International Immunology, Vol. 17, No. 8, pp. 1009–1021
doi:10.1093/intimm/dxh283

Global analysis of IL-2 target genes: identification of chromosomal clusters of expressed genes

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Keywords: genomics, microarray, target gene, SAGE, interleukin-2

Abstract

T lymphocytes play a central role in controlling adaptive immune responses. IL-2 critically regulates both T cell growth and death and is involved in maintaining peripheral tolerance, but the molecules involved in these and other IL-2 actions are only partially known. We now provide a comprehensive compendium of the genes expressed in T cells and of those regulated by IL-2 based on a combination of DNA microarrays and serial analysis of gene expression (SAGE). The newly identified IL-2 target genes include many genes previously linked to apoptosis in other cellular systems that may contribute to IL-2-dependent survival functions. We also studied the mRNA expression of known regulators of signaling pathways for their induction in response to IL-2 in order to identify potential novel positive and/or negative feedback regulators of IL-2 signaling. We show that IL-2 regulates only a limited number of these genes. These include suppressors of cytokine signaling (SOCS) 1, SOCS2, dual-specificity phosphatase (DUSP) 5, DUSP6 and non-receptor type phosphatase-7 (PTPN7). Additionally, we provide evidence that many genes expressed in T cells locate in chromosomal clusters, and that select IL-2-regulated genes are located in at least two clusters, one at 5q31, a known cytokine gene cluster, and the other at 6p21.3, a region that contains genes encoding the tumor necrosis factor (TNF) superfamily members TNF, LT-α and LT-β.

Introduction

T lymphocytes play a central role in orchestrating adaptive immune responses to pathogens such as bacteria and viruses. T cell development, maturation and responsiveness are regulated in part by secreted proteins called cytokines. Cytokines are essential for the integrity of the immune system, and abnormal cytokine function can result in immunodeficiency or autoimmunity (1, 2). One cytokine that has an essential role in regulating immune responses is IL-2 (1), which is produced mainly by activated $CD4^+$ T lymphocytes in response to antigen stimulation (1). The actions of IL-2 are mediated by signal transduction cascade(s) that are initiated by IL-2-induced oligomerization of IL-2Rα chain, IL-2Rβ and the common cytokine receptor γ chain (γ_c) on activated T cells (2, 3). This juxtaposes cytoplasmic Janus family tyrosine kinases Jak1 and Jak3 that associate with IL-2Rβ and γ_c, respectively (2–4). Activated Jak kinases phosphorylate specific tyrosine residues in IL-2Rβ that serve as docking sites for SH2 domain-containing signaling molecules, such as Stat5a, Stat5b and Shc (2). Eventually, activation of multiple signaling pathways, including phosphatidylinositol 3kinase (PI3-K), Jak-STAT and Ras-MAP kinase (MAPK)-coupled pathways leads to the transcription of target

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Received 7 February 2005, *accepted* 11 May 2005
Transmitting editor: T. Hirano
Advance Access publication 24 June 2005

genes that contribute to IL-2-dependent biologic actions (2, 3).

IL-2 has multiple roles in regulating T cell function (1). During T cell clonal expansion after initial antigen encounter, IL-2 functions as a growth and survival factor. Additionally, IL-2 can sensitize proliferating T cells to death triggered by secondary TCR stimulation in a process known as activation-induced cell death (AICD) (4). IL-2 is also necessary for the development and maintenance of regulatory T cells, which are a population of $CD4^+$ T cells that suppress other T cells (5, 6). Loss of IL-2 function results in defective AICD and in a decrease of regulatory T cells, resulting in the expansion of autoreactive T lymphocytes and autoimmunity (6–10). Thus, an essential role for IL-2 is to serve as a key guardian of peripheral tolerance.

Although many functions of IL-2 are known and the early signaling events induced by IL-2 are well established, the genes that mediate IL-2-dependent biological actions remain poorly characterized. IL-2 is known to regulate the function of transcription factors such as Stat5a, and Stat5b and Stat3, and these are important for IL-2-dependent functions (11–14). For example, T cells from mice deficient either in Stat5a (13) or Stat5b (14) show decreased IL-2-dependent proliferation, and mice deficient in both Stat5a and Stat5b show a more severe defect in IL-2-dependent proliferation (15). Thus, IL-2-activated Stat5-regulated genes such as cyclin D2, Pim-1, Fas ligand (FasL), CIS and suppressors of cytokine signaling (SOCS) 1 are implicated in mediating the biologic actions of IL-2 (3). IL-2 presumably also regulates gene expression through other factors, as it also activates Ras-MAPK and PI3-K/Akt-signaling pathways but the genes regulated by these pathways are less well characterized (3, 15). In order to understand how IL-2-regulated signaling pathways converge to mediate biologic actions, we sought to identify the genes regulated by those pathways.

The identification of genes regulated by IL-2 was long hindered by lack of suitable methodologies. However, the development of technologies such as microarrays and serial analysis of gene expression (SAGE) has allowed the comprehensive analysis of genes involved in different cellular processes (16, 17). When combined with large-scale transcript mapping, novel insights into disease pathogenesis as well as gene regulation have emerged from these methodologies in other systems (18, 19). Recently, microarrays were used to identify ~200 putative IL-2 target genes (20, 21). We now extend this analysis and use both DNA microarrays and SAGE to generate a broader compendium of genes expressed in T cells and in particular those regulated by IL-2. We have studied the genomic organization of these genes and present evidence that many genes expressed in T cells are organized into chromosomal clusters, with some IL-2-regulated genes locating in clusters at chromosomes 5q31 and 6p21 as well. The relevance of these findings from the viewpoint of gene regulation is discussed.

Methods

Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers at the National Institutes of Health Blood Bank. PBMC were isolated by centrifugation on Ficoll-Paque density gradient (Pharmacia, Uppsala, Sweden), suspended in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (U.S. Biotechnologies, Inc., Parkerford, PA, USA), 100 $\mu g\ ml^{-1}$ streptomycin, 100 U ml^{-1} penicillin and 2 mM glutamine (all from Cellgro, VA, USA), and stimulated for 18 h with 2 $\mu g\ ml^{-1}$ PHA (Boehringer Mannheim). T lymphocytes were selectively expanded for 10 days in complete RPMI medium supplemented with 500 ng ml^{-1} PHA and 50 U ml^{-1} IL-2 (Tecin™, Roche, Nutley, NJ, USA). The cells were then washed twice, resuspended in complete RPMI medium and rested for 3 days. T cells (>95% $CD3^+$, IL-$2R\alpha^+$) were stimulated or not stimulated with 100 U ml^{-1} of IL-2 for 4 h to induce gene expression, and then pelleted and stored at −80°C. In some experiments $CD4^+$ or $CD8^+$ T cells were isolated prior to culture using positive selection and magnetic beads (Miltenyi Biotec, GmBH, Germany). For TCR stimulation experiments, T cells were purified using magnetic beads (Pan-T cell isolation kit, Miltenyi Biotec) and stimulated for 4 h on plates that had been coated for 1 h with anti-CD3 (5 $\mu g\ ml^{-1}$) and anti-CD28 (1 $\mu g\ ml^{-1}$) (both from PharMingen, San Diego, CA, USA), after which the cells were pelleted and stored at −80°C. Four replicate experiments were performed.

T cells from wild-type, $Stat5a^{-/-}$, and $Stat5b^{-/-}$ mice were activated with anti-CD3 (5 $\mu g\ ml^{-1}$) and anti-CD28 (1 $\mu g\ ml^{-1}$) mAbs and cultured in the presence of IL-2 (100 U ml^{-1}) for 7 days. T cells from $Stat5a/b^{-/-}$ double-knockout mice were activated for only 18 h, because the cells do not proliferate in response to anti-CD3 and IL-2 (22). The generation of $Stat5a^{-/-}$, $Stat5b^{-/-}$ and Stat5a/b double-deficient mice has been described (23–25). After activation, cells were washed twice with PBS, and rested for 18 h in RPMI complete medium without IL-2, then stimulated with IL-2 (100 U ml^{-1}) for 4 h, lysed with Trizol (GIBCO) and the lysates were stored at −20°C.

mRNA preparation and GeneChip hybridization

The mRNA was extracted from frozen cells using either RNeasy (Qiagen, Valencia, CA, USA) or FastTrac 2.0 (Invitrogen, Carlsbad, CA, USA) RNA-extraction kits.

Identification of constitutively expressed and differentially expressed genes by GeneChip microarrays

RNA was processed to cRNA probes for GeneChip analysis (Affymetrix Inc., Santa Clara, CA, USA). The probes were hybridized to U95A GeneChips (Affymetrix), washed and scanned (Hewlett Packard, Gene Array scanner G2500A) according to procedures outlined by the manufacturer (Affymetrix). Scanned images were processed using the Microarray Suite™ version 4 (Affymetrix) which yielded for each probe set average difference values, as well as 'absent' or 'present' calls. Four similar experiments were performed, each of which consisted of one control and one IL-2-treated sample. A transcript was considered to be 'present' when the corresponding probe set gave a 'present call' in at least three of four experiments for either control or IL-2-treated T cells. Data were further analyzed for differential expression using methods and programs developed by some of us (P.J.M.), and

these are available from our website, http://abs.cit.nih.gov/main/CIT_Bioinformatics_Cooper.htm or http://affylims.cit.nih.gov, as scripts for the JMP-software's (SAS Inc, Cary, NC, USA) statistics package. Average difference values were normalized, as described previously (26–28). The statistical significance of change in expression was determined using a consistency test for each gene (29). This statistical test is similar to a paired Student's *t*-test, but has greater power with small replication numbers. A set of differentially expressed genes was chosen to have an estimated false discovery rate (FDR) of <5% (i.e. at most 5% of the set is likely due only to chance) (30, 31). The analysis of TCR-regulated genes was performed as described above, using U133A Genechips™ (Affymetrix).

Genomic localization of expressed genes

Affymetrix target nucleotide sequences (~400 bases in length) for 12 453 of the 12 625 probe sets were compared to the public human genome sequence (NCBI build, December 22, 2001 release) using BLAT (32–34). A target sequence was considered to be located on the genome if its alignment score (number of matched bases minus number of mismatches minus gap penalties) (33) was ⩾100 and its percent identity to the genomic locus was ⩾60%. A total of 11 115 target sequences were so located. Of these, 36 target sequences mapped to more than one genomic locus and were excluded, leaving 11 079 probe sets with unique genome locations. When multiple probe sets represented a single gene (i.e. same UniGene cluster or same GenBank identifier), we chose the probe set of the highest apparent quality (lowest calculated FDR for differential expression). In this fashion, we obtained expression measurements for 8582 distinct genes.

Clusters of differentially or constitutively expressed genes

We sought to determine if the distribution of expressed genes along the chromosome was random or whether the expressed genes were clustered. A set of genes was considered to form a cluster if the size of the genomic region they occupied was less than would be expected by chance, or alternatively a region represented a cluster if it contained more genes than it should by chance. More specifically, we looked for gene clusters by observing the length of chromosome segments occupied by each set of k consecutive differentially (or constitutively) expressed genes. We define k genes as clustered if the length of their occupied segment (k-span) is shorter than would be expected by chance. To determine the statistical significance for an observed k-span, we generated numerous copies of the genome with the actual gene locations, but with the differentially expressed genes marked at random, keeping the number of marked genes per chromosome constant. For each k, and each randomly marked copy of the genome, the smallest k-span was computed, forming a distribution of values for the minimal k-span under the null hypothesis that the differentially expressed genes are distributed randomly among the genes in each chromosome. The statistical significance of a cluster of k genes under consideration was then evaluated by comparing its *span* to this distribution. Since the number of genes, k, was kept fixed in this simulation, we call the significance calculated as above, the 'k-specific' P value. In fact, we did not know, *a priori*, what size clusters to look for, and therefore scanned over all values of k from 2 to 10 genes (i.e. we searched for clusters comprising up to 10 genes). A correction on the significance was therefore required and was obtained by retaining the minimum k-specific P value for each iteration, over the range $2 \leqslant k \leqslant 10$. This second distribution allows for correction for the fact that k is, in general, unspecified. We refer to the significance calculated in this fashion as the 'k-unspecified P value'. No restrictions were placed on k for clusters of constitutively expressed genes. Careful simulations done in this manner protect from finding false clusters solely due to the non-uniform spacing of genes in the genome, or due to the fact that neither the size nor the length of clusters being sought could be specified ahead of time.

Generation of SAGE libraries

SAGE libraries were generated from 10 μg mRNA from IL-2-stimulated or unstimulated T lymphocytes. We used the protocol available at www.SAGENET.org/sage_protocol.htm, except that streptavidin-coated tubes (Boehringer Mannheim) were used instead of streptavidin-coated magnetic beads, and the elution step was done using an Elutrap chamber (S&S). Clones containing different numbers of tags were screened by PCR, and those with an insert size of ⩾500 bp were sequenced by the National Institutes of Health Intramural Sequencing core. SAGE tag analysis was done using eSAGE (http://genome.nhgri.nih.gov/eSAGE/) and the SAGE2000 (www.SAGENET.org/sage_protocol.htm) software. Replicate ditags as well as tags corresponding to the linker sequence were excluded. The tags were identified by linking them with UniGene clusters (build #146, ftp://ncbi.nlm.nih.gov), and tag counts were summed for each cluster. UniGene identifiers linked by one or more SAGE tags were mapped to the UCSC build of the genome (UCSC, December 22, 2001), using the UCSC Human Genome Project annotation database for UniGene identifiers and the same criteria as for the microarray data. Relative positions from the different genome builds were used to compare UniGene clusters identified from SAGE data with those from Affymetrix data. Of the entire UniGene database of 52 783 identifiers that could be mapped, our SAGE tags corresponded to 8883 of these (http://genome-archive.cse.ucsc.edu/goldenPath/22dec2001/database/uniGene.txt.gz).

Real-time PCR

Real-time PCR was performed using an ABI Prism Instrument (Applied Biosystems). Total RNA was reverse transcribed using the RNA PCR gold kit (Applied Biosystems). Ten nanograms of cDNA was amplified for 40 cycles with primers for Bcl-2, Bcl-XL, nuclear factor (NF)-κB1, STK17A, Caspase 3, FasL, TRAIL or tumor necrosis factor (TNF) and normalized against mRNA for 18S rRNA, a housekeeping gene. All primers were ordered ready-made from the 'Gene expression assays' system (Applied Biosystems). In the case of genes encoding for IL-2Rα, LT-α, LT-β or TNF, total RNA was reverse transcribed (Superscript II, Invitrogen) and 10 ng of cDNA was amplified for 40 cycles with primers from Applied Biosystems. A standard curve was prepared by amplifying different amounts of cDNA prepared from IL-2-stimulated

murine T cells. Results obtained with specific primers were normalized against hypoxanthine guanine phosphoribosyl transferase, a housekeeping gene.

Results

Identification of IL-2-regulated genes using GeneChip microarrays

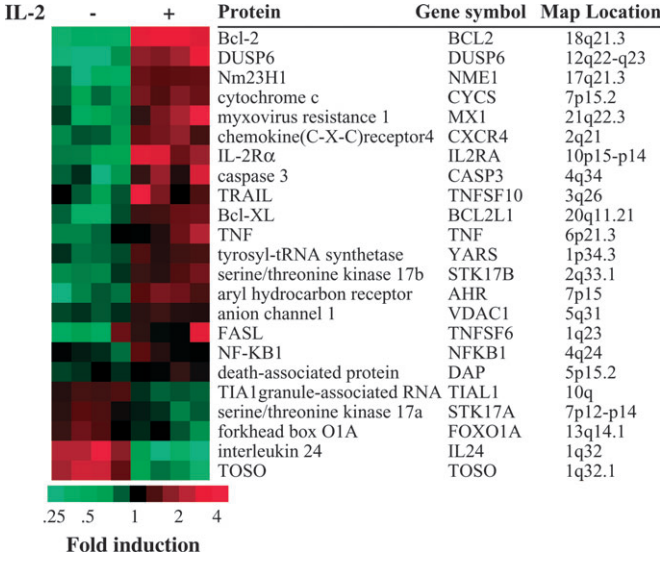
Recently, we reported a limited comparative study of genes regulated by IL-2, IL-4, IL-7 and IL-15 (21). We sought to identify a more comprehensive set of IL-2-regulated genes, and we used U95A GeneChip microarrays, which correspond to >9000 genes or transcripts (12 625 probe sets), as well as SAGE analysis. For the microarray analysis, we analyzed mRNAs from resting and IL-2-stimulated mRNA samples from four separate experiments. The transcript profiles were compared at 4 h, a time at which we found that most known IL-2-regulated genes are induced or repressed (21). We defined a set of IL-2-regulated genes using a false discovery rate (FDR) of ⩽0.05. The 12 625 probe sets on the GeneChip corresponded to 8582 UniGene clusters. Of these, 460 were up-regulated and 419 down-regulated. Among these 879 differentially expressed genes, we identified many previously reported IL-2 target genes (e.g. IL-2Rα, Pim-1, Bcl-2, Bcl-X_L and granzyme B), but most of them had not been previously reported as IL-2-regulated genes. The complete list of IL-2-regulated genes is available at dir.nhlbi.nih.gov/labs/supplements/ (Supplementary Tables 1 and 2, available at *International Immunology* Online).

Functional annotation of IL-2-regulated genes identified by expression profiling

IL-2 has pleiotropic effects on human T cells. To identify factors possibly related to known IL-2 functions, we linked Affymetrix probe set IDs to the corresponding 'functional' Gene Ontology annotations using the websites http://netaffx.com and http://www.geneontology.org. We thus identified, for example, the list of IL-2-regulated genes corresponding to term 'apoptosis' (Fig. 1). Some of these genes, such as the anti-apoptotic molecules Bcl-2 and Bcl-X_L (35), and pro-apoptotic cytokines FasL and TNF have known functions, whereas most have not been functionally evaluated in T cells. We further studied the kinetics of expression of select anti- and pro-apoptotic genes in purified $CD4^+$ and $CD8^+$ T cells; these included Bcl-2, Bcl-X_L, NF-κB1, STK17A, Caspase 3, FasL, TNF and TRAIL (Fig. 2). The kinetics of induction of both anti- and pro-apototic genes was similar with the peak of induction after 4 or 8 h of IL-2 treatment. The genes were also similarly regulated in both $CD4^+$ and $CD8^+$ cells. Thus, neither the kinetics of induction nor the specific pattern of expression in $CD4^+$ versus $CD8^+$ T cells readily explains the survival versus death (AICD)-inducing functions of IL-2.

IL-2 regulation of genes possibly involved in feedback regulation of IL-2 signaling

To identify potential novel regulators of IL-2 signaling, we collected microarray data for members of gene families involved in the regulation of signaling pathways, including



Protein	Gene symbol	Map Location
Bcl-2	BCL2	18q21.3
DUSP6	DUSP6	12q22-q23
Nm23H1	NME1	17q21.3
cytochrome c	CYCS	7p15.2
myxovirus resistance 1	MX1	21q22.3
chemokine(C-X-C)receptor4	CXCR4	2q21
IL-2Rα	IL2RA	10p15-p14
caspase 3	CASP3	4q34
TRAIL	TNFSF10	3q26
Bcl-XL	BCL2L1	20q11.21
TNF	TNF	6p21.3
tyrosyl-tRNA synthetase	YARS	1p34.3
serine/threonine kinase 17b	STK17B	2q33.1
aryl hydrocarbon receptor	AHR	7p15
anion channel 1	VDAC1	5q31
FASL	TNFSF6	1q23
NF-KB1	NFKB1	4q24
death-associated protein	DAP	5p15.2
TIA1granule-associated RNA	TIAL1	10q
serine/threonine kinase 17a	STK17A	7p12-p14
forkhead box O1A	FOXO1A	13q14.1
interleukin 24	IL24	1q32
TOSO	TOSO	1q32.1

Fig. 1. Shown are microarray expression data of four T cell samples not stimulated or stimulated with IL-2. Shown are data for 23 IL-2-regulated genes that are annotated as being involved in 'apoptosis' at the gene ontology database (www.geneontology.org). Green squares correspond to genes with relatively low-level expression and red squares to genes with relatively high expression as compared to the average expression of all genes on the microarray (see scale at bottom of page).

dual-specificity phosphatases (DUSPs) (Fig. 3A), SOCS and protein inhibitor of activated Stats (PIAS) gene family members (Fig. 3B) and other phosphatases (Fig. 3C); these are listed in Fig. 3 according to the level of IL-2 inducibility. We also collected corresponding microarray data from T cells stimulated with anti-CD3 plus anti-CD28 (Figs 3A, B and C, left panel; Supplementary Table 3, available at *International Immunology* Online). DUSPs are phosphatases that dephosphorylate both tyrosine and serine/threonine residues and are known to negatively regulate MAPK (36, 37). Of 12 DUSPs represented on the microarrays, 2 of these, DUSP5 and DUSP6, were induced by IL-2 whereas DUSP2 was repressed by IL-2. DUSP11 mRNA was highly expressed in both resting and IL-2-stimulated T cells. In naive T cells, TCR stimulation strongly induced the expression of DUSP5 and DUSP2, whereas DUSP6 was not induced (Fig. 3A). SOCS1 was strongly induced, whereas SOCS2 was only weakly induced by both stimuli (Fig. 3B). The other SOCS mRNAs and the PIAS mRNAs were not regulated in T cells by IL-2 nor were they highly expressed. From the many phosphatases represented on the microarrays, IL-2 regulated nine as defined by the FDR, albeit weakly in most cases. Only protein tyrosine phoshatase non-receptor type 7 was induced by IL-2, but not by TCR stimulation.

SAGE analysis of IL-2-regulated genes

The microarray analysis covered ~30% of the predicted number of human genes (38, 39). To obtain a more comprehensive view of IL-2-regulated transcripts, we also constructed SAGE libraries from mRNA of resting and IL-2-activated T cells and sequenced ~20 000 SAGE tags from each library. Only ~4000 of the 40 000 sequenced tags were

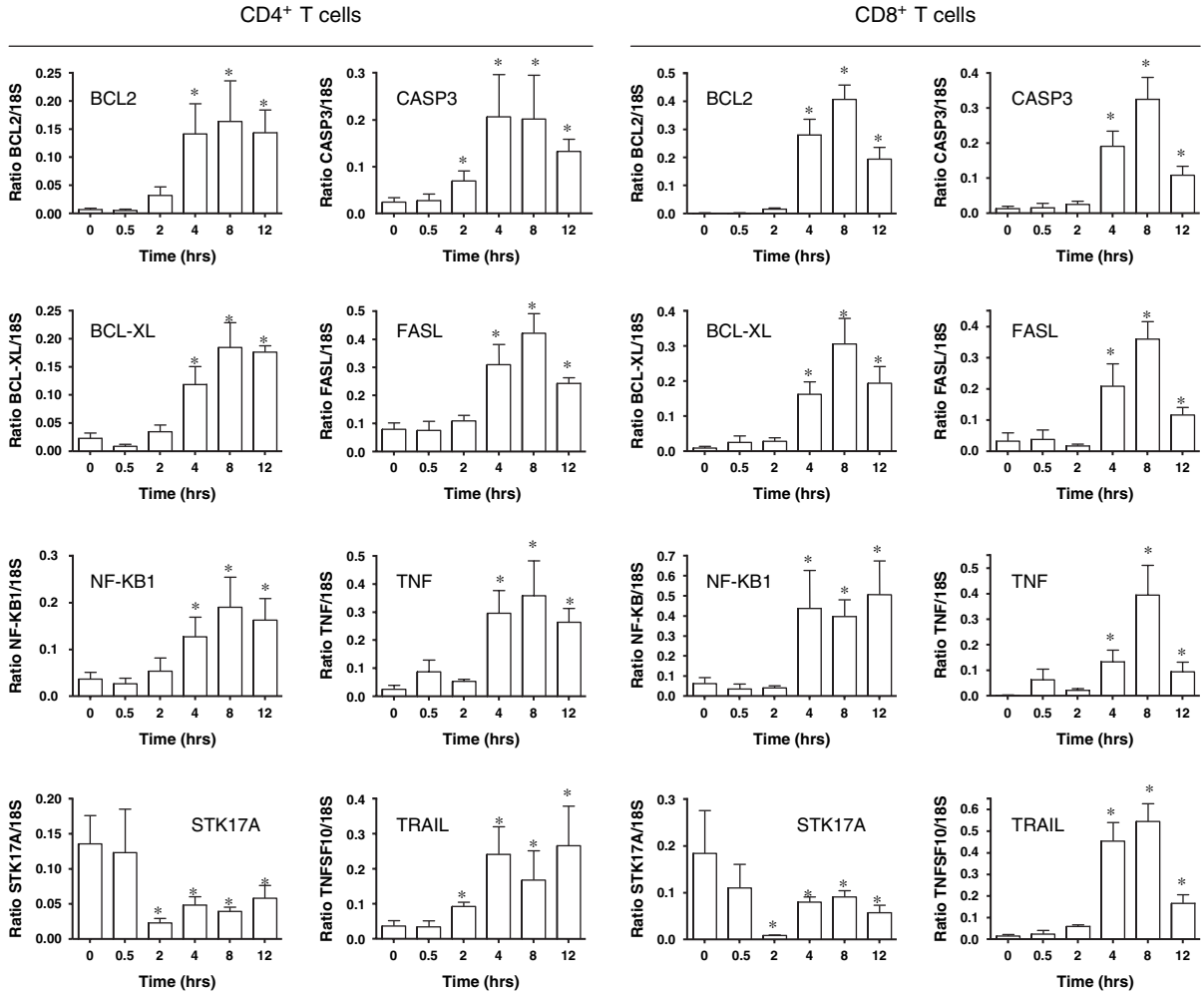


Fig. 2. Shown are real-time PCR data demonstrating the kinetics of IL-2 induction of three anti-apoptotic (Bcl-2, Bcl-XL and NF-κB1) genes and suppression of one pro-apoptotic gene (STK17A), as well as the kinetics of induction of four known pro-apoptotic genes (CASP3, FasL, TNF and TRAIL) in purified $CD4^+$ and $CD8^+$ T cells. Data represent average expression of genes in $CD4^+$ and $CD8^+$ T cells isolated from six different donors and cultured as in Fig. 1. The asterisk marks statistically significant difference in expression when compared to control (time 0) using the Student's *t*-test.

observed more than once (data not shown), consistent with most mammalian transcripts being expressed at low copy number. The most abundant tags in both SAGE libraries are at dir.nhlbi.nih.gov/labs/supplements/ (Supplementary Table 4, available at *International Immunology* Online). As expected, the most highly expressed tags correspond to housekeeping genes. After eliminating tags derived from linkers and duplicate tags, ~20 000 unique tags remained out of the 40 000 initially sequenced tags. We further removed those tags that did not map to any UniGene cluster, and when different tags mapped to the same UniGene cluster, the expression counts were combined. This resulted in 6349 different UniGene clusters for the SAGE data of which 100 were differentially expressed ($P < 0.05$), with 52 being induced by IL-2 and 48 being repressed (see Supplementary Table 5 at dir.nhlbi.nih.gov/labs/supplements/). When SAGE data were compared to the microarray analysis, 22 genes were found in common and similarly regulated by IL-2, indicating statistically significant concordance between the two data sets ($P < 0.0014$ by Fisher's exact test). An even better concordance would likely be achieved by sequencing more SAGE tags. Table 1 lists the 10 most induced (top 10 genes) and the 10 most repressed genes (bottom 10 genes) corresponding to SAGE tags. In the SAGE analysis, we also identified eight SAGE tags corresponding to IL-2-up-regulated transcripts and 13 SAGE tags corresponding to down-regulated transcripts that were not identified by microarrays (i.e. no oligonucleotides corresponding to these transcripts were on the microarray) (Table 2), demonstrating the advantage of using complementary approaches to identify differentially regulated genes.

Genomic localization of genes expressed in T cells, and identification of gene clusters

We scanned the human genome sequence for evidence of an uneven, possibly clustered distribution of IL-2-regulated

genes. As noted in Methods, 8582 distinct genes including 460 IL-2-induced and 419 repressed genes represented on the Affymetrix U95A chip could be located in the genome. Potential clusters were identified by cataloging the spans of all possible clusters and calculating statistical significance (see Methods). We identified non-random clusters in both differentially expressed and constitutively expressed genes (Table 3; Fig. 4).

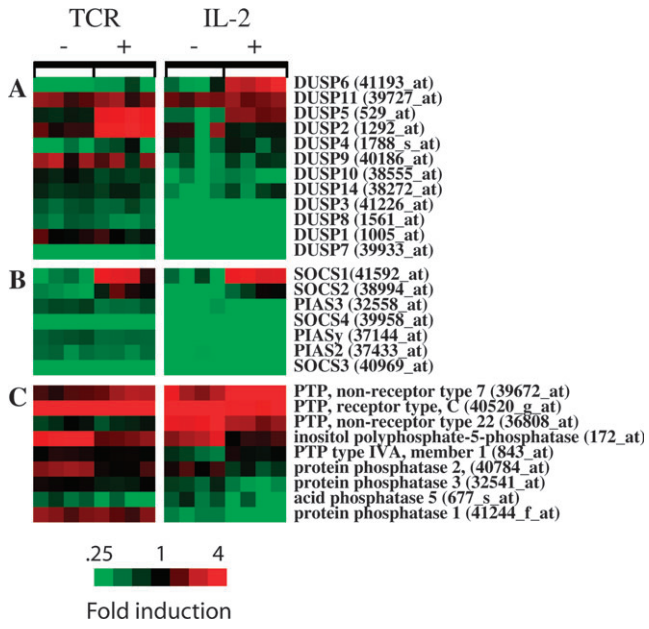


Fig. 3. Shown are microarray expression data from naive (TCR−) or anti-CD3 plus anti-CD28 (TCR+)-stimulated T cells (left), or T cells stimulated (+) or not stimulated (−) with IL-2 (right). Shown are data of expression for DUSP (A), SOCS (B) and PIAS (B) gene family members and IL-2-regulated phosphatases (C). Green squares correspond to genes with relatively low expression and red squares to genes with relatively high expression as in Fig. 1.

Clusters of IL-2-regulated genes and the role for Stat5a and Stat5b proteins in the regulation of LTA and TNF genes in 6p21.3 cluster

For the IL-2-regulated genes, two clusters were identified [Tables 3, A, and 4; Fig. 4, green arrow heads]. The 5q31 cluster spans 635 kb and overlaps the 5q31 cytokine gene cluster including those encoding IL-3, IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF). The cluster at 6p21.3 spans 7 kb and consists of three TNF superfamily members (TNF, LT-α and LT-β). Stat5a and Stat5b transcription factors regulate the transcription of several IL-2 target genes, including IL-2Rα, perforin, CIS and cyclin D2 (3). To investigate if Stat5 proteins are involved in the regulation of TNF, LT-α and LT-β in the 6p21.3 cluster, we studied the expression of these genes in normal and in mouse T cells deficient in expression of Stat5a and/or Stat5b (Fig. 5). Although LT-β was IL-2-regulated in human cells, it was constitutively expressed and not regulated by IL-2 in mouse T cells (data not shown). However, TNF and LT-α mRNAs were potently induced by IL-2 in wild-type murine lymphocytes, but were less well induced in T cells derived from Stat5a- or Stat5b-deficient animals, and not induced at all in T cells lacking both Stat5a and Stat5b (Fig. 5). This indicates that IL-2-dependent Stat5 activation is critical for the IL-2-mediated regulation of TNF and LT-α in the 6p21.3 cluster.

Clusters of expressed genes in T lymphocytes

We also identified chromosomal clusters of genes expressed in T cells but not necessarily regulated by IL-2 in T cells [Tables 3, B, and 5; Fig. 4, red arrow heads]. Some of these clusters were large, but the methodology also identified clusters with smaller spans on chromosomes 3 (7 Mb), 6 (16 Mb), 9 (9 Mb), 21 (3 Mb) and clusters with spans

Table 1. Ten most highly IL-2-induced and repressed transcripts identified by SAGE

P value	IL-2−	IL-2+	UniGene[a]	Protein	Gene symbol
0.000009	0	17	Hs.36	Lymphotoxin alpha (TNF superfamily, member 1)	LTA
0.000069	0	14	Hs.159161	Rho GDP dissociation inhibitor alpha	ARHGDIA
0.000069	0	14	Hs.91146	Protein kinase D2	PKD2
0.000112	74	131	Hs.890	Lymphotoxin beta (TNF superfamily, member 3)	LTB
0.000138	0	13	Hs.285313	Core promoter element-binding protein	COPEB
0.000138	0	13	Hs.289101	Glucose-regulated protein, 58kD	GRP58
0.000258	2	18	Hs.1051	Granzyme B	GZMB
0.000258	2	18	Hs.75748	Proteasome (prosome, macropain) subunit, beta type 1	PSMB1
0.000273	0	12	Hs.81256	S100 calcium-binding protein A4	S100A4
0.000369	4	22	Hs.428f	Ms-related tyrosine kinase 3 ligand	FLT3LG
0.000001	95	40	Hs.241392	Small inducible cytokine A5 (RANTES)	SCYA5
0.000171	180	118	Hs.184108	Ribosomal protein L21	RPL21
0.000348	17	2	Hs.58685	CD5 antigen (p56–62)	CD5
0.000579	49	21	Hs.241471	RNB6	RNB6
0.000618	28	8	Hs.79356	Lysosomal-associated multispanning membrane protein-5	LAPTM5
0.000637	16	2	Hs.1741	Integrin, beta 7	ITGB7
0.000637	16	2	Hs.63489	Protein tyrosine phosphatase, non-receptor type 6	PTPN6
0.000889	10	0	Hs.154365	E74-like factor 1 (ets domain transcription factor)	ELF1
0.000889	10	0	Hs.16291	*Homo sapiens* cDNA FLJ33028 fis,	
0.000889	10	0	Hs.289064	Hypothetical protein FLJ22251	FLJ22251

[a]Build 146.

Table 2. IL-2-regulated genes identified by SAGE, that were not represented on microarrays (U95A GeneChips)

P value	IL-2−	IL-2+	UniGene ID[a]	Protein	Gene symbol
0.0141	2	11	Hs.149923	X-box-binding protein 1	XBP1
0.0414	2	9	Hs.154993	Expressed sequence tags (ESTs)	
0.0414	1	7	Hs.175613	Homolog of Xenopus Claspin	CLASPIN
0.0165	0	6	Hs.180059	*Homo sapiens* cDNA FLJ31360	
0.0017	3	17	Hs.274535	Small inducible cytokine A3-like 1	SCYA3L1
0.0011	0	10	Hs.44163	13 kDa differentiation-associated protein	DAP13
0.0382	3	11	Hs.7104	Kruppel-like factor 13	KLF13
0.0027	6	22	Hs.83848	Triosephosphate isomerase 1	TPI1
0.0023	18	4	Hs.123164	ESTs	
0.0009	10	0	Hs.16291	*Homo sapiens* cDNA FLJ33028	
0.0201	8	1	Hs.176626	Hypothetical protein EDAG-1	EDAG-1
0.0298	5	0	Hs.197922	Hypothetical protein PRO1489	PRO1489
0.0201	8	1	Hs.202091	ESTs	
0.0020	32	12	Hs.238707	Hypothetical protein FLJ22457	FLJ22457
0.0380	20	9	Hs.240443	*Homo sapiens* cDNA: FLJ23538	
0.0006	49	21	Hs.241471	RNB6	RNB6
0.0009	10	0	Hs.289064	Hypothetical protein FLJ22251	FLJ22251
0.0298	5	0	Hs.304619	ESTs, weakly similar to I38022	
0.0298	5	0	Hs.48349	ESTs, weakly similar to I78885	
0.0036	8	0	Hs.7189	Pleckstrin homology, Sec7 and coiled/coil domains 4	PSCD4
0.0058	10	1	Hs.77667	Lymphocyte antigen 6 complex, locus E	LY6E

[a]Build 146.

Table 3. Clusters of expressed genes identified from U95A GeneChip microarray expression data

Experiment	Chr	*k*	*n*	Span (bp)	Address 1	Address *k*	p(*k*, chr)	p(*k*-chr)
A. IL-2-stimulated T cells	5	6	12	634 636	137 519 012	138 153 648	0.0018	0.01
	6	3	3	6884	33 874 814	33 881 698	0.0123	0.044
B. T lymphocytes	1	311	659	166 337 962	636 015	166 973 977	0.0012	0.042
	2	156	299	123 026 373	9 524 189	132 550 562	0.0004	0.015
	3	48	77	6 983 095	46 063 793	53 046 888	0.0014	0.036
	6	96	181	16 471 917	28 475 685	44 947 602	0.0012	0.036
	8	118	265	135 420 103	10 025 271	145 445 374	0.0013	0.028
	9	38	60	94 555 011	17 372 638	126 828 139	<0.0001	0.005
	11	10	10	269 879	65 823 696	66 093 575	0.0008	0.026
	19	218	447	65 805 210	906 140	66 711 350	0.0017	0.043
	21	19	28	2 964 681	41 620 365	44 585 046	0.0016	0.019
	X	16	21	665 846	149 839 137	150 504 983	0.0007	0.02
C. Heart	2	74	196	72 577 258	25 450 621	98 027 879	0.001	0.028
	6	41	86	353 345	132 029 115	35 562 566	0.0002	0.011
	11	147	352	80 225 435	342 252	80 567 687	0.0001	0.009
	15	7	9	590 142	70 716 273	71 306 415	0.0025	0.035
	18	17	47	20 708 631	1 196 508	21 905 139	0.002	0.016
	19	177	450	66 230 761	906 140	67 136 901	0.0004	0.009
	X	15	23	698 817	149 896 835	150 595 652	0.0001	0.006
D. Brain	22	47	90	10 181 576	26 451 080	36 632 656	0.003	0.048

Address 1 refers to the first nucleotide and Address *k* to the final nucleotide of the span.

<1 Mb on chromosomes 11 and X [Tables 3, B, and 5]. The cluster on chromosome 6 overlaps the 6p21.3 cluster of IL-2-regulated genes. We performed a similar analysis using SAGE data and identified 22 non-random clusters of expressed genes (see Methods for details, Table 6). Clusters from the analysis of SAGE data overlapped with those from microarray data, except that on chromosome 11, generally validating our analysis. Identification of more clusters by SAGE than by microarrays is consistent with the SAGE data set being considerably larger.

To determine if the clusters we identified were unique for T cells, we also analyzed the organization of expressed genes derived from human heart and brain [publicly available from www.ncbi.nlm.nih.gov/geo as data sets GSM2829, GSM2856, GSM2828 and GSM2837 or from expression.gnf.org (40)]. We identified seven clusters of expressed genes from human heart (Table 3, C), and one from brain tissue (Table 3, D). Of the seven clusters found in heart tissue, only the clusters on chromosomes 6 and X overlapped with those identified from T cells. These results indicate that some expressed genes

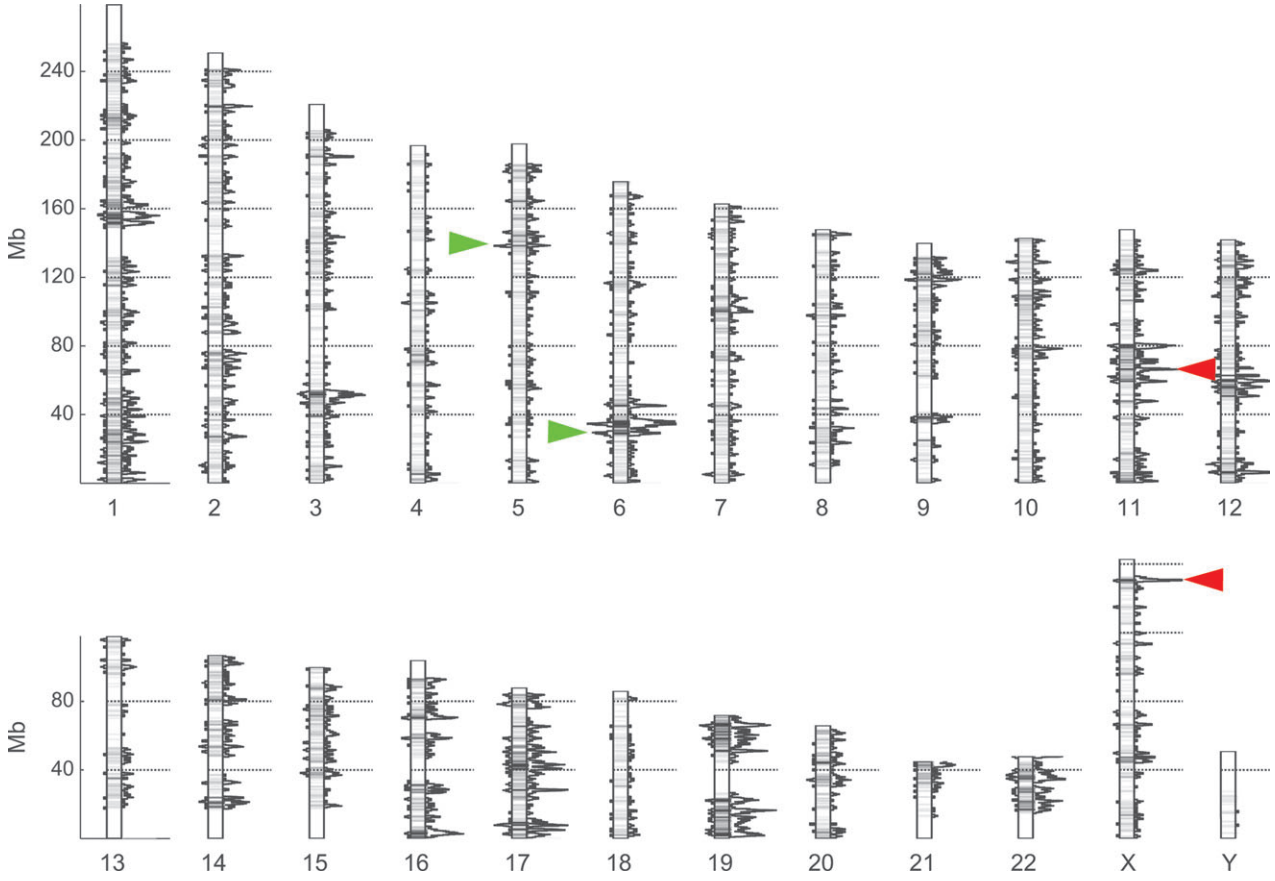


Fig. 4. Locations of induced and repressed genes on chromosomes of the human genome. The density of locations of the genes mapped from the Affymetrix U95A array is represented by a gray scale on the chromosome plot. The locations of the induced and repressed genes are represented by the peaks extending to the left of each chromosome. The height of each peak corresponds to the number of such differentially expressed genes found within a 1-Mb window along the chromosome. The locations of the 'present' genes are represented by peaks extending to the right of each chromosome using the same window size. The length of each chromosome is based on estimates from the International Human Genome Sequencing Consortium (38). The green arrow heads represent non-random clusters of IL-2-regulated genes and red arrow heads clusters of genes (less than 1 megabase) expressed in T cells.

Table 4. Clusters of IL-2-regulated genes identified from U95A GeneChip microarray data

Chr	Start	Strand	Protein	Gene symbol
5	137519012	+	IL-3	IL3
	137530582	+	GM-CSF	CSF2
	137649284	+	Procollagen-proline 2-oxoglutarate 4-dioxygenase	P4HA2
	137727986	+	LIM domain protein	RIL
	138131394	+	IL-4	IL4
	138153648	+	Kinesin family member 3A	KIF3A
6	33874814	+	Lymphotoxin alpha (TNF superfamily, member 1)	LTA
	33878788	+	TNF (superfamily, member 2)	TNF
	33881698	+	Lymphotoxin beta (TNF superfamily, member 3)	LTB

appear in chromosomal clusters, but the genomic distribution of clusters shows tissue specificity.

Discussion

Recent advances in genomics have provided new approaches for studying biological systems. High-throughput methods for mRNA expression profiling such as DNA microarrays and SAGE have enabled quantitative analysis of gene expression at the genomic scale. Recently, combining large-scale expression profiling and genomic mapping has been used to define candidate genes for pathological conditions such as retinal diseases and schizophrenia (18, 41). Moreover, an understanding of genes expressed in a particular cell type

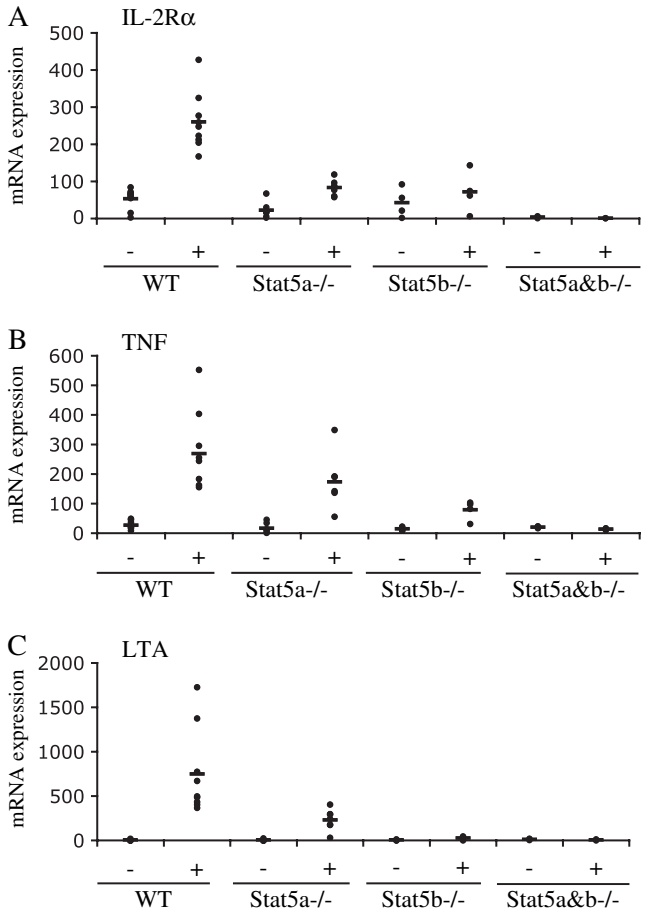


Fig. 5. IL-2 induction of TNF and LT-α mRNA is dependent on Stat5a and Stat5b. Mouse T cells derived from wild-type mice or mice lacking Stat5a or Stat5b or both were stimulated with IL-2 for 4 h. Expression of IL-2Rα, TNF and LT-α mRNA was analyzed by real-time PCR, and normalized relative to the expression of the hypoxanthine guanine phosphoribosyl transferase, a housekeeping gene.

and regulated within a biological pathway can facilitate studies aimed at identifying novel molecules that are important to these pathways (16, 42–44). We have used DNA microarrays and SAGE to identify genes expressed in T cells as well as those regulated by IL-2. The resulting compendium of genes can be utilized for a systematic study of their role in IL-2 responses and T cell biology and as a reference database for candidate gene searches for T cell-associated diseases.

Using microarrays and SAGE, we identified close to 900 genes whose expression is regulated by IL-2, extending earlier studies aimed at identifying IL-2-regulated genes (20, 21). The complete list of IL-2-regulated genes is provided at dir.nhlbi.nih.gov/labs/supplements/. This website also includes the genomic locations of these genes as well as their functional annotations as defined in the gene ontology database (http://www.geneontology.org). Combining expression data with functional annotations allowed us to define subsets of IL-2-regulated genes and to identify, among others, a subset of 23 IL-2-regulated genes that are involved in apoptosis in different cellular systems. Some of these (Bcl-2 and Bcl-X_L) have known pro- or anti-apoptotic functions in T cells, whereas others are poorly studied in T lymphocytes. For example, TNF-dependent NF-κB activation supports survival in a variety of cell types (45, 46), but the possible functional relevance of IL-2 induction of TNF and NF-κB1 is not yet known. Similarly, the significance of the induction by IL-2 of molecules such as aryl hydrocarbon receptor and cytochrome c, as well as the inhibition by IL-2 of a pro-apoptotic serine/threonine kinase 17a (STK17A) remains to be determined.

IL-2 is required for the maintenance of peripheral tolerance (4, 47, 48). This likely involves IL-2-dependent generation of $CD4^+CD25^+$ regulatory T cells, and IL-2-mediated sensitization of proliferating T cells to TCR-dependent apoptotic stimuli (AICD) (4, 6). The molecules involved in these processes are largely not known. TCR-mediated induction of FasL is critical for AICD, and mice deficient in Fas or FasL show defective AICD, uncontrolled lymphocyte proliferation and autoimmunity (49, 50). In combination with TCR signaling, IL-2 stimulation potently induces FasL and TNF, and this may partly explain the IL-2-dependent sensitization of T cells to AICD (51, 52). We also found that IL-2 induces a number of other pro-apoptotic genes as well, including those encoding TRAIL (TNF gene family member, (45, 46), Caspase 3 [an essential intracellular mediator of apoptosis (53)], death-associated protein (DAP), and serine/threonine kinase 17b (STK17B). DAP and STK17B have been shown to mediate IFN-dependent apoptotic signals (54). TOSO is a potent inhibitor of Fas-mediated apoptosis (55), and TOSO mRNA expression was repressed by IL-2. Thus, we found that IL-2 modulates the expression of a set of apoptosis-related molecules that have not previously been linked to the survival or cell death-inducing functions of IL-2.

Recently, several gene families have been identified that regulate signaling through cytokine receptors and modulate immune responses. The best-characterized negative regulators of cytokine signaling include SOCS proteins, PIAS proteins (protein inhibitors of activated STATs) and phosphatases. The SOCS family consists of eight members (CIS and SOCS1–7) (56). They are induced by cytokines and regulate cytokine signaling by at least two distinct mechanisms; SOCS1 and SOCS3 directly inhibit cytokine receptor-associated Jak kinases and provide general inhibitory signals, whereas CIS and SOCS2 more specifically inhibit cytokine-induced STAT activation, likely by preventing their association with cytokine receptors (56). We identified SOCS1 as strongly induced by IL-2 as previously noted (57, 58), while SOCS2 was moderately induced, which is a novel finding that requires further evaluation. PIAS proteins regulate cytokine signaling by interacting with and inhibiting the activation of STATs (59). We did not observe IL-2 regulation of PIAS1, PIASx (PIAS2), PIAS3 or PIASy by microarray or SAGE analysis.

Protein phosphatases are essential regulators of cellular functions, but their role in IL-2 signaling is largely unknown. We recently characterized DUSP5 as a negative regulator of IL-2-induced MAPK activity and identified DUSP6 as another strongly IL-2-induced potential regulator of MAPK activity (21). The DUSP gene family consists of 61 genes, 11 of which are designated as typical MAPK phosphatases and 19 as atypical MAPK phosphatases that lack MAPK-targeting motifs (60). DUSP5 and DUSP6 are specific for Erk-1/2, the primary MAPKs regulated by IL-2 in primary T cells. Of the many known protein phosphatases, only the mRNA of non-receptor protein

Table 5. Chromosome 11 and X clusters of genes constitutively expressed in T lymphocytes identified from U95 microarray data

Chr	Start	Strand	Protein	Gene symbol
11	65823696	–	Tumor suppressor deleted in oral cancer	DOC-1R
	65844882	–	NADH dehydrogenase	NDUFV1
	65907084	–	Glutathione S-transferase	GSTP1
	65975379	–	Phosphatidylinositol transfer protein	PITPNM
	65976504	–	Aryl hydrocarbon receptor-interacting protein	AIP
	66021066	–	RAD9 (*S. pombe*) homolog	RAD9
	66021355	–	Protein phosphatase 1, catalytic subunit	PPP1CA
	66057388	–	Ribosomal protein S6 kinase	RPS6KB2
	66058521	–	Protein tyrosine phosphatase C-associated protein	PTPRCAP
	66093575	–	Polymerase (DNA-directed), delta 4	POLD4
X	149839137	–	Inhibitor of kappa light polypeptide gene	IKBKG
	149896835	–	Glucose-6-phosphate dehydrogenase 2.19 gene	G6PD
	149915660	–	Solute carrier family 10, member 3	SLC10A3
	149925487	–	DNA segment on chromosome X (unique) 98	DXS9879E
	149960121	–	GDP dissociation inhibitor 1	GDI1
	149967004	–	ATPase, H+ transporting, lysosomal (vac	ATP6S1
	149981781	–	Tafazzin (cardiomyopathy, dilated 3A (X	TAZ
	150001114	–	Deoxyribonuclease I-like 1	DNASE1L1
	150002614	–	Ribosomal protein L10	RPL10
	150021949	–	Emerin (Emery-Dreifuss muscular dystrophy	EMD
	150270144	–	Methyl CpG binding protein 2 (Rett synd	MECP2
	150308952	–	Chromosome X open reading frame 12	CXorf12
	150344093	–	IL-1R-associated kinase	IRAK1
	150384158	–	Rho GTPase activating protein 4	ARHGAP4
	150493633	–	Signal sequence receptor, delta (transl	SSR4
	150504983	–	Isocitrate dehydrogenase 3 (NAD+) gamma	IDH3G

Table 6. Clusters of expressed genes in T cells identified by SAGE data

Chr	*k*	*n′*	Span (bp)	Address 1	Address *k*	p(*k*, chr)	p(chr)
1	297	1269	48 783 275	129 982	48 913 257	<0.0002	0.015
	119	395	10 952 406	151 077 634	162 030 040	0.0002	0.022
2	14	27	330 016	218 932 786	219 262 802	<0.0002	0.009
	75	333	13 417 460	62 457 380	75 874 840	0.0002	0.019
3	14	26	352 881	53 074 122	53 427 003	<0.0002	0.014
6	21	51	409 707	41 470 877	41 880 584	<0.0002	0.01
7	23	50	946 419	153 626 836	154 573 255	<0.0002	0.006
	28	78	1 556 567	102 315 647	103 872 214	<0.0002	0.006
8	273	2043	137 698 456	7 169 919	144 868 375	0.0006	0.031
9	62	221	6 516 510	121 593 429	128 109 939	<0.0002	0.011
10	77	348	13 612 479	97 217 133	110 829 612	<0.0002	0.007
11	17	33	378 412	59 630 458	60 008 870	<0.0002	0.013
	27	64	1 072 457	57 759 161	58 831 618	<0.0002	0.013
12	22	38	545 957	6 398 289	6 944 246	<0.0002	0.006
14	18	47	468 041	21 961 666	22 429 707	0.0002	0.01
15	238	1365	59 133 810	29 744 524	88 878 334	0.0008	0.032
16	99	317	5 863 649	223 689	6 087 338	<0.0002	0.013
17	28	51	669 750	7 591 670	8 261 420	<0.0002	0.008
19	451	1646	67 016 037	889 228	67 905 265	<0.0002	0.013
21	85	526	14 946 714	29 609 337	44 556 051	<0.0002	0.008
X	9	10	85 535	149 921 832	150 007 367	<0.0002	0.006
	33	75	2 545 953	44 350 009	46 895 962	<0.0002	0.006

The span, Address 1 and Address *k* are as described in Table 3.

tyrosine phosphatase 7 (PTPN7, also known as LCPTP or HePTP) was strongly induced by IL-2 in microarray data. Interestingly, PTPN7 has also been shown to be a specific Erk-2 phosphatase (61, 62).

Taken together, IL-2 appears to regulate the mRNA levels of relatively few known feedback regulators of signaling; SOCS1, SOCS2, CIS, DUSP5, DUSP6 and PTPN7. SOCS1 regulates the activity of Jaks and provides general inhibitory signals, whereas CIS and SOCS2 likely regulate the activity of Stat5. IL-2 mediated Erk-1/2 signaling is presumably controlled by DUSP5, DUSP6 and PTPN7.

The completion of human and other genome projects together with the availability of data from genome-wide gene expression studies have provided new insights into regulation of

gene expression. Transcribed genes are not always randomly distributed in the genome, but are clustered in chromosomes. Such clusters have been detected among highly transcribed genes, including housekeeping genes in the human genome as well as in *Drosophila* (63–66). Tissue-specific genes also appear to be organized in part in chromosomal clusters (67–72), and functionally related genes sometimes form chromosomal clusters of co-regulated genes, somewhat analogous to the clustering of operons in *Caenorhabditis elegans* (73–77). We identified two significant clusters of IL-2-regulated genes as well as several chromosomal clusters of T cell-expressed genes. The 5q31 cluster of IL-2-regulated genes overlaps with the cytokine gene cluster consisting of cytokines IL-3, GM-CSF, IL-5, IL-13 and IL-4 (78), and the 6q21 cluster consists of TNF, LT-α and LT-β genes. We found that Stat5 is important for the regulation of TNF and LT-β in the 6q21 cluster of IL-2-regulated genes, suggesting a partial mechanism for their co-regulation. Such clustering of transcription factor recognition sequences has been detected in the *Drosophila* genome (79–81). We also observed that genes expressed in T cells (constitutively expressed, but not necessarily IL-2 regulated) formed clusters. Interestingly, these appear similar to and co-localize with previously observed domains of highly expressed genes (63, 64). Although the significance of these domains remains unclear, they have been hypothesized to be segments of chromatin that are particularly accessible for transcriptional activation (63, 64, 82).

In summary, we have used microarrays and SAGE analysis and obtained a genome-wide view of genes expressed in T cells of those induced by IL-2. Our detailed mapping data may facilitate candidate gene searches for T cell-associated diseases. Moreover, we have identified a number of novel target genes for IL-2, including genes potentially involved in IL-2-dependent survival functions and in AICD. The characterization of these molecules should allow a better understanding of and provide novel target molecules for the modulation of T cell immune responses.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Acknowledgements

We thank Hyoung-Pyo Kim for advice in regard to real-time PCR and Jian-Xin Lin for critical reading of the manuscript. P.E.K. was funded in part with grants from the Academy of Finland, Emil Aaltonen Foundation and Finnish Cultural Foundation.

Abbreviations

AICD	Activation-induced cell death
DAP	Death-associated protein
DUSP	Dual-specificity phosphatase
FasL	Fas ligand
FDR	False discovery rate
GM-CSF	Granulocyte macrophage colony-stimulating factor
MAPK	MAP kinase
PBMC	Peripheral blood mononuclear cells
PI 3-K	Phosphatidylinositol-3-kinase
PIAS	Protein inhibitor of activated Stats
SAGE	Serial analysis of gene expression
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TNF	Tumor necrosis factor

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