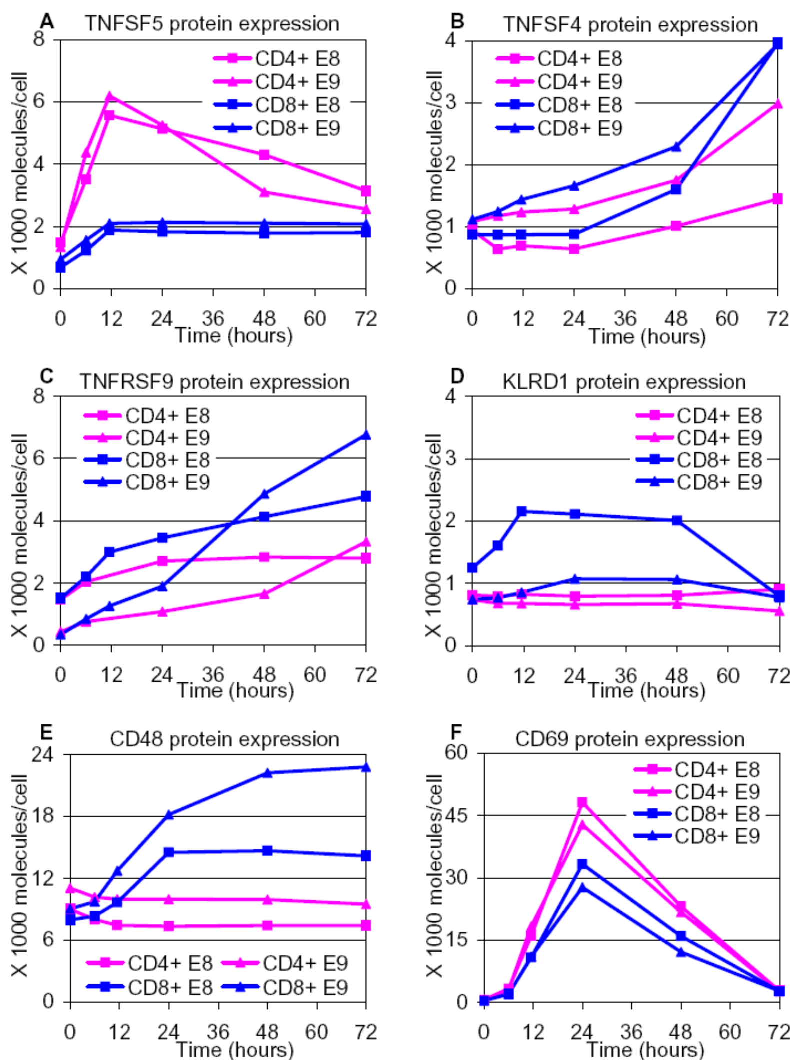


Figure 2.4. Protein expression profiles supported the different transcription patterns between CD4+ and CD8+ subsets.

CD4+ T cells and CD8+ T cells were selected, stimulated (with anti-CD3/anti-CD28 antibodies), cultured separately and harvested at the indicated timepoints of culture. Flow cytometric assays were carried out for the selected genes with different transcription patterns between CD4+ and CD8+ subsets ((A) TNFSF5, (B) TNFSF4, (C) TNFRSF9, (D) KLRD1, (E) CD48, and (F) CD69). Data from two independent experiments, E8 and E9, are shown.

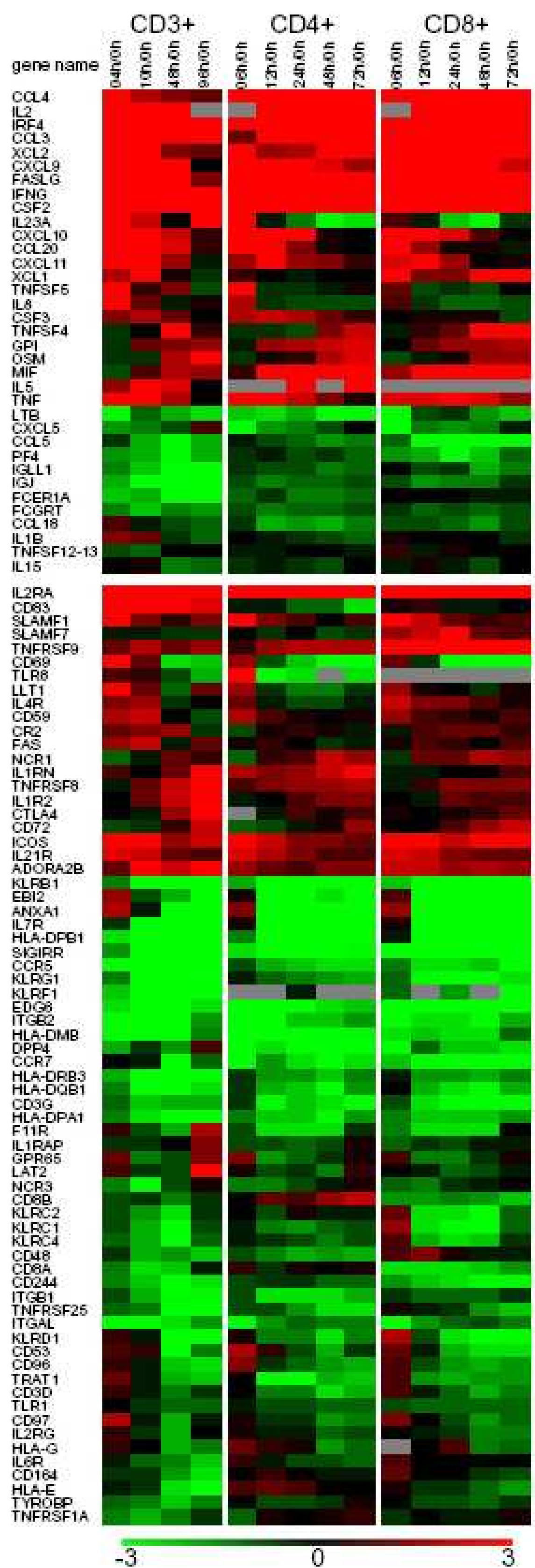


Figure 2.5. Transcription profile of significant (A) cytokines and (B) cytokine receptors.

Cytokines and cytokine receptors, belonging to the 'immune response' Gene Ontology category, were sorted. Membership to these sets was manually curated from the corresponding gene pages in NCBI (68) and references therein. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population.

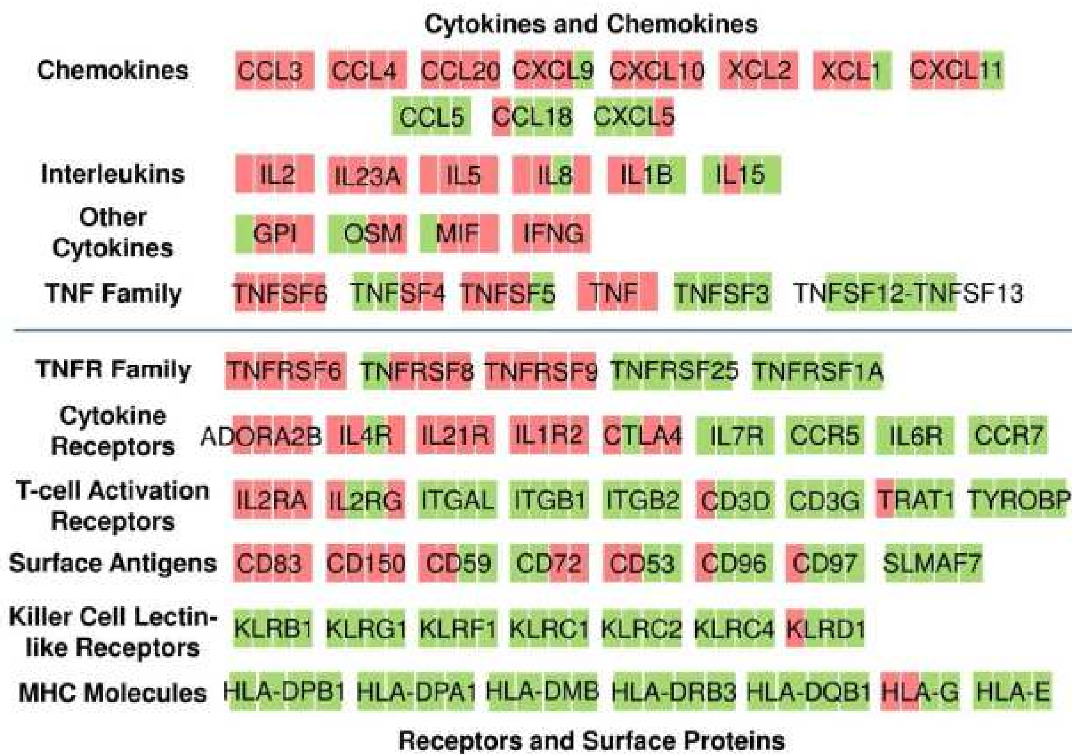


Figure 2.6. Schematic showing the significantly regulated genes of cytokines and receptors.

Membership to these sets was manually curated from the corresponding gene pages by NCBI website (http://www.ncbi.nlm.nih.gov/) and references therein. The regulation of gene transcription in CD3+ T cells, compared to 0 hour, is denoted by different color (green: downregulation; red: upregulation) at each timepoint in the sequence of 4, 10, 48 and 96 hours.

2.3.3 Cytokines and Chemokines

A group of chemokines (CCL3, CCL4, CCL20, CXCL9, -10, -11, XCL1 and XCL2) showed a steady increasing expression upon anti-CD3/anti-CD28 stimulation in all 3 populations with the exception that XCL1 was not upregulated in CD4+ T cells. The strong upregulation of these genes is likely responsible for the proinflammatory response of T cells, including the recruitment of T cells as well as other leukocytes to the sites of inflammation. CCL20 is mainly secreted by epithelial cells and macrophages (69), and CXCL9, -10, -11 are mainly secreted by dendritic cells and macrophages (70, 71); their expressions have not been reported in T cells. Supernatant ELISA assays confirmed the significant continuous transcription upregulation of CCL20 (Figure 2.7), suggesting the induction of CCL20 secretion in T-cell activation. The preferential expression of XCL1 and XCL2 in CD8+ T cells suggests that these proteins might have roles in activation and/or functions of cytotoxic T cells (72). A few chemokines showed high expression in resting T cells (CCL5, CCL18 and CXCL5), suggesting their importance in the homeostasis of resting T cells in the peripheral immune system. Interferon gamma (IFNG), INFG-inducible protein 16 (IFI16), IFN regulatory factor 4 (IRF4), -1, and IFNG-inducible Guanylate binding proteins (GBP1 and -2) were all upregulated (Figure 2.5 and Figure 2.8A). Supernatant ELISA assays revealed that the secreted IFNG protein level continuously increased throughout the 96 hours (Figure 2. 8B). IFNG has important immunoregulatory functions such as antiviral and anti-tumor activity, and as an activator of macrophages (73), yet its functions in T-cell activation remains unknown. The orchestrated transcriptional regulation of IFN regulatory factors, IFNG, and INFG-inducible proteins and the significant induction of IFNG protein secretion suggest that IFNG secreted by T cells has an important role in T-cell activation.

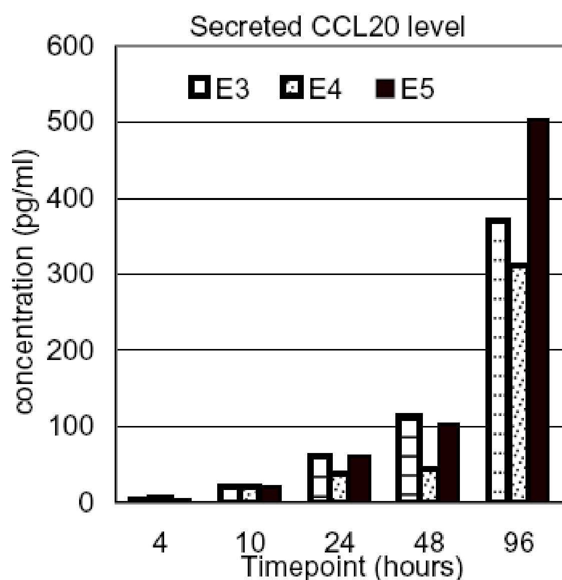


Figure 2.7. Supernatant ELISA analysis of CCL20 secretion in three independent CD3+ T-cell experiments.

CD3+ T cells were selected, stimulated (by anti-CD3/anti-CD28 antibodies) and supernatants harvested at the indicated timepoints of culture. Data from three independent experiments E3-E5 are shown.

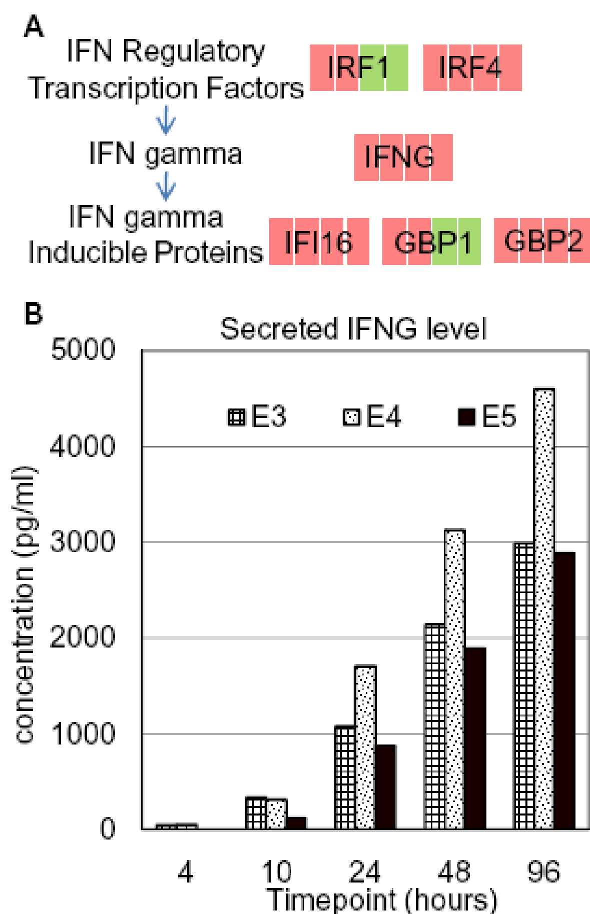


Figure 2.8. Regulation of IFNG in T-cell activation.

(A) Schematic showing the significantly regulated genes associated with IFNG. The regulation of gene transcription in CD3+ T cells, compared to 0 hour, is denoted by different color (green: downregulation, red: upregulation) at each timepoint in the sequence of 4, 10, 48 and 96 hours. (B) Supernatant ELISA analysis of IFNG secretion in three independent CD3+ T-cell experiments, E3-E5. CD3+ T cells were selected, stimulated (by

anti-CD3/anti-CD28 antibodies) and the supernatants were harvested at the indicated timepoints of culture.

Some of the interleukins (IL2, -23A, -5 and -8) were also upregulated, and with distinct patterns. The expression pattern of IL23A in CD3+ T cells was significantly different than that in CD4+ or CD8+ T cells. Expressed by activated dendritic cells, IL23 has anti-tumor effects through inducing CD4+ T-cell proliferation and its anti-tumor effects are reportedly to be inhibited by the depletion of CD4+ or CD8+ subset (74). These expression differences among the three populations suggest that IL23A might be implicated in the communication between CD4+ and CD8+ T cells. Early upregulation of IL5 (reportedly a B-cell and eosinophil differentiation factor) (75) and IL8 (a neutrophil-activating factor) (76) suggests that they might have important roles in T cells as activating/differentiation factors. The downregulation (at 48-96 hours) of IL1B, mainly secreted by macrophages (77), and IL15, an important cytokine in lymphocyte survival (78), upon anti-CD3/anti-CD28 stimulation was unexpected.

A few members of the TNF family (TNFSF6 (FASLG), -4, -5 and TNF) were upregulated while others (LTB (TNFSF3) and TNFSF12-TNFSF13) were downregulated. A preferential expression in CD8+ T cells especially at 72 hours was observed for TNFSF4. TNFSF4 has been hypothesized to have costimulatory functions in both CD4+ and CD8+ subsets (79, 80). However, no subset-specific functions of TNFSF4 have been reported. Flow-cytometric analysis confirmed its significant upregulation at 72 hours in CD8+ T cells (Figure 2.4B). This preferential expression suggests that in CD8+ T cells, TNFSF4 might play an important role, possibly with cytotoxic effector functions besides the reported costimulatory functions.

A few cytokines (GPI, OSM and MIF) also displayed increased expression mainly at 10-96 hours. Neither the expression nor the functions of these genes have been previously reported in T cells.

2.3.4 Receptors

The upregulation of several key receptors (IL2RA (CD25), ICOS, and IL21R) was expected and confirms the validity of our data. However, the temporal expression patterns of these upregulated receptors, including those expressed throughout these experiments (IL2RA (CD25), ADORA2B), early (IL4R, IL21R, FAS) or late (IL1R2, TNFRSF8 and CTLA4), provide new insights that reflect their roles in T-cell activation. For instance, the late upregulation of CLTA4 is consistent with its inhibitory functions in T-cell activation (79). The upregulation of FAS at 4-10 hours implies that FAS might have facilitating functions in early T-cell activation in addition to its known role in inducing apoptosis in fully activated cells (81). The IL4 receptor is critical for inducing the development of the Th2 lineage of effector T cells (82). The simultaneous early upregulation of IL4R and IL5 (a signature cytokine of Th2 cells) suggests that the Th1/Th2 balance might be biased towards the Th2 direction in our experiments. Significant differential expression of receptors, which have not been reported in T cells, call for attention to their possible role in T-cell activation. These include the upregulation of IL1R2 (as well as its binding proteins IL1RN and IL1RAP) at 96 hours and the constant upregulation of ADORA2B (G protein-coupled adenosine A2b receptor). Extensive upregulation of TNFRSF9 was observed in CD8+ subsets through 6-72 hours, but not in CD3+ T cells or CD4+ T cells. Flow cytometry analysis supported this preferential expression of TNFRSF9 in CD8+ T cells at the protein level (Figure 2.4C). TNFRSF9 has been reported as a costimulatory receptor in T

cells, but not in a subset specific manner [25]. This continuous strong upregulation of TNFRSF9 in CD8+ T cells indicates its specific involvement in the activation, proliferation and differentiation of cytotoxic T cells.

Transcription of a number of other receptors, not previously reported in the context of T-cell action, was downregulated, likely to help achieve an efficient T-cell activation. These include IL7R (which shares the IL2 receptor gamma chain (IL2RG) with IL2RA) (83), CCR5, TNFRSF25 and TNFRSF1A (apoptosis inducing receptors) (84, 85), CCR7 (enabling cells for secondary lymphoid organ homing) (86) and IL6R (regulating cell growth and differentiation in neutrophils) (87). The unexpected downregulation of IL2RG (as opposed to the strong upregulated IL2RA) and of integrins (ITGAL, ITGB1 and ITGB2), the components of LFA-1 (reportedly a receptor for costimulatory signal in T-cell activation (88)) was somewhat surprising and deserve attention. .

Our data reveal the transcription dynamics of a few preciously reported upregulated cell-surface antigens including CD83 (89) (expressed throughout) and SLAMF1 (CD150) (90) (expressed early), and novel ones such as CD59 (expressed early) and CD72 (expressed late). Interestingly, CD83 showed increased expression upon stimulation in CD3+ T cells, decreased expression at 24-72 hours in CD4+ T cells, but no change in CD8+ T cells. CD83 expressed on dendritic cells delivers costimulatory signals to T cells for activation (91), and CD83 expressed on T cells has been hypothesized to be involved in T-cell activation with its detailed function yet to be defined (89). These apparently different expression patterns among the three populations indicate that CD83 might have different roles in CD4+ versus CD8+ subsets, and may be possibly implicated in the communication of the subsets. In contrast to CD3+ or CD4+ T cells,

SLAMF7 showed significant increased expression in CD8+ T cells at 6-24 hours. SLAMF7 regulates the cytotoxity of NK cells (92), and migration/adhesion of B cells (93); however, its function in T cells has not been reported. This preferential expression of SLAMF7 in CD8+ T cells suggests that it might be involved in the development of the cytotoxicity of CD8+ T cells.

Among the mainly downregulated cell-surface antigens, the simultaneous downregulation of CD3D, CD3G, TRAT1 (TCR-associated transmembrane adaptor 1) and TYROBP (ZAP-70 binding protein) along with the downregulation of LCK and ZAP-70 (TCR associated tyrosine kinases) are likely part of the orchestrated regulation of T-cell activation. CD53, CD96, and CD97 shared similar expression patterns (upregulated at 4 hours and then downregulated at 48-96 hours). However, little is known about their roles in T-cell activation. We also observed the downregulation of CD8B and CD8A in CD8+ T-cell, possibly as a part of the CD8+ T-cell activation machinery. Interesting, binding partners, cell-surface antigens CD48 and CD244, demonstrated opposite expression patterns. CD48 was continuously downregulated in CD4+ T cells, but not in CD8+ T cells; while CD244 was continuously downregulated in CD8+ T cells, but not in CD4+ T cells. It has been hypothesized that T cells costimulate each other through the interactions of CD244 and CD48 (94). This cell-type-specific downregulation of CD48 (CD4+ T-cell) and CD244 (CD8+ T-cell) suggests that this CD244-CD48 interaction might be cell-type-specific as well between the CD244 expressing CD8+ T cells and CD48 expressing CD4+ T cells.

Upon activation, killer cell lectin-like receptors (KLRB1, KLRG1, KLRF1, KLRC1, -2, -4, and KLRD1) showed mainly decreased expression. First discovered in NK cells, the expression of inhibitory receptors (KLRG1, KLRB1, KLRC1, and KLRD1) (95, 96), and

activating receptor KLRC2 (97) has also been reported in activated CD8+ T cells, involved in TCR signaling, but not in activated CD4+ T cells or naive T cells. The shared downregulation of these KLRs along with that of some less-well-studied members (KLRF1, and KLRC4) suggests that their function might not be limited to effector CD8+ T cells, and that they are implicated more broadly in T-cell activation. Surprisingly, members of both MHC classes I and II were expressed in resting T cells (0 hour; data not shown). Several genes encoding MHC class II members (HLA-DPB1, HLA-DMB, HLA-DRB3, HLA-DQB1, and HLA-DPA1) showed decreased expression upon stimulation, while some MHC class I molecules (HLA-G and HLA-E) were downregulated at 48-96 hours.

2.3.5 Granzymes

Secretion of cytotoxic granules is one of the major effector functions of cytotoxic T cells to induce apoptosis in target cells(98). Perforins, granulysin and granzymes are the core components of the dense cytotoxic granules responsible for target cell lysis. Most of the granzyme genes (GZMB, -A, -H, -K, except for GZMM), but not GNLY and PRF1, were significantly regulated (Figure 2.9A). GZMA and GZMK were considerably downregulated throughout the experiments. It is likely that T cells have not acquired the full cytotoxic effector functions at this stage of the activation. However, GZMB was transcriptionally upregulated in both CD4+ and CD8+ T cells, and this was supported by data from a protein abundance assay (Figure 2.9B).

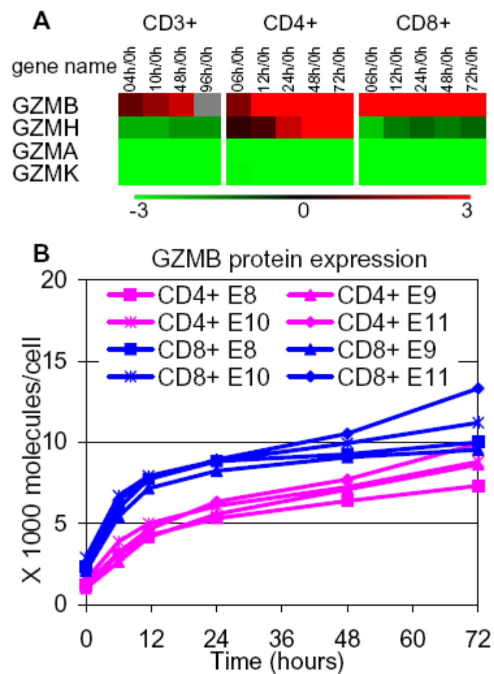


Figure 2.9. GZMB was upregulated continuously in all three populations.

(A) Transcription profile of significantly regulated granzymes (GZMB, -A, -H, and –K). Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population. (B) Intracellular protein expression profiles of GZMB in the CD4+ and CD8+ subsets. CD4+ T cells and CD8+ T cells were selected, stimulated (by anti-CD3/anti-CD28 antibodies), cultured separately and harvested at the indicated timepoints of culture to analyze the protein expression via by flow cytometric assays. Data from four independent experiments, E8-E11, are shown.

2.4 DISCUSSION

Genome-scale transcriptional profiling can add significant new information for better understanding of T-cell activation as an important biological process of the immune response. Previous efforts had examined T-cell activation at one single time point (50, 51) or addressed costimulatory signal effects using only a single experiment (37, 38). Using multiple donors, in this study we focused on the temporal, donor-independent gene expression patterns not only in the CD3+ T cells, but also in the CD4+ and CD8+ subsets. We identified donor-independent significantly regulated genes in CD3+ T cells (from the activation of co-cultures of CD4+ and CD8+ T cells in their natural ratio), and the CD4+ and CD8+ subsets. The CD3+ T cells had far more significantly differentially expressed genes than those in the activation of the CD4+ and CD8+ T cells (Figure 2.2). Regardless, the transcription profiles of the pooled significant genes in T-activation shared largely similar patterns among the three populations.

Anti-CD3/anti-CD28 stimulation induced expression pattern changes of ‘immune response’ genes which are consistent with the important roles of T cells in adaptive immune response. Not only well-known cytokines (e.g., IL2, IFNG, TNFSF6 (FASLG)) and cytokine receptors (IL2RA (CD25), CD69, ICOS), but also numerous novel ones, for T-cell activation, were differentially expressed. Among the novel cytokines, strongly upregulated genes (e.g., CCL3, -4, CCL20, CXCL9, -10, -11) might have important roles in enhancing T-cell activation in addition to their functions in cell trafficking. Downregulated genes (CCL5, IL5) might rather be involved in maintaining the homeostasis of resting T cells.

A number of cell-surface receptors not previously associated with T-cell activation, were differentially expressed including downregulated CCR5, TNFRSF25, TNFRSF1A, CCR7 and IL6R, and upregulated CD59 and CD72. A number of receptors involved in TCR activation (CD3D, CD3G, TRAT1, ITGAL, ITGB1, ITGB2, CD8A and CD8B (CD8+ T-cell specific) along with LCK, ZAP-70 and TYROBP, were all simultaneously downregulated. Little is known about the expression and functions of KLRs in resting T cells. The downregulated KLR receptors are likely involved in the homeostasis of resting T cells. It is also possible that they regulate T-cell activation through TCR signaling. Surprisingly, both MHC class I and class II molecules were transcriptionally expressed in resting T cells (0 hour) (data not shown), and MHC class II molecules were significantly downregulated upon T-cell activation. MHC Class I and class II molecules are receptors on APCs, but not T cells, for the activation of CD8+ and CD4+ T cells, respectively.

Comparison of the expression patterns among the three populations provided further insights. Different expression patterns between CD4+ and CD8+ T cells (XCL1, -2, SLAMF7, CD244, CD48, TNFRSF9, TNFSF4, -5, CSF3, and GZMH) were observed, suggesting their subset specific involvement/functions in T-cell activation. Among these genes, very little is known about XCL2 and SLAMF7 in T cells. Genes (IL23A, NR4A2, CD83, PSME2, PSMB8, MIF) with similar expression patterns between CD4+ and CD8+ T cells, but different than those in CD3+ T cells are likely involved in the communication between CD4+ and CD8+ subsets. These and the large number of novel genes in the context of T-cell activation that were identified in this study offer new research targets fro a more complete understanding of T-cell activation.

2.5 CONCLUSIONS

In conclusion, our study captured novel temporal patterns of previously known but many novel, in the context of T-cell activation, genes ontologically classified under the term ‘immune response’. These patterns were reproducibly and robustly identified as donor independent, and were partially confirmed by Q-RT-PCR and protein-level assays. Comprehensively integrating previous knowledge, we identified novel significant genes associated with immune response in T cells, as well as subset specific genes, and genes implicated in the communication between CD4+ and CD8+ T cells. This study improves our understanding of the biology and the underlying regulation of T-cell activation in the natural combined CD3+ population, as well as in the CD4+ and CD8+ subsets.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3: A GLOBAL TRANSCRIPTIONAL VIEW OF APOPTOSIS IN HUMAN T-CELL ACTIVATION

3.1 INTRODUCTION

The adaptive immune response starts with the activation of the naive CD4+ and CD8+ T cells in the peripheral immune system. Successful T-cell activation requires the T-cell receptor complex (TCR) and the co-receptor CD28 (47), the ligation of which leads to several downstream signaling events, including activation of protein kinases such as LCK and ZAP-70, activation of MAP kinase cascades, and activation and nuclear localization of crucial transcription factors including AP-1, NFAT, and NF-κB (3). In contrast, TCR signaling alone without CD28 co-stimulation results in anergy and eventual cell death (99).

Apoptosis has been extensively examined in T cells post activation, such as activation-induced cell death (AICD), due to its essential role in eliminating unwanted lymphocytes and maintaining the homeostasis after fighting infection and inflammation (100). However, the regulation of apoptosis and the balance between the anti-apoptotic and pro-apoptotic signaling (which is an essential part of the surveillance machinery) during the process of T-cell activation have not been examined.

Genome-scale transcriptional analysis is a powerful tool for understanding complex processes such as T-cell activation (37, 38). In a previous effort, using ontological analysis coupled with a comparative analysis of primary human T-cell activation in the CD3+ T cells and the two subsets, CD4+ and CD8+ T cells, we probed the common and potentially subset-specific

immune response-associated transcriptome in T-cell activation (101). In this study we focus on the differentially expressed genes associated with regulation of apoptosis, as well as essential apoptotic signaling pathways: the NF-κB signaling pathway, and MAP kinase signaling. We identified several potentially important apoptotic genes based on their patterns of expression and examined the protein expression of a select set of genes, most of which have not been previously discussed in T-cell activation.

3.2 MATERIALS AND METHODS

3.2.1 Cells and culture system

CD3+, CD4+ and CD8+ T-cell cultures were set up as previously described (101). Briefly, negatively-selected T cells (CD3+, CD4+, and CD8+) were activated with anti-CD3/anti-CD28 Mab conjugated to magnetic beads. Cell counting and sampling for flow cytometry and microarray analysis were carried out at 0, 4, 10, 48 and 96 hours in the CD3+ T-cell experiments, and at 0, 6, 12, 24, 48 and 72 hours in the CD4+ T-cell and CD8+ T-cell experiments. This study was approved by the Northwestern University IRB.

3.2.2 Flow cytometry

The following monoclonal antibodies (Mabs) for flow cytometry were purchased from BD Biosciences (San Jose, CA) unless otherwise stated and included CD3 (FITC+PE), active CASP3 PE, phospho-NFκB-p65 PE, phospho-p38 (MAPK14) PE, phospho-ERK1 (MAPK3) PE, PUMA (BBC3) (Cell Signaling Technology, Danvers, MA), BCL2A1 (Abcam, Cambridge, MA) and goat anti rabbit IgG PE (Jackson ImmunoResearch Laboratories, West Grove, PA). Flow cytometry was carried out as described (54, 55). Briefly, all samples were gated on forward scatter and on propidium iodide negative (PI-) to eliminate debris and dead cells. For intracellular detections, cells were first stained with anti-CD3-FITC and then fixed, permeabilized, and stained as previously described (56). Quantibrite beads (BD Biosciences

Immunocytometry Systems) labeled with different amounts of PE molecules were used to quantify surface or intracellular protein levels and normalize measurements between timepoints.

3.2.3 RNA extraction and quality control

Total RNA was extracted from frozen cells using the NucleoSpin RNA II kit (Clontech, Palo Alto, CA). RNA samples were resuspended in RNase-free water and stored at –80°C. RNA yield and purity were assessed by UV spectrophotometric measurements at 260 and 280 nm (Biomate 3, Thermo Spectronic, Marietta, OH). Furthermore, RNA integrity was evaluated using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

3.2.4 Microarray experiments and analysis

Total RNA was extracted, RNA integrity was evaluated and microarray experiments and data analysis were carried out as preciously described (101). Briefly, microarray data were normalized and further analyzed (identification of significant genes, hierarchical clustering, and Gene Ontology assignment) with 'MultiExperiment Viewer (MeV)' from The Institute for Genomic Research (TIGR) (58). Raw and normalized data were deposited in the Gene Expression Omnibus (GSE6607 (CD3+ T-cell experiment), GSE7571 (CD4+ T-cell experiment) and GSE7572 (CD8+ T-cell experiment)) (59). Within each population (three biological replicates using cells from three different donors), multi-class SAM (Significance Analysis of Microarrays) with a false discovery rate of <1% was used to select genes that show statistically different expression between groups. A SAM group is defined here as all the samples belonging

to the same timepoint regardless of donor. Briefly, there were 5 groups (0 hour, 4, 10, 48 and 96 hours) in the set of CD3+ experiments and 6 groups (0 hour, 6, 12, 24, 48 and 72 hours) in the set of CD4+ experiments and CD8+ experiments. Gene expression at each time point was compared to that of 0 hour in each experiment. Gene Ontology annotations, as curated by European Bioinformatics Institute, were retrieved from the Gene Ontology Consortium website (60). The EASE (Expression Analysis Systematic Explorer) score in ontological analysis is a modified Fisher Exact Probability p-value (102) indicating the probability of finding by chance the same degree of enrichment on a Gene Ontology term in a set of genes. The lower the EASE score, the more significant is the enrichment, i.e. the less likely that degree of enrichment can be found by chance. Hierarchical clustering analysis was performed with the Euclidean distance metric. The list of genes associated with NF-κB signaling pathway was curated based on the information of Gene Ontology Consortium (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) per 'positive regulation of I-kappaB kinase/NF-kappaB cascade' and superarray (http://www.superarray.com/) per 'NF-κB Signaling Pathway'. Information of superarray enriched our list with members of the Rel, NF-κB, and IκB families. The list of NF-κB target genes was curated based on the information of website (http://people.bu.edu/gilmore/nf-kb/), a collective information source of NF-κB research based on updated publications. The gene list of MAP kinase signaling pathway was curated and sorted based on information of Kegg website (http://www.genome.jp/kegg/) per 'MAPK signaling pathway', superarray (http://www.superarray.com/) per 'MAP Kinase Signaling Pathway', and NCBI website (http://www.ncbi.nlm.nih.gov/).

3.2.5 AP-1 activity assay

DNA-binding activity of AP-1 was assessed using the TransBinding AP-1 ELISA kit (Panomics; Fremont, CA) as described (54). Briefly, nuclear extracts were incubated with biotinylated AP-1-consensus-binding-sequence oligonucleotides and complexes were detected using a primary AP-1 antibody and a secondary antibody conjugated to horseradish peroxidase. This assay is analogous to the traditional electrophoretic mobility shift assay in that it measures the ability of a transcription factor from nuclear lysates to bind to a consensus-binding sequence of that transcription factor, and has been extensively validated (103, 104).

3.3 RESULTS

3.3.1 Anti- and pro-apoptotic genes in T-cell activation

As previously reported, within each population (CD3+, CD4+ and CD8+ T cells), T cells from three independent biological donors (three biological replicates) exhibited overall similar phenotypic characteristics (101). Briefly, the surface expression of the early T-cell activation marker CD69 and the middle activation marker CD25 (IL2RA) (105) were rapidly upregulated within 10 hours and 24 hours respectively; T-cell proliferation did not start until 48 hours and cell numbers doubled by 96 hours following T-cell activation. Accordingly, we divided our experimental time course into early T-cell activation (0-10 hours), middle and late T-cell activation (10-48 hours), and T-cell proliferation (48-96 hours). Our microarray results have been validated by Q-RT-PCR assays with a selection of fifteen significant genes covering a broad range of expression patterns and intensities (101). We have also established the reproducibility of our genome scale transcription data within each population (CD3+, CD4+ and CD8+ T cells) and across the three populations (101). SAM analysis identified a total of 4167 unique, significant regulated genes in T-cell activation, with similar transcription patterns in three replicate biological experiments within each population (101).

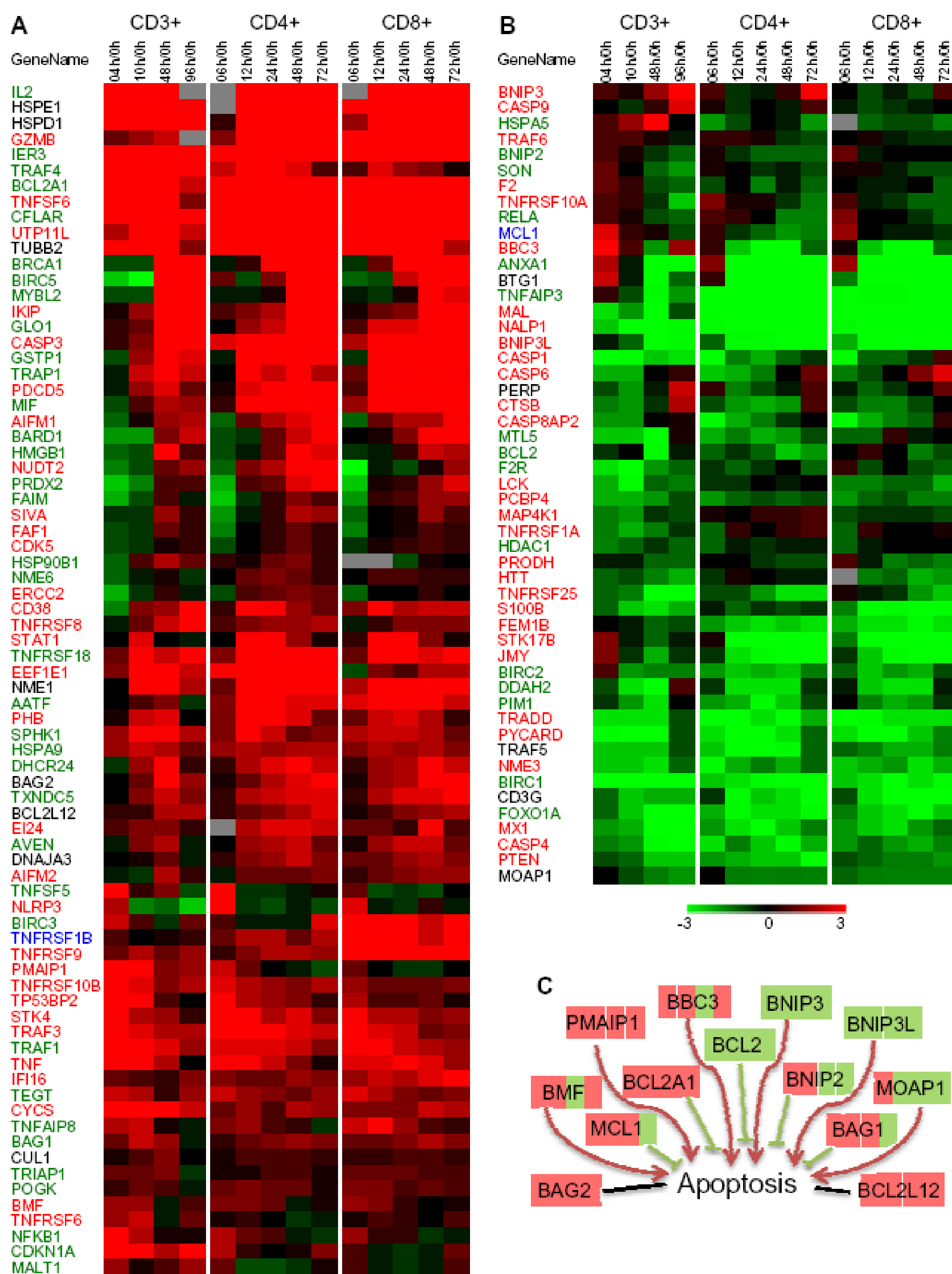
Following SAM analysis, ontological analysis using the MeV EASE module identified 125 significantly regulated genes, associated with ‘regulation of apoptosis’ (EASE score: 7.65E-08), suggesting an active involvement of apoptosis during T-cell activation and proliferation. Made up of both anti- and pro-apoptotic genes, these 125 genes shared well-preserved expression patterns among the three T-cell populations (Figure 3.1A and Figure 3.1B). The mainly

upregulated cluster (Figure 3.1A) contains both anti- and pro-apoptotic genes, and so does the mainly downregulated cluster (Figure 3.1B). This suggests an essential role of the balance between anti- apoptotic and pro- apoptotic signaling in T-cell activation.

Members of the BCL2 family are key regulators of apoptosis. The balance between pro- and anti-apoptotic BCL2 family members determines the cellular fate in response to survival cues and stress signals (106). The functions and transcriptional regulation of these BCL2 family genes in T-cell activation remain largely unexplored. Alves et al. reported several significantly regulated BCL2 family genes in T-cell activation without discussion (107). Our microarray data identified a set of significantly regulated BCL2 family genes reported by Alves (107), but with different transcriptional patterns (Figure 3.1). These included continuously upregulated BCL2A1 (anti-apoptotic), early upregulated MCL1 (myeloid cell leukemia sequence 1) (anti-apoptotic), BMF (BCL2 modifying factor) (pro-apoptotic) and PMAIP1 (pro-apoptotic), late (48 hours) downregulated BCL2 and dynamically (up-down-up) regulated BBC3 (pro-apoptotic). Flow cytometric assays demonstrated the continuous upregulation of BCL2A1 (Figure 3.2A) and upregulation of BBC3 at 0-10 hours and 24-96 hours at the protein level (Figure 3.2B), both of which are consistent with their transcriptional patterns. The BCL2A1 gene has been reported as a direct target of transcription factor NF-κB complex, p65/p50, in T cells (108). This indicates a continuous involvement of BCL2A1 and a constant activity of the NF-κB (p65/p50) complex in T-cell activation. BCL2 has been hypothesized to be able to block T-cell death (109). Several genes of the BCL2 protein family and their interacting proteins, whose functions and transcription regulation have not been discussed in the context of T-cell activation, were differentially expressed. These include upregulated early (anti-apoptotic BNIP2

Figure 3.1. Expression profiles of genes associated with regulation of apoptosis.

Genes that were differentially expressed temporally in T-cell activation of the three (CD3+, CD4+ and CD8+) populations were divided into two groups (**A** with mostly upregulated genes, and **B** with mostly downregulated genes) according to their distinct expression patterns based on hierarchical clustering using the Euclidean distance metric. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population. Pro-apoptotic genes names and descriptions are shown in red, anti-apoptotic genes are shown in green and genes with both pro- and anti-apoptosis roles are shown in blue. Genes with unknown functions in apoptosis are shown in black. **(C)** Schematic view of significantly regulated genes of BCL2 family. Green and red connections denote negative and positive regulation of apoptosis, respectively, based on information of NCBI website (http://www.ncbi.nlm.nih.gov/). Regulation of gene transcription in CD3+ T cells, compared to 0 hour, is denoted by different color (green: downregulation, red: upregulation) at each timepoint in the sequence of 4, 10, 48 and 96 hours.


A
CD3+
CD4+
CD8+
GeneName
04h/0h
10h/0h
48h/0h
96h/0h
06h/0h
12h/0h
24h/0h
48h/0h
72h/0h
06h/0h
12h/0h
24h/0h
48h/0h
72h/0h
IL2
HSPE1
HSPD1
GZMB
IER3
TRAF4
BCL2A1
TNFSF6
CFLAR
UTP11L
TUBB2
BRCA1
BIRC5
MYBL2
IKIP
GLO1
CASP3
GSTP1
TRAP1
PDCD5
MIF
AIFM1
BARD1
HMGB1
NUDT2
PRDX2
FAIM
SIVA
FAF1
CDK5
HSP90B1
NME6
ERCC2
CD38
TNFRSF8
STAT1
TNFRSF18
EEF1E1
NME1
AATF
PHB
SPHK1
HSPA9
DHCR24
BAG2
TXNDC5
BCL2L12
EI24
AVEN
DNAJA3
AIFM2
TNFSF5
NLRP3
BIRC3
TNFRSF1B
TNFRSF9
PMAIP1
TNFRSF10B
TP53BP2
STK4
TRAF3
TRAF1
TNF
IFI16
TEGT
CYCS
TNFAIP8
BAG1
CUL1
TRIAP1
POGK
BMF
TNFRSF6
NFKB1
CDKN1A
MALT1
B
CD3+
CD4+
CD8+
GeneName
04h/0h
10h/0h
48h/0h
96h/0h
06h/0h
12h/0h
24h/0h
48h/0h
72h/0h
06h/0h
12h/0h
24h/0h
48h/0h
72h/0h
BNIP3
CASP9
HSPA5
TRAF6
BNIP2
SON
F2
TNFRSF10A
RELA
MCL1
BBC3
ANXA1
BTG1
TNFAIP3
MAL
NALP1
BNIP3L
CASP1
CASP6
PERP
CTSB
CASP8AP2
MTL5
BCL2
F2R
LCK
PCBP4
MAP4K1
TNFRSF1A
HDAC1
PRODH
HTT
TNFRSF25
S100B
FEM1B
STK17B
JMY
BIRC2
DDAH2
PIM1
TRADD
PYCARD
TRAF5
NME3
BIRC1
CD3G
FOXO1A
MX1
CASP4
PTEN
MOAP1
-3
0
3
C
PMAIP1
BBC3
BNIP3
BNIP3L
BCL2
BMF
BCL2A1
BNIP2
MOAP1
MCL1
BAG1
BAG2
Apoptosis
BCL2L12

at 10-48 hours (anti-apoptotic BAG1 (BCL2-associated athanogene)), and late BAG2, BCL2L12, and pro-apoptotic BNIP3, and downregulated pro-apoptotic BNIP3L and pro-apoptotic MOAP1 (Figure 3.1).

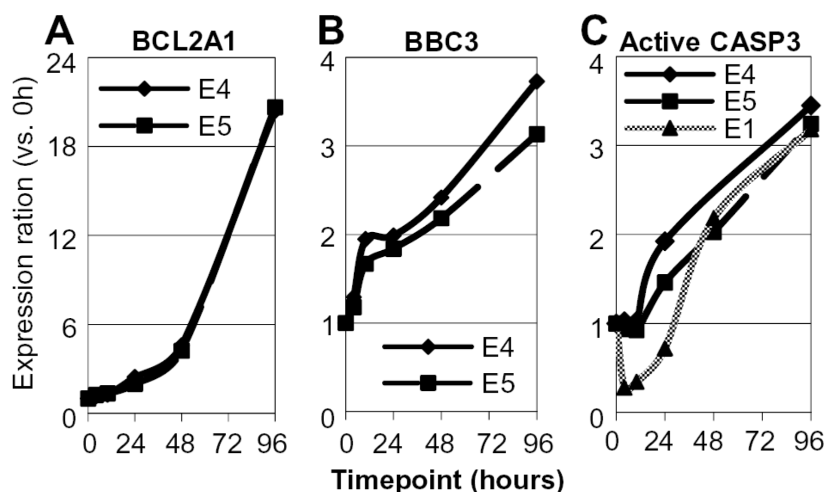


Figure 3.2. Protein expression profiles of (A) BCL2A (B) BBC3 and (C) active CASP3. Protein expression kinetics supports the transcriptional patterns of selected genes demonstrated by microarray analysis. CD3+ T cells were selected, stimulated (with anti-CD3/anti-CD28 antibodies), cultured and harvested at the indicated timepoints of culture to analyze the protein expression by flow cytometric assays. For BCL2A and BBC3, data from two independent experiments, E4 and E5, are shown; for CASP3, data from three independent experiments, E1, E4 and E5, are shown.

Caspases play a central role as executioners in most types of apoptosis, including activation induced cell death (AICD). However they have not been discussed during T-cell activation. Our microarray data show that numerous caspase genes were differentially expressed. Contrary to the signaling mechanism whereby CASP9 becomes activated first, and then in turn

activates CASP3 and CASP6 (100), CASP3 was upregulated at 48-96 hours, earlier than CASP9 and CASP6, which were only upregulated at 96 hours. The flow cytometric assay specific for the active form of caspase 3 protein supported the involvement of CASP3 at 48-96 hours (Figure 3.2C). CASP8AP2, which is required in CASP8 mediating apoptosis (110), was downregulated at 4-10 hours and then upregulated to the same level of resting T cells at 48-96 hours. CASP1, as well as its adaptor PYCARD (111), and CASP4 displayed decreased expression throughout, suggesting that CASP1 and CASP4 might play important roles in the homeostasis of resting T cells, but not in T-cell activation. Caspase regulatory protein AVEN (reportedly an inhibitor of CASP9 activation) (112) was upregulated at 10-48 hours, concomitantly with the upregulation of CASP9 at 96 hours. CFLAR (CASP8 and FADD-like apoptosis regulator) was significantly and continuously upregulated. CFLAR has different roles in T cells at different stages. It has been reported that CFLAR is induced by restimulaiton in activated T cells, inhibiting FAS-mediated apoptosis (113), while overexpression of CFLAR in naive T cells decreased T-cell proliferation upon anti-CD3/anti-CD28 stimulation (114). The strong transcriptional upregulation of CFLAR in T-cell activation, not previously reported, suggests an important role in T-cell activation.

Some heat shock proteins are involved in apoptosis, but none has been discussed in the context of T-cell activation. HSPE1 and HSPD1 shared continuously elevated expression at 4-96 hours. It has been hypothesized that HSPE1 and HSPD1 form a complex with and facilitate the activation of pro-caspase 3 (115). Anti-apoptotic interacting heat shock proteins HSPA9 and HSP90B1 (116, 117) were also upregulated; their role remains unknown in T-cell activation. Of note, reportedly anti-apoptotic HSPA5 (118), demonstrated distinct transcription patterns in the

three populations: it was upregulated in CD3+ T cells especially at 48 hours, but overall downregulated in both CD4+ and CD8+ T cells. HSPA5 has been reported to retain T-cell antigen receptor alpha chain (TCR-alpha) within the endoplasmic reticulum (119). These different expression patterns among the three T-cell populations indicate that HSPA5 might be involved in the interaction between CD4+ and CD8+ T cells in T-cell activation.

Other significantly regulated, and not-previously reported, apoptotic genes in T-cell activation included members of inhibitor of apoptosis protein (IAP) family and genes of programmed cell death (PDCD) proteins. IAPs inhibit apoptosis by interfering with activation of caspase proteins (120). The differentially expressed genes of the IAP family displayed different expression patterns: BIRC1 and BIRC2 were downregulated, BIRC5 was down-then-upregulated, and BIRC3 was up-down-upregulated. Of note, pro-apoptotic PDCD5 and AIF1 (PDCD8) were upregulated at 10-96 hours.

Non-caspase executor proteases, such as Granzyme B (GZMB) and CTSB (cathepsin B), were also significantly regulated. Besides its function in inducing target cell apoptosis, intracellular degranulated GZMB has also been implicated in AICD in TH2 cells (121). Our microarray data revealed that GZMB was continuously upregulated with similar transcription patterns in CD4+ T cell and CD8+ T cells, suggesting an involvement of GZMB in T-cell activation. CTSB has been reported to promote T-cell apoptosis by immune-suppressive anti-T cell agents, mitogen antithymocyte globulins (ATGs) (122). Transcription of CTSB was downregulated first (at 4-48 hours) then upregulated at 96 hours, thus suggesting a role of CTSB in the early stage of T-cell proliferation.

The TNF receptor family plays important roles in extrinsically induced apoptotic pathways. Some TNF receptors have death domains and are directly involved in apoptosis. Transcription of several TNF receptors (TNFRSF6, -8, -9, -18, -1A, -1B, -10A, -10B, and -25) was differentially regulated. Of note, TNFRSF6 (FAS), the well-known AICD receptor, was upregulated at 4-10 hours, but not the other two components of the death-inducing signaling complex (DISC): FADD and CASP8 (123). FAS has also been implicated in multiple pathways including NF-κB, extracellular signal-regulated protein kinase (ERK) 1 and -2, and p38; however, the function of FAS in early T-cell activation has not been reported (100). Contrary to the reported induction of death-receptors TNFRSF1A and TNFRSF25 (DR3) in T-cell activation (124, 125), our microarray data show that both are downregulated, together with TRADD (TNFRSF1A-associated death domain protein), their common adaptor protein. These data suggest that the apoptosis pathways mediated by TNFRSF1A/TRADD and TNFRSF25/TRADD are suppressed upon T-cell activation. TNFRSF1B was upregulated and more significantly so in CD8+ T cells. The function of TNFRSF1B in T cells remains controversial, with both anti-apoptotic and pro-apoptotic functions reported (126, 127). Furthermore, TNFRSF1B has not been reported to be T-cell subset (CD4+ or CD8+) specific. BIRC3 (also known as IAP1), component of the TNFRSF1B signaling complexes inducing apoptosis (128), was more significantly upregulated in CD8+ T cells, similarly to TNFRSF1B. This suggests that the TNFRSF1B signaling might have CD8+ specific functions. A few TNFSF and TNFRSF related proteins, the function of which remain largely unknown, were significantly regulated. These included FAIM (FAS apoptotic inhibitory molecule), FAF1 (Fas associated factor 1), and SIVA

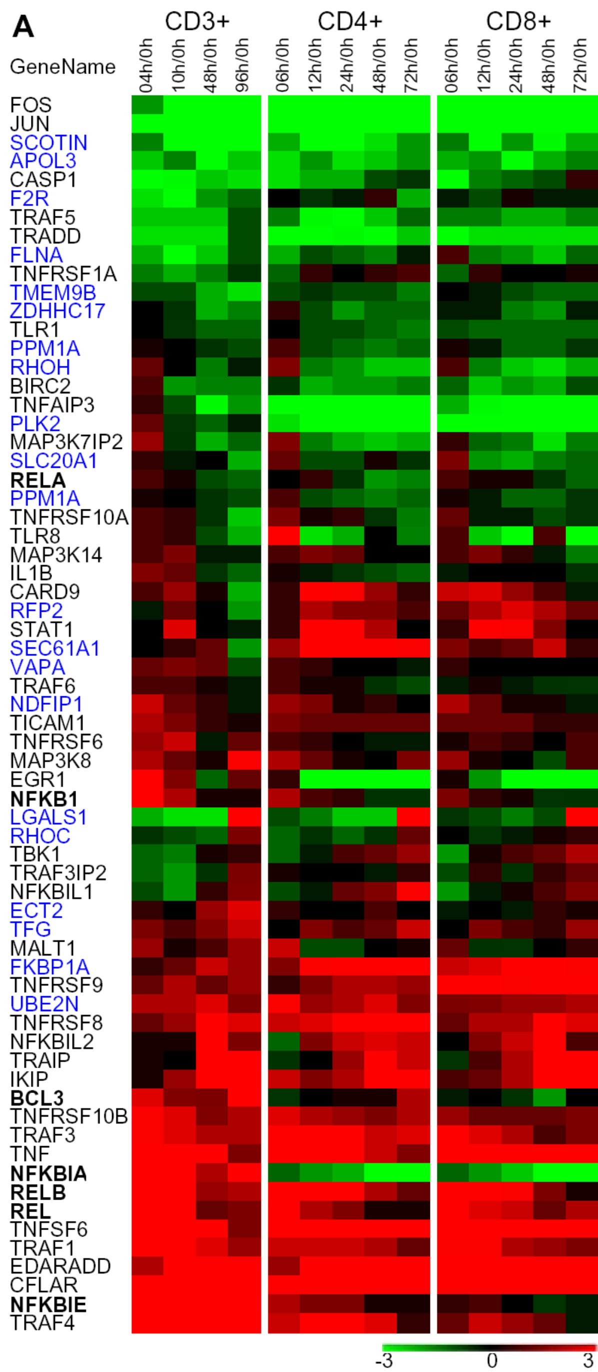
(CD27 (TNFRSF7)-binding protein)), which were downregulated first and then upregulated; and TNFAIP8, which was upregulated first and then downregulated.

Numerous TNF receptors and related proteins involved in NF-κB signaling pathway were significantly regulated in T-cell activation. TNFRSF8, reportedly able to promote apoptosis through inducing the activation of NF-κB complex (129), was significantly upregulated at 48-96 hours. TNFRSF9, reportedly able to promote apoptosis and suppress the activation of NF-κB complex (130), was mainly upregulated. TNFRSF10A (TRAILR1) and TNFRSF10B (TRAILR2), which can interact with several members of TRAFs to activate NF-κB complex (131), together with several members of the TRAF family (TRAF4, TRAF1, TRAF3, and TRAF6) were upregulated. A detailed examination of the transcriptional orchestration of NF-κB signaling pathway in T-cell activation is presented next.

3.3.2 NF-κB signaling pathway

The transcription factor NF-κB complex, a collection of several homodimers or heterodimers of Rel proteins (REL, RELA (p65), RELB, p50 and p52), plays a key role for the regulation of T-cell activation by mediating the induction of various genes that control T-cell proliferation, activation and survival (132). A wide array of stimuli including IL1, and TNF as well as TCR stimulation lead to the onset of cascades that ultimately lead to NF-κB activation (3). Due to the broad range of the upstream signalings and the complexity of the dimers, our knowledge of the orchestrated regulation of NF-κB signaling pathway and activity of the different NF-κB dimers in T-cell activation is far from complete. The transcriptional regulation of significantly regulated genes associated with the NF-κB signaling pathway is shown in Figure

3.3A and Figure 3.5A. NF-κB family genes (REL, RELA (p65), and RELB) and IκB family genes (NFKBIA (the inhibitor of RELA), NFKBIE (inhibitor of REL) and NFKB1 (p105, precursor of p50)) shared similar transcription patterns, an early upregulation at 4-10 hours was followed by a decrease at 48-96 hours. IKIP (IKK interacting protein) was significantly upregulated at 48-96 hours (Figure 3.3A). We also examined the intracellular protein expression of phosphorylated p65, the major active component of the NF-κB complex. Flow cytometric analysis demonstrated an increase of the phosphorylated p65 at 48-96 hours (Figure 3.3B). This activation delay is likely the result of the strong upregulation of NFKBIA at 4 and 10 hours. It is also possible that p65 might quickly become activated within 4 hours leading to the transcriptional induction of NFKBIA, one of the target genes of the NF-κB complex (133). Of



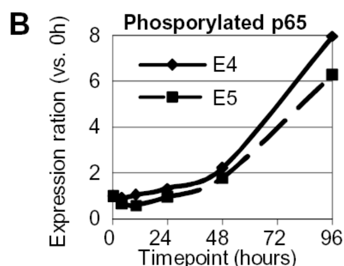


Figure 3.3. Significantly regulated genes in NF-κB signaling pathway.

(A) Expression profiles of genes involved in NF-κB signaling pathway. Color denotes degree of differential expression compared to 0 hour

(saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population. Genes, whose transcription can induce the activation of the NF-κB complex, identified in a large scale screening study (134) were shown in blue. **(B)** Protein expression profile of phosphorylated p65 of NF-κB complex. CD3+ T cells were selected, stimulated (with anti-CD3/anti-CD28 antibodies), cultured and harvested at the indicated timepoints of culture to analyze the protein expression by flow cytometric assays. Data from two independent experiments, E4 and E5, are shown.

note, not only the transcriptional regulation of RELB (maximum fold change of 7.4) was more significant than that of RELA (maximum fold change of 6.5), but also RELB had higher transcriptional levels than RELA. BCL3, a transcriptional coactivator of NF-κB homodimer p50/p50 and p52/p52 (135), was upregulated at 4 and 96 hours.

TLR1 (toll-like receptor) and TICAM1 (toll-like receptor adaptor), which promote activation of the NF-κB complex (136), showed decreased expression at 48-96 hours, suggesting that this upstream activation cascade of the NF-κB complex might not be active during early T-cell proliferation. Also involved in NF-κB complex activity regulation, members of the TNF receptor super family (TNFRSF6, -8, -9, -10A, and -10B), their associated proteins (TRAF1, -3, -4 and -6, EDARADD (ectodysplasin A receptor-associated)) and TRAF interacting protein (TRAIP) showed increased expression. TNFRSF-10A, -10B, TRAF1, -3, and -6 were upregulated at 4-10 hours and TRAIP, which inhibits the TRAF-mediated NF-κB activation (137), was upregulated at 48-96 hours. This orchestrated gene expression regulation suggests that the TRAF-mediated upstream signaling of NF-κB activation is active at 4-10 hours. Furthermore, the early upregulation of MAP3 Kinases (MAP3K14, MAP3K8, MAP3K7IP2) supports the early involvement of the TNF receptor pathway since MAP3 Kinases activate IκB kinases recruited by TRAFs to the TNF receptor complex (138). In contrast, TNFRSF1A, TRADD (TNFRSF1A-associated via death domain) and TRAF5 were downregulated, suggesting that the reported TNFRSF1A-TRADD-TRAF2 cascade (139) and TRAF5 mediated cascade (140), regulating the activation of NF-κB complex, might not be active in T-cell activation.

TCR specific signaling protein MALT1 (through the CARD11-BCL10-MALT1 complex) was recently reported to be required for optimal NF-κB activation through proteolysis of the NF-κB inhibitor TNFAIP3 (141, 142). However the transcriptional regulation of MALT1 and TNFAIP3 in T-cell activation has not been reported. Our microarray data demonstrated that MALT1 was upregulated at 4 and 96 hours, and TNFAIP3 was upregulated at 4 hours and downregulated at 10-96 hours. CARD11 and BCL10 were not identified as significantly regulated, however. CARD9, the equivalent gene of CARD11 in dendritic cells (143), was upregulated at 4 and 10 hours, suggesting its involvement in T-cell activation.

Matsuda et al. identified genes whose transcription can induce the activation of the NF-κB complex by introducing cDNA clones of full-length human cDNA libraries to HEK 293 cells (134). Our microarray data demonstrated that several of these genes were significantly regulated with different transcription patterns (gene names shown in blue in Figure 3.3A). Some of these genes were upregulated, such as UBE2N, ECT2, TFG, and FKBP1A; while others were downregulated, such as SCOTIN, APOL3 and FLNA. Two NF-κB inhibitor-like proteins (NFKBIL1 and NFKBIL2), whose function has not been determined, were significantly upregulated at 96 hours and 48 hours respectively, suggesting that they are involved in early T-cell proliferation and late T-cell activation, respectively.

Some genes (EGR1, NFKBIA and NFKBIE) had significantly different expression patterns in CD4+ and CD8+ T cells compared to CD3+ T cells. NFKBIA and NFKBIE are inhibitors of REL proteins, and EGR1 has been reported to inhibit the activity of RELA (144). It is possible that the communication between CD4+ and CD8+ T cells may affect the activation of the NF-κB complex.

Activation of the NF-κB complex is further supported by the significant regulation of many target genes, whose transcription is regulated by NF-κB complex upon anti-CD3/anti-CD28 stimulation (Figure 3.4). Interestingly, some of these target genes showed higher expression in resting T cells.

Figure 3.4. Expression profile of NF-κB target genes in T-cell activation of the three (CD3+, CD4+ and CD8+) populations. Hierarchical clustering (Euclidean distance metric) shows the temporal expression. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population.

3.3.3 MAP kinase signaling pathway

Mitogen-activated protein (MAP) kinases are important signaling mediators in regulation of apoptosis, including the anti-apoptotic ERKs, the anti-/pro-apoptotic c-Jun N-terminal kinases (JNKs), and anti-/pro-apoptotic p38-MAPKs. Yet the mechanisms as to how these MAP kinases regulate apoptosis remain controversial (145). It has been suggested that the three main mammalian cell MAP kinase cascades, JNK, p38 and ERK, are essential for T-cell functions (146, 147). However, the temporal regulation of these MAP kinase cascades in T-cell activation remains largely unexplored. Thus, we focused on the significantly regulated genes involved in MAP kinase signaling pathway (Figure 3.5B and Figure 3.6). A few genes of the RAS family and RAS regulating protein were upregulated upon anti-CD3/anti-CD28 T-cell stimulation, including HRAS (at 10-48 hours), KRAS (at 4 hours), NRAS (at 4-96 hours) and ADORA2B (at 4-96 hours). HRAS, KRAS and NRAS have been reported to be activated shortly after TCR stimulation (148), however the regulation of their expression (either at the transcriptional or protein level) has not been reported. ADORA2B reportedly regulates ERK and p38 MAP kinase cascades in mast cells (149), but its function in T cells is not known. Contrary to upregulated RAS genes, several upstream RAS regulating proteins (RASGRP1, -2, ADRB2, GRAP, ERBB2) showed higher expression in resting T cells (Figure 3.6A). The downregulation of RASGRP1 and GRAP does not correlate with their reportedly positive roles in T-cell receptor signaling (150, 151). Little is known about RASGRP2, ADRB2, and ERBB2 in the context of T-cell activation. Contrary to the reported activation of RAC proteins CDC42 and RAC2 (152, 153) in T-cell activation, here we found that their transcription was downregulated.

Several kinases in the MAP kinase signaling pathway were differentially expressed. A few kinases upstream of the JNK cascade (MAP4K2 (154), MAP4K1 (155), MAP3K12 (156),

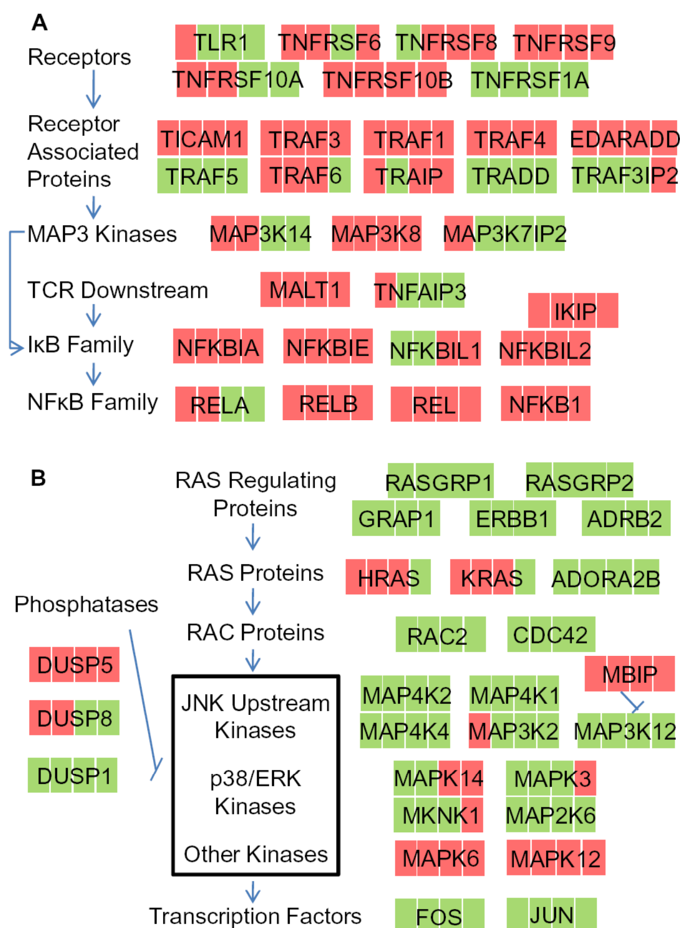


Figure 3.5. Pathway schematic of significantly regulated genes in (A) NF-κB signaling and (B) MAP kinase signaling.

Membership was manually determined from the corresponding gene pages in NCBI (http://www.ncbi.nlm.nih.gov/) and references therein. The regulation of gene transcription in CD3+ T cells, compared to 0 hour, is denoted by different color (green: downregulation, red: upregulation) at each timepoint in the sequence of 4, 10, 48 and 96 hours.

MAP4K4 (157), MAP3K2 (158)) were downregulated, and MBIP (MAP3K12 binding inhibitory protein), which inhibits the MAP3K12 mediated JNK activation (159), was upregulated, suggesting that the JNK cascade might not be active at 4-96 hours. MAPK14 (p38), MAPK3 (ERK1), MKNK1 (the interacting protein of both p38 and ERK1 (160)) and MAP2K6 (p38 specific MAP kinase kinase (161)) displayed similar regulation patterns: downregulated at 4-10 hours and then upregulated at 48-96 hours. The flow cytometric assays of phosphorylated p38

and ERK1 confirmed their transcriptional patterns, namely that there was no significant increase of phosphorylated p38 until 24 hours and phosphorylated ERK1 until 48 hours, but large increases after that until 96 hours (Figure 3.6B and Figure 3.6C). Kinases involved in positive regulation of activity of the NF-κB complex, MAP3K14 (162) and MAP3K8 (163), showed increased expression at 4-10 hours which is consistent with expression patterns of several members of NF-κB and IκB family genes (Figure 3.3A). Two less examined kinases, MAPK6 and MAPK12, were mainly upregulated at 4-48 hours and at 10-96 hours respectively, suggesting that they are actively involved in T-cell activation.

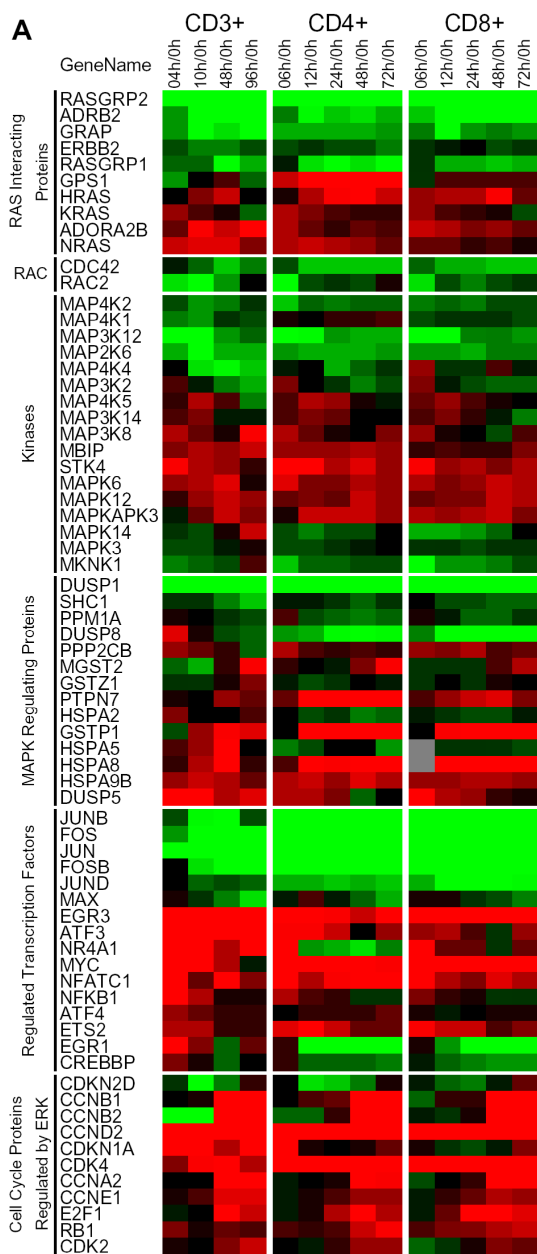
Several MAP kinase regulating proteins were also differentially expressed upon anti-CD3/anti-CD28 activation of T cells. Members of the dual specificity phosphatase family, negatively regulating members of the MAP kinase family, show different expression patterns. DUSP1, able to inactivate ERK1, JNK and p38 (164), was significantly downregulated throughout. In contrast, DUSP5, specific inhibitor of ERK1 (165), was strongly upregulated at 4-10 hours and decreased thereafter in concert with the upregulation of ERK1 at 48-96 hours at both the transcriptional and protein level (Figure 3.6A and Figure 3.6C). DUSP8, of which little is known, was significantly upregulated at 4 hours and then downregulated at 48-96 hours, which is opposite to the transcriptional pattern of MAPK14 and MAPK3.

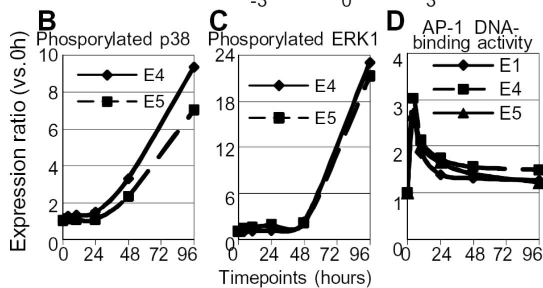
Some transcription factors regulated by MAP kinase pathway were downregulated, and most intensely so were FOS and JUN. FOS and JUN proteins are the main components of the transcription factor complex AP-1. AP-1 has been reported to be quickly activated in response to T-cell activation (166), which is contradictory to the significant downregulation of FOS and JUN. The temporal activity of AP-1 in T-cell activation is not known. Thus, we examined the

DNA-binding activity of AP-1, which rapidly increased within 4 hours and then rapidly decreased (Figure 3.6D). It is possible that FOS and JUN were immediately and transiently regulated upon anti-CD3/anti-CD8 stimulation, and that their upregulation was not captured by our first timepoint following T-cell activation.

Figure 3.6. Significantly regulated genes involved in MAP kinase signaling.

(A) Expression profiles of genes that belong to the list of MAP kinase pathways. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population. **(B)** Protein expression profiles of phosphorylated p38 and of **(C)** phosphorylated ERK1 agree with the late transcription upregulation of MAPK8 and MAPK3. CD3+ T cells were selected, stimulated (with anti-CD3/anti-CD28 antibodies), cultured and harvested at the indicated timepoints of culture to analyze the protein expression by flow cytometric assays. Data from two independent experiments, E4 and E5, are shown. **(D)** DNA-binding activity profile of transcription factor AP-1 captured the immediate and transient activation of AP-1 in T-cell activation. CD3+ T cells were selected, stimulated (with anti-CD3/anti-CD28 antibodies), cultured and harvested at the indicated timepoints of culture. Data from three independent experiments, E1, E4 and E5, are shown.


A
CD3+
CD4+
CD8+
GeneName
04h/0h
10h/0h
48h/0h
96h/0h
06h/0h
12h/0h
24h/0h
48h/0h
72h/0h
RAS Interacting Proteins
RASGRP2
ADRB2
GRAP
ERBB2
RASGRP1
GPS1
HRAS
KRAS
ADORA2B
NRAS
RAC
CDC42
RAC2
Kinases
MAP4K2
MAP4K1
MAP3K12
MAP2K6
MAP4K4
MAP3K2
MAP4K5
MAP3K14
MAP3K8
MBIP
STK4
MAPK6
MAPK12
MAPKAPK3
MAPK14
MAPK3
MKNK1
MAPK Regulating Proteins
DUSP1
SHC1
PPM1A
DUSP8
PPP2CB
MGST2
GSTZ1
PTPN7
HSPA2
GSTP1
HSPA5
HSPA8
HSPA9B
DUSP5
Regulated Transcription Factors
JUNB
FOS
JUN
FOSB
JUND
MAX
EGR3
ATF3
NR4A1
MYC
NFATC1
NFKB1
ATF4
ETS2
EGR1
CREBBP
Cell Cycle Proteins Regulated by ERK
CDKN2D
CCNB1
CCNB2
CCND2
CDKN1A
CDK4
CCNA2
CCNE1
E2F1
RB1
CDK2
-3
0
3


B
Phosporylated p38
C
Phosporylated ERK1
D
AP-1 DNA-binding activity
Expression ratio (vs.0h)
E4
E5
E1
0
24
48
72
96
Timepoints (hours)

3.4 DISCUSSION

In this work, we sought to improve our understanding of regulation of apoptosis in T-cell activation. We approached this problem by analyzing global, temporal microarray data from ex vivo CD3+, CD4+ and CD8+ T-cell cultures, and leveraging Gene Ontology associations and prior knowledge. This approach extended our knowledge base in three ways: we presented detailed kinetic gene expression information on genes with previously hypothesized or presumed important roles in apoptosis; we identified a new set of genes not previously associated with T-cell activation; and, we integrated and connected the previous knowledge with temporal transcription profiles to build a more comprehensive picture of regulation of apoptosis in T-cell activation. Gene expression data were further explored by examining protein expression (active form/phosphorylated form) and functional activity levels as a first assessment of their functional role.

Genome-scale transcription profiling provides the opportunity to more holistically evaluate the regulation of a group of genes, of the same family, or with analogous functions, or associated in specific pathways. Composed of several members, the BCL2 family proteins are key players in regulation of apoptosis. BCL2, BAX, BAK have been reported to play important roles in apoptosis post activation in T cells (100). Knockout of pro-apoptotic BBC3 and PMAIP1 decreased DNA damage-induced apoptosis in mice fibroblasts, but only loss of BBC3 protected lymphocytes from cell death (167). However, the function and transcriptional regulation of most of the BCL2 family members and their regulatory proteins remain unexplored in T-cell

activation. Our data suggest the distinct stages that BCL2 family members are involved in the process of T-cell activation, thus providing directions for future studies. For instance, validated by protein abundance assays (Figure 3.2A and Figure 3.2B), the functions of continuously upregulated BCL2A1 and dynamically regulated BBC3 deserve further studies in T-cell activation. The synchronized early transcriptional upregulation of MCL1 and its inhibitory interacting proteins PMAIP1 (107) and BMF (168) imply their involvement in T-cell activation quickly upon TCR ligation.

In typical apoptosis signaling, CASP9 is activated first, which leads to activation of downstream effectors CASP3, and CASP6. Here, CASP3 was upregulated (at both the transcriptional and protein levels) at 48-96 hours, compared to the upregulation of CASP9 only at 96 hours. These findings suggest that active CASP3 protein might have a different role in T-cell activation and thus its activity might be regulated by other proteins, such as HSPE1 and HSPD1 (Figure 3.1A), rather than CASP9. Surprisingly, the well-known AICD receptor, TNFRSF6 (FAS), was upregulated at 4-10 hours, suggesting the involvement of FAS immediately upon T-cell activation.

Our data show that numerous significantly regulated genes associated with apoptosis are involved NF-κB signaling pathway and MAP kinase signaling pathway. Supported by the increase of phosphorylated p65 at 48-96 hours (Figure 3.3B), the simultaneous upregulation of NF-κB family genes (REL, RELA, and RELB) and IκB family genes (NFKBIA, NFKBIE and NFKB1) at 48-96 hours (Figure 3.3A) suggests that multiple versions of the NF-κB dimmer complex are active during this time period in our experiments. Lack of early detection of phosphorylated p65 could be the result of the strong upregulation of NFKBIA at 4-10 hours. It is

also possible that p65 might immediately and transiently become activated, and then quickly deactivated within 4 hours, our first timepoint. Of note, IKIP (IκB kinase interacting protein) was significantly upregulated at 48-96 hours (Figure 3.3A). To date, the function of IKIP remains unknown. Its transcriptional kinetics suggests that it might have a positive role in NF-κB activity regulation.

Validated by the increase of phosphorylated p38 and ERK1 at 24-96 hours and 48-96 hours, respectively, as measured by flow cytometry assays (Figure 3.6B and Figure 3.6C), the similar transcriptional patterns of MAPK14 (p38), MAPK3 (ERK1), MKNK1 (the interacting protein of both MAPK14 and MAPK3 (160)) and MAP2K6 (p38 specific MAP kinase kinase (161)) (downregulated at 4-10 hours and then upregulated at 48-96 hours) suggest that cascades of p38 and ERK1, but not JNK, are synergistically activated during late T-cell activation and early proliferation. Activity of transcription factor AP-1, mostly regulated by JNK and p38 cascades (169), increased immediately, but only transiently, upon anti-CD3/anti-CD28 stimulation, suggesting a potentially rapid but transient activation of JNK and p38 cascades in T-cell activation. Significantly, there was no activity increase of AP-1 at 48-96 hours, in contrast to the increase of phosphorylated p65. It has been suggested that MAP kinases have different roles in CD4+ and CD8+ T cells (170). However most of the significant regulated genes in MAP kinase signaling pathway shared similar expression patterns between the CD4+ and CD8+ subsets. It is possible that the MAP kinase signaling pathway is regulated similarly between the two subsets in the context of T-cell activation. It is also possible that the MAP kinases might function differently due to regulation at protein level.

3.5 ACKNOWLEDGMENTS

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CHAPTER 4: GENOMIC-SCALE ANALYSIS OF HUMAN T-CELL ACTIVATION IDENTIFIES SIGNIFICANT CELL CYCLE GENES AND MITOCHONDRIA GENES

4.1 INTRODUCTION

4.1.1 Cell cycle

The cell cycle is remarkably conserved among eukaryotes, composed of series of steps, including G0, the phase at which cells are nonproliferative; G1, which defines entry into the cell cycle and is characterized by the synthesis of various enzymes that are required in S phase; S, in which the whole chromosome of the cell is replicated; G2, where the cell finishes significant protein synthesis necessary for the division; and M (mitosis), where the parent cell with the double genetic content divides into two daughter cells. The G1 to S transition is the key step in cell cycle progression. The fidelity of cell cycle progression is strictly regulated by the cell cycle machinery (171).

The cell cycle machinery, mediated by the balance between the proliferative and anti-proliferative signals determining the fate of each cell to enter and go through the cell cycle process in an orderly fashion and then exit the cell cycle, or to undergo programmed cell death (172), is mainly composed of two protein families, the regulatory cyclins and the catalytic cyclin-dependent kinases (CDKs). The CDKs, namely CDK4, CDK6, and CDK2, control the

G1/S transition (173). The activation of CDKs starts with the association with a cyclin subunit, followed by phosphorylation/dephosphorylation of specific amino acids. The cyclins that are active in different parts of the cell cycle and regulate the activity of corresponding CDKs. CDKs have been characterized into several different subtypes, including Cyclin A, Cyclin B, Cyclin D, Cyclin E and Cyclin F. Of note, CDK4 and CDK6 are only able to complex with D-type cyclins. The activity of CDKs is also regulated by the cyclin kinase inhibitors (CKIs) including p27, p21, and p19 (174).

4.1.2 Mitochondrion

Mitochondria, the membrane-enclosed organelle found in most eukaryotic cells, are the cellular power plant. Mitochondrion generates most of the cellular supply of adenosine triphosphate (ATP), used as the main source of chemical energy. Mitochondria are composed of compartments that carry out specialized functions. These compartments include the outer membrane, the intermembrane space, the inner membrane, the cristae and matrix. Significantly, Mitochondria have their own independent genome. In addition to supplying cellular energy, mitochondria are also involved in a range of other processes, such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth (175).

Mitochondria are required for all cell processes due to common energetic requirements. It has been reported that low glucose and ATP leads to cell cycle arrest (176). A low-energy cell-cycle checkpoint has been hypothesized monitoring the metabolic activity of the mitochondria before the cell can commit to cell division (175). AMPK, a sensor of the AMP:ATP ratio, is a heterotrimeric kinase that is activated under high AMP conditions (177). Activated AMPK

initiates a phosphorylation cascade that switches on catabolic pathways and switches off processes that consume ATP. Activated AMPK also phosphorylates transcription factor p53 and then promotes cell-cycle arrest during DNA damage and aberrant growth factor signaling (178).

Mitochondria have essential functions in the apoptotic cascades. Mitochondrial fragmentation is a fundamental step for cytochrome c release and cell death (179). During cell death, a group of proteins translocate to the mitochondria in a highly synchronized fashion, in addition to those that are systematically released from mitochondria in the dramatic apoptosis cascades. For instance, upon phosphorylation by protein kinase C, K-Ras translocates to the mitochondria and interacts with Bcl-XL to promote activation-induced apoptosis of T cells (180). Another example is transcription factor P53. The p53 translocated to the mitochondria interacts with the anti-apoptotic proteins Bcl2, Bcl-XL and participates in the cytosolic activation of Bax, oligomerization of Bax and Bak and subsequent cytochrome c release (181, 182).

4.2 MATERIALS AND METHODS

4.2.1 Cells and culture system

CD3+, CD4+ and CD8+ T-cell cultures were set up as previously described (101). Briefly, negatively-selected T cells (CD3+, CD4+, and CD8+) were activated with anti-CD3/anti-CD28 Mab conjugated to magnetic beads. Cell counting and sampling for flow cytometry and microarray analysis were carried out at 0, 4, 10, 48 and 96 hours in the CD3+ T-cell experiments, and at 0, 6, 12, 24, 48 and 72 hours in the CD4+ T-cell and CD8+ T-cell experiments. This study was approved by the Northwestern University IRB.

4.2.2 Flow cytometry

The following monoclonal antibodies (Mabs) for flow cytometry were purchased from BD Biosciences (San Jose, CA) unless otherwise stated and included CD3 (FITC+PE), CD25 PE, CD28 PE. Flow cytometry was carried out as described (54, 55). Briefly, all samples were gated on forward scatter and on propidium iodide negative (PI-) to eliminate debris and dead cells. For intracellular detections, cells were first stained with anti-CD3-FITC and then fixed, permeabilized, and stained as previously described (56). Quantibrite beads (BD Biosciences Immunocytometry Systems) labeled with different amounts of PE molecules were used to quantify surface or intracellular protein levels and normalize measurements between timepoints.

4.2.3 RNA extraction and quality control

Total RNA was extracted from frozen cells using the NucleoSpin RNA II kit (Clontech, Palo Alto, CA). RNA samples were resuspended in RNase-free water and stored at –80°C. RNA yield and purity were assessed by UV spectrophotometric measurements at 260 and 280 nm (Biomate 3, Thermo Spectronic, Marietta, OH). Furthermore, RNA integrity was evaluated using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

4.2.4 Microarray experiments and analysis

Total RNA was extracted, RNA integrity was evaluated and microarray experiments and data analysis were carried out as preciously described (101). Briefly, microarray data were normalized and further analyzed (identification of significant genes, hierarchical clustering, and Gene Ontology assignment) with 'MultiExperiment Viewer (MeV)' from The Institute for Genomic Research (TIGR) (58). Raw and normalized data were deposited in the Gene Expression Omnibus (GSE6607 (CD3+ T-cell experiment), GSE7571 (CD4+ T-cell experiment) and GSE7572 (CD8+ T-cell experiment)) (59). Within each population (three biological replicates using cells from three different donors), multi-class SAM (Significance Analysis of Microarrays) with a false discovery rate of $<1\%$ was used to select genes that show statistically different expression between groups. A SAM group is defined here as all the samples belonging to the same timepoint regardless of donor. Briefly, there were 5 groups (0 hour, 4, 10, 48 and 96 hours) in the set of CD3+ experiments and 6 groups (0 hour, 6, 12, 24, 48 and 72 hours) in the set of CD4+ experiments and CD8+ experiments. Gene expression at each time point was

compared to that of 0 hour in each experiment. Gene Ontology annotations, as curated by European Bioinformatics Institute, were retrieved from the Gene Ontology Consortium website (60). The EASE (Expression Analysis Systematic Explorer) score in ontological analysis is a modified Fisher Exact Probability p-value (102) indicating the probability of finding by chance the same degree of enrichment on a Gene Ontology term in a set of genes. The lower the EASE score, the more significant is the enrichment, i.e. the less likely that degree of enrichment can be found by chance. Hierarchical clustering analysis was performed with the Euclidean distance metric.

4.3 RESULTS AND DISCUSSION

4.3.1 Significant cell cycle regulation in T-cell activation

Upon stimulation, T cells get activated, go through the balance of apoptosis regulation, entry into cell cycle and start to proliferate. The disruption of the proper pathways of T-cell cycle control may cause decreased immune competence, immunosuppression and diseases such as lymphohematopoietic cancer. However, the temporal transcription regulation of the genes associated with this orderly orchestrated progress has not been reported in the context of T-cell activation. Ontological analysis using the MeV EASE module identified 142 significantly regulated genes associated ‘cell cycle’ (EASE score: 2.28E-15). Transcription profiling demonstrated highly reserved transcriptional patterns shared by the three populations, CD3+, CD4+ and CD8+ T cells: (A) genes generally downregulated; (B) genes generally upregulated; (C) genes upregulated at 48-96 hours (Figure 4.1). The transcription dynamics indicate the stages of cell cycle these genes are involved in.

Several cell division cycle (CDC) proteins (CDC25A (G1/S), CDC25C (G2/M), CDC7 (G1/S), CDC23 (G1/S), CDC45L (G1/S), CDC20 (G1/S), CDC6 (G1/S), CDCA5 (G1/S), and CDC2 (G1/S and G2/M)) showed synchronized increased expression at 48 hours or 48-96hours except for CDC42. This is consistent with the phenotype data that T-cell expansion did not start till 48 hours (Figure 2.1). Surprisingly, CDC42, which has been reported to be activated upon T-cell activation (183), was rather higher expressed in resting T cells. It is possible that CDC42 protein might be activated independent of transcription regulation. Cyclin-dependent kinases (CDKs) were upregulated at different stages. CDK4 was significantly

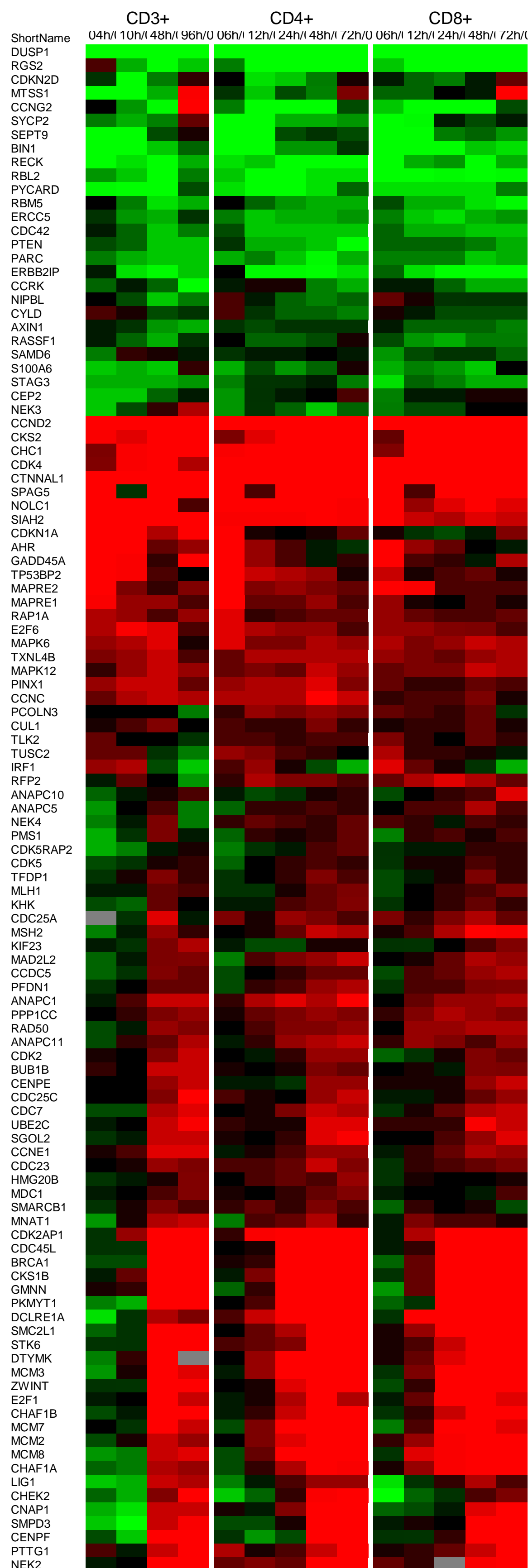


Figure 4.1. Expression profiles of significant genes associated with the Gene Ontology term 'cell cycle'.

Genes that were differentially expressed temporally in T-cell activation of the three (CD3+, CD4+ and CD8+) populations were divided into three groups (genes generally downregulated; genes generally upregulated; genes upregulated at 48-96 hours) according to their distinct expression patterns based on hierarchical clustering using the Euclidian distance metric. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population.

upregulated at 10-48 hours and CDK2 was significantly upregulated at 96 hours, consistent with their hypothesized functions in T-cell cycle entry and/or early G1 progression and the G2/M transition respectively (184). CDK7 was significantly upregulated at 48-96 hours, contrary to its reported constant expression throughout cell cycle (185). Our microarray data also captured the transcription regulation patterns shared by CDK5 and CDK5 regulatory subunit associated protein 2 (CDK5RAP2): downregulated at 4-10 hours and then upregulated at 96 hours, oscillation of which have not been reported in cell cycle. Inhibitors of cyclin-dependent kinase (CDKNs) were regulated differently. CDKN2D (inhibitor of CDK4 and CDK6) was downregulated at 10 hours; CDKN3 (inhibitor of CDK2) was upregulated at 48-96 hours, simultaneously with the upregulation at 48-96 hours of CDK2 and CDK2AP1. Interestingly, CDKN1A (also called P21, inhibitor of CDK2 and CDK4), which has been reported to inhibit lymphocyte proliferation (186), was upregulated throughout the T-cell activation process in CD3+ T cells, possibly with functions in strict control of cell cycle progression. However, CDKN1A was only upregulated at 4 hours in CD4+ T cells and of no significant change in CD8+ T cells, suggesting its different roles in the CD4+ and CD8+ T-cell subsets. Different than the upregulation at 48-96 hours of several cyclins (CCNE1, -A2, -B2, and -B1), CCND2 and CCNC were continuously upregulated, suggesting that they may have different functions at the earlier phases of cell cycle. Several genes critical for DNA replication in the S-phase (CDT1 (chromatin licensing and DNA replication factor 1), MCM3, -2, -7, -8, -6 (minichromosome maintenance complex component), and CHAF1A, -B (chromatin assembly factor) showed simultaneous increased expression at 48-96 hours.

Besides those general cell cycle regulation genes, some have been specifically implicated in T-cell activation. MAPRE2 has been hypothesized to be involved in control of the signal transduction cascade downstream of the TCR (187). Highly homologous members of microtubule-associated protein, RP/EB family (MAPRE1 and MAPRE2), with functions in mitotic spindle function and in late mitotic checkpoint (188, 189), were significantly upregulated at only 4 hours. The simultaneous transcription upregulation of MAPRE1 and MAPRE2 suggests that they might be immediate and transient response genes in T cells upon anti-CD3/anti-CD28 stimulation. NME2 (non-metastatic cells 2, protein (NM23B) expressed in), together with KCNN4 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4), has been reportedly required in T-cell activation in CD4+ cells (190). Indeed, KCNN4 was more significantly upregulated in CD4+ T cells than in CD8+ T cells; however, NME2 was similarly upregulated in the CD3+, CD4+ and CD8+ T cells, so as another NM23 genes, NME1. It has been reported that transcription factor MYC is able to induce expression of NME1 and NME2, which suppresses the activity of CDC42 and prevent CDC42 mediated cell differentiation (191, 192). The synchronized upregulation of MYC at 4-48 hours (Appendix A4), upregulation of NME1 and NME2 and downregulation of CDC42 at 10-96 hours suggest the involvement of this pathway in T-cell activation. Transcription factor, aryl hydrocarbon receptor (AHR) was significantly upregulated at 4-10 hours. The function of AHR in cell cycle is controversial. It has been reported that AHR facilitated G1 cell cycle progression (193), however overexpression of AHR inhibited T-cell proliferation (194), yet the transcription kinetics of AHR in T-cell activation is unknown. The early upregulation of AHR suggests that

AHR might be of help to bring cells out of phase G0 to phase G1 into the cell cycle and no longer needed during the cell cycle progression.

The MeV self-organizing tree algorithm (SOTA) module identified a cluster of genes that were significantly upregulated at 48-96 hours, but not at 4 or 10 hours (Appendix A6) (the centroid graph see Appendix A7). Many of these genes have been associated with cell cycle (per corresponding gene pages in NCBI http://www.ncbi.nlm.nih.gov/ and references therein). We hypothesize that this cluster of gene are cell cycle signature genes in T-cell activation, which provides a valuable platform for immune system disorder and malignancies.

4.3.2 Mitochondria in T-cell activation

Ontological analysis using the MeV EASE module identified 246 genes associated with 'mitochondrion' among the 4167 significant genes (EASE score: 1.91E-35), suggesting its essential role in T-cell activation, proliferation and differentiation. Hierarchical clustering revealed distinct expression patterns for these 246 genes and allowed us to divide them into three clusters (Appendix A8): (A) mainly downregulated compared to resting T cells (0 hour); (B) mainly upregulated compared to resting T cells (0 hour); (C) mainly downregulated at 4-10 hours and then upregulated at 48-96 hours. These 246 genes were manually curated into subcategories based on their functions listed by NCBI website (http://www.ncbi.nlm.nih.gov/).

Numerous mitochondrial ribosomal proteins were significantly regulated: some were generally upregulated throughout the T-cell activation process of our experiment period; some were upregulated at 48-96 hours (Figure 4.2). The mammalian mitochondrial ribosomal proteins are products of nuclear genes and help synthesize proteins within the mitochondrion (195). Few

genes have been reported to have functions in other area, such as DAP3 (MRPS29), which participates in apoptotic pathways initiated by TNF, FasL, and interferon- gamma (196). As discussed in Chapter 3, T-cell proliferation did not start until 48 hours upon stimulation. The down/no change at 4-10 hours and then upregulation at 48-96 hours of a number of the mitochondrial ribosomal proteins is agreeing with the T-cell proliferation phenotype. Of note, several gene encoding mitochondrial ribosomal proteins were upregulated at 4 or 10 or 4-10 hours, including MRPL50, MRPL32, MRPL47, MRPL23, MRPS18A, MRPS6 and MRPL44, suggesting their, non-previously reported, involvement in T-cell activation.

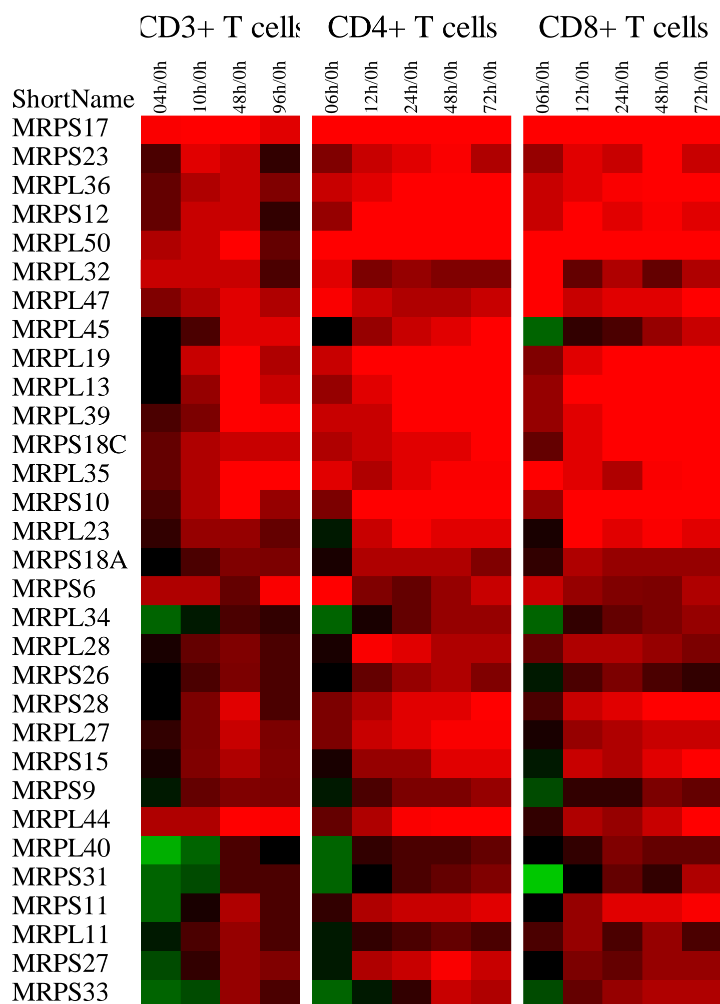


Figure 4.2. Expression profiles of significant mitochondrial ribosomal protein genes.

Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population.

The mitochondrial electron transport chain, composed of four complexes: mitochondrion (NADH dehydrogenases), complex II (succinate dehydrogenases), complex III (cytochrome bc1 complex), and complex IV (cytochrome c oxidases), produces the majority of ATP in mammalian cells. Numerous NADH dehydrogenases, the components of mitochondrion, were significantly regulated. The roles and functions of NADH dehydrogenases are mainly to transport of electrons from NADH to ubiquinone, accompanied by translocation of protons from the mitochondrial matrix to the intermembrane space (197). Interestingly, they demonstrated different transcription patterns (Figure 4.3). As opposed to the upregulation of most of the NADH dehydrogenases, the early upregulation of NDUFV2, NDUFA6, NDUFAB1, NDUFS2, NDUFAF1, NDUFB8 suggests their involvement in early T-cell activation. No subunit of the complex II was identified as significantly regulated. UQCRFS1, UQCRC1, UQCRH, CYC1 of the complex III and COX (cytochrome c oxidase) 17, SCO1, COX5A, COX15 and COX8A of the complex VI shared similar transcriptional pattern: not significantly regulated at 4-10 hours and significantly upregulated at 48-96 hours. Of note, genes of voltage-dependent anion channels (VDACs) also shared this particular transcriptional pattern with components of complex III and complex VI. The main functions of VDACs are to control the Ca^{2+} exchange between mitochondria and cytoplasm (198). VDAC1 has been reported to be able to bind to cytochrome c oxidase (COX) in vitro (199). Their similar transcription patterns suggest that COXs and VDACs might be binding proteins in control of Ca^{2+} exchange in T-cell activation.

A number of oxidoreductases, not component of electron transport chain, were significantly regulated. PRODH (proline dehydrogenase (oxidase) 1) was downregulated at 48-

96 hours. It has been reported that PRODH is able to generate reactive oxygen species (ROS), induce apoptosis and regulate nuclear factor of activated T cells (NFAT) signaling and MEK/ERK pathway (200). The downregulation of PRODH suggests that this oxidase and its related pathways are not active during our T-cell proliferation (at 48-96 hours). The MTHFD2 protein is a NADP+ dependent, bifunctional, methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase, while the MTHFD1 protein is a NADP+ dependent, trifunctional methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase and formyltetrahydrofolate synthetase. Interestingly, the gene MTHFD2 was significantly upregulated at 4-96 hours while MTHFD1 was downregulted at 4 hours and then significantly upregulated at 48-96 hours. The comparison of the functions and transcriptional patterns of MTHFD1 and MTHFD2 suggests that formyltetrahydrofolate synthetase activity might not be needed in the early stage of T-cell activation. Components of the pyruvate dehydrogenase complex, which catalyzes the irreversible conversion of pyruvate into acetyl-CoA, and components of Acyl-Coenzyme A dehydrogenase complex, which catalyzes the first step of β-oxidation in fatty acid metabolism, were similarly regulated: not significantly regulated at 4-10 hours but significantly upregulated at 48-96 hours, including DLD (E3 component of pyruvate dehydrogenase complex), PDHA1 (pyruvate dehydrogenase (lipoamide) alpha 1), PDHX (pyruvate dehydrogenase complex, component X), ACAD9 (Acyl-Coenzyme A dehydrogenase family, member 9), ACADVL (Acyl-Coenzyme A dehydrogenase, very long chain (ACADVL)), ACADS (Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain (ACADS)) and HADH (hydroxyacyl-Coenzyme A dehydrogenase).

Different than the most of components of the electron transport chain (as discussed above), CYCS (cytochrome c), which transfers electrons between complexes III and IV, was continuously upregulated. SOD2, superoxide dismutase 2, was significantly upregulated at 4-10 hours. The SOD2 protein has been reported to participate in the response to oxidative stress, converting superoxide to hydrogen peroxide and diatomic oxygen (201). Even though not directly measured, the intracellular reactive oxygen species (iROS) has been suggested to be induced in T-cell activation (202). This significant upregulation of SOD2 at 4-10 hours suggests an immediate induction of iROS in T-cell activation. PRDX3, encoding peroxiredoxin 3 withantioxidant function, was significantly upregulated at 10-96 hours. It has been reported that deletion of PRDX3 led to increased intracellular levels of H_2O_2 and sensitized cells to induction of apoptosis. The upregulation of PRDX3 at 10-96 hours suggests its anti-apoptotic and antioxidant functions in T-cell activation during this time period.

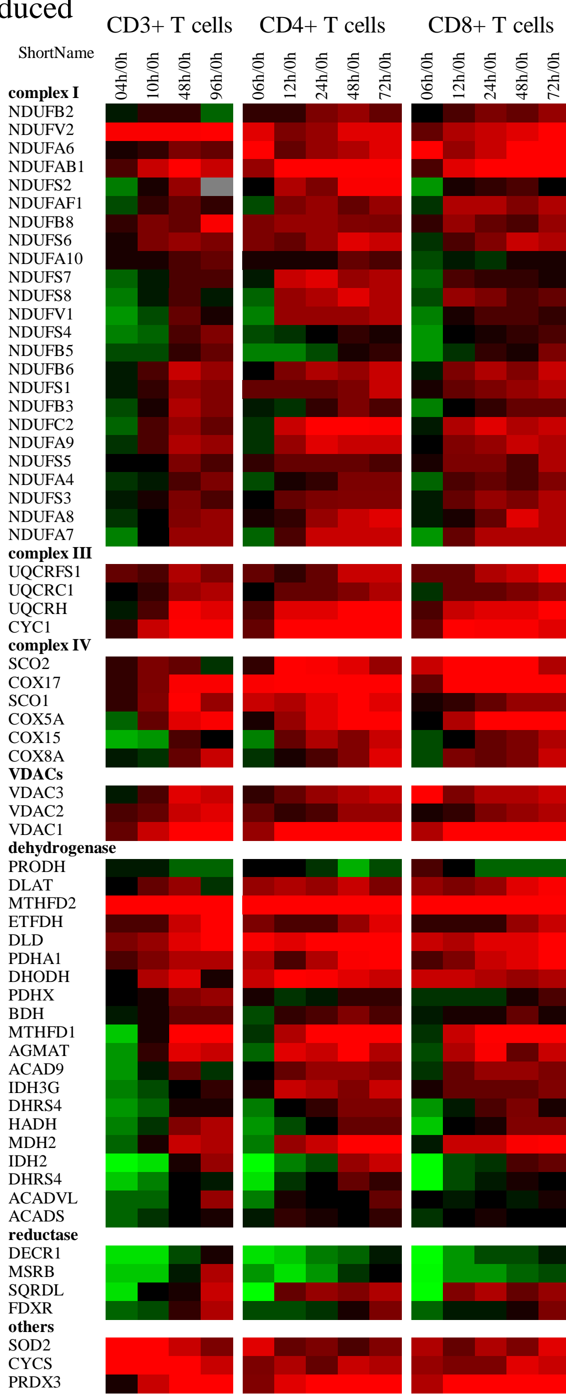


Figure 4.3. Expression profiles of significant genes encoding oxidoreductases in 'Mitochondrion'.

The membership of oxidoreductases is based on the NCBI website (http://www.ncbi.nlm.nih.gov/) and information thereby. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population.

CHAPTER 5: TRANSCRIPTIONAL PROFILING OF T-CELL RESPONSE TO H_2O_2 STRESS

5.1 INTRODUCTION

Oxidative stress has been associated with aging and certain disease conditions, executed by intracellular reactive oxygen species (iROS) (203). Persistent increase in iROS production may cause a dysregulation of redox-sensitive signaling pathways in addition to direct oxidative damage and eventually aging, which will lead to changes in gene expression and subsequent cellular events.

There are three main iROS: the superoxide anion ($\bullet O_2^-$), the hydroxyl radical (OH•) and the hydrogen peroxide (H_2O_2). The concentrations of these iROS are determined by the balance between the rates of production and the rates of clearance by various antioxidant scavengers (203). At low concentrations, iROS positively regulate numerous signaling cascades, including membrane receptor signaling pathways (such as EGF), inhibition of protein tyrosine phosphatases, activation of cytoplasmic protein kinases, activation of mitogen-activated protein (MAP) kinase cascades, activation of protein kinase c isoforms and activation of the transcription factors AP-1, NF-κB (203). In immune system, reactive oxygen species can also be beneficial as a way to attack pathogens in infections. Under aging and various clinical conditions, such as malignant diseases, chronic inflammation, human immunodeficiency virus (HIV) infection, extra iROS are produced, causing oxidative stress and damage (203).

Up to date, the molecular and cellular mechanisms through which T cells respond to oxidative stress remain poorly understood. Elucidating these mechanisms will be key to understanding oxidative stress as a frequent complication in disease conditions as well as aging under physiological and pathophysiological conditions.

The present study focused on T-cell response to moderate oxidative stress, implemented by incubation with H_2O_2. The global transcription profiling revealed the main transcriptional events in T-cell response to oxidative stress, improved our understanding of T-cell specific oxidative stress response and provided a platform for future research leads in aging, inflammation and immune dysfunctions.

5.2 MATERIALS AND METHODS

5.2.1 Cells and culture system

Healthy-donor peripheral blood mononuclear cells (PBMCs) (AllCells, Berkeley, CA) were used to select CD3+ T cells (Pan T-cell Isolation Kit II, Miltenyi Biotech, Sunburn, CA). After selection, T cells for 0 hour sampling (flow assay and microarray) were collected. The rest cells were equally divided and incubated with H_2O_2 at different final concentrations (0 μM (control), 25 μM, 50 μM and 75 μM for experiment 1 (E1) and 0 μM (control), 25 μM, 35 μM and 50 μM for experiment 2 (E2)) and incubated at 37°C for 10min, and then washed once with AIM-V medium (Invitrogen, Carlsbad, CA). Cell cultures were setup separately, the control samples and H_2O_2 stressed samples, seeded at $1x10^6$ cells/mL in T-flasks and cultivated for 96 hours in AIM-V medium with 100U/mL IL2 (Chiron, Emeryville, CA) and 2% human serum (Sigma-Aldrich St. Louis, MO). T cells were activated polyclonally with anti-CD3/anti-CD28 Mab (1:1)-coated magnetic beads (500 fmol/bead) (Dynabeads M-450 Epoxy, Dynal Biotech, Lake Success, NY). The ratio of beads to cells was 3:1. Cell counting and cell sampling for microarray analysis were carried out at 0, 4, 10, 48 and 96 hours. Cells were counted using a Coulter Multisizer 3 (Beckman Coulter, Fullerton, CA). Specific proliferation rates (μ) of T cells were calculated as described (53).

5.2.2 Flow cytometry

The following monoclonal antibodies (Mabs) for flow cytometry were purchased from BD Biosciences (San Jose, CA) unless otherwise stated and included CD3 (FITC+PE), active CASP3 PE, phospho-NFκB-p65 PE, phospho-p38 (MAPK14) PE, phospho-ERK1 (MAPK3) PE, PUMA (BBC3) (Cell Signaling Technology, Danvers, MA), BCL2A1 (Abcam, Cambridge, MA) and goat anti rabbit IgG PE (Jackson ImmunoResearch Laboratories, West Grove, PA). Flow cytometry was carried out as described (54, 55). Briefly, all samples were gated on forward scatter and on propidium iodide negative (PI-) to eliminate debris and dead cells. For intracellular detections, cells were first stained with anti-CD3-FITC and then fixed, permeabilized, and stained as previously described (56). 10,000 gated events from each tube were acquired using a FACscan (BD Biosciences) or LSRII flow cytometer (Becton Dickinson). Quantibrite beads (BD Biosciences Immunocytometry Systems) labeled with different amounts of PE molecules were used to quantify surface or intracellular protein levels and normalize measurements between timepoints.

5.2.3 RNA extraction and quality control

Total RNA was extracted from frozen cells using the Total RNA Isolation Mini Kit (Agilent, Wilmington, DE). RNA samples were re-suspended in RNase-free water and stored at -80°C. RNA yield and purity were assessed spectrophotometrically at 260 and 280 nm (Biomate 3, Thermo Spectronic, Marietta, OH). RNA integrity was evaluated using a Bioanalyzer 2100 (Agilent).

5.2.4 DNA-microarray experiments and data analysis

Microarray-based transcriptional analysis was carried out for samples at 0 hour (prior to stimulation), and 4, 10, 48 and 96 hours post stimulation for three experiments (E3, E4, E5) using a 'reference' design (55), with Human Thymus Total RNA (Ambion, Austin, TX) as the reference RNA. Detailed experimental procedures and the use of the SNNLERM-algorithm (57) for data normalization were described (55). Gene expression at each time point was compared to that of 0 hour in each experiment. To select genes consistently differentially expressed, the following selection criterion was used: a gene had to have a minimum of 1.8-fold difference compared to the 0 hour, in at least 3 out of the total 12 timepoints (4, 10, 48 and 96 hours for 3 experiments) in order to be considered for further analysis. Further analysis (hierarchical clustering and gene ontology assignment) was carried out using 'MultiExperiment Viewer (MeV)' from The Institute for Genomic Research (TIGR) (58). Gene Ontology annotations, as curated by European Bioinformatics Institute, were retrieved from the Gene Ontology Consortium website (60). Hierarchical clustering was performed with the Euclidian distance metric. The Fisher score in ontological analysis is a is a Fisher Exact Probability p-value (102) indicating the probability of finding by chance the same degree of enrichment on a Gene Ontology term in a set of genes. The lower the EASE score, the more significant is the enrichment, i.e the less likely that degree of enrichment can be found by chance. Raw and normalized data were deposited in the Gene Expression Omnibus (GSE6607; http://www.ncbi.nlm.nih.gov/geo/).

5.2.5 Supernatant ELISA assay of CCL20 and IFNG

Culture supernatants were collected at 4, 10, 48 and 96 hours in three CD3+ T-cell experiments and analyzed for CCL20 and IFNG concentrations by ELISA (R&D Systems, Minneapolis) following the manufacturer's instructions.

5.2.6 AP-1 activity assay

DNA-binding activity of AP-1 was assessed using the TransBinding AP-1 ELISA kit (Panomics; Fremont, CA) as described (54). Briefly, nuclear extracts were incubated with biotinylated AP-1-consensus-binding-sequence oligonucleotides and complexes were detected using a primary AP-1 antibody and a secondary antibody conjugated to horseradish peroxidase. This assay is analogous to the traditional electrophoretic mobility shift assay in that it measures the ability of a transcription factor from nuclear lysates to bind to a consensus-binding sequence of that transcription factor, and has been extensively validated (103, 104).

5.3 RESULTS AND DISCUSSION

5.3.1 H_2O_2 stress delays T-cell activation and proliferation

In spite of the important roles of iROS, little is known about physiological concentrations of iROS in vivo. It was estimated that T cells might be exposed to 10-100 μM H_2O_2 in the physiological microenvironment of an inflammation site (204). In this study, we aim to understand T-cell response to oxidative stress by H_2O_2 application in a manner that extreme enough to cause oxidative damage, but not necrosis. We did concentration titration with 25 μM, 35 μM, 50 μM and 75 μM H_2O_2. H_2O_2 with final concentrations of 35 μM, 50 μM and 75 μM induced cell death in most of the cells within 24 hours (Figure 5.1B). H_2O_2 with final concentration of 25 μM caused ca. 50% T cells non-viable, delayed T-cell activation and proliferation, compared to non-stress control (Figure 5.1), but not extreme rapid necrosis. Therefore, we chose 25 μM H_2O_2 to investigate the T-cell global transcriptional response to oxidative stress.

5.3.2 Ontological analysis identifies significant 'response to stimulus' genes in T-cell response to H_2O_2 stress

To capture significant regulated genes, gene expression of the 25 μM H_2O_2 stressed samples was compared to that of non-stressed control samples at each timepoint in each experiment accordingly (see materials and methods). 1329 genes were identified as differentially

expressed by selection criterion (see materials and methods). Ontological analysis of MeV module associated the significantly regulated genes with Gene Ontology terminology and

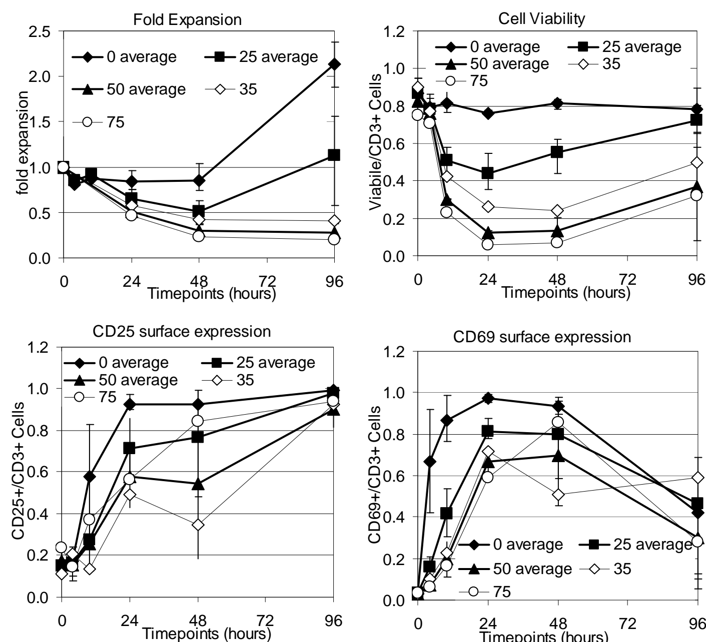


Figure 5.1. Phenotypic analysis of T-cell response to H_2O_2 stress in ex vivo activation upon anti-CD3/anti-CD28 stimulation.

CD3+ T cells were negatively selected from PBMCs of healthy donors, stressed by indicated concentrations of H_2O_2 for 10 minutes and activated with anti-CD3/anti-CD28 antibodies. **(A)** T-cell expansion as assessed by cell numbers; **(B)** The percentage of the viable T cells as determined by flow cytometry; **(C)** The percentage of the viable cells expressing CD69; **(D)** The percentage of the viable cells expressing CD25. Data from two independent experiments, E1 and E2, are shown.

provided information regarding the major cellular activities regulated by H_2O_2 stress (Appendix A9), such as ‘response to biotic stimulus’ (Fisher score: 2.28E-17), ‘immune response’ (Fisher score: 2.29E-16). A few defense related associations including ‘defense response’ (Fisher score: 4.26E-15), ‘response to stress’ (Fisher score: 9.05E-15), ‘response to pest, pathogen or parasite’ (Fisher score: 9.99E-14), ‘response to stimulus’ (Fisher score: 1.84E-12), ‘response to external

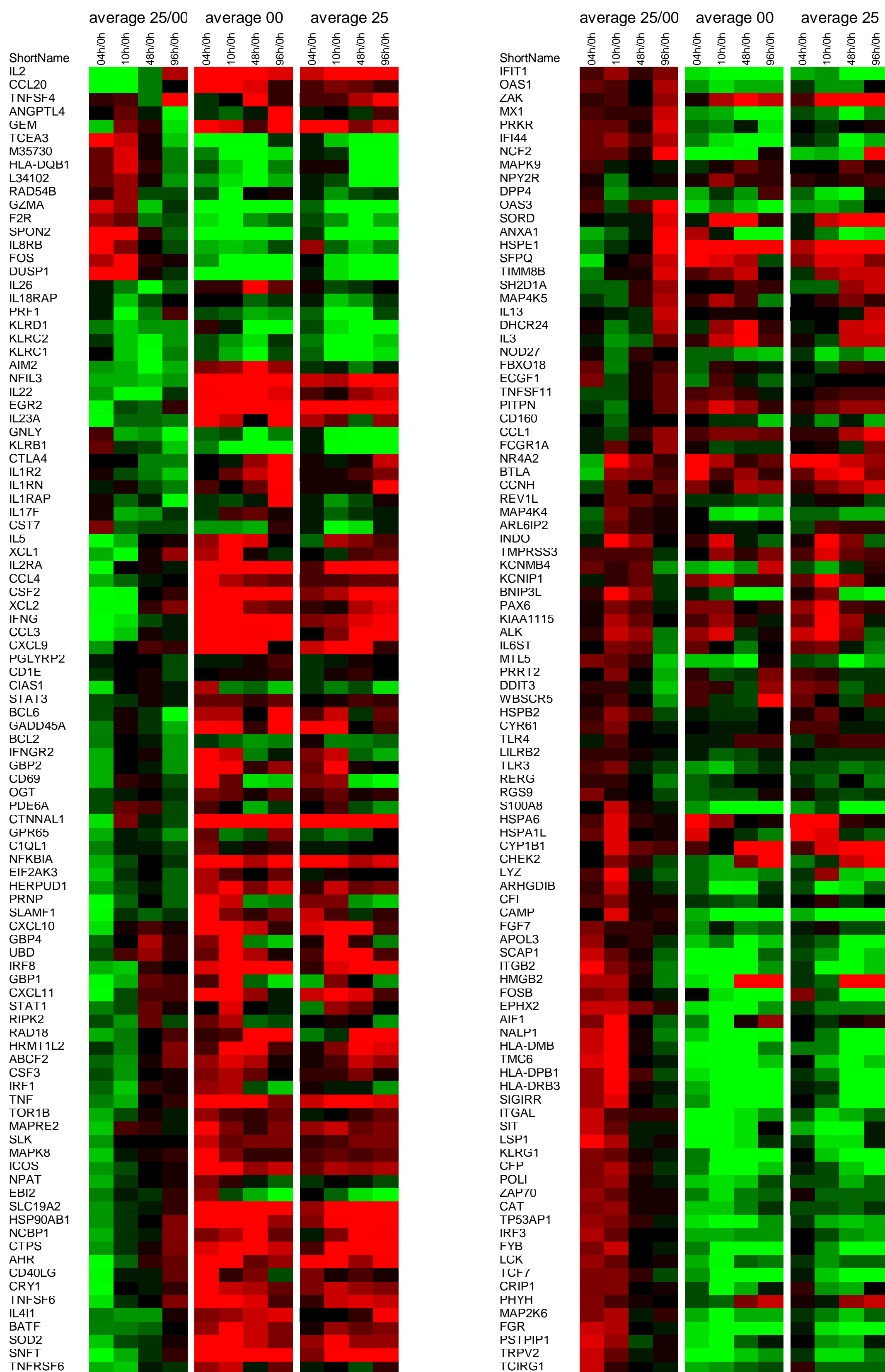
stimulus' (Fisher score: 1.09E-08), shared numerous genes in common. Therefore we focused on stimulus responsive genes. Transcription profiles of (194) genes associated with 'response to stimulus' were demonstrated in Figure 5.2. These genes were sorted based on their functions given by NCBI website (http://www.ncbi.nlm.nih.gov/) and information thereby (Appendix A10), and discussed in details in the following.

Several transcription factors were regulated by H_2O_2 stress. KLF2, JUN, FOS, DUSP1, FOSB and TCF7 shared similar transcription patterns. They were consistently downregulated in T-cell activation while this downregulation was suppressed in stressed samples. FOS, FOSB, and JUN (purple colored in Appendix A10) are components of transcription factor AP-1 complex. AP-1 has been reported to be activated in T-cell activation (51, 205), which is contrary to the synchronized transcriptional downregulation of AP-1 component genes. We therefore measured the AP-1 transcription factor DNA-binding activity, which rapidly increased at 4 hours (Figure 5.3) and decreased thereafter. AP-1 in T cells stressed by H_2O_2 demonstrated the similar activity trend, but at a less extent, indicating that AP-1 complex is immediately and transiently activated in T-cell activation while the oxidative stress hinders the activation of transcription factor activity of AP-1 in T-cell activation. It is likely that AP-1 complex was activated in T-cell activation even though the transcription of its components was downregulated. It is also possible that the transcription of FOS, FOSB, and JUN was quickly upregulated and our first timepoint did not capture the transient upregulation. KLF2 is an immediate-early transcription factor regulating IL2 expression in T-cell activation (206). It has been reported that its transcription was upregulated within 2 hours and then downregulated in T-cell activation (207). Consistently, our microarray data captured the transcription downregulation of KLF2 in T-cell activation at 4-

96 hours. Dual specificity phosphatase (DUSP1) specifically inactivates the kinase activity of JNK1 (MAPK8), which regulates the transcription factor activity of AP-1 (164). Interestingly, DUSP1 shared similar expression patterns with FOS and JUN. Protein encoded by BATF (purple colored in Appendix A10)

Figure 5.2. Expression profiles of genes associated with the Gene Ontology term 'response to stimulus'.

Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples.



average 25/00
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04h/0h
10h/0h
48h/0h
96h/0h
ShortName
IL2
CCL20
TNFSF4
ANGPTL4
GEM
TCEA3
M35730
HLA-DQB1
L34102
RAD54B
GZMA
F2R
SPON2
IL8RB
FOS
DUSP1
IL26
IL18RAP
PRF1
KLRD1
KLRC2
KLRC1
AIM2
NFIL3
IL22
EGR2
IL23A
GNLY
KLRB1
CTLA4
IL1R2
IL1RN
IL1RAP
IL17F
CST7
IL5
XCL1
IL2RA
CCL4
CSF2
XCL2
IFNG
CCL3
CXCL9
PGLYRP2
CD1E
CIAS1
STAT3
BCL6
GADD45A
BCL2
IFNGR2
GBP2
CD69
OGT
PDE6A
CTNNAL1
GPR65
C1QL1
NFKBIA
EIF2AK3
HERPUD1
PRNP
SLAMF1
CXCL10
GBP4
UBD
IRF8
GBP1
CXCL11
STAT1
RIPK2
RAD18
HRMT1L2
ABCF2
CSF3
IRF1
TNF
TOR1B
MAPRE2
SLK
MAPK8
ICOS
NPAT
EBI2
SLC19A2
HSP90AB1
NCBP1
CTPS
AHR
CD40LG
CRY1
TNFSF6
IL4I1
BATF
SOD2
SNFT
TNFRSF6
average 25/00
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04h/0h
10h/0h
48h/0h
96h/0h
ShortName
IFIT1
OAS1
ZAK
MX1
PRKR
IFI44
NCF2
MAPK9
NPY2R
DPP4
OAS3
SORD
ANXA1
HSPE1
SFPQ
TIMM8B
SH2D1A
MAP4K5
IL13
DHCR24
IL3
NOD27
FBXO18
ECGF1
TNFSF11
PITPN
CD160
CCL1
FCGR1A
NR4A2
BTLA
CCNH
REV1L
MAP4K4
ARL6IP2
INDO
TMPRSS3
KCNMB4
KCNIP1
BNIP3L
PAX6
KIAA1115
ALK
IL6ST
MTL5
PRRT2
DDIT3
WBSCR5
HSPB2
CYR61
TLR4
LILRB2
TLR3
RERG
RGS9
S100A8
HSPA6
HSPA1L
CYP1B1
CHEK2
LYZ
ARHGDIB
CFI
CAMP
FGF7
APOL3
SCAP1
ITGB2
HMGB2
FOSB
EPHX2
AIF1
NALP1
HLA-DMB
TMC6
HLA-DPB1
HLA-DRB3
SIGIRR
ITGAL
SIT
LSP1
KLRG1
CFP
POLI
ZAP70
CAT
TP53AP1
IRF3
FYB
LCK
TCF7
CRIP1
PHYH
MAP2K6
FGR
PSTPIP1
TRPV2
TCIRG1

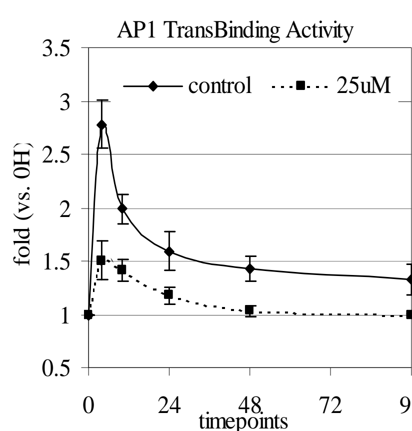

Figure 5.3. Transient Induction of AP-1 transcription binding activity in T-cell activation was decreased by H_2O_2 stress. CD3+ T cells were selected, incubated with 25μM H_2O_2 for 10mins, or let alone as control, stimulated (by anti-CD3/anti-CD28 antibodies), cultured separately and harvested at the indicated timepoints of culture to analyze the transcription binding activity. Data from three independent experiments were averaged.

is a negative regulator of AP-1 transcription factor activity, forming inhibitory DNA binding heterodimers with JUN proteins (208). The transcriptional pattern of BAFT was the opposite to that of FOS and JUN, upregulated in T-cell activation and this upregulation was attenuated by H_2O_2 stress. It is possible that this upregulation of BAFT in cooperation with the downregulation of FOS and JUN are involved in the downregulation of AP-1 activity at 4-96 hours in T-cell activation. SNFT (also called BATF3, purple colored in Appendix A10) shared similar transcription pattern with BATF. Little known is about the functions of SNFT. It is possible that it functions similarly as BAFT. TCF7, transcription factor 7 (T-cell specific) has been reported to effectively limit Antigen-driven T-cell proliferation, cytokine production, and changes in surface receptor expression (209). Consistently, our microarray data show that the transcription of TCF7 is continuously downregulated in T-cell activation and this downregulation of TCF7 is attenuated by H_2O_2 stress. HSP90AB1 and AHR (blue colored in Appendix A10) shared similar expression pattern that the strong upregulation in T-cell activation was suppressed by H_2O_2 stress. The

cytosolic AHR complex exists as a transcriptionally cryptic complex, consisting of the HSP90AB1 and AHR (210). The similar transcription patterns of HSP90AB1 and AHR suggest that the AHR complex is activated in T-cell activation while the H_2O_2 stress abates its activation.

Numerous genes encoding cytokines and cytokine receptors were significantly regulated. IL1R2, IL1RN, and IL1RAP (orange colored in Appendix A10) are interacting proteins, forming a decoy receptor for interleukin 1 (IL1) (211, 212). IL1R2, IL1RN, and IL1RAP shared similar expression patterns. They were significantly upregulated in T-cell activation at 48-96 hours and this upregulation was suppressed by H_2O_2 stress. This suggests that IL1 blocking receptor activity might be upregulated in T-cell activation, while H_2O_2 stress attenuates this upregulation. Of note, no significant gene regulation of components of IL1-Type-I-activating receptor was observed. This indicates that IL1-Type-II-blocking receptor activity might be activated in T-cell activation rather than IL1-Type-I-activating receptor activity. SIGIRR is a negative regulator of IL1 and lipopolysaccharide (LPS) signaling (213). SIGIRR was downregulated in T-cell activation immediately, while it was only downregulated at 48-96 hours in H_2O_2 stressed sample. This suggests that as a negative regulator of cytokine mediating signaling pathways (213), SIGIRR is involved in H_2O_2 stress delayed T-cell activation. A number of interferon related genes were regulated in T-cell activation and by H_2O_2 stress. IFNGR2 (interferon gamma receptor 2), GBP2 (guanylate binding protein 2), GBP1, GBP4, IRF8 (interferon regulatory factor 8) and IRF1 (yellow colored in Appendix A10) shared similar expression patterns. They were upregulated at 4-10 hours in T-cell activation and this upregulation was attenuated by H_2O_2 stress. Interferon induced genes, IFIT1, MX1, PRKR, OAS1, OAS3 and IFI44 (pink color coded in Appendix A10), shared similar transcription patterns. They were consistently downregulated

in T-cell activation and this downregulation was attenuated by H_2O_2 stress. All these interferon-induced genes, except for IFIT1, have been reported to be involved in the antiviral action. OAS1 and OAS3 can activate latent RNase L in the OAS/RNase L system of innate viral resistance (214) and PRKR is a dsRNA-dependent protein kinase with a key antiviral role against hepatitis C virus (215). The orchestrated transcription regulation suggests that those interferon-induced genes related anti-virus actions might not be active in T-cell activation, however the interferon related signal pathways in T-cell activation could be regulated by oxidative stress.

Interestingly, HSPA6 and HSPA1L (blue color coded in Appendix A10) were upregulated in T-cell activation and this upregulation was enhanced by H_2O_2 stress, especially at 10 hours. It has been reported that they could be induced in response to unfolded protein (216, 217). This unique transcription upregulation induced by H_2O_2 stress suggests that they may play important roles in T-cell response to oxidative stress. HLA-DQB1, HLA-DRB3, HLA-DPB1, and HLA-DMB are the components of major histocompatibility complex II. The transcription of these genes was consistently induced in H_2O_2 stressed samples at 4-10 hours, which suggests the regulation of MHC II molecules by H_2O_2 stress.

5.3.3 Ontological analysis identifies significant 'regulation of apoptosis' genes in T-cell response to H_2O_2 stress

Ontological analysis of MeV module identified several gene groups related to the negative regulation of cellular process, such as 'negative regulation of cellular process' (Fisher score: 4.12E-06), 'negative regulation of cellular physiological process' (Fisher score: 2.15E-05), 'apoptosis' (Fisher score: 8.01E-04), and 'regulation of apoptosis' (Fisher score: 8.26E-05). The

genes associated with the aforementioned groups were combined and their transcription profile is shown as Figure 5.4.

Transcriptional expression of several B-cell CLL/lymphoma genes, including BBC3, BCL2A1, MCL1, BCL3, BCL2 and BCL6 (blue color coded), were suppressed by H_2O_2. BCL2A1 is a direct transcription target of NF-κB (218), and its encoding protein has a cytoprotective function essential for lymphocyte activation as well as cell survival (219). The protein expression kinetics of BCL2A1 measured by flow cytometry assays supported its transcription pattern demonstrated by our microarray data. In T-cell activation, protein expression of BCL2A1 was upregulated, but H_2O_2 stress delayed this T-cell activation induced protein expression upregulation of BCL2A1 (Figure 5.5). BBC3 (also called PUMA) is known as a pro-apoptosis gene. Our microarray data revealed a dynamic transcription pattern of BBC3 in T-cell activation. It was significantly upregulated at 4 hours, and then downregulated at 48 hours, and upregulated again at 96 hours. This transcriptional regulation of BBC3 in T-cell activation was suppressed by H_2O_2 stress, especially at 4 and 96 hours. The protein expression kinetics of BBC3 measured by flow cytometry assays supported its transcription pattern demonstrated by our microarray data. While in H_2O_2 stressed samples, there was similar regulation but at less extent (Figure 5.6). CDC2L1 has been reported to be involved in regulation of cell growth and apoptosis (220). However, in control samples, CDC2L1 was not significantly regulated. Transcription of CDC2L1 was induced by H_2O_2 stress at 10 hours. This indicates that CDC2L1 is H_2O_2 stress inducible, but not actively involved in T-cell activation.

Protein encoded by R9AP, RGS2, ARHGDIB, RGS10, MTSS1, MAP2K6, SIPA1, EVL, VILL and BIN1 (yellow color coded in Appendix A10) are GTP-binding/actin cytoskeleton

related. They were downregulated in T-cell activation, and relatively upregulated by H_2O_2 stress, indicating the change of GTP/GDP balance and cell cytoskeleton in T-cell activation and response to H_2O_2 stress.

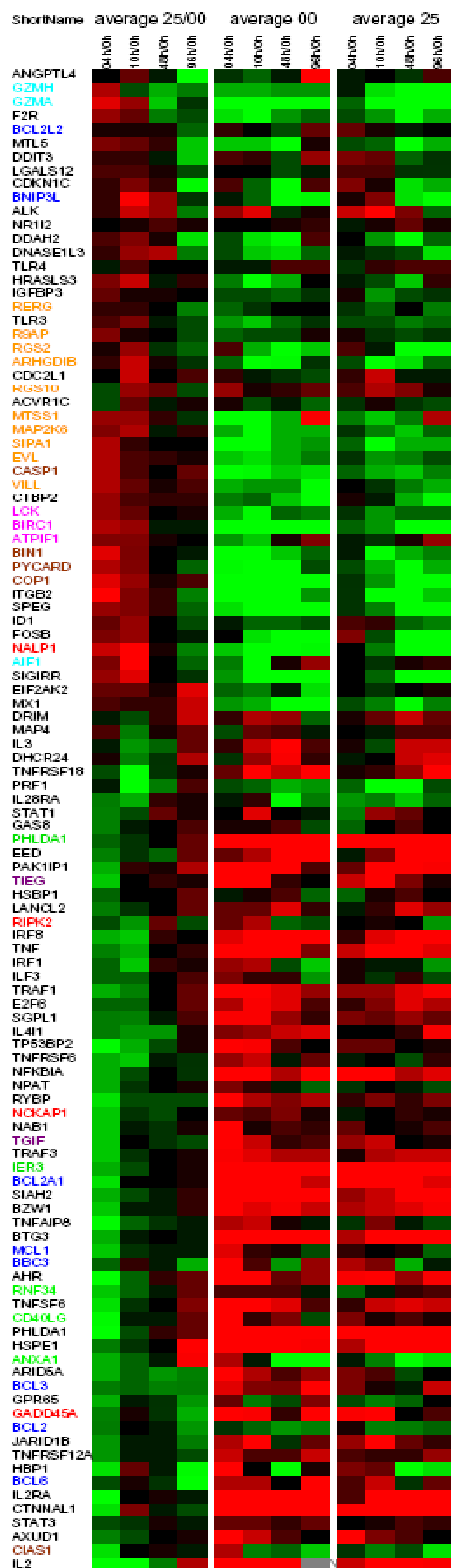

Figure 5.4. Expression profiles of significant genes associated with negative regulation of cellular process.

Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples. The gene names are color coded for the convenience of review.

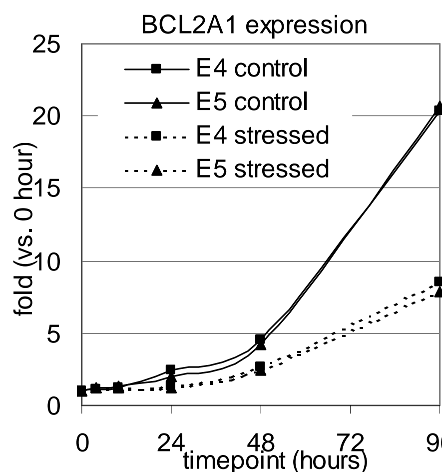

Figure 5.5. Induction of protein expression of BCL2A1 in T-cell activation was decreased by H_2O_2 stress.

Intracellular protein expression profiles of BCL2A1 in the control and stressed sampels. CD3+ T cells were selected, incubated with 25μM H_2O_2 for 10mins, or let alone as control, stimulated (by anti-CD3/anti-CD28 antibodies), separately and harvested at the indicated timepoints of culture to analyze the protein expression by flow cytometric assays. Data from two independent experiments, E4 and E5, are shown.

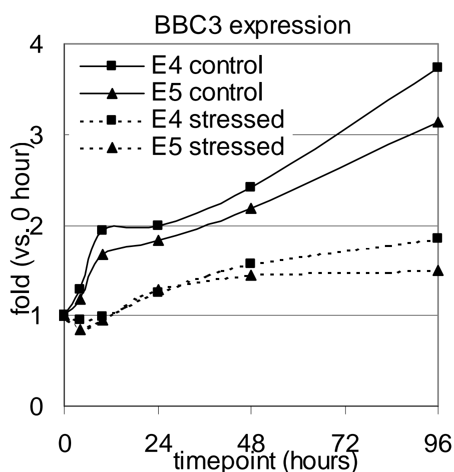

Figure 5.6. Induction of protein expression of BBC3 in T-cell activation was decreased by H_2O_2 stress.

Intracellular protein expression profiles of BBC3 in the control and stressed sampels. CD3+ T cells were selected, incubated with 25 μM H_2O_2 for 10mins, or let alone as control, stimulated (by anti-CD3/anti-CD28 antibodies), cultured separately and harvested at the indicated timepoints of culture to analyze the protein expression via flow cytometric assays. Data from two independent experiments, E4 and E5, are shown.

Several genes encoding TNF and TNF related proteins were significantly regulated by H_2O_2 stress. TNF has been reported to be involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, and apoptosis (221).

Transcription of TNF was strongly upregulated in T-cell activation, and this upregulation was attenuated by H_2O_2 stress, indicating that the transcription of TNF is under influence of H_2O_2 stress, negatively. TRAF1 and TRAF2 can form a heterodimeric complex, which is required for TNF-mediated activation of MAPK8/JNK and NF-κB complex (222). TRAF1 shared similar transcription patterns with TNF, suggesting the involvement of MAPK8/JNK and NF-κB complex in T-cell activation and H_2O_2 response. TRAF3 and TNFRSF18 shared similar expression patterns with TRAF1. Protein encoded by TNFRSF18 can bind protein TRAF1 and TRAF3, and induce the activation of NF-κB complex (223). It has also been reported that receptor encoded by TNFRSF18 is involved in the regulation of TCR-driven T-cell activation (224) and programmed T-cell death (225). Transcription of TNFRSF18 was upregulated in T-cell activation while the upregulation was abated by H_2O_2 stress, indicating its positive role in T-cell activation and negative role in apoptosis. Similar to TNFRSF18, TNFRSF12A are binding proteins of TRAF1 and TRAF3 (226). Our data revealed that the transcription upregulation of TNFRSF12A in T-cell activation was cancelled by H_2O_2 stress, supporting that TRAF1 and TRAF3 and their downstream events are involved in T-cell response to H_2O_2 stress. Additionally, it has been reported that TRAF1 and TRAF2 cooperate in CD40 signaling (227). The similar expression patterns shared by TRAF1, TRAF3 and CD40LG suggest that this CD40LG signaling is involved in T-cell activation and suppressed by H_2O_2 stress.

5.3.4 Ontological analysis identifies significant 'cytokine' and 'chemokine' genes in T-cell response to H_2O_2 stress

Ontological analysis of MeV module identified significantly regulated genes associated with 'cytokine activity' (Fisher score: 9.99E-05) and 'chemokine activity' (Fisher score: 2.11E-03) (Figure 5.7). Most of the genes were upregulated in T-cell activation, but H_2O_2 stress attenuated this upregulation, indicating that the delay of T-cell activation by H_2O_2 stress suppresses the secretion of cytokines and chemokines.

CCL20 shared similar transcription patterns with IL2, suggesting that it might have a positive role in T-cell activation like IL2. Reportedly, CCL20 is mainly secreted by epithelial cells and macrophages (69). The transcription upregulation of both CCL20 and IL2 was significantly abated by H_2O_2 stress, which was supported by secreted supernatant CCL20 assay (Figure 5.8). TNFSF4 along with CD70 has been reported to provide CD28-independent costimulatory signals to T cells (228). TNFSF4 was even more upregulated in H_2O_2 stressed sample. This indicates that TNFSF4 may play an important role in response to H_2O_2 stress besides its function as a costimulatory signal. IL3 is a potent growth promoting cytokine (229). It was significantly upregulated in T-cell activation at 10-48 hours, while H_2O_2 stress abated this upregulation, which supports its positive regulation of cell proliferation.

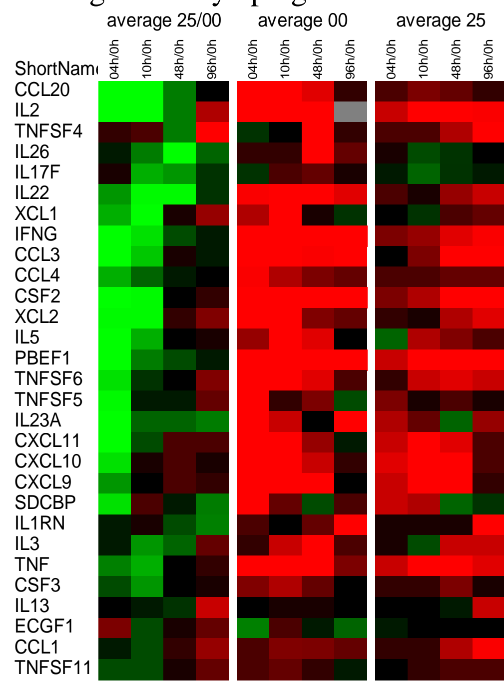

Figure 5.7. Expression profiles of significant genes associated with the Gene Ontology term 'cytokine activity' and chemokine activity'.

Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples.

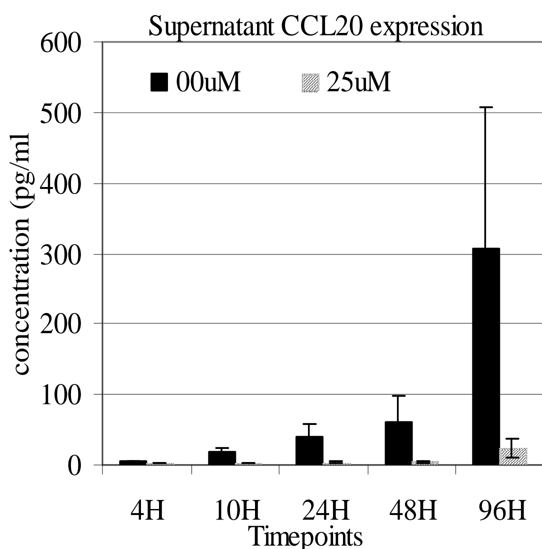

Figure 5.8. Induction of secretion of CCL20 in T-cell activation was decreased by H_2O_2 stress. CD3+ T cells were selected, incubated with 25µM H_2O_2 for 10mins, or let alone as control, stimulated (by anti-CD3/anti-CD28 antibodies), cultured separately. Supernatants were harvested at the indicated timepoints of culture to analyze the protein expression via ELISA assay. Data from three independent experiments were averaged.

5.3.5 Other significant genes identified by Ontological analysis in T-cell response to H_2O_2 stress

Ontological analysis identified several genes associated with 'MAPKKK cascade' (Fisher score: 1.43E-03) significantly regulated in response to H_2O_2 stress (Figure 5.9). MAPK3 and MAPK8 have been reported to play key roles in T-cell proliferation, apoptosis and differentiation

(146, 147). Transcription of MAPK3 and MAPK8 shared similar patterns; they were upregulated in T-cell activation and this upregulation was attenuated by H_2O_2 stress (Figure 5.9). Additionally, MAP2K4 shared similar expression patterns with MAPK8, which is consistent with its known function as a direct activator of MAP kinases such as MAPK8/JNK1 and MAPK14/p38 (230). The MAPKAPK2 kinase can be phosphorylated by p38 (231), and both of them are known to be involved in many cellular processes including stress and inflammatory responses. Protein encoded by MAP2K6 can phosphorylate and activate p38 MAP kinase in response to inflammatory cytokines or environmental stress (232). GADD45A is a pro-apoptotic protein, responding to environmental stresses by mediating activation of the p38/JNK pathway via MTK1/MEKK4 kinase (233). The transcription of GADD45A was upregulated in T-cell activation, and the H_2O_2 stress strongly attenuated this upregulation at 96 hours. Both phosphorylated protein p38 and phosphorylated protein ERK1 were upregulated upon T-cell activation, and this upregulation was suppressed by H_2O_2 stressed (Figure 5.10). Together, we hypothesize that MAPK3/ERK1 and MAPK14/p38 not only play important roles in T-cell activation, especially in proliferation, but are also involved in T-cell response to H_2O_2 stress.

Figure 5.9. Expression profiles of significant genes associated with the Gene Ontology term 'MAPKKK cascade'.

Color denotes degree of differential expression (saturated red = 3-fold up-

regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples.

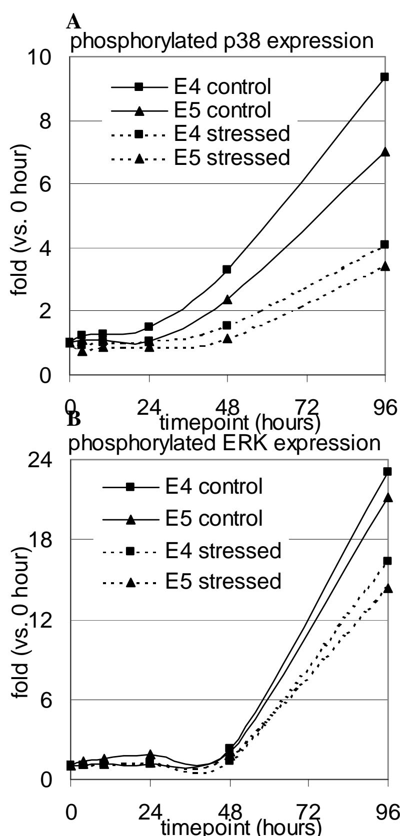

Figure 5.10. Induction of phosphorylated protein expression of (A) p38 and (B) ERK1 in T-cell activation was decreased by H_2O_2 stress.

CD3+ T cells were selected, incubated with 25µM H_2O_2 for 10mins, or let alone as control, stimulated (by anti-CD3/anti CD28 antibodies), cultured separately and harvested at the indicated timepoints of culture to analyze the protein expression via flow cytometric assays. Data from two independent experiments , E4 and E5, are shown.

Ontological analysis identified seven significantly regulated genes associated with 'Regulation of caspase activity' (Fisher score: 1.77E-03) (Figure 5.11). HSPE1 has been reported to induce the activation of pro-caspase 3 (115). Transcription of HSPE1 was upregulated in T-cell activation, and this upregulation was increased by H_2O_2 stress at 96 hours. Flow cytometry

assays revealed that active caspase 3 protein was upregulated in T-cell activation, but not affected by H_2O_2 stress (Figure 5.12). CASP1, PYCARD and COP1 shared similar transcription patterns, consistently downregulated in T-cell activation, while relatively upregulated by H_2O_2 stress. Both PYCARD and COP1 are activating adaptor and inhibitor of CASP1, respectively (234, 235). A simultaneous downregulation of PYCARD, COP1 and CASP1 were observed in T-cell activation. Taken together, our data suggested that CAPSP1 may not be active in T-cell activation, but play a role in response to H_2O_2 stress.

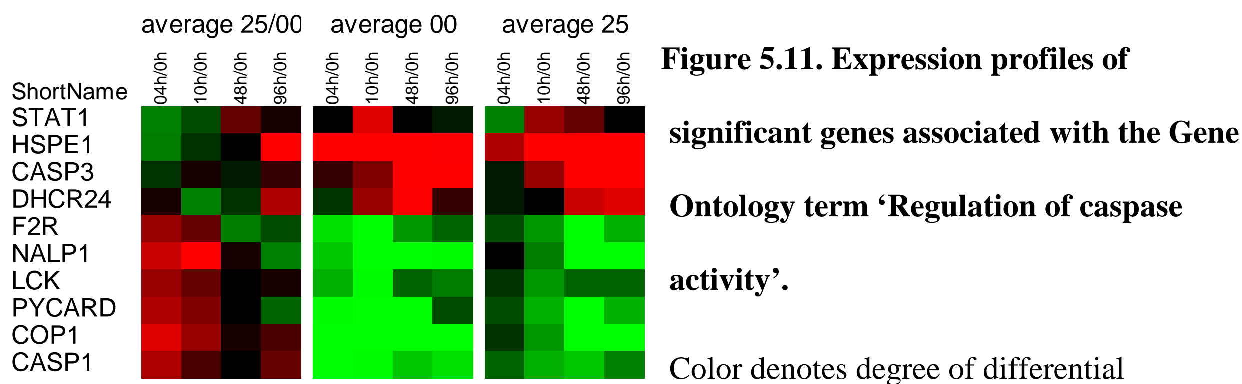

Figure 5.11. Expression profiles of significant genes associated with the Gene Ontology term 'Regulation of caspase activity'. Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples.

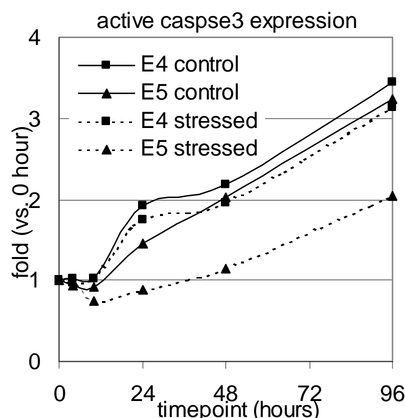

Figure 5.12. Induction of protein expression of active form of caspase 3 in T-cell activation was decreased by H_2O_2 stress.

CD3+ T cells were selected, incubated with 25µM H_2O_2 for 10mins, or let alone as control, stimulated (by anti-CD3/anti-CD28 antibodies), cultured separately and harvested at the indicated timepoints of culture to analyze the protein expression via flow cytometric assays. Data from two independent experiments, E4 and E5, are shown.

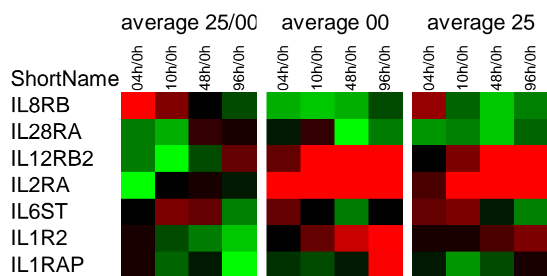

Figure 5.13. Expression profiles of significant genes associated with the Gene Ontology term 'Interleukin receptor activity'.

Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples.

Ontological analysis identified several significantly regulated genes associated with ‘Interleukin receptor activity’ (Figure 5.13) (Fisher score: 3.94E-03). IL8RB and IL6ST were relatively upregulated by H_2O_2 stress. Transcription of IL8RB, receptor binding to chemokine (C-X-C motif) ligand 1 (CXCL1/MGSA) (236) and IL8 (237), was downregulated in T-cell activation, while H_2O_2 stress abated this downregulation. IL2RA (CD25) was upregulated by T-cell activation and H_2O_2 suppressed this upregulation as well (Figure 5.1D). IL12RB2 shared similar transcription pattern with IL2RA. This suggests that IL12, similar to IL2, might be a pro-activation Interleukin and its receptor, IL12RB2, might be another surface maker of T-cell activation.

Ontological analysis identified several significantly regulated genes associated with ‘Positive regulation of I-kappaB kinase/NF-κB cascade’ (Fisher score: 1.10E-02) (Figure 5.14). This suggests that the NF-κB cascade is involved in T-cell response to H_2O_2 stress. The NF-κB complex has been of study focus and its activity is complex and cell type dependent (132).

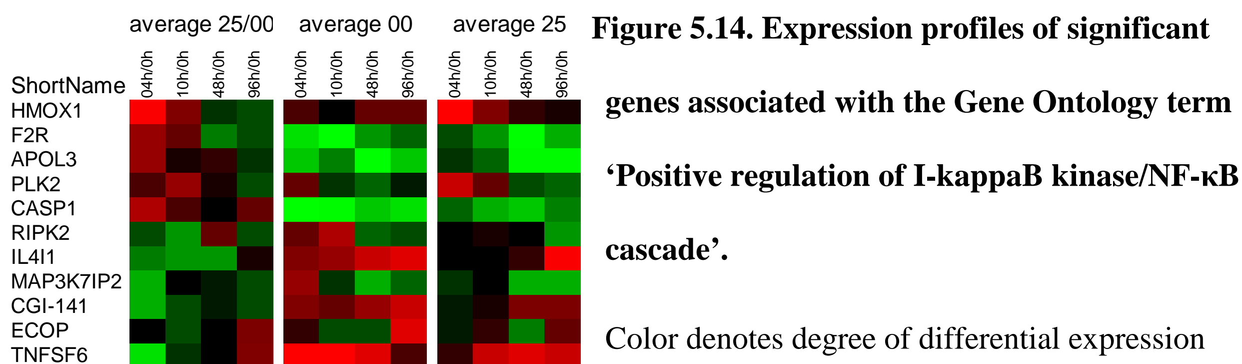

Figure 5.14. Expression profiles of significant genes associated with the Gene Ontology term ‘Positive regulation of I-kappaB kinase/NF-κB cascade’.

Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments. The first column is the ratios of stressed samples vs. the

control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples.

RIPK2 has been reported as a potent activator of NF-κB (134). Transcription of RIPK2 was upregulated in T-cell activation, while this upregulation was abated by H_2O_2 stress. Similarly, Transcription of NFKBIA was upregulated upon T-cell activation and this upregulation was attenuated by H_2O_2 stress. We therefore measured the protein level of phosphorylated RELA (p65), which is the major component of NF-κB complex. In control primary T cells, protein level of phosphorylated p65 increased in T-cell activation; while H_2O_2 stress suppressed this increase (Figure 5.15).

Ontological analysis identified several significantly regulated genes associated with 'Oxygen and reactive oxygen species metabolism' (Fisher score: 1.57E-02) (Figure 5.16). Protein encoded by SOD2 catalyzes the reaction that converts superoxide to hydrogen peroxide

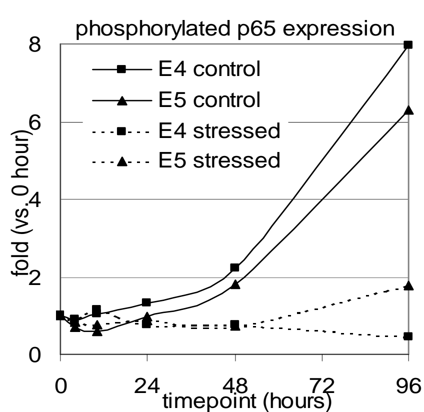

Figure 5.15. Induction of protein expression of phosphorylated p65 in T-cell activation was decreased by H_2O_2 stress.

CD3+ T cells were selected, incubated with 25µM H_2O_2 for 10mins, or let alone as control, stimulated (by anti-CD3/anti-CD28 antibodies), cultured separately and harvested at the indicated timepoints of culture to analyze the protein expression via flow cytometric assays. Data from two independent experiments, E4 and E5, are shown.

and diatomic oxygen ($2O2^- + 2H^+ \rightarrow H_2O_2 + O2$) (201). Transcription of SOD2 was upregulated in T-cell activation. Interestingly, this upregulation was attenuated by H_2O_2 stress, probably because of the abundance of intracellular H_2O_2, no need for the SOD2. IL4I1 catalyzes the reaction: L-amino acid + H_2O + O_2 = a 2-oxo acid + NH_3 + H_2O_2, producing iROS (238). IL4I1 was upregulated in T-cell activation, but relatively downregulated by H_2O_2 stress. Catalase (CAT) catalyzes the decomposition of hydrogen peroxide, producing H_2O and O_2 (239). Transcription of CAT was downregulated in T-cell activation, which could be one of the reasons leading to iROS accumulation in T-cell activation (240). H_2O_2 stress caused more iROS, which explains CAT was upregulated in H_2O_2 stressed sample compared to control. SORD catalyzes the reaction: L-iditol + NAD^+= L-sorbose + NADH, providing more NADH as substrates for oxidase (241). NCF2 is p67phox, cytosolic subunit of NADPH oxidase complex (242). Transcription of NCF2 was significantly downregulated in T-cell activation, while the upregulated by H_2O_2 stress. S100A8/S100A9 dimmer has been reported as a positive mediator of activation of phagocyte NADPH oxidase complex (243). Transcription of S100A8 and S100A9 was downregulted in T-cell activation, while relatively upregulated by H_2O_2 stress at either 4 or 10 hours. HBP1 has been suggested to contribute to the regulation of NADPH oxidase-dependent superoxide production through transcriptional repression of the p47phox gene (244). Taken together, our data suggests that NADPH oxidases and other enzymes involved in iROS regulation play important roles in T-cell activation and T-cell response to H_2O_2 stress.

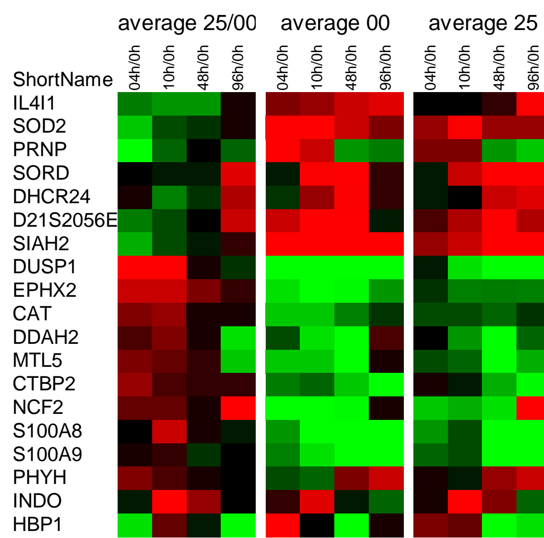

Figure 5.16. Expression profiles of significant genes associated with the Gene Ontology term 'Oxygen and reactive oxygen species metabolism'.

Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples.

CHAPTER 6: CONCLUDING REMARKS AND RECOMMENDATIONS

The immune system defends the host against pathogens and infectious diseases. When the immune system malfunctions, it can cause a broad range of severe disorders and diseases, including allergy, autoimmune disease, immune complex diseases, AIDS and leukemia. Understanding T-cell biology is fundamental to our understanding of immune system and therapeutic research targeting immune system disorders and diseases. Furthermore, a complete understanding of T-cell activation will provide insights in optimizing ex vivo T-cell expansion in application of immunotherapy, which has become more widespread and receives more attention in science and industry. Thus, the goal of this study was to investigate T-cell activation and T-cell response to oxidative stress, on a global, molecular level by genome-scale transcriptional analysis and moreover, use this information as platform as future research leads in immune system disorder and disease studies, as well as improve expansion protocols.

We employed a Gene-Ontology-driven transcriptional analysis coupled with protein abundance assays in order to identify novel T-cell activation genes and cell-type-specific genes involved in ‘immune response’. We identified potential genes involved in the communication between the two subsets (including IL23A, NR4A2, CD83, PSMB2, -8, MIF, IFI16, TNFAIP1, POU2AF1, and OTUB1) and would-be effector-function-specific genes (XCL2, SLAMF7, TNFSF4, -5, -9, CSF3, CD48 and CD244). Chemokines induced during T-cell activation but not previously identified in T cells include CCL20, CXCL9, -10, -11 (in all three populations), and XCL2 (preferentially in CD8+ T cells). Increased expression of other unexpected cytokines

(GPI, OSM and MIF) suggests their involvement in T-cell activation with their functions yet to be examined. Differential expression of many receptors, not previously reported in the context of T-cell activation, includes CCR5, CCR7, IL1R2, IL1RAP, IL6R, TNFRSF25 and TNFRSF1A, thus suggesting their role in this immune process. Several receptors involved in TCR activation (CD3D, CD3G, TRAT1, ITGAL, ITGB1, ITGB2, CD8A and B (CD8+ T-cell specific) along with LCK, ZAP-70 and TYROBP were synchronously downregulated. Members of cell-surface receptors (HLA-Ds and KLRs), none previously identified in the context of T-cell activation, were also downregulated. This comparative genome-scale, transcriptional analysis of T-cell activation in the CD4+ and CD8+ subsets and the mixed CD3+ populations made possible the identification of many immune-response genes not previously identified in the context of T-cell activation. Significantly, it made possible to identify the temporal patterns of many previously known T-cell activation genes, and also identify genes implicated in effector functions of and communication between CD4+ and CD8+ T cells.

The equivalence (the quantity and expression patterns) of anti-apoptotic and pro-apoptotic genes suggests a balance between the anti-apoptotic and pro-apoptotic signalings in T-cell activation. We identified significantly regulated apoptotic genes in key protein families and detailed their transcriptional kinetics, validated by protein expression patterns of selected genes (BCL2A1, BBC3 and CASP3). The simultaneous upregulation of NF-κB and IκB family genes (REL, RELA, and RELB, NFKBIA, NFKBIE and NFKB1) at 48-96 hours, supported by the increase of phosphorylated RELA (p65) at 48-96 hours, suggests an active involvement of the NF-κB complex during this time period in our experiments. A close examination of significant regulated genes revealed an increase of p38 and ERK1 signalings at 48-96 hours, which was

explored using phosphorylation assays for p38 (MAPK14) and ERK1 (MAPK3). A quickly acquired, transient activity of AP-1 measured by DNA-binding activity assay suggests an immediate and transient activation of p38 and/or JNK MAP kinase cascades in T-cell activation. Ontology analysis also identified significantly regulated genes associated with 'cell cycle' and 'mitochondria'. A group of genes sharing the similar transcriptions pattern (significantly upregulated at 48-96 hours, but not at 4 or 10 hours) with crucial cell cycle genes were identified by the self-organizing tree algorithm (SOTA) analysis. We hypothesize that this cluster of gene are cell cycle signature genes in T-cell activation, which provides a valuable platform for immune system disorder and malignancies.

T cells face oxidative stress, executed by intracellular reactive oxygen species (iROS) in aging, infections and certain disease conditions. We observed that T-cell activation was delayed in a dose dependent manner when exposed to 25–75 μM H_2O_2, the estimated iROS level in the physiological microenvironment of an inflammation site. Ontology analysis identified significantly regulated genes by H_2O_2 stress including genes associated with 'response to stimulus', 'regulation of apoptosis', 'cytokine activity', 'chemokine activity', 'MAPKKK cascade', 'Positive regulation of I-kappaB kinase/NF-κB cascade', and 'Oxygen and reactive oxygen species metabolism'.

In summary, DNA microarray technology was successfully applied to monitor and characterize ex vivo T-cell activation, expansion and T-cell response to H_2O_2 stress. Comprehensively integrating previous knowledge, this genome scale transcriptional study in conjunction with complementary methodologies improves our understanding of T-cell activation

and suggests substantial future research leads in studies of immune system disorder, immune suppression in transplants, and ex vivo T-cell activation and expansion.

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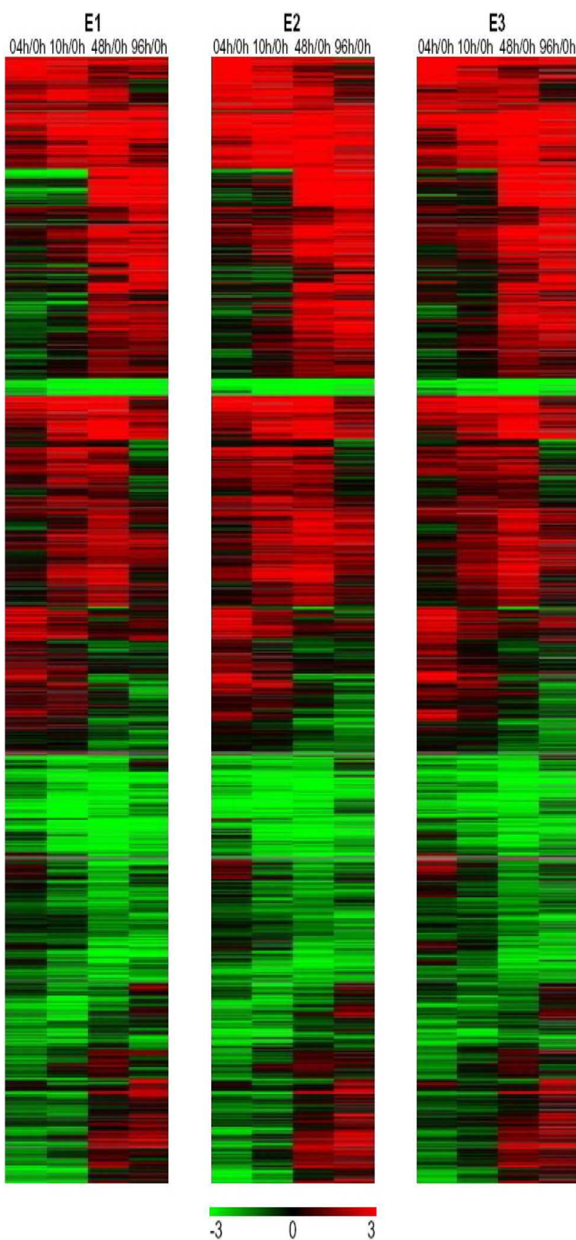
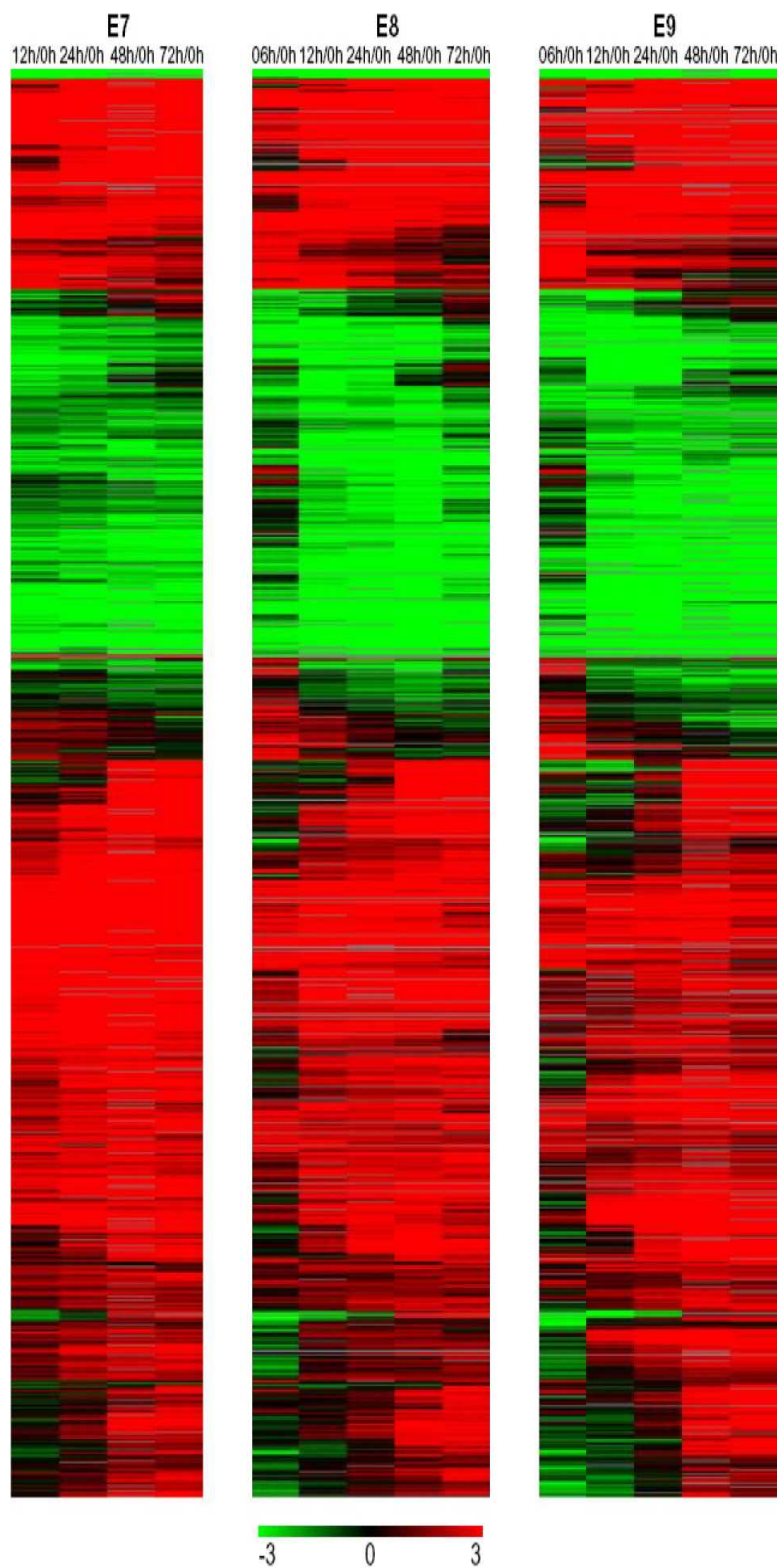
APPENDICES



Appendix A1. Chapter 2 Reproducibility of expression profiles of the T-cell activation in CD3+ cells.

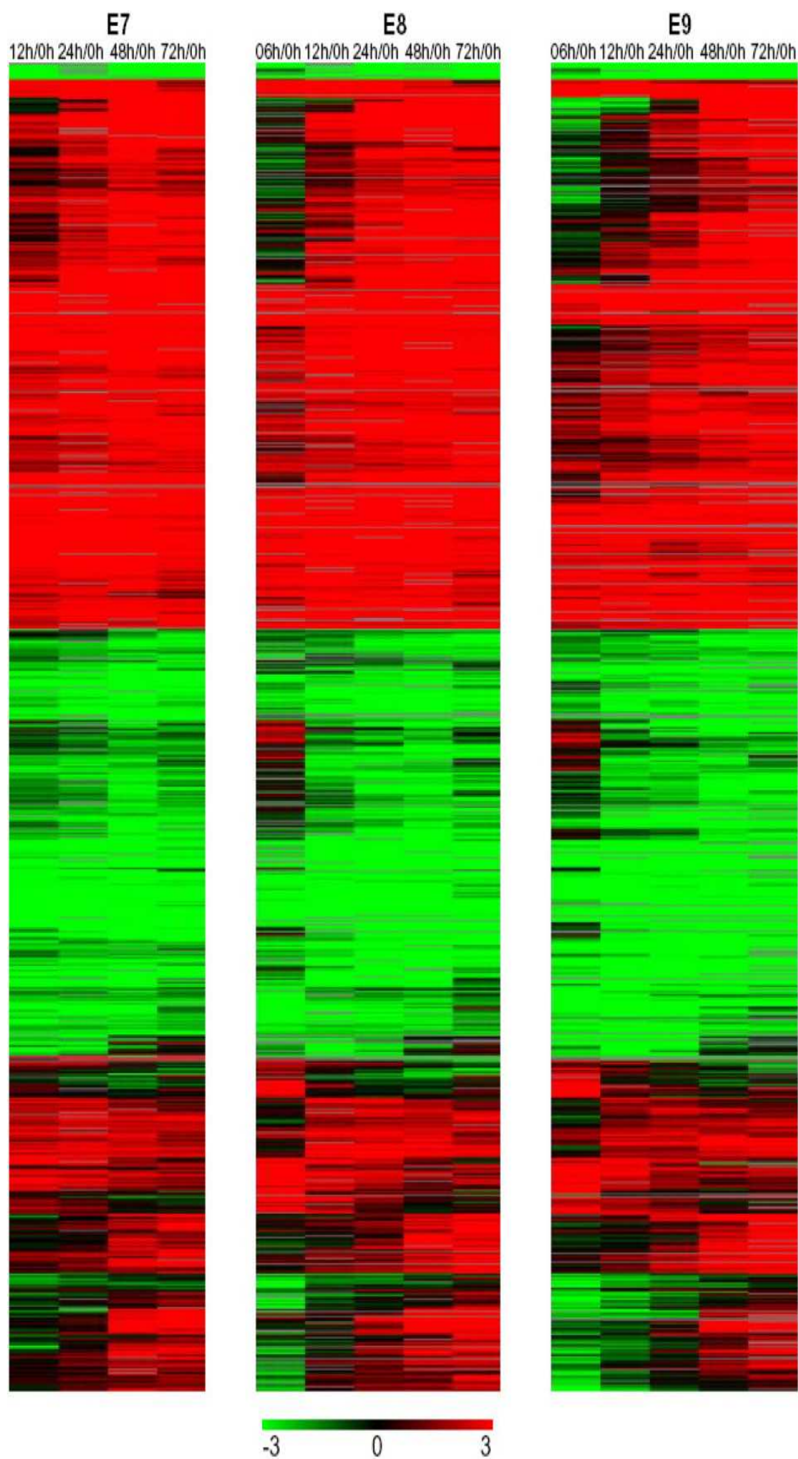
Hierarchical clustering (using the Euclidian distance metric) of the 3793 significant genes in T-cell activation of CD3+ cells in three independent biological experiments, E1-E3, (timepoints at 4, 10, 48 and 96 hours) demonstrated high reproducibility. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold upregulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available).



Appendix A2. Chapter 2 Reproducibility of expression profiles of the T-cell activation in CD4+ cells.

Hierarchical clustering (using the Euclidian distance metric) of the 1463 significant genes in T-cell activation of CD4+ cells in three independent biological experiments, E7-E9, (timepoints at 12, 24, 48 and 72 hours in one experiment; and timepoints at 6, 12, 24, 48 and 72 hours in the other two experiments) demonstrated high reproducibility. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold upregulation, saturated green = 3-fold down-

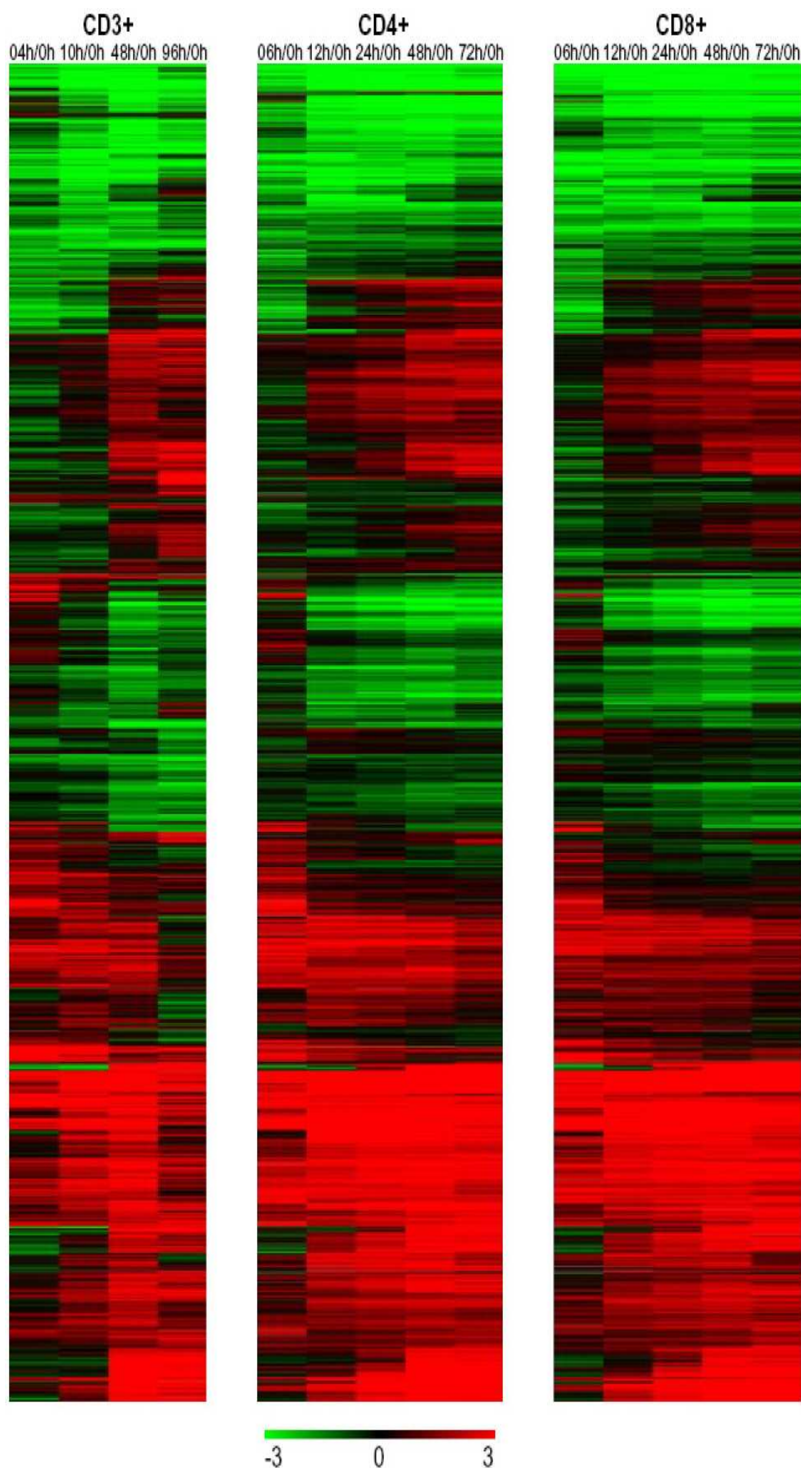
regulation, black = unchanged, gray = no data available).



Appendix A3. Chapter 2 Reproducibility of expression profiles of the T-cell activation in CD8+ cells.

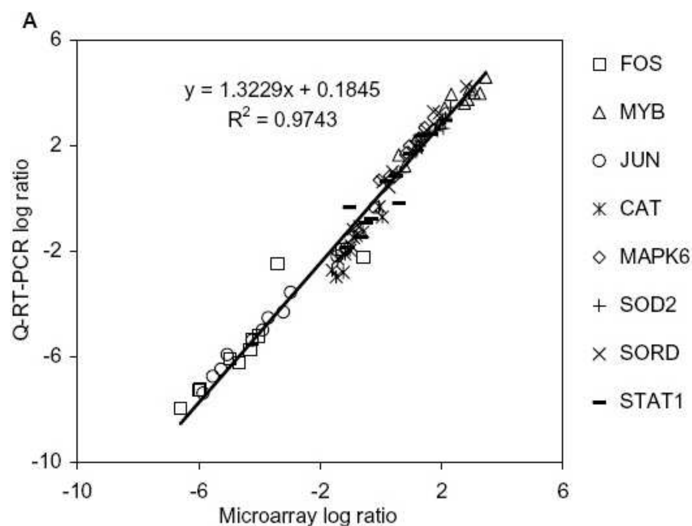
Hierarchical clustering (using the Euclidian distance metric) of the 1258 significant genes in T-cell activation of CD8+ cells in three independent biological experiments, E7-E9, (timepoints at 12, 24, 48 and 72 hours in one experiment; and timepoints at 6, 12, 24, 48 and 72 hours in the other two experiments) demonstrated high reproducibility. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold upregulation, saturated green = 3-fold down-regulation, black =

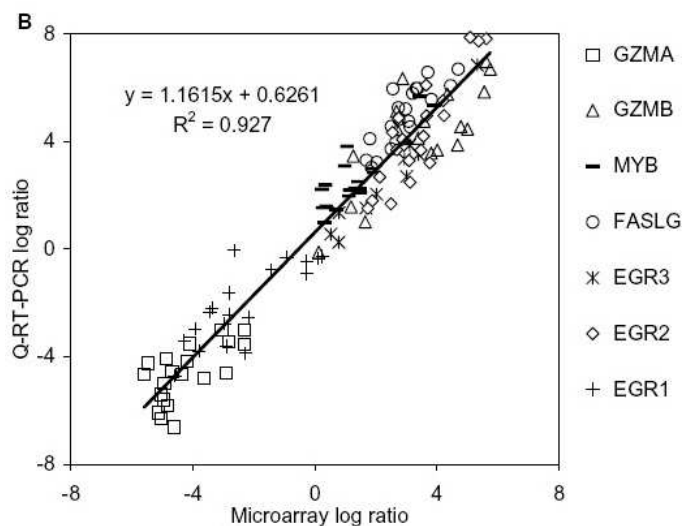
unchanged, gray = no data available).



Appendix A4. Chapter 2 The three populations, CD3+, CD4+ and CD8+ T cells, shared largely conserved expression patterns for the significant genes.

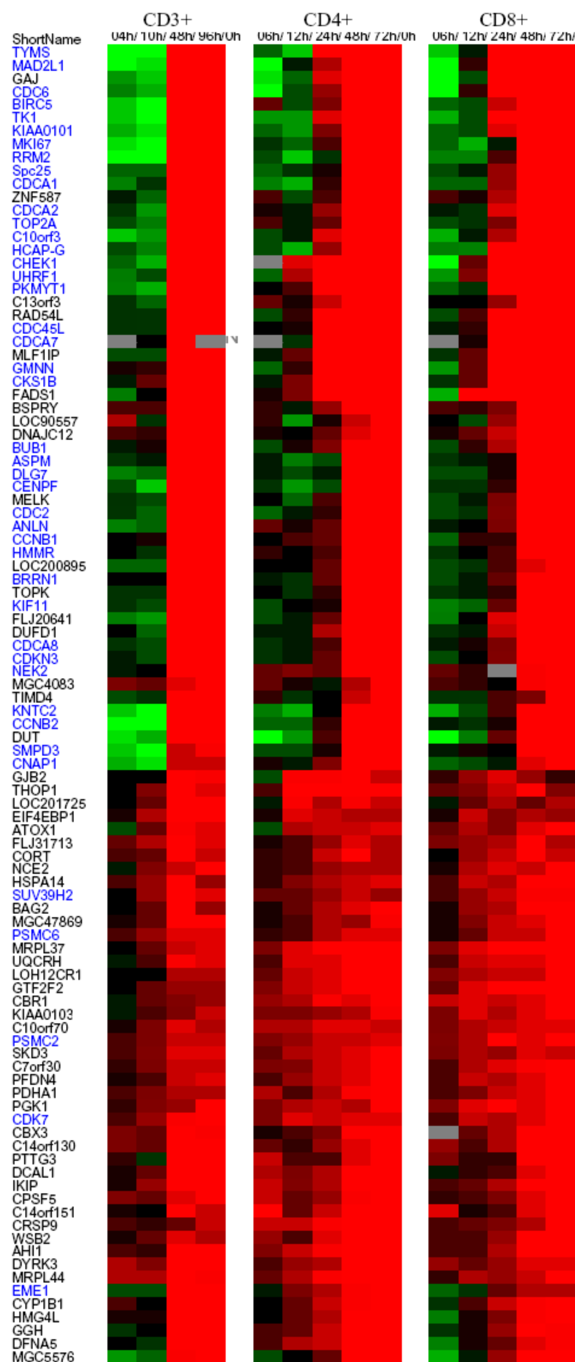
Demonstrated by the hierarchical clustering (using the Euclidian distance metric) of the combined 4167 significant genes upon T-cell activation in CD3+, CD4+ and CD8+ populations (average of three biological-replicate experiments for each population). Color denotes degree of differential expression comparing to 0 hour (saturated red = 3-fold upregulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available).





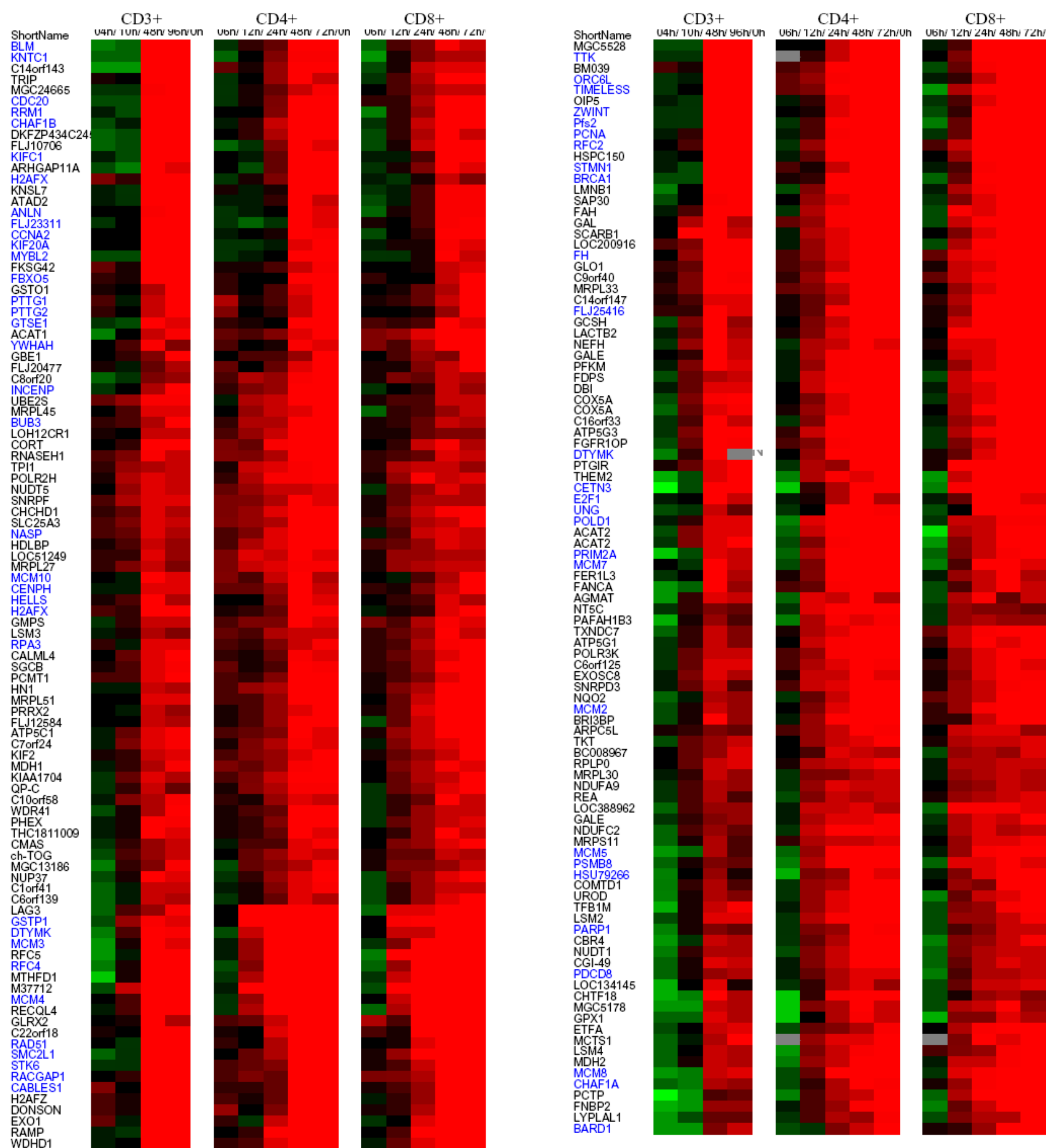
Appendix A5. Chapter 2 Q-RT-PCR validation of microarray results across multiple culture samples.

Q-RT-PCR validation of microarray results across multiple culture samples. (**A**) Q-RT-PCR versus microarray log expression ratios (timepoint vs. 0 hour) from CD3+ T-cell activation experiments, E1-E3, (for all 12 (= 3x4) timepoints: 4, 10, 48 and 96 hours of 3 experiments) for each of the 8 selected genes (FOS, MYB, JUN, CAT, MAPK6, SORD, SOD2, and STAT1). (**B**) Q-RT-PCR versus microarray log expression ratios (timepoint vs. 0 hour) from CD4+ and CD8+ T-cell activation experiments, E8 and E9, (for all 20 (= 2x2x5) timepoints: 6, 12, 24, 48 and 72 hours of 2 experiments) for each of the 7 selected genes (EGR1, EGR2, EGR3, FASL, GZMA, GZMB, and MYB).

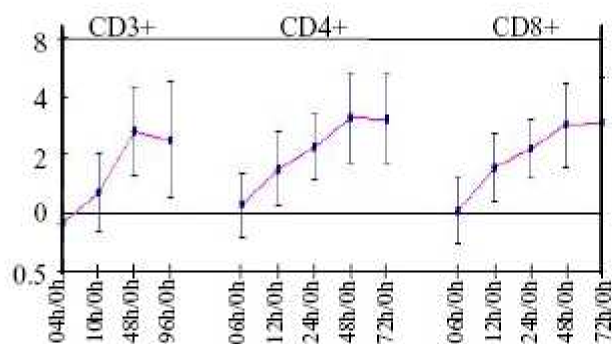


Appendix A6. Chapter 4 Signature T-cell activation cell cycle genes.

The MeV self-organizing tree algorithm (SOTA) module identified a cluster of potential signature T-cell activation cell cycle genes base on their transcription kinetics: significantly upregulated at 48-96 hours, but not at 4 or 10 hours. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell population. Genes have been associated with cell cycle (per corresponding gene pages in NCBI http://www.ncbi.nlm.nih.gov/ and references therein) are highlighted blue.

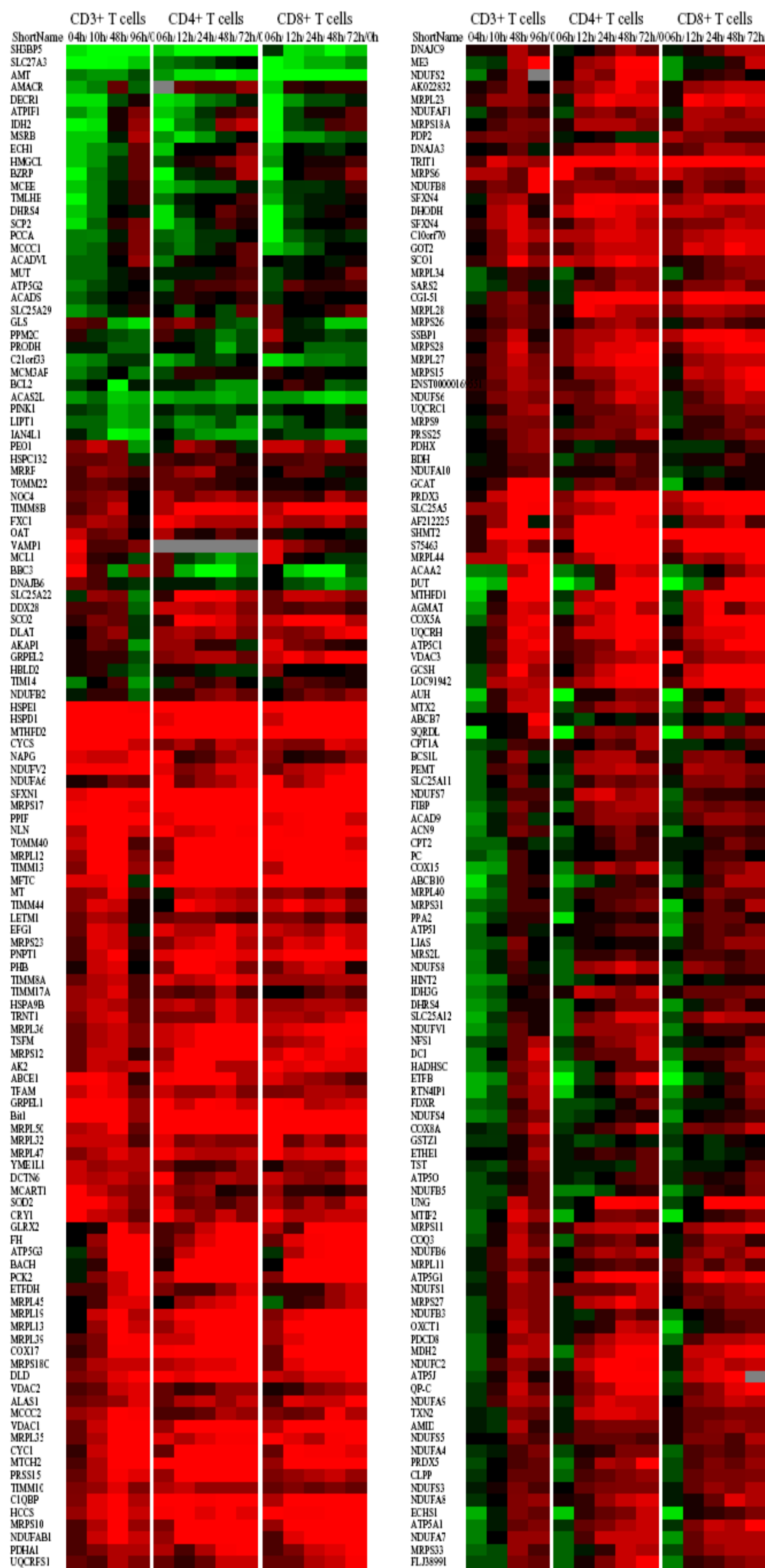


Appendix A6. Chapter 4 Signature T-cell activation cell cycle genes continued.



Appendix A7. Chapter 4 The centroid graph of the signature T-cell activation cell cycle genes produced by the MeV self-organizing tree algorithm (SOTA) module.

Based on the expression averages from three independent biological experiments for each T-cell population.



Appendix A8. Chapter 4

Expression profiles of genes associated with 'mitochondion'.

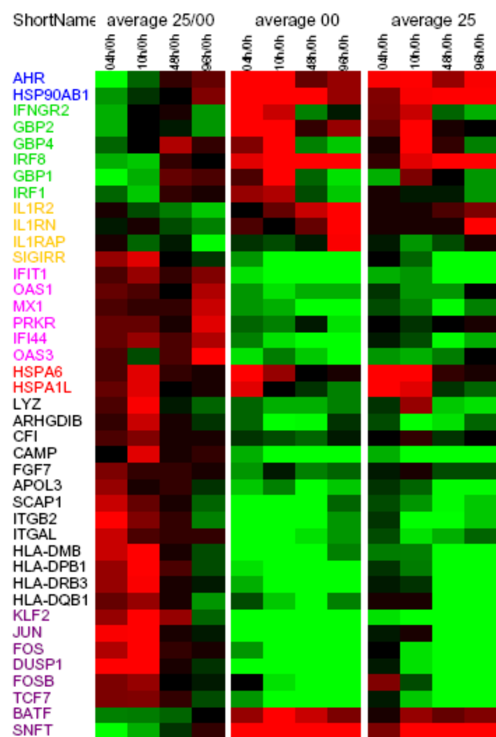
Expression patterns of genes that were differentially expressed temporally in T-cell activation of the three (CD3+, CD4+ and CD8+) populations, based on hierarchical clustering using the Euclidian distance metric. Color denotes degree of differential expression compared to 0 hour (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments for each T-cell

population.

File	Term	List Hits	List Size	Pop. Hits	Pop. Size	Fisher's exact
biological_process	response to biotic stimulus	127	762	835	10721	2.28E-17
biological_process	immune response	110	762	694	10721	2.29E-16
biological_process	defense response	117	762	791	10721	4.26E-15
biological_process	response to stress	120	762	829	10721	9.05E-15
biological_process	response to pest, pathogen or parasite	77	762	444	10721	9.99E-14
biological_process	response to stimulus	194	762	1712	10721	1.84E-12
biological_process	response to wounding	46	762	262	10721	7.77E-09
biological_process	response to external stimulus	57	762	365	10721	1.09E-08
biological_process	negative regulation of cellular process	77	762	648	10721	4.12E-06
biological_process	cellular defense response	17	762	75	10721	1.50E-05
biological_process	inflammatory response	29	762	177	10721	1.86E-05
biological_process	negative regulation of cellular physiological process	69	762	589	10721	2.15E-05
biological_process	negative regulation of biological process	79	762	702	10721	2.30E-05
biological_process	negative regulation of physiological process	69	762	612	10721	7.34E-05
biological_process	regulation of apoptosis	42	762	321	10721	8.26E-05
biological_process	regulation of programmed cell death	42	762	323	10721	9.52E-05
molecular_function	cytokine activity	30	824	206	11771	9.99E-05
biological_process	antimicrobial humoral response	16	762	79	10721	1.13E-04
biological_process	anti-apoptosis	19	762	105	10721	1.32E-04
biological_process	negative regulation of apoptosis	21	762	124	10721	1.60E-04
biological_process	negative regulation of programmed cell death	21	762	125	10721	1.80E-04
biological_process	detection of stimulus	9	762	33	10721	3.60E-04
biological_process	response to abiotic stimulus	41	762	334	10721	3.97E-04
biological_process	death	37	762	292	10721	4.09E-04
biological_process	cell death	37	762	292	10721	4.09E-04
biological_process	response to chemical stimulus	37	762	294	10721	4.67E-04
biological_process	programmed cell death	33	762	260	10721	8.01E-04
biological_process	apoptosis	33	762	260	10721	8.01E-04
biological_process	MAPKKK cascade	8	762	32	10721	1.43E-03
biological_process	rRNA metabolism	13	762	72	10721	1.51E-03
biological_process	regulation of caspase activity	7	762	26	10721	1.77E-03
molecular_function	chemokine receptor binding	9	824	42	11771	2.11E-03
molecular_function	chemokine activity	9	824	42	11771	2.11E-03
molecular_function	oxygen transporter activity	5	824	15	11771	2.76E-03
molecular_function	interleukin receptor activity	7	824	30	11771	3.94E-03
molecular_function	MHC class II receptor activity	4	824	11	11771	5.28E-03
biological_process	positive regulation of caspase activity	6	762	24	10721	5.62E-03
molecular_function	SH3/SH2 adaptor activity	8	824	42	11771	7.82E-03
molecular_function	hematopoietin/interferon-class (D200-domain) cytokine re	8	824	42	11771	7.82E-03
molecular_function	NF-kappaB binding	3	824	7	11771	9.66E-03
biological_process	positive regulation of I-kappaB kinase/NF-kappaB cascade	11	762	71	10721	1.10E-02
biological_process	oxygen and reactive oxygen species metabolism	10	762	65	10721	1.57E-02

Appendix A9. Chapter 5 Gene ontology analysis of significantly regulated genes in T-cell response to H_2O_2 stress.

Ontological analysis of MeV module associated significantly regulated genes with Gene Ontology terminologies. Statistic significance of the Gene Ontology terminologies was demonstrated by p-value based Fisher’s score.



Appendix A10. Chapter 5 Expression profiles of selected significant genes associated 'response to stimulus'.

Color denotes degree of differential expression (saturated red = 3-fold up-regulation, saturated green = 3-fold down-regulation, black = unchanged, gray = no data available). Expression data shown are averages from three independent biological experiments. The first column is the ratios of stressed samples vs. the control samples; the second column is the ratios of timepoints vs. 0 hour in control samples; the third column is the ratios of timepoints vs. 0 hour in stressed samples. The gene names are color coded for the convenience of review.