Molecular profiling of $CD3^-CD4^+$ T cells from patients with the lymphocytic variant of hypereosinophilic syndrome reveals targeting of growth control pathways

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The clonal $CD3^-CD4^+$ T-cell population characterizing lymphocytic variant hypereosinophilic syndrome (L-HES) persists for years, with a subgroup of patients ultimately progressing to T lymphoma. The molecular changes associated with the premalignant clone and the emergence of malignant subclones are unknown, precluding the development of targeted therapy for this HES variant. In this study, we used whole genome arrays to examine gene expression in the $CD3^-CD4^+$ T cells and found that 850 genes were differentially regulated during chronic disease compared with $CD3^+CD4^+$ T cells from healthy donors. Changes in the expression of 349 genes were altered in association with the clinical progression from chronic L-HES to T lymphoma in 1 patient, with 87 of 349 genes representing further changes in genes whose expression was altered in all chronic disease patients (87 of 850). Array analysis after CD2/CD28-mediated activation revealed that the major gene expression changes observed in the $CD3^-CD4^+$ T cells do not reflect activation induced alterations but rather pathways involved in T-cell homeostasis, including transforming growth factor-β signaling, apoptosis, and T-cell maturation, signaling, and migration. Examination of microRNA expression in the $CD3^-CD4^+$ T cells from patients with chronic disease identified 23 microRNAs that changed significantly, among which miR-125a further decreased in association with one patient's evolution to T lymphoma. (Blood. 2009;114:2969-2983)

Introduction

Patients with lymphocytic variant hypereosinophilic syndrome (L-HES) are distinguished by the presence of abnormal T-cell populations ($CD3^-CD4^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, or $CD3^+CD4^-CD8^-$) that are frequently monoclonal.[1] These clonal T cells secrete various combinations of interleukin-4 (IL-4), IL-5, and IL-13, resulting in hypereosinophilia and, in many cases, increased serum IgE levels.[2-6] Some patients with L-HES eventually develop peripheral T lymphoma[2,4,7] with detection of an abnormal karyotype[3,8,9] and resistance to apoptosis[10] observed at preneoplastic disease stages. Despite this knowledge, T cell–mediated HES remains a heterogeneous group of diseases lacking definition of the molecular mechanisms underlying the persistence and expansion of the T-cell clone during chronic disease as well as the generation of increasingly abnormal subclones leading to T lymphoma. This contrasts with the discovery of the disease-inducing *FIP1L1/PDGFRA* fusion gene in HES patients with features of myeloproliferative disease, who are now treated with and remarkably responsive to the tyrosine kinase inhibitor imatinib mesylate.[11]

The goal of this study was to establish a molecular profile for $CD3^-CD4^+$ T cell–mediated L-HES by comparing gene expression (both mRNA and microRNA) in the abnormal T cells relative to normal $CD3^+CD4^+$ T cells. These analyses established a comprehensive immunophenotype/genotype that reflects the cells' Th2 nature as well as specific characteristics of polarization, signaling, and function. In patients with chronic disease, significant changes in gene expression were detected in critical growth control pathways of potential clinical relevance, including the IL-25 receptor and genes from the transforming growth factor-β (TGF-β) superfamily. Our previous studies found that the $CD3^-CD4^+$ T cells are dependent on exogenous T-cell receptor (TCR/CD3)–independent activation signals for Th2 cytokine expression.[12] Assessment of gene expression changes associated with CD2/CD28-mediated costimulation revealed that the molecular alterations found in quiescent T cells associated with chronic disease did not simply reflect activation-associated changes in the abnormal T cells. Finally, we explored molecular changes linked with the outgrowth of a 6q-deleted subclone as one patient developed T lymphoma[8] and found that approximately one-third of these genes were also altered in patients during chronic disease, suggesting that they may be of particular interest in terms of conferring a selective survival and growth advantage to the $CD3^-CD4^+$ T cells.

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The online version of this article contains a data supplement.

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Methods

Detailed methods are provided in supplemental data (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Patients

Patients with hypereosinophilic syndrome were selected for cohort inclusion based on the presence of a monoclonal $CD3^-CD4^+$ T-cell population in their peripheral blood. The clinical characteristics of all patients analyzed are summarized in supplemental Table 1. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and this study was approved by the ethics committees at Institut Jules Bordet and Hôpital Erasme.

Cell purification

Circulating leukocytes were obtained from the peripheral blood of patients and healthy donors (controls) by venipuncture or cytapheresis, and peripheral blood mononuclear cells (PBMCs) were isolated and frozen as previously described.[12] PBMCs were thawed and resuspended in X-vivo-20 medium (Lonza Braine SA). Patient $CD3^-CD4^+$ and donor $CD3^+CD4^+$ T cells were isolated by negative selection using magnetic beads (Miltenyi Biotec).[8] The isolated cell populations were checked for purity by flow cytometry and were consistently more than 95% pure $CD3^-CD4^+$ (patients) or more than 90% pure $CD3^+CD4^+$ (controls). Thawed and purified T cells were incubated in X-vivo-20 at 37°C/5% CO_2 for 18 hours to eliminate dying cells before RNA extraction. For the costimulation experiments, purified $CD3^-CD4^+$ T cells were cultured for 18 hours in X-vivo-20 supplemented with rhIL-2 (100 U/mL) and anti-CD28 (CLB-CD28/1; 1 μg/mL) plus 2 anti-CD2 antibodies (CLB-T11.1/1 and CLB-T11.2/1; 5 μg/mL each; Sanquin). In the IL-25 experiments, purified $CD3^-CD4^+$ (patient) and $CD3^+CD4^+$ (patient and control) T cells were stimulated for 48 hours with phorbol ester (10 ng/mL) and anti-CD28 in the absence or presence of increasing concentrations of rhIL-25 (R&D Systems).

RNA extraction and gene expression microarray procedures

RNA was extracted by the single-step method of isolation using Trizol (Invitrogen). RNA quantity and quality were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a Bioanalyzer (Agilent); 1.5 μg of total RNA was labeled following the manufacturer's protocols for probe preparation and hybridization (Affymetrix); 15 μg of cRNA was hybridized onto a U133 Plus 2.0 GeneChip.

Statistical analysis of microarray data

Raw data were analyzed using the *SScoreBatch*[13,14] function from the *SScore* package (Version 1.5.1) in the R statistical environment (Version 2.3.0; http://www.r-project.org/; http://www.bioconductor.org). To identify genes consistently deregulated in the 3 patients relative to the 4 controls, we selected genes where each patient's *P* value (generated by S-score algorithm and Z transformation of mean S-score values) was inferior to .05 in comparison with individual controls (supplemental text and supplemental Table 2). A further filter was applied to probe sets of low significance. Similar analyses were performed to determine the genes deregulated during P1's evolution from chronic disease to T lymphoma (supplemental Table 3).

Flow cytometry

Flow cytometric analysis was performed by labeling 2 to 5×10^5 cells with 5 μL fluorescein isothiocyanate–conjugated anti-CD45RO, 5 μL peridinin chlorophyll protein-conjugated anti-CD3, 5 μL allophycocyanin-conjugated anti-CD4, and 5 μL of the phycoerythrin-conjugated test antibody (BD Biosciences; supplemental Table 4) in 50 μL X-vivo-20. A total of 10 000 viable cells were acquired on a FACS Calibur (BD Biosciences). Measurement of cytokine concentrations in culture supernatants was performed using BD Cytometric Bead Array Flex Sets.

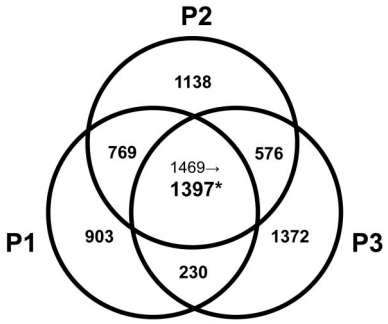

Figure 1. Venn diagram of altered gene probe sets in the L-HES patients (P1-P3) vs controls. Significant changes in the expression of gene probe sets were based on a $P < .05$ after Z transformation of the mean S-score values obtained from all possible 2-chip comparisons between triplicates of P1-yr 0 or duplicates of P2-yr 0 and P3-yr 0 and individual arrays from 4 controls (supplemental data). The number of altered probe sets is shown, and the individual genes are listed in supplemental Table 2. *Among the 1469 commonly changed probe sets detected in the comparison of P1 to P3 vs controls, only 1397 probe sets passed a further filter that was applied to probe sets with a $P > .01$ and/or with fold change < 2 for at least 1 patient.

Real-time quantitative RT-PCR

Total RNA (200 ng to 1 μg) was reverse-transcribed with random hexanucleotides using the SuperScript III First-Strand Synthesis System (Invitrogen) and standard protocols. Primers specific for the *MAP3K8, RUNX1, RUNX2, DIABLO, TGFBR1, TGFBR2, TGFBR3, KIT, SMAD5, SMAD7, NOG, ACVR2A*, and *CYSLTR1* genes were purchased from QIAGEN (QuantiTect Primer Assays). Primers for *ABL* (endogenous control) were kindly provided by Dr J.-L. Vaerman. Quantitative reverse-transcribed polymerase chain reaction (RT-PCR) was performed on a Roche LightCycler 480 (Roche Applied Science). Analyses (supplemental Table 5) were performed using LightCycler Basic software, Version 1.5 (Roche).

MicroRNA quantification

Quantification of mature microRNAs was achieved using stem-loop-mediated RT-PCR with the TaqMan microRNA assay-early access kit or with individual microRNA assay mixes using the manufacturer's protocols (Applied Biosystems; supplemental Table 6). Standard real-time PCR was performed on an ABI Prism 7900HT (Applied Biosystems). Putative microRNA target genes were predicted using MiRanda algorithm-associated MirBase software (http://microrna.sanger.ac.uk).

Results

Comprehensive gene expression analysis of $CD3^-CD4^+$ T cells from L-HES patients

We compared the gene expression profiles of clonal $CD3^-CD4^+$ T cells isolated from L-HES patients (P1-P3; supplemental Table 1) during chronic disease with $CD3^+CD4^+$ T cells from controls. We also evaluated changes in gene expression associated with CD2/CD28 activation of their $CD3^-CD4^+$ T cells in vitro, an antibody combination targeting costimulatory receptors previously shown to mediate their Th2 cytokine production and proliferation.[12] We further analyzed changes in gene expression linked with P1's clinical progression[8,15] by assessing $CD3^-CD4^+$ T cells at diagnosis (yr 0), yr +4 (both premalignant stages of chronic L-HES), and yr +6 (concurrent with T lymphoma diagnosis). After comprehensive and stringent statistical analyses, we detected 850 genes (1397 probe sets) that were differentially regulated in all 3 patients $CD3^-CD4^+$ T cells compared with control $CD3^+CD4^+$ T cells (Figure 1; supplemental Table 2), 312 genes (411 probe sets) that were altered in all 3 patients $CD3^-CD4^+$ T cells after CD2/CD28 costimulation (supplemental Table 2), and 349 genes

(450 probe sets) whose expression was altered in concert with P1's malignant evolution (supplemental Table 3). The original data for all 54 675 probes from each array are provided at Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/, under accession number GSE12079.

Gene expression changes in CD3⁻CD4⁺ T cells from L-HES patients compared with CD3⁺CD4⁺ T cells from controls

Immunophenotype/genotype. Based on the microarray data, we compiled a comprehensive phenotype/genotype of the CD3⁻CD4⁺ cells that extends previous characterizations of L-HES (Figure 2, supplemental Table 7). Prior studies of surface receptors on the CD3⁻CD4⁺ T cells[2,5,9,10,12,15-17] validated parallel changes observed in this study, including reduced or lost CD3 (*CD3γ,CD3ζ*), *CD7*, CD27 (*TNFRSF7*), and *CD69* mRNA transcripts and increased *CD5*, CD95 (*FAS*) and HLA class II antigen mRNA transcripts. We determined whether altered mRNA expression corresponded to increased surface protein expression for numerous previously unexplored immunophenotypic markers using flow cytometry (supplemental Table 4) and an enlarged patient cohort (P1-P7; supplemental Table 1). Some gene expression changes can be attributed to the clonal Th2 nature of the patient's CD3⁻CD4⁺ T cells compared with the heterogeneous CD3⁺CD4⁺ T-cell population from controls.[18] These include down-regulation of the Th1 genes *BTLA, CCL5, IL-18R, NOTCH2, JUN, SLAMF7*, and integrin α6 (*ITGA6*) and up-regulation of the established Th2 genes *IL4R, CCR3, CCR8, GATA3*, CRTH2 (CD294, *GPR44*), and *IL17RB* in the abnormal T cells.

IL-17RB, the receptor for IL-25 (*IL17E*), is expressed on memory Th2 cells[19] and as recently shown also on human CD14⁺ cells.[20] IL-25 induces Th2 inflammation in mice and, when bound to human Th2 cells, enhances Th2 cytokine production in response to T cell–stimulating agents.[19] In this study, we detected increased expression of the *IL17RB* gene not only in all patients CD3⁻CD4⁺ T cells during chronic L-HES (Figure 2) but also observed further increases on P1's T lymphoma cells (Table 1) and after CD2/CD28 costimulation of P1 to P3's CD3⁻CD4⁺ T cells (Table 2). Flow cytometry detected a significantly higher proportion of P3's CD3⁻CD4⁺CD45RO⁺ T cells expressing membrane IL-17RB compared with CD3⁺CD4⁺CD45RO⁺ or CD45RO⁻ T cells from controls (Figure 3A). P3's CD3⁻CD4⁺ T cells cultured with rhIL-25 induced a dose-response increase in the production of IL-5 and IL-13 but not interferon-γ and enhanced their proliferation in response to phorbol ester/anti-CD28 (Figure 3B). The further up-regulation of *IL17RB* mRNA observed on P1's T lymphoma cells and after CD2/CD28 costimulation of CD3⁻CD4⁺ T cells from chronic disease suggests that eosinophil-produced IL-25 may facilitate sustained and preferential expansion of the abnormal T-cell clone. Thus, increased *IL17RB* expression may provide a selective advantage to the CD3⁻CD4⁺ T-cell clone (or a subclone), thereby contributing to the maintenance of chronic disease and malignant progression.

Modulation of surface receptor expression is a common mechanism for controlling T cell–mediated immune responses that are characterized by cytokine production and proliferation. mRNA expression for several immunomodulatory genes other than the TCR/CD3 complex were altered in the patient's CD3⁻CD4⁺ T cells (Figure 2, supplemental Table 7), and some with apparent relevance include (1) decreases in the membrane complement regulatory proteins, which have been associated with autoimmune and inflammatory disease as well as the regulation of T-cell activation responses[21]; (2) decreased expression of genes involved in negatively regulating T-cell responses, including *CTLA4* (CD152) and *IL27RA*, both shown to play critical roles in murine Th2 cell homeostasis[22,23] and whose downmodulation potentially contributes to persistence and expansion of the CD3⁻CD4⁺ T-cell clone; and (3) increases in several immunoregulatory receptors, which perhaps provide a more pertinent characterization of costimulatory and regulatory pathways operating in the CD3⁻CD4⁺ T cells, including: (1) SLAMF5 (*CD84*), an inhibitory receptor for the high affinity IgE receptor[24]; (2) DCAL1 (*CLECL1*), a type II transmembrane C-type lectin-like protein expressed on dendritic cells and B cells (no previous reports of T-cell expression) and whose interaction with T cells has been shown to enhance their IL-4 production[25]; (3) *CD99*, a T-cell costimulatory receptor capable of fully activating cells stimulated with a suboptimal TCR/CD3 signal[26]; and (4) *CD200R1*, an inhibitory receptor regulating the activation threshold of inflammatory immune responses, which might also affect CD3⁻CD4⁺ T-cell activation.

The lack of TCR/CD3 expression on the abnormal T-cell surface dictates the potential loss of this important signaling pathway, although the CD3⁻CD4⁺ T cells do remain responsive to costimulatory signals.[12] Some critical TCR/CD3 downstream signals were found decreased in unstimulated CD3⁻CD4⁺ T cells, including inhibitory receptors, PI3K-associated or family proteins, tyrosine and MAP3 kinases, and activation responsive transcription factors (supplemental Table 7). Interestingly, gene expression analysis of the abnormal T cells after CD2/CD28 costimulation revealed that relatively few of the gene expression changes detected in "quiescent" CD3⁻CD4⁺ T cells isolated from patient blood involved genes whose expression was affected by activation (Table 2, supplemental Table 2). Thus, induction of the Th2 cytokine genes *IL5*, *IL13*, and other immune response genes by costimulation in vitro apparently induces transient signals that may be elicited by a sustained stimulus present in local immune microenvironments in vivo.

G-protein–coupled receptors. Numerous G-protein–coupled receptors were altered on the CD3⁻CD4⁺ T cells (Figure 2) and include 2 of particular significance. A 19-fold decrease in cysteinyl leukotriene receptor 1 (*CYSLTR1*) gene expression was observed in the CD3⁻CD4⁺ T cells with the greatest degree of downmodulation detected in P1-yr 0. Decreased CYSLTR1 expression was confirmed by quantitative RT-PCR (P1-P5, P7; Figure 4A, supplemental Table 5) but revealed a disparity in expression between the patients (ie, it is not expressed in P1 and low expression levels were detected in P2, P3, and P5). CYSLTR1 is normally expressed on myeloid cells, including eosinophils, and induced on CD4⁺ T cells by Th2 cytokines and TCR/CD3-mediated activation. The CYSLTR1 ligand, leukotriene D4, is produced by eosinophils and other myeloid cells and plays an active role both in cell survival and leukocyte recruitment to inflamed tissues.[27] In contrast to the decreased expression observed on the CD3⁻CD4⁺ T cells, CYSLTR1 is significantly up-regulated and functional on CD4⁺ T cells from mice carrying an LAT gene mutation. These mice develop a Th2 lymphoproliferative disorder characterized by marked infiltration of CD3^{lo}CD4⁺ T cells in secondary lymphoid organs.[28] Lack of CYSLTR1 on patients CD3⁻CD4⁺ T cells may render them less responsive to some eosinophil-derived survival signals and thereby contribute to the more indolent nature of L-HES.

The prostaglandin D2 receptor CRTH2, a G-protein–coupled receptor selectively expressed by Th2 cells, eosinophils, and basophils, is currently considered the most reliable marker for memory Th2 cells.[29] Two CRTH2 (*GPR44*) gene probes revealed a 5- and 22-fold increase in expression in the patients abnormal T cells, which was confirmed by flow cytometry (supplemental

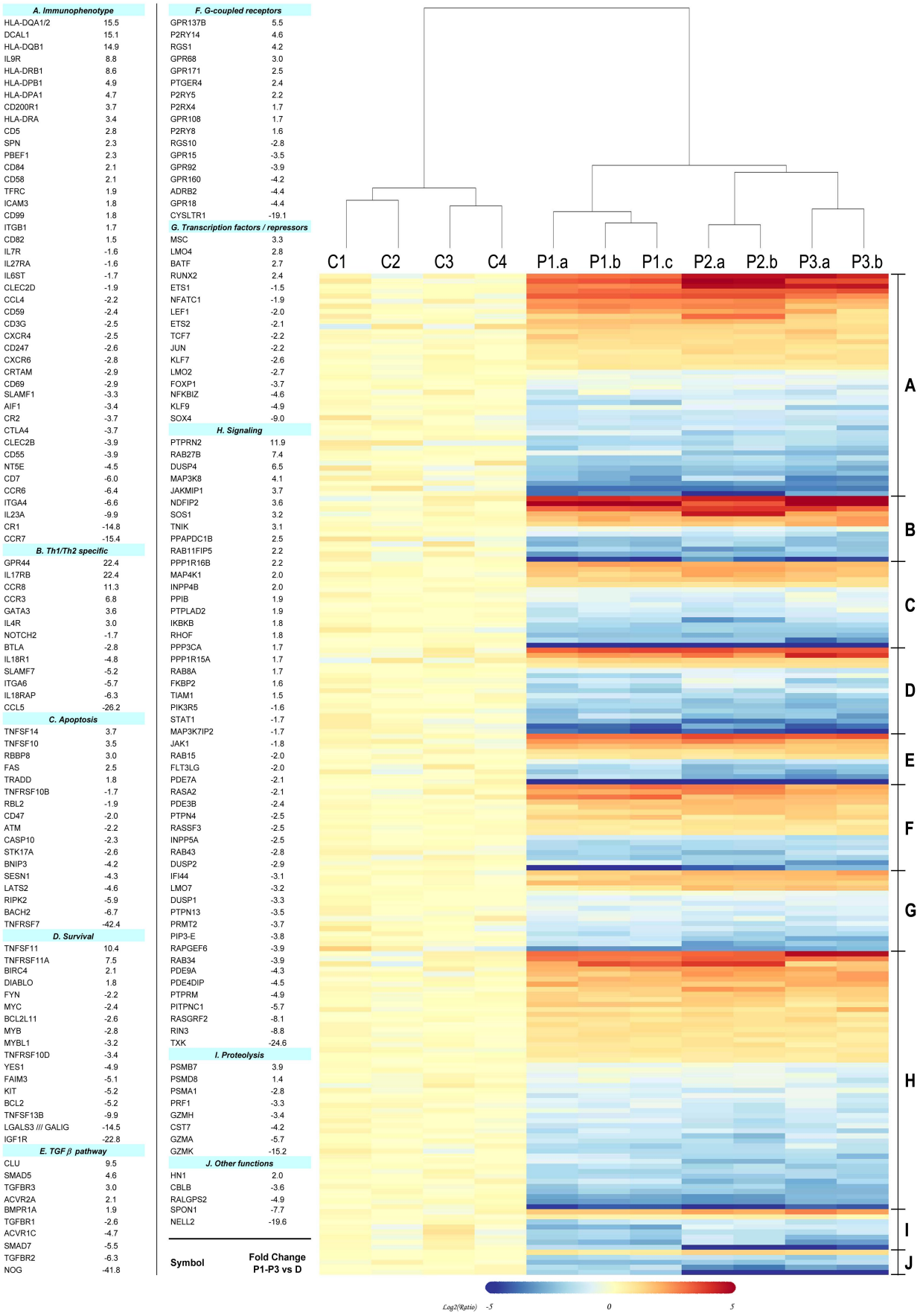

Figure 2. Heat map of selected gene alterations detected in the comparison of L-HES patients (P1-P3) vs controls. A total of 198 of the commonly altered genes detected in all 3 patients were selected from supplemental Table 2 based on their functional relevance (one probe set/gene is shown and represents the greatest absolute fold change in patients relative to controls). The genes are classified in functional groups and listed in numerical order based on fold change with the same order and group numbers maintained in the heat map. Each column in the heat map represents the expression from an individual gene chip and includes the 4 healthy controls (C1-C4), triplicates of P1-yr 0 (a-c), and duplicates of P2 and P3 (a-b). The clustering and the heat map were generated using R 2.5.1. The dendrogram was derived from a group of selected genes using the hierarchical clustering method and shows the relatedness of gene expression patterns in the L-HES patients relative to the controls.

Table 1. Gene expression changes detected in CD3⁻CD4⁺ T cells from chronic L-HES patients relative to controls and in association with P1's evolution to T lymphoma

Symbol	Name	Probe set	P1-P3 vs C[a,b]	P1 yr+6 vs P1-yr0[b,c]
ABLIM1	actin binding LIM protein 1	200965_s_at	-2.26	1.48
		210461_s_at	-2.44	
ANKRD57	ankyrin repeat domain 57	227034_at	3.13	-1.69
		219496_at	2.96	
APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2 (X11-like)	209870_s_at	-3.81	-1.56
		209871_s_at	-12.51	
AQP3	aquaporin 3 (Gill blood group)	39248_at	-3.07	1.56
		39249_at	-2.16	
		203747_at	-2.14	
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	236796_at	-6.70	-1.78
		221234_s_at	-5.59	
		1556451_at	-4.38	
		236307_at	-4.53	
		227173_s_at	-4.86	
		215907_at	-1.77	
BCAT1	branched chain aminotransferase 1, cytosolic	225285_at	26.07	2.58
		226517_at	27.21	2.52
		214452_at	6.56	3.11
		214390_s_at		1.94
BCL2	B-cell CLL/lymphoma 2	232210_at	-5.23	
		232614_at	-4.83	
		244035_at	-3.84	
		203685_at		-1.64
BEXL1	brain expressed X-linked-like 1	215440_s_at	-2.19	-1.90
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	201849_at	-4.24	-2.27
		201848_s_at	-3.40	
C18orf1	chromosome 18 open reading frame 1	242551_at	-2.90	2.38
		207996_s_at	-2.47	
C9orf40	chromosome 9 open reading frame 40	222781_s_at	1.81	2.01
CCR7	chemokine (C-C motif) receptor 7	206337_at	-15.39	3.13
		243107_at	-3.58	
CCR8	chemokine (C-C motif) receptor 8	208059_at	11.33	1.50
CDCA7	cell division cycle associated 7	230060_at	5.24	2.63
		224428_s_at	6.32	2.61
CEP55	centrosomal protein 55kDa	218542_at	3.07	2.08
ChGn	chondroitin β1,4 N-acetylgalactosaminyltransferase	1569387_at	1.93	
		219049_at		1.36
CLEC2B	C-type lectin domain family 2, member B	209732_at	-3.88	
		1556209_at		-1.59
COTL1	coactosin-like 1 (Dictyostelium)	1556346_at	4.25	1.51
		224583_at	4.28	
		221059_s_at	3.39	
CST7	cystatin F (leukocystatin)	210140_at	-4.21	3.61
CTSC	cathepsin C	201487_at	2.09	1.45
		225647_s_at	2.17	1.43
		225646_at	2.13	
DCAL1	CLECL1, dendritic cell-associated lectin-1	244413_at	15.14	1.56
DIABLO	diablo homolog (Drosophila)	219350_s_at	1.79	2.18
DKFZp761P0423	homolog of rat pragma of Rnd2	235085_at	-6.77	1.49
		240690_at	-9.70	
EMR1	egf-like module containing, mucin-like, 1	207111_at	4.35	1.36
ETFB	electron-transfer-flavoprotein, beta polypeptide	202942_at	2.58	1.76
F5	coagulation factor V (proaccelerin, labile factor)	204713_s_at	-3.53	4.70
		204714_s_at	-3.06	4.90
FAIM3	Fas apoptotic inhibitory molecule 3	221601_s_at	-4.61	-1.62
		221602_s_at	-5.13	
FAM13A1	family with sequence similarity 13, member A1	232628_at	-2.75	2.53
		202973_x_at	-2.43	2.10
		217047_s_at		2.29
FAM50B	family with sequence similarity 50, member B	205775_at	1.82	1.53
FANK1	fibronectin type III and ankyrin repeat domains 1	232968_at	9.90	1.58
FGF9	fibroblast growth factor 9 (glia-activating factor)	206404_at	-3.48	-2.10
FLJ20152	hypothetical protein FLJ20152	218532_s_at	-9.93	-1.91
		218510_x_at	-6.90	
FLJ21272	hypothetical protein FLJ21272	220467_at	-3.35	-1.57
FLJ42957	FLJ42957 protein	237591_at	2.40	2.04
GLIPR1	GLI pathogenesis-related 1 (glioma)	204222_s_at	2.13	
		214085_x_at	2.27	
		204221_x_at	2.24	
		233515_at		-1.89
GLUL	glutamate-ammonia ligase (glutamine synthetase)	215001_s_at	1.97	1.38
		200648_s_at	2.02	
GPR68	G protein-coupled receptor 68	229055_at	3.02	1.50
GTPBP8	GTP-binding protein 8 (putative)	223486_at	2.29	1.93
		221046_s_at	2.19	
GZMA	granzyme A (granzyme 1)	205488_at	-5.70	2.40
HLA-DQA1/2	major histocompatibility complex, class II, DQα1/2	212671_s_at	15.46	3.07
HLA-DQB1	major histocompatibility complex, class II, DQβ1	212998_x_at	14.90	2.49
		211656_x_at	4.23	2.02
		209823_x_at	5.17	2.17
		211654_x_at	3.54	1.60
HLA-DRA	major histocompatibility complex, class II, DRα	208894_at	3.45	3.01
HLA-DRB1	major histocompatibility complex, class II, DRβ1	215193_x_at	8.60	1.64
		208306_x_at	7.51	1.41
		209312_x_at	8.34	1.52
		204670_x_at	7.01	1.52
HN1	hematological and neurological expressed 1	222396_at	2.05	1.36
		217755_at	2.00	
HPCAL4	hippocalcin like 4	219671_at	-4.90	-3.01
HRBL	HIV-1 Rev binding protein-like	222126_at	1.77	
		1554618_at		-2.23
IGSF9B	immunoglobulin superfamily, member 9B	215255_at	2.48	1.64
IL17RB	interleukin 17 receptor B	219255_x_at	22.24	1.55
		224156_x_at	22.37	1.45
		224361_s_at	16.85	1.47
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	244599_at	-6.56	
		213416_at		2.08
		205884_at		2.55
		205885_s_at		2.78
KIAA1199	KIAA1199	212942_s_at	6.10	1.79
KLF9	Kruppel-like factor 9	203543_s_at	-4.86	-2.12
		203542_s_at	-4.19	-2.66
KLHL3	kelch-like 3 (Drosophila)	221221_s_at	-2.64	1.77
LASS6	LAG1 longevity assurance homolog 6 (S. cerevisiae)	212442_s_at	-7.49	2.01
		212446_s_at	-5.80	
LOC283174	hypothetical protein LOC283174	229734_at	2.71	1.71
LOC89944	hypothetical protein BC008326	213713_s_at	2.80	1.77
LYZ /// LILRB1	lysozyme (renal amyloidosis) /// leukocyte Ig-like receptor, subfamily B, member 1	213975_s_at	-3.26	10.01
LYZ		1555745_a_at		3.41
MAN1C1	mannosidase, alpha, class 1C, member 1	218918_at	-3.14	-1.66
		214180_at	-2.75	
MAP3K8	mitogen-activated protein kinase kinase kinase 8, COT, TPL2	235421_at	2.90	1.90
		205027_s_at	4.10	
MGAT5	mannosyl (alpha-1,6-)-glycoprotein e beta-1,6-N-acetyl-glucosaminyltransferase	241893_at	-2.75	
		215528_at		1.56
MSC	musculin (activated B-cell factor-1)	209928_s_at	3.28	1.95
NELL2	NEL-like 2 (chicken)	203413_at	-19.57	-6.10
P2RX4	purinergic receptor P2X, ligand-gated ion channel, 4	204088_at	1.75	1.64
P4HB	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	200656_s_at	2.05	1.46
		1564494_s_at	1.81	1.59
		200654_at	2.04	
PBEF1	pre-B-cell colony enhancing factor 1	243296_at	2.07	1.98
		1555167_s_at	1.76	1.82
		217739_s_at	2.33	1.93
		217738_at	2.08	1.88
PDE4DIP	phosphodiesterase 4D interacting protein (myomegalin)	236704_at	-4.48	
		212390_at		-2.59
PPP3CA	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	202457_s_at	1.70	
		1562467_at		-3.53
PRSS21	protease, serine, 21 (testisin)	220051_at	7.10	1.44
PTPLAD2	protein tyrosine phosphatase-like A domain 2	244050_at	1.85	1.34
PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	203029_s_at	11.85	2.20
		203030_s_at	9.14	2.49
		211534_x_at	2.68	1.99
RBBP8	retinoblastoma binding protein 8	203344_s_at	3.02	1.73
RGS10	regulator of G-protein signalling 10	204319_s_at	-2.81	1.40
		204316_at	-2.02	
RNF130	ring finger protein 130	217865_at	-3.41	-2.08
RUNX2	runt-related transcription factor 2	232231_at	2.41	1.86
SCML1	sex comb on midleg-like 1 (Drosophila)	235652_at	-11.11	-2.61
		218793_s_at	-12.21	
		222747_s_at	-4.39	
SLC16A6	solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	230748_at	2.86	2.10
		207038_at	2.76	
SLC1A4	solute carrier family 1, member 4 (glutamate/neutral amino acid transporter)	212811_x_at	6.71	2.20
		209610_s_at	9.11	2.07
		212810_s_at	6.62	2.18
		244377_at	5.53	2.05
		235875_at	5.03	1.89
		209611_s_at		2.14
SLC2A3	solute carrier family 2, member 3 (facilitated glucose transporter)	202499_s_at	1.62	
		236180_at		2.16
SLC39A10	solute carrier family 39 (zinc transporter), member 10	225295_at	-1.93	1.60
		226444_at	-2.38	
SLC44A2	solute carrier family 44, member 2	224609_at	-2.17	-1.65
		225175_s_at	-2.12	
SOS1	son of sevenless homolog 1 (Drosophila)	212780_at	2.97	1.45
		229261_at	3.08	
		230337_at	3.23	
		232883_at	2.37	
		242018_at	2.53	
		212777_at		1.51
SPON1	spondin 1, extracellular matrix protein	213994_s_at	-7.71	-2.57
		209436_at	-5.41	-2.03
		213993_at	-7.27	
		209437_s_at	-3.37	
TBL1X	transducin (beta)-like 1X-linked	213400_s_at	-2.68	-1.80
		201869_s_at	-2.01	-1.57
		201867_s_at		-2.01
TCEAL4	transcription elongation factor A (SII)-like 4	202371_at	-6.95	-3.00
WWTR1	WW domain containing transcription regulator 1	202133_at	6.17	2.15
ZNF30	zinc finger protein 30	232014_at	-2.61	-1.53
ZNF439	zinc finger protein 439	236562_at	-4.82	-1.80
		237441_at	-3.46	-1.76
ZNF447	zinc finger protein 447	218312_s_at	-7.77	
		217593_at		-1.89

[a]Fold change comparing the mean expression of duplicate arrays from P1, P2, and P3 with the mean expression from 4 controls; data are from supplemental Table 2.
[b]Consistently up-regulated genes are highlighted in dark gray; and consistently down-regulated genes in light gray.
[c]Fold change comparing the mean expression of triplicate arrays from P1-yr 0 and P1-yr +6; data are from supplemental Table 3.

Table 2. Changes in the expression of immune response genes associated with anti-CD2/CD28 activation of the CD3⁻CD4⁺ T cells from P1-P3

Symbol	Name	Probe set ID	Mn fold chg: P1-P3 vs C[b]	Mn fold chg: P1 yr+6 vs yr0[c]	Mn fold chg: P1-P3 S vs NS[d,e]
ATF4	activating transcription factor 4	200779_at	nc[f]	nc	1.55
BCL2	B-cell CLL/lymphoma 2	232210_at	-5.23	nc	18.44
		203685_at	nc	-1.64	8.91
BCL2L1	BCL2-like 1	212312_at	nc	nc	1.70
BCL2L11	BCL2-like 11 (apoptosis facilitator)	225606_at	-2.62	nc	nc
CCL5	chemokine (C-C motif) ligand 5 (RANTES)	1405_i_at	-26.17	nc[g]	nc
CCR2	chemokine (C-C motif) receptor 2 (CD192)	206978_at	nc	2.15	2.01
CCR3	chemokine (C-C motif) receptor 3 (CD193)	208304_at	6.79	nc	nc
CCR5	chemokine (C-C motif) receptor 5 (CD195)	206991_s_at	nc	1.80[g]	nc
CCR6	chemokine (C-C motif) receptor 6 (CD196)	206983_at	-6.40	nc	nc
CCR7	chemokine (C-C motif) receptor 7 (CD197)	206337_at	-15.39	3.13	nc
		243107_at	-3.58	nc	nc
CCR8	chemokine (C-C motif) receptor 8 (CD198)	208059_at	11.33	1.50	nc
CCR10	chemokine (C-C motif) receptor 10	220565_at	nc	2.81	nc
CD27	CD27 molecule	206150_at	-42.39	nc	nc
CD3G	CD3g molecule, gamma (TCR/CD3)	206804_at	-2.47	nc	nc
CD47	CD47 molecule	227259_at	-1.99	nc	nc
CD5	CD5 molecule	230489_at	2.78	nc	nc
CD53	CD53 molecule	203416_at	nc	nc	1.38
CD55	CD55 molecule, DAF for complement	243395_at	-3.93	nc	nc
CD58	CD58 molecule	243931_at	2.10	nc	nc
CD59	CD59 molecule, complement regulatory	212463_at	-2.43	nc	nc
CD69	CD69 molecule	209795_at	nc	nc	2.36
		237009_at	-2.88	nc	nc
CD7	CD7 molecule	214551_s_at	-6.03	nc	nc
CD74	CD74 molecule, MHC, class II invariant	209619_at	nc	nc	2.73
CD80	CD80 molecule	1554519_at	nc	nc	3.33
CD82	CD82 molecule	203904_x_at	1.53	nc	nc
CD84	CD84 molecule	205988_at	2.11	nc	nc
CD96	CD96 molecule	1555120_at	nc	1.62	nc
CD99	CD99 molecule	201028_s_at	1.78	nc	nc
CD200R1	CD200 receptor 1	1552875_a_at	3.69	nc	nc
CD247	CD247 molecule	210031_at	-2.56	nc	nc
CIITA	class II, MHC, transactivator	205101_at	nc	nc	2.87
CISH	cytokine inducible SH2-containing protein	223961_s_at	nc	nc	11.83
DUSP1	dual specificity phosphatase 1	201041_s_at	-3.25	nc	nc
DUSP2	dual specificity phosphatase 2	204794_at	-2.86	nc	nc
DUSP4	dual specificity phosphatase 4	204014_at	6.51	nc	nc
DUSP5	dual specificity phosphatase 5	209457_at	nc	nc	5.76
DUSP6	dual specificity phosphatase 6	208891_at	nc	nc	6.93
DUSP16	dual specificity phosphatase 16	224336_s_at	nc	nc	-2.14
EGR1	early growth response 1	227404_s_at	nc	nc	7.82
EGR2	early growth response 2	205249_at	nc	nc	8.02
EGR3	early growth response 3	206115_at	nc	nc	6.54
EPHA4	EPH receptor A4	206114_at	nc	nc	-1.75
EPHB6	EPH receptor B6	204718_at	-2.98	nc	nc
FAIM3	Fas apoptotic inhibitory molecule 3	221601_s_at	-4.61	-1.62	-1.94
		221602_s_at	-5.13	nc	-1.83
FLT3LG	fms-related tyrosine kinase 3 ligand	206980_s_at	-2.03	nc	3.36
		210607_at	nc	nc	4.01
FYB	FYN binding protein (FYB-120/130)	227266_s_at	nc	nc	-1.44
FYN	FYN oncogene related to SRC, FGR, YES	212486_s_at	-2.18	nc	nc
HLA-DMA	MHC, class II, DM alpha	217478_s_at	nc	nc	2.42
HLA-DMB	MHC, class II, DM beta	203932_at	nc	nc	2.31
HLA-DPA1	MHC, class II, DP alpha 1	211990_at	4.68	nc	nc
HLA-DPB1	MHC, class II, DP beta 1	201137_s_at	4.89	nc	nc
HLA-DQA1	MHC, class II, DQ alpha 1	212671_s_at	15.46	3.07	2.49
HLA-DQB1	MHC, class II, DQ beta 1	212998_x_at	14.90	2.49	2.03
HLA-DRA	MHC, class II, DR alpha	208894_at	3.45	3.01	4.17
IFI44	interferon-induced protein 44	214453_s_at	-3.08	nc	nc
IFI6	Interferon, alpha-inducible protein 6	224533_s_at	-22.00	nc	nc
IFITM1	interferon induced transmembrane 1	201601_x_at	nc	1.57	2.17
		214022_s_at	nc	nc	2.10
IFITM2	interferon induced transmembrane 2	201315_x_at	nc	1.59	nc
IL2RA	interleukin 2 receptor, alpha (CD25)	211269_s_at	nc	nc	4.22
IL2RB	interleukin 2 receptor, beta (CD122)	205291_at	nc	nc	1.73
IL4R	interleukin 4 receptor (CD124)	203233_at	2.98	nc	nc
IL5	interleukin 5 (CSF, eosinophil)	207952_at	nc	nc	6.82
IL6ST	Interleukin 6 signal transducer (CD130)	212195_at	-1.71	nc	nc
IL7R	interleukin 7 receptor (CD127)	205798_at	-1.59	nc	-1.50
IL9R	interleukin 9 receptor (CD129)	214950_at	8.81	nc	nc
IL13	interleukin 13	207844_at	nc	nc	7.34
IL17RB	interleukin 17 receptor B	219255_x_at	22.24	1.55	2.97
IL18R1	interleukin 18 receptor 1 (CD218a)	206618_at	-4.85	nc	nc
IL18RAP	interleukin 18 receptor AP (CD218b)	207072_at	-6.27	nc	nc
IL23A	interleukin 23, alpha subunit p19	234377_at	-9.93	nc	nc
IL27RA	interleukin 27 receptor, alpha	222062_at	-1.61	nc	nc
IL32	interleukin 32	203828_s_at	nc	2.50	nc
INPP4B	inositol polyphosphate-4-phosphatase	205376_at	1.99	nc	1.78
IRF4	interferon regulatory factor 4	204562_at	nc	nc	6.12
ISGF3G	interferon-stimulated transcript. factor 3	203882_at	nc	nc	1.83
ITGA4	integrin, alpha 4 (CD49D, VLA-4)	205885_s_at	nc	2.78	nc
		213416_at	nc	nc	2.05
ITGA6	integrin, alpha 6	244599_at	-6.56	nc	nc
ITGB1	integrin, beta 1 (CD29)	215177_s_at	-5.67	nc	nc
LGALS1	lectin, galactoside-binding 1 (galectin 1)	1553678_a_at	1.70	nc	nc
LGALS3	lectin, galactoside-binding 3 (galectin 3)	201105_at	nc	2.40	nc
LIF	leukemia inhibitory factor	208949_s_at	-14.53	nc	nc
LMAN1	lectin, mannose-binding, 1	205266_at	nc	nc	2.13
LMNA	lamin A/C	224629_at	1.49	nc	1.46
LMNB1	lamin B1	1554600_s_at	nc	nc	2.13
LTA	lymphotoxin alpha (TNFSF1)	203276_at	nc	nc	1.76
LTB	lymphotoxin beta (TNFSF3)	206975_at	nc	nc	3.33
MAL	mal, T-cell differentiation protein	207339_s_at	nc	nc	1.58
MAP2K5	mitogen-activated protein kinase kinase 5	204777_s_at	nc	nc	2.19
MAP3K7IP2	mitogen-activated protein 3x kinase 7 IP2	216765_at	nc	-1.75	nc
MAP3K7IP3	mitogen-activated protein 3x kinase 7 IP3	212184_s_at	-1.72	nc	1.68
MAP3K8	mitogen-activated protein 3x kinase 8	227357_at	nc	1.45	nc
		205027_s_at	4.07	nc	nc
MAPKAPK3	mitogen-activated protein kinase-act 3	235421_at	2.91	1.93	nc
MAP4K1	mitogen-activated protein 4x kinase 1	202788_at	nc	nc	1.54
NDFIP2	Nedd4 family interacting protein 2	206296_x_at	2.00	nc	nc
		224802_at	3.62	nc	8.10
		224799_at	nc	nc	6.62
NFIA	nuclear factor I/A	224970_at	nc	1.76	nc
NFIL3	nuclear factor, interleukin 3 regulated	203574_at	nc	2.73	2.69
NFATC1	NFAT, cytoplasmic 1	211105_s_at	-1.92	nc	nc
NFKBIZ	NF-kB inhibitor, zeta	223218_s_at	-4.56	nc	nc
OSM	oncostatin M	230170_at	nc	nc	3.52
PAK1IP1	PAK1 interacting protein 1	218886_at	nc	nc	1.82
PBXIP1	pre-B-cell leukemia TF interact. protein 1	214177_s_at	nc	nc	-1.62
PDE3B	phosphodiesterase 3B, cGMP-inhibited	214582_at	-2.12	nc	-1.80
		222330_at	-2.37	nc	nc
PDE4DIP	phosphodiesterase 4D interacting	236704_at	-4.48	nc	nc
		212390_at	nc	-2.59	nc
PDE7A	phosphodiesterase 7A	1552343_s_at	nc	nc	-1.57
		224046_s_at	-2.06	nc	nc
PDE9A	phosphodiesterase 9A	205593_s_at	-4.32	nc	nc
PIM1	pim-1 oncogene	209193_at	nc	nc	2.22
PIM2	pim-2 oncogene	204269_at	nc	nc	2.25
PMAIP1	PMA-induced protein 1	204285_s_at	nc	nc	5.69
PPIB	peptidylprolyl isomerase B (cyclophilin B)	200968_s_at	1.91	nc	nc
PPIF	peptidylprolyl isomerase F (cyclophilin F)	201490_s_at	nc	nc	1.66
PPIL1	peptidylprolyl isomerase, cyclophilin-like 1	222500_at	nc	nc	1.72
PRDM1	PR domain containing 1, with ZNF domain	228964_at	nc	2.07	1.98
PTGER2	prostaglandin E receptor 2 (subtype EP2)	206631_at	nc	nc	4.05
PTGER4	prostaglandin E receptor 4 (subtype EP4)	204897_at	2.37	nc	nc
RGS1	regulator of G-protein signalling 1	202988_s_at	4.21	nc	nc
RGS10	regulator of G-protein signalling 10	204319_s_at	-2.81	1.40	nc
RGS10	regulator of G-protein signalling 10	204316_at	-2.02	nc	nc
RGS16	regulator of G-protein signalling 16	209324_s_at	nc	nc	2.29
RUNX1	runt-related transcription factor 1	209360_s_at	nc	nc	1.35
RUNX2	runt-related transcription factor 2	232231_at	2.41	1.86	nc
SNFT	Jun dimerization protein p21SNFT	220358_at	nc	nc	2.28
SOCS1	suppressor of cytokine signaling 1	210001_s_at	nc	nc	5.15
SOCS2	suppressor of cytokine signaling 2	203373_at	nc	nc	4.44
SOCS3	suppressor of cytokine signaling 3	227697_at	nc	nc	4.49
SORL1	sortilin-related receptor, L(DLR class)	230707_at	2.07	nc	-1.85
SOS1	son of sevenless homolog 1	212780_at	2.97	1.45	1.65
SOX4	SRY (sex determining region Y)-box 4	201417_at	-9.01	nc	nc
SOX6	SRY (sex determining region Y)-box 6	235526_at	nc	1.59	nc
STAT1	signal transducer & act. transcription 1	AFFX-HUMISG	-1.67	nc	4.44
		209969_s_at	nc	nc	6.74
STAT3	signal transducer & act. transcription 3	208992_s_at	nc	nc	1.61
TFRC	transferrin receptor (CD71)	208691_at	1.89	nc	1.84
TNF	TNF superfamily, member 2	207113_s_at	nc	nc	5.16
TNFAIP3	TNF, alpha-induced protein 3	202644_s_at	nc	nc	-1.58
TNFRSF4	TNF receptor superfamily, member 4	214228_x_at	nc	1.65	3.30
TNFRSF10B	TNF receptor superfamily, member 10b	209295_at	-1.71	nc	nc
TNFRSF10D	TNF receptor superfamily, member 10d	227345_at	-3.39	nc	nc
TNFRSF11A	TNF, member 11a, NFKB activator	238846_at	7.45	nc	nc
TNFSF4	TNF (ligand) superfamily, member 4	207426_s_at	nc	2.77	nc
TNFSF8	TNF (ligand) superfamily, member 8	235735_at	nc	nc	2.30
TNFSF10	TNF (ligand) superfamily, member 10	214329_x_at	2.92	nc	1.63
		202688_at	3.47	nc	nc
TNFSF11	TNF (ligand) superfamily, member 11	210643_at	10.35	nc	nc
TNFSF13B	TNF (ligand) superfamily, member 13b	223502_s_at	-9.87	nc	nc
TNFSF14	TNF (ligand) superfamily, member 14	207907_at	3.72	nc	nc
TNIK	TRAF2 and NCK interacting kinase	211828_s_at	2.05	nc	-1.62
TNIK	TRAF2 and NCK interacting kinase	213107_at	3.06	nc	nc

[a]One probe (greatest fold change) is shown for each gene, except where differences were detected in individual probes to the same gene.

[b]Fold change comparing the mean expression of duplicate arrays from P1, P2, and P3 nonstimulated with the mean expression from 4 controls; data are from supplemental Table 2.

[c]Fold changes detected in the mean expression from triplicate arrays of P1 nonstimulated CD3⁻CD4⁺ T cells from yr +6 compared with yr 0; data are from supplemental Table 3.

[d]Fold change comparing the mean expression of duplicate arrays from stimulated (S) vs nonstimulated (NS) CD3⁻CD4⁺ T cells from P1 to P3; data are from supplemental Table 2.

[e]Genes whose expression is similarly altered both after activation and in L-HES patient cells (either chronic disease or T lymphoma) are highlighted in gray.

[f]NC indicates no change.

[g]In P1, CCL5 levels are decreased yr 0 vs C and yr +4 vs yr 0 but then increase in yr +6 vs yr 4 (supplemental Table 3).

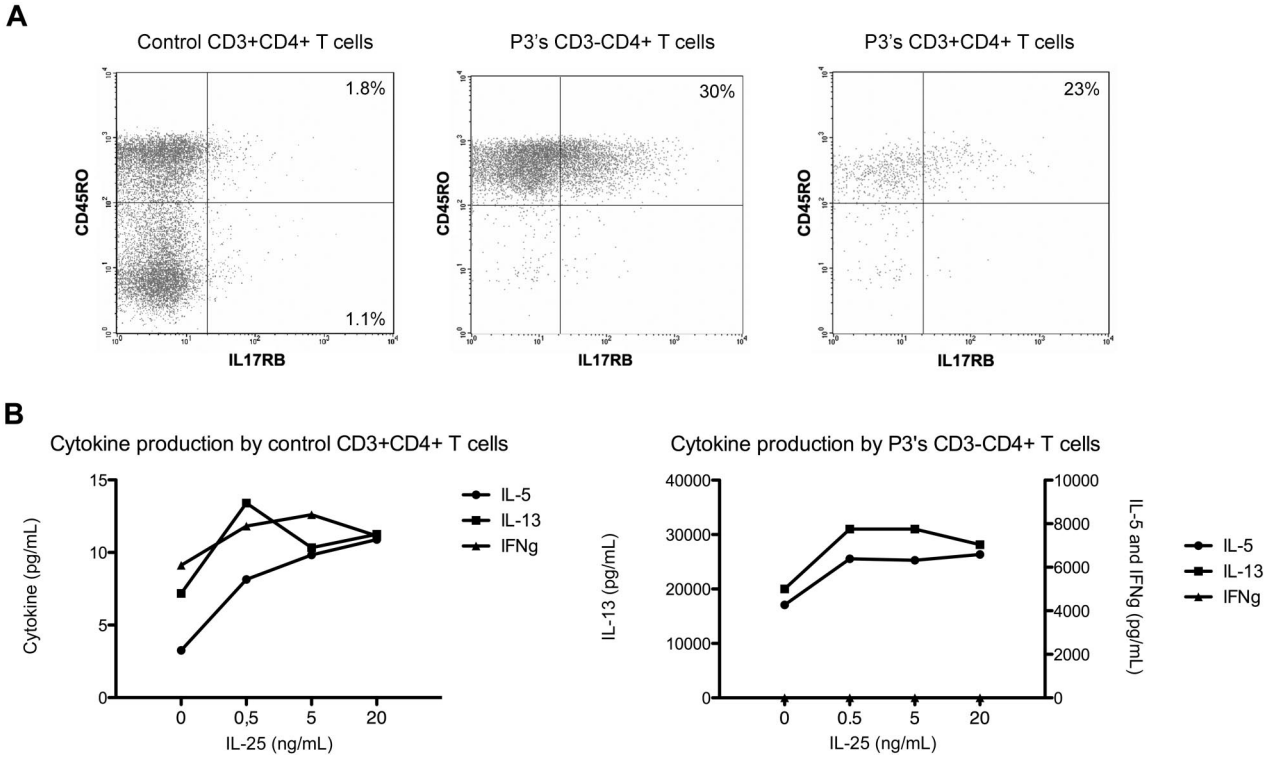

Figure 3. IL-17RB (IL-25 receptor) and cytokine expression by L-HES CD3⁻CD4⁺ T cells. (A) Four-color immunofluorescent labeling of control and P3's PBMCs. The lymphocyte populations were gated on CD4 and CD3 positivity/negativity. (B) Purified CD3⁻CD4⁺ T cells from P3 were cultured for 48 hours with phorbol ester and anti-CD28 in the absence or presence of increasing concentrations of rhIL-25, and cytokine concentrations were determined using BD Cytometric Bead Array Flex Sets. A representative experiment is shown.

Table 4). CRTH2 is involved in Th2 cell migration, GATA3 up-regulation, and induction of Th2 cytokine production.[30] Our experiments found that GATA3 nuclear binding is up-regulated in patients CD3⁻CD4⁺ T cells only after CD2/CD28 costimulation[31]; however, the arrays detected a 3.6-fold increase in *GATA3* transcripts in the patient's quiescent CD3⁻CD4⁺ T cells, which contrasts with the reported lack of differences in a microarray study of quiescent human Th1 and Th2 cells.[32] Taken together, our data suggest that significant levels of GATA3 may be present in the cytoplasm waiting for activation signals that rapidly induce phosphorylation and nuclear translocation of this Th2 transcription factor, followed by cytokine gene up-regulation. The gene expression profiles of unstimulated versus CD2/CD28 costimulated CD3⁻CD4⁺ T cells clearly show that their Th2 cytokine expression is dependent on activation (Table 2). Together with our previous studies,[12] these data suggest that, despite high CRTH2 and GATA3 expression levels, activating signals from local microenvironments in vivo are required to bring the circulating cells out of standby.

Apoptosis. The clonal CD3⁻CD4⁺ T cells persist at relatively stable levels for many years in vivo, suggesting equilibrium between cell proliferation and apoptosis during chronic disease. The death domain containing tumor necrosis factor (TNF) superfamily plays critical roles in controlling the induction and progression of cell death, and the microarrays revealed altered expression of several TNF family member genes in the CD3⁻CD4⁺ T cells, including both proapoptotic genes and antiapoptotic (pro-proliferation) genes (Figure 2, supplemental Table 7); some were confirmed by quantitative RT-PCR (Figure 4A-B). Increased surface expression of FAS and a lack of CD27 in L-HES have been previously described[2,10] and were reconfirmed in this cohort (supplemental Table 4). RANKL (*TNFSF11*; 10-fold increase) augments the costimulatory properties of antigen-presenting cells and thus could be important for CD3⁻CD4⁺ T cell activation in vivo. A 10-fold increase in RANKL mRNA was also detected in microarrays of CD4⁺ T cells from patients with Sezary syndrome.[33] Interestingly, there was no further increase in RANKL expression associated with P1's progression to lymphoma or on costimulation. Further investigation into the functional implications of increased RANKL expression in L-HES is warranted given the ongoing development of humanized monoclonal antibodies for clinical use. The altered expression in specific subsets of genes involved in programmed cell death observed in this study suggests that there is a controlled balance that potentiates the increased survival and persistent expansion of the CD3⁻CD4⁺ T-cell clone.

T-cell homeostasis and the TGF-β family. Altered expression among the TGF-β superfamily (TGF-β, activins, inhibins, growth differentiation factors, and bone morphogenetic proteins [BMP]) has been described for a variety of epithelial-derived solid tumors and hematologic malignancies.[34] A microarray study revealed that TGF-β is the major signaling pathway that constitutively keeps human CD4⁺ T cells in a resting state.[35] In this study, we detected numerous changes in TGF-β family gene expression in the L-HES CD3⁻CD4⁺ T cells during chronic disease, with a subset of these genes changing further during P1's evolution to T lymphoma (Figure 5, supplemental Table 7); in contrast, no additional changes were observed in the patients' abnormal T cells after CD2/CD28 costimulation. Decreased expression of the type I TGF-β receptor genes, TGF-βRI (*TGFBR1*) and *ACVRIC*, and the type II receptor gene TGF-βRII (*TGFBR2*) in the CD3⁻CD4⁺ T cells was confirmed by quantitative RT-PCR (Figure 4A). A previous study of CD4⁺ T-cell lines derived from T lymphoma patients found that decreased TGF-βRI and TGF-βRII expression was related to reduced responsiveness to TGF-β1-mediated growth inhibition,[36]

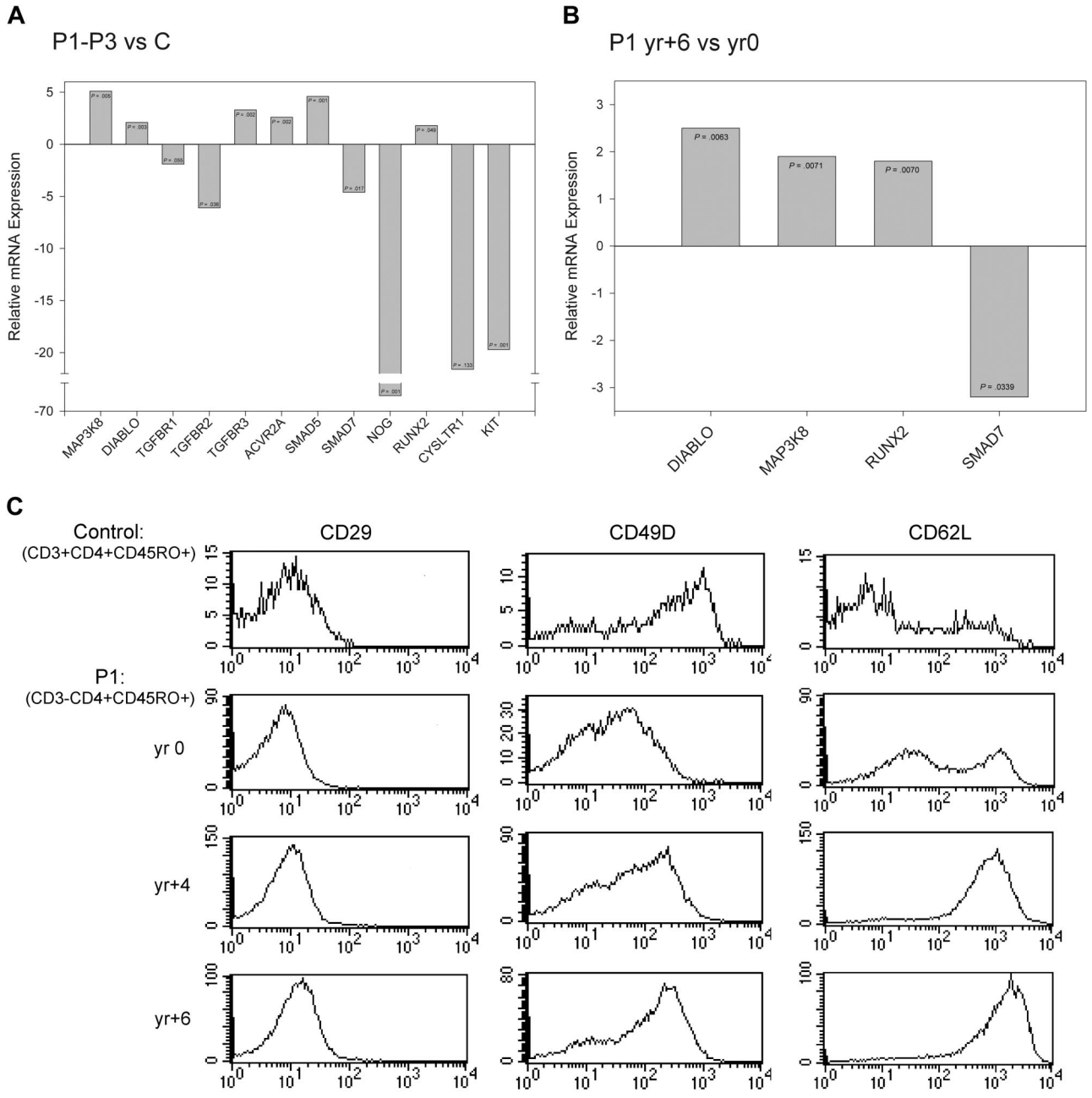

Figure 4. Validation of changes in gene expression using quantitative RT-PCR and flow cytometry. (A-B) Fold change in the expression of selected genes measured by quantitative RT-PCR for (A) patients (P1-P3) relative to controls[4] and (B) P1-yr +6 relative to P1-yr 0. *P* values were calculated based on 3 independent experiments using the Student *t* test and are indicated in the corresponding bar. (C) Histograms showing the surface expression of CD29 (*ITGB1*), CD49D (*ITGA4*), and CD62L (*SELL*) on control $CD3^+CD4^+CD45RO^+$ T cells and P1-yr 0, P1-yr +4, and P1-yr +6 $CD3^-CD4^+CD45RO^+$ T cells. Isotype controls (not shown) for each sample were set between 10^0 and 10^1.

whereas microarrays of Sezary T cells detected *TGFBR2* gene down-regulation.[33]

Studies have shown that a third TGF-β receptor, TGF-βRIII (*TFGBR3*; betaglycan) is frequently down-regulated in solid tumors[34] in contrast to B chronic lymphocytic leukemia where its up-regulation has been reported.[37] We detected increased *TGFBR3* in the $CD3^-CD4^+$ T cells from patients with chronic disease. One study reported that corticosteroids can selectively stimulate TGF-βRIII expression in hepatic stellate cells[38]; and although corticosteroids are standard therapy for symptomatic L-HES patients, this treatment is probably not responsible for the *TGFBR3* up-regulation observed because the fold changes for P2 and P3 (treated; supplemental Table 1) were lower than P1 (untreated).

TGF-βRIII binds all TGF-β isoforms and presents them to TGF-βRII, thereby initiating the recruitment and phosphorylation of TGF-βRI that leads to kinase activation. However, evidence indicates that TGF-βRIII also functions independently from TGF-β ligand presentation by working as a coreceptor with type 2 activin receptors (*ACVR2A* and *ACVR2B*, both genes increased in the $CD3^-CD4^+$ T cells). Activins and inhibins are structurally related members of the TGF-β superfamily that act as antagonists, with the former providing positive and the latter negative intracellular signals. High affinity binding of inhibin by TGF-βRIII is favored in cells coexpressing ACVR2A, thereby inhibiting the activin pathway. We also detected an increase in the BMP type I receptor gene, *BMPRIA*, which can interact with ACVR2A to bind BMPs. Finally,

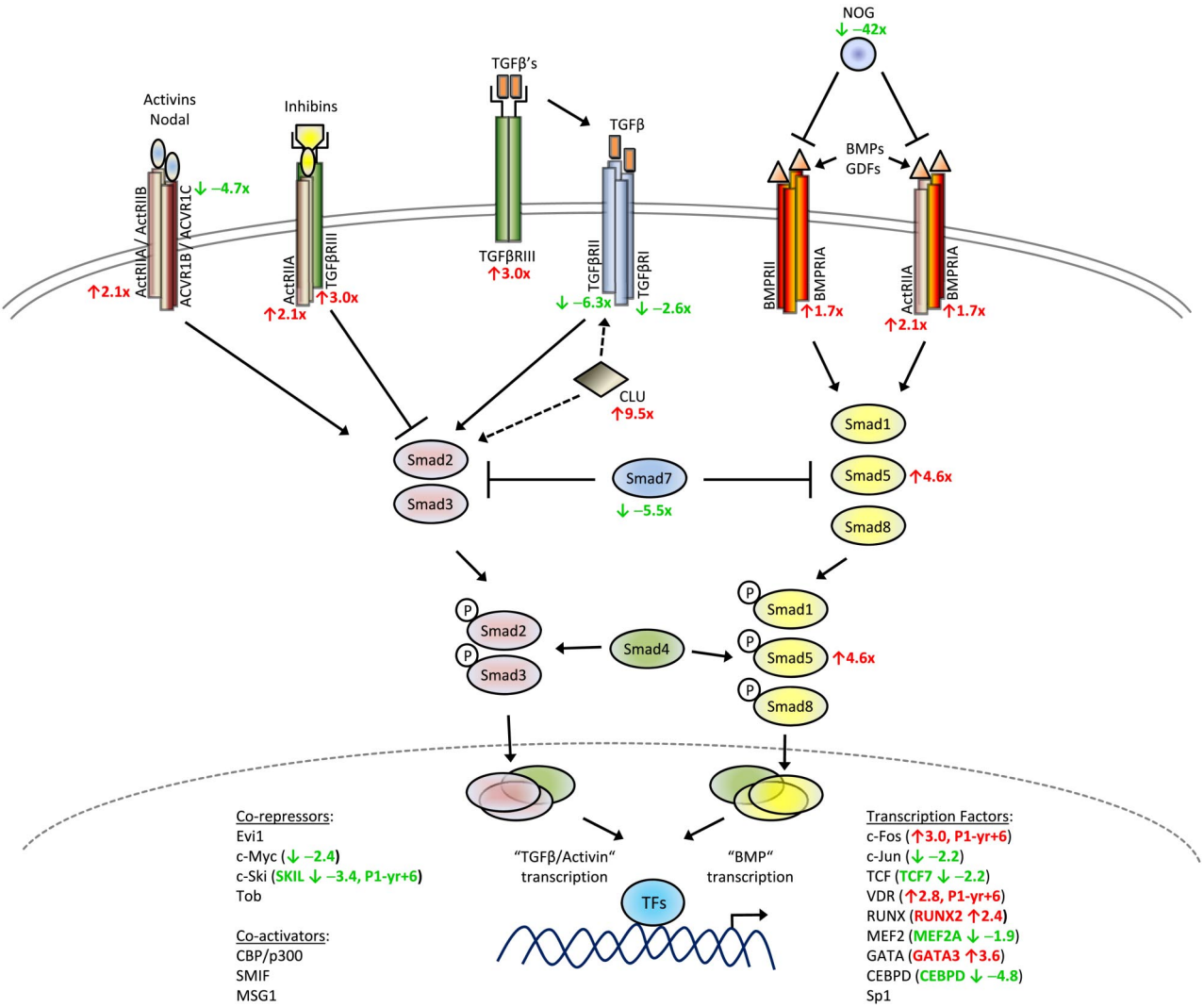

Figure 5. Schematic diagram of TGF-β signaling pathway. Altered expression in several TGF-β superfamily signaling genes was detected in L-HES patients CD3⁻CD4⁺ T cells. The mean fold changes detected in the microarrays for P1 to P3 relative to the controls are indicated in red (up-regulated genes) and green (down-regulated genes).

the noggin gene (*NOG*) was substantially decreased in the abnormal T cells and acts as an antagonist for the TGF-β superfamily members, BMP2 and BMP4, both of which play a role in early thymocyte differentiation.[39] Altogether, these alterations in gene expression reflect a shift in the balance of TGF-β superfamily–dependent intracellular pathways in the CD3⁻CD4⁺ T cells, with uncontrolled signaling via the BMP pathway possibly disrupting normal homeostasis and favoring abnormal cell survival and growth.

This hypothesis is further substantiated by altered expression of Smad proteins, which transmit signals downstream from the TGF-β superfamily receptors. We observed increased receptor regulated *SMAD5* gene expression together with decreases in the inhibitory *SMAD7* gene, both confirmed by quantitative RT-PCR. Although their function in hematopoietic cells is not as well defined as Smad2, Smad3, and Smad4, Smad5 is involved in regulating BMP signaling whereas Smad7 negatively regulates receptor regulated Smad signaling and has been implicated in mature hematopoietic cell development.[40] Receptor regulated Smad proteins specific for the BMP pathway, such as Smad5, interact with a variety of proteins, including Runx family transcription factors. The Runx genes have been shown to function as tumor suppressors in human cancer, although their overexpression in murine models revealed an oncogenic role in the development of hematopoietic tumors, including T lymphomas.[41] Runx2 mediates cellular responses to signaling pathways hyperactive in tumors, including TGF-β family pathways, by forming coregulatory complexes with Smads and other coactivator and corepressor proteins to regulate gene transcription. Runx2, better known for its role in bone development and maintenance, was up-regulated in all patients and then again during P1's evolution to T lymphoma (Table 1). *RUNX2* and RANKL (*TNFSF11*) are targets of transcriptional regulation by the vitamin D receptor (*VDR*; up-regulated in P1 yr +6) and were up-regulated in the CD3⁻CD4⁺ T cells from chronic disease. In addition, several target genes known to be induced by TGF-β were also decreased, including *JUN, MYB, FLT3LG*, and *CXCR4* (the latter confirmed by flow cytometry). The clusterin gene (*CLU*), which interacts with TGF-βRII to modulate Smad signaling,[42] was also significantly up-regulated in the abnormal T cells. Further investigation into the perturbations detected in the TGF-β superfamily signaling pathways and the role they play in the persistence of the CD3⁻CD4⁺ T-cell clone in L-HES are ongoing.

Gene expression changes in the CD3$^-$CD4$^+$ T cells associated with the evolution from chronic L-HES to T lymphoma

Cryopreserved blood samples from P1 spanning her 6-year progression from chronic disease to T lymphoma provided a very rare opportunity to assess changes in gene expression associated with malignant transformation in vivo. Our previous studies found that over time the initial CD3$^-$CD4$^+$ T-cell clone spawned subclones containing 2 independent 6q deletions (6q11-6q23.1 and 6q13-6q22.1) with progressive outgrowth of the 6q13q22.1-deleted subclone detected in 91% of the malignant T cells.[2,8,15] Gene expression profiles of P1's CD3$^-$CD4$^+$ T cells at diagnosis (yr 0), during chronic disease (yr +4), and with T lymphoma (yr +6) revealed 349 genes (450 probe sets) that were differentially expressed in the malignant compared with the premalignant T cells (Table 3 and supplemental Table 3). Remarkably, approximately one-third of the probes (126 of 450), corresponding to 87 of 349 genes, were also initially altered in patients with chronic L-HES (Table 1), with the majority of these genes displaying stepwise alterations in expression (decreases or increases), first in all patients with chronic disease and then with P1's T lymphoma development. Progressive decreases were observed in several apoptosis genes, growth factors, and transcription factors along with progressive increases in surface receptors, signaling proteins, as well as additional growth factors and transcription factors. Among the genes whose expression changed progressively from chronic to malignant L-HES, only 6 genes (*BCAT1, HLA-DQA1/2, HLA-DQB1, HLA-DRA, IL17RB*, and *SOS1*) also increased and only the *FAIM3* gene decreased after in vitro activation (Table 2). These data suggest that the genes whose expression is altered in the abnormal T cells from chronic and malignant L-HES reflect genuine changes in CD3$^-$CD4$^+$ T-cell physiology and not a transient response to external stimuli, such as signaling molecules, surface receptors, and cytokine production.

Genes with potential relevance to malignant transformation include progressive increases in surface receptors, growth factors, transcription factors, and signaling proteins, as shown in Table 3. The limited number of genes whose expression decreases in the T lymphoma cells is distinguished by signaling proteins and transcription factors. One potentially relevant gene is the IL-9 receptor (*IL9R*), which was up-regulated on the CD3$^-$CD4$^+$ T cells from chronic L-HES; however, in contrast to its increased expression in mice overexpressing IL-9 that develop thymomas[43] and some Hodgkin lymphomas, we did not observe further up-regulation on P1's T lymphoma cells. Overall, these alterations reflect progressive activation, altered signaling, and/or homing of the CD3$^-$CD4$^+$ T cells to specific sites and/or their adaptation to a specific microenvironment. The stepwise-modulated genes as well as those newly deregulated in the malignant T cells are of particular interest and relevance as potential therapeutic targets and new diagnostic markers.

T-cell trafficking and migration. Leukocyte migration is mediated by a network of trafficking receptors expressed both on lymphoid and nonlymphoid tissues such that specific combinations of these adhesion and chemoattractant molecules act as traffic signals for directing extravasion and migration.[44] Trafficking genes play distinct roles as leukocytes migrate through blood vessels. The initial step is mediated by selectins; and although we did not detect statistically significant changes in *SELL* (L-selectin; CD62L) gene expression, flow cytometry revealed surface receptor up-regulation on the CD3$^-$CD4$^+$ T cells, which continued to increase as P1 progressed to T lymphoma (Figure 4C). Rolling over endothelial cells exposes leukocytes to chemokines, which in turn provoke conformational changes in integrins that increase their affinity. The $\alpha4\beta1$ integrin (VLA-4) is composed of 2 subunits, CD49D ($\alpha4$; *ITGA4*) and CD29 ($\beta1$; *ITGB1*), both required for VLA-4 surface expression. Down-regulation of CD49D in association with a slight increase in CD29 was observed in the CD3$^-$CD4$^+$ T cells during chronic disease (Figures 2,4C and supplemental Tables 2,4). CD49D was reexpressed in concert with increased VLA-4 surface expression as P1 developed enlarged lymph nodes and progressed to T lymphoma (Table 1, Figure 4C).

Changes in other trafficking receptor genes were detected both in chronic disease and during the evolution to T lymphoma. Down-regulation of *CXCR4, CXCR6, CCR6*, and *CCR7* was detected in patients during chronic disease with some *CCR7* expression returning as P1 progressed to T lymphoma (Tables 1, 3). Increases in *CCR3, ICAM3, LFA-3, CD82*, and *CD99* were observed in all patients with up-regulation of *CCR2* in P1-yr +4 and *CCR5, CCR10, CD96*, and *PECAM1* in P1-yr +6. *CCR8* expression levels increased stepwise, 10-fold in chronic patients and a further 1.5-fold in P1-yr +6. CCR8 binds CCL1, which like CCL17 can be induced in bronchial epithelial cells by the Th2 cytokines IL-4 and IL-13.[45] Although we have previously shown that serum CCL17 levels are extremely high in patients with L-HES,[16] serum CCL1 levels and the functional role of CCR8 on CD3$^-$CD4$^+$ T cells remain unknown. The altered expression of trafficking receptors and ligands observed on the CD3$^-$CD4$^+$ T cells probably directs their movement to specific sites during premalignant and malignant L-HES disease,[46] exposing the cells to external activation signals and/or costimulatory cells present locally.

Differential microRNA expression in the CD3$^-$CD4$^+$ T cells

MicroRNAs are endogenously expressed noncoding RNAs that regulate gene expression via mRNA degradation, mRNA destabilization, or translation inhibition. There is growing evidence that deregulated microRNA expression contributes to oncogenesis with an increasing number of identified microRNAs targeting genes involved in immune development, proliferation, and apoptosis.[47] We extended the molecular profile of L-HES using quantitative RT-PCR to quantify changes in mature microRNA expression. Initially, we compared the expression of 156 microRNAs in CD3$^-$CD4$^+$ T cells from P1-yr.6 with control CD3$^+$CD4$^+$ T cells (supplemental Table 6). Thirty-eight microRNAs that decreased or increased greater than 2-fold in 2 independent experiments were selected for further analysis in CD3$^-$CD4$^+$ T cells from 6 chronic L-HES patients (P1-P5, P7) and CD3$^+$CD4$^+$ T cells from the same 4 controls. Using the nonpaired Student *t* test, we identified 23 microRNAs that were differentially expressed in the abnormal T cells (Table 4). The majority (19 of 23) of the selected microRNAs were down-regulated with increases found for only 4 microRNAs.

One effect of the interaction between a microRNA and its target mRNA can be transcript cleavage and degradation. We searched for a correlation between global changes in mRNA and microRNA expression in the CD3$^-$CD4$^+$ T cells but did not observe a statistically significant correlation. We then used Ingenuity Pathways Analysis to assess the potential biologic importance of the predicted target genes as a group and determined that the best scored functional networks included the cell cycle, cell death, and hematologic system development and function. Individual microRNAs and their putative gene targets were generated using MirBase and included some of notable interest and potential relevance. The expression of 3 Th2 genes in the CD3$^-$CD4$^+$ T cells inversely paralleled several microRNAs predicted to target them, including increases in

Table 3. Selected gene expression changes in the CD3⁻CD4⁺ T cells during P1's evolution from chronic L-HES to lymphoma

Probe set	Symbol	Name	P1 yr0 vs C[d]	P1 yr+4 vs yr0[c]	P1 yr+6 vs yr+4[b]	P1 yr+6 vs yr0[a,e]
236796_at	BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	-4.31			-1.78
203685_at	BCL2	B-cell CLL/lymphoma 2				-1.64
232210_at			-3.65			
201849_at	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-3.10			-2.27
204655_at	CCL5	chemokine (C-C motif) ligand 5 (RANTES)	-33.99	-2.09	3.65	
206978_at	CCR2	chemokine (C-C motif) receptor 2 (CD192)		2.69		2.15
208304_at	CCR3	chemokine (C-C motif) receptor 3 (CD193)	3.70	3.09		
206991_s_at	CCR5	chemokine (C-C motif) receptor 5 (CD195)		2.67		1.80
206337_at	CCR7	chemokine (C-C motif) receptor 7 (CD197)	-13.03		1.91	3.13
208059_at	CCR8	chemokine (C-C motif) receptor 8 (CDw198)	9.75			1.50
220565_at	CCR10	chemokine (C-C motif) receptor 10				2.81
1555120_at	CD96	CD96 molecule				1.62
1556209_at	CLEC2B	C-type lectin domain family 2, member B		-1.49		-1.59
209732_at			-1.59			
207630_s_at	CREM	cAMP responsive element modulator		1.50		1.99
210140_at	CST7	cystatin F (leukocystatin)	-4.44		2.99	3.61
244413_at	DCAL1	CLECL1, dendritic cell-associated lectin-1	10.34			1.56
221601_s_at	FAIM3	Fas apoptotic inhibitory molecule 3	-3.68			-1.62
224840_at	FKBP5	FK506 binding protein 5				1.60
224856_at				1.33		
225262_at	FOSL2	FOS-like antigen 2			2.52	2.95
229055_at	GPR68	G protein-coupled receptor 68	2.88			1.50
205488_at	GZMA	granzyme A (granzyme 1, CTL-associated serine esterase 3)	-6.65		2.20	2.40
226878_at	HLA-DOA	major histocompatibility complex, class II, DO alpha				1.86
203290_at	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	5.73	1.84	1.81	3.33
212998_x_at	HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	6.02		1.56	2.49
208894_at	HLA-DRA	major histocompatibility complex, class II, DR alpha	2.41	2.08		3.01
215193_x_at	HLA-DRB1	major histocompatibility complex, class II, DR beta 1	9.74	1.35		1.64
221491_x_at	HLA-DRB1,3,4,5	major histocompatibility complex, class II, DR beta	9.47			1.77
222396_at	HN1	hematological and neurological expressed 1	1.62			1.36
1554618_at	HRBL	HIV-1 Rev binding protein-like	3.16			-2.23
201601_x_at	IFITM1	interferon induced transmembrane protein 1 (9-27)	-1.88		1.32	1.57
201315_x_at	IFITM2	interferon induced transmembrane protein 2 (1-8D)				1.59
210095_s_at	IGFBP3	insulin-like growth factor binding protein 3				-2.44
201508_at	IGFBP4	insulin-like growth factor binding protein 4			2.92	3.45
219255_x_at	IL17RB	interleukin 17 receptor B	30.68	1.42		1.55
203828_s_at	IL32	interleukin 32	-2.02		2.19	2.50
213416_at	ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-7.38		1.85	2.08
1554306_at	ITPKB	inositol 1,4,5-trisphosphate 3-kinase B				-2.15
203542_s_at	KLF9	Kruppel-like factor 9			-2.12	-2.66
203543_s_at			-2.72			-2.12
201105_at	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)			1.64	2.40
208949_s_at	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	-19.96		2.93	
35974_at	LRMP	lymphoid-restricted membrane protein	-3.23		1.62	1.81
206584_at	LY96	lymphocyte antigen 96	1.70			1.52
206363_at	MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	-2.04	1.68	2.06	3.45
218918_at	MAN1C1	mannosidase, alpha, class 1C, member 1	-2.09		-2.16	-1.66
216765_at	MAP2K5	Mitogen-activated protein kinase kinase 5	2.06			-1.75
227357_at	MAP3K7IP3	mitogen-activated protein kinase kinase kinase 7 interacting protein 3				1.45
235421_at	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	3.49	1.42		1.93
212022_s_at	MKI67	antigen identified by monoclonal antibody Ki-67		1.99		2.37
209928_s_at	MSC	musculin (activated B-cell factor-1)	2.60			1.95
202364_at	MXI1	MAX interactor 1				-1.60
203413_at	NELL2	NEL-like 2 (chicken)	-4.22	-2.40	-2.54	-6.10
224975_at	NFIA	nuclear factor I/A				1.88
203574_at	NFIL3	nuclear factor, interleukin 3 regulated			2.19	2.73
213028_at	NFRKB	nuclear factor related to kappaB binding protein	-1.34			1.34
204088_at	P2RX4	purinergic receptor P2X, ligand-gated ion channel, 4	1.56		1.43	1.64
217738_at	PBEF1	pre-B-cell colony enhancing factor 1	1.82	1.53		1.88
212390_at	PDE4DIP	phosphodiesterase 4D interacting protein (myomegalin)				-2.59
236704_at			-2.62			
208983_s_at	PECAM1	platelet/endothelial cell adhesion molecule (CD31)				2.00
202014_at	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A		-1.33	1.61	
37028_at			1.64			
1562467_at	PPP3CA	Protein phosphatase 3, catalytic subunit, alpha isoform (calcineurin A alpha)	2.94			-3.53
228964_at	PRDM1	PR domain containing 1, with ZNF domain	1.92		1.49	2.07
204061_at	PRKX	protein kinase, X-linked				1.61
233314_at	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)				4.61
244050_at	PTPLAD2	protein tyrosine phosphatase-like A domain containing 2	1.97			1.34
204201_s_at	PTPN13	protein tyrosine phosphatase, non-receptor type 13 (CD95 (Fas)-associated)	-3.64		1.57	
203029_s_at	PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	7.50	1.51	1.45	2.20
206039_at	RAB33A	RAB33A, member RAS oncogene family				1.62
228113_at	RAB37	RAB37, member RAS oncogene family				1.54
215070_x_at	RABGAP1	RAB GTPase activating protein 1				-1.71
203344_s_at	RBBP8	retinoblastoma binding protein 8	2.41			1.73
202988_s_at	RGS1	regulator of G-protein signalling 1	7.13		-1.49	
204319_s_at	RGS10	regulator of G-protein signalling 10	-2.31			1.40
232231_at	RUNX2	runt-related transcription factor 2				1.86
204351_at	S100P	S100 calcium binding protein P			1.73	1.91
217591_at	SKIL	SKI-like			-2.88	-3.37
212780_at	SOS1	son of sevenless homolog 1 (Drosophila)	2.38		1.42	1.45
235526_at	SOX6	SRY (sex determining region Y)-box 6				1.59
213994_s_at	SPON1	spondin 1, extracellular matrix protein	-3.59		-2.07	-2.57
241860_at	STK17B	Serine/threonine kinase 17b (apoptosis-inducing)				-1.64
234050_at	TAGAP	T-cell activation GTPase activating protein				-1.46
208944_at	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	-3.08		-1.59	
210166_at	TLR5	toll-like receptor 5		1.65	1.94	3.21
214228_x_at	TNFRSF4	tumor necrosis factor receptor superfamily, member 4 (CD134, OX40)				1.65
207426_s_at	TNFSF4	tumor necrosis factor ligand superfamily, member 4 (CD252, OX40L)			2.79	2.77
204529_s_at	TOX	thymus high mobility group box protein TOX				2.20
224412_s_at	TRPM6	transient receptor potential cation channel, subfamily M, member 6		3.53	7.09	25.05
204254_s_at	VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor			2.71	2.74

[a]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr 0 compared with 4 controls; data are from supplemental Table 3. Progressively up-regulated genes are highlighted in dark gray and progressively down-regulated genes are highlighted in light gray.

[b]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr +4 compared with yr 0; data are from supplemental Table 3.

[c]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr +6 compared with yr +4; data are from supplemental Table 3.

[d]Fold changes detected in the mean expression from triplicate arrays of P1 CD3⁻CD4⁺ T cells from yr +6 compared with yr 0; data are from supplemental Table 3.

Table 4. microRNAs that are differentially expressed in the CD3$^-$CD4$^+$ T cells from L-HES patients compared with CD3$^+$CD4$^+$ T cells from controls

	Patients vs controls		
miRNA name	*P**	Fold change†	Chromosomal location‡
hsa-let-7b	.032	3.2	22q13.31
hsa-miR-26a	.019	−2.3	3p22.3
hsa-miR-31	.004	−111.4	9p21.3
hsa-miR-95	.025	−2.6	4p16.1
hsa-miR-99a	.011	−60.9	21q21.1
hsa-miR-100	.010	−57.6	11q24.1
hsa-miR-126	.030	−9.1	9q34.3
hsa-miR-130a	.034	−6.2	11q12.1
hsa-miR-135b	.011	−11.8	1q32.1
hsa-miR-135a	.008	−10.9	3p21.1
hsa-miR-151	.019	−12.1	8q24.3
hsa-miR-181a	.010	−34.6	1q31.3
hsa-miR-181b	.010	−19.3	1q31.3
hsa-miR-193a	.017	−4.6	17q11.2
hsa-miR-213	.011	−78.8	1q31.3
hsa-miR-215	.019	−3.1	1q41
hsa-miR-221	.010	3.4	Xp11.3
hsa-miR-222	.010	3.7	Xp11.3
hsa-miR-335	.010	−8.0	7q32.2
hsa-miR-340	.019	−4.9	5q35.3

**P* values were corrected using the false discovery rate calculation.

†Fold change in the L-HES patients' CD3$^-$CD4$^+$ T cells (P1-P5 + P7) relative to controls (4).

‡Chromosomal locations were obtained from Ensembl.

GATA3 with decreases in miR-10a, miR-95, and miR-130a, *IL4R* increases in concert with decreased miR-126 and miR-340, and increased *CCR3* in parallel with decreased miR-181a, miR-181b, and miR-335. Genes whose mRNA expression changed in the abnormal T cells that were also predicted targets of 2 or 3 altered microRNAs included: *IL18RAP* (let7b, miR-221), *CD99* (miR-31, miR-95, miR-135a), *TRADD* (miR-31, miR-125a), *CD58* (miR-95, miR-135b), *PPP3CA* (miR-99a, miR-100), RANKL (*TNFSF11;* miR-126, miR-335), *DMN3* (miR-126, miR-151), *RGS1* (miR-130a, miR-335), and *PRMT2* (miR-221, miR222).

Perhaps of greatest potential biologic significance were 3 genes whose expression increased in patients with chronic disease (*RBBP8, CLU*, and *MAP3K8*) with further increases associated with P1's evolution to T lymphoma (*RBBP8* and *MAP3K8*) that were also predicted targets of 4 different down-regulated microRNAs. The retinoblastoma binding protein 8 gene (*RBBP8*), a predicted target of miR-31, miR-126, miR-130a, and miR-335, is thought to function as a tumor suppressor in conjunction with the transcriptional corepressor CTBP and BRCA1. Clusterin (*CLU*) is a calcium regulated protein whose expression has been associated with tumorigenesis and malignant progression, perhaps in part by modulating TGF-βRII signaling (Figure 5). The nuclear form is proapoptotic and the secretory form is antiapoptotic,[48] with both forms involved in DNA repair and cell cycle regulation. Clusterin expression was significantly up-regulated in the CD3$^-$CD4$^+$ T cells in concert with the down-regulation of miR-99a, miR-100, miR-126, and miR-335. Thus, miR-126 and miR-335 potentially target both *RBBP8* and *CLU*. A recent study of breast cancer found that miR-126 expression reduced tumor growth, whereas miR-335 suppressed lung and bone metastasis,[49] with miR-335 loss leading to *SOX4* and tenascin C (*TNC*) activation, which are both implicated in the acquisition of metastatic properties. We detected a 9-fold decrease in *SOX4* (no change in *TNC*), which paralleled a 9-fold decrease in miR-335, suggesting that this microRNA targets other critical genes in T cells.

Many of the gene changes detected in patients relative to controls and then during P1's evolution to T lymphoma are involved in cell signaling. The *MAP3K8* (Tpl2/Cot) oncogene expression increased stepwise first in patients during the chronic disease phase and again during P1's evolution to T lymphoma. The *MAP3K8* gene is a predicted target of miR-135a, miR-135b, miR-181a, and miR-181b, which were all decreased in the CD3$^-$CD4$^+$ T cells. Studies have shown that decreased miR-181b expression in B chronic lymphocytic leukemia patients is associated with up-regulation of the TCL1 oncogene.[50] The *MAP3K8* gene is of particular interest because studies have shown that it is differentially regulated in hematopoietic cells and plays a role in tumor development. Overexpression and truncation of MAP3K8 lead to the activation of several T-cell signaling pathways and have been associated with large granular lymphocyte proliferative disorders.[51] miR-181a also positively modulates TCR/CD3 sensitivity and affinity by suppressing phosphatases involved in negatively regulating TCR/CD3 signaling.[52] The miR-181 family is involved in controlling hematopoietic cell differentiation and maturation with miR-181a levels fluctuating during thymopoiesis and its repression shown to diminish T-cell sensitivity in both primed and stimulated naive T cells.[53] MiR-181a has also been shown to inhibit *CD69*, *BCL2*, and TCRα gene transcription.[52] Intriguingly, our data revealed a substantial decrease in miR-181a and miR-181b associated with low CD69, *BCL2*, and TCR/CD3 expression levels in the CD3$^-$CD4$^+$ T cells, suggesting that the complex interactions between the TCR/CD3 signaling genes and the miR-181 family require further analysis. Experiments designed to approach the functional relevance of decreased expression of miR-135 family members, about which little is known, were accomplished by transfecting miR-135a and miR-135b mimics together in the CD4$^+$ Jurkat T-cell line. These preliminary data indicate that the miR-135 mimics decrease *MAP3K8* (−2.6-fold) and *SMAD5* (−2.3-fold) expression compared with irrelevant sequence controls (data not shown).

Changes in microRNA expression during P1's clinical evolution

We also quantified expression of the same 38 microRNAs in association with P1's evolution to T lymphoma and found that only miR-125a changed significantly. miR-125a levels were 5.7-fold lower ($P = .059$) in the L-HES patient cohort relative to controls; and as P1 evolved to T lymphoma, miR-125a expression progressively decreased with an additional 2.8-fold drop ($P = .003$) detected at yr +6. Predicted gene targets of miR-125a were generated using MirBase and compared with the mRNA expression profiles of P1's CD3$^-$CD4$^+$ T cells (supplemental Table 6). The up-regulated target genes included another gene involved in signaling, *PTPRN2*, which is a member of the receptor-type protein tyrosine phosphatase family. *PTPRN2* expression increased in parallel with the progressive decrease of miR-125a expression in the CD3$^-$CD4$^+$ T cells from chronic and malignant disease. *PTPRN2* (IA-2β) is a pancreatic β-cell autoantigen for type 1 diabetes; and although its function is largely unknown, our studies suggest its role in L-HES warrants further investigation. A second miR-125a target gene, the Abelson helper integration site 1 (*AHI1*), was significantly up-regulated in the latter stages of P1's evolution to T lymphoma. AHI1 has been implicated in the development of T- and B-cell malignancies with increased expression detected in CD4$^+$CD7$^-$ T cells from Sezary syndrome patients.[54] Although the function of miR-125a remains unknown, its homolog miR-125b

has been shown to posttrancriptionally target TNF-α and decrease cell proliferation.[55] The stepwise down-regulation of miR-125a detected in L-HES disease suggests a potential role for this microRNA in the persistence and progressive transformation of the CD3⁻CD4⁺ T cells.

Discussion

L-HES associated with a clonal population of CD3⁻CD4⁺ Th2 cells is a rare benign lymphoproliferative disorder that can progress to malignant T lymphoma after a prolonged period of chronic disease. During the chronic phase, patients generally seek medical attention because of cutaneous symptoms, including severe eczema, angioedema, and urticaria. The Th2 nature of the underlying T-cell disorder is responsible for the marked hypereosinophilia, which also leads to frequent patient follow-up by physicians and treatment well before the development of malignancy. However, the mechanisms underlying clonal T-cell persistence and transformation remain unknown, precluding the development of a targeted therapy capable of eradicating the abnormal T cells during the chronic disease phase and thereby short-circuiting malignant transformation.[1,56] The abnormal T cells persist for many years in vivo during which their levels are frequently stable in conjunction with corticosteroid treatment. The CD3⁻CD4⁺ T cells may eventually become refractory to treatment in parallel with malignant progression, which currently leaves only allogeneic stem cell transplantation as possibly curative.[57]

Our earlier work identified a CD3⁻CD4⁺ T-cell population as the source of Th2 cytokines in a symptomatic L-HES patient.[17] We further demonstrated that the CD3⁻CD4⁺ T-cell population in patients was monoclonal based on its T-cell receptor rearrangement[2] and that loss of surface TCR/CD3 expression was the result of a defect in *CD3*γ gene transcripts.[15] We detected multiple chromosomal aberrations within the clonal T-cell population and found that evolution to malignancy in vivo was associated with the emergence of a subclone characterized by a specific 6q deletion in one patient.[8] The present global gene expression study was undertaken to identify, in an unbiased manner, the specific genes and cellular pathways involved in the complex interplay between persistence and control of the CD3⁻CD4⁺ T cells in chronic L-HES and during their progression to full-blown malignancy.

The microarray analysis of patients with chronic disease provides a detailed immunophenotype/genotype, confirming the Th2 nature of the abnormal T-cell clone and offering insight on activation pathways and their homing state. Comparison of gene expression profiles from patients CD3⁻CD4⁺ T cells during chronic L-HES versus CD3⁺CD4⁺ T cells from healthy controls, activated or not by CD2/CD28 costimulation, demonstrated that altered gene expression in the abnormal T-cell clone does not simply reflect an activated memory T-cell phenotype. In addition, these data confirm that other previously reported functional characteristics of the CD3⁻CD4⁺ T cells, such as Th2 cytokine production and altered surface receptor expression, occur on engagement of membrane costimulatory receptors. We further assessed the importance of increased IL-25 receptor (IL-17RB) expression on the CD3⁻CD4⁺ T cells given the expected significant in vivo exposure to eosinophil-derived IL-25 in L-HES patients. These data demonstrate that the CD3⁻CD4⁺ T-cell response to IL-25 is characterized by Th2 cytokine production and increased proliferation in vitro. Given the premalignant nature of the CD3⁻CD4⁺ T cells during chronic disease, our findings indicate that controlling eosinophil levels should be a therapeutic endpoint for these patients, even though their frequently isolated cutaneous manifestations may not appear to warrant systemic therapy. Taken together, our data suggest that the blood-derived CD3⁻CD4⁺ T cells are in a transient state of ingress and egress with tissue microenvironments where they receive the signals for aberrant cytokine production and expansion.

We also identified genes whose expression deviated from the normal pattern of checks and balances controlling T-cell signaling and survival and thereby maintaining homeostasis. One of the more intriguing findings is the apparent switch in TGF-β superfamily signaling from TGF-β/Activin-directed to BMP-directed gene expression (Figure 5). TGF-β has been extensively characterized for its immune suppressive functions and is known to play critical roles in controlling thymocyte development and limiting effector/memory T-cell responses. Activin A is produced by activated Th2 cells and plays a role in Th2-mediated responses of B cells and macrophages.[58] BMPs were initially identified for their growth factor effects on bone formation but have since been shown to regulate neurogenesis and hematopoiesis during embryonic development; and although little is known about BMP-mediated control of mature T-cell responses, BMPs have been shown to play a role in T-cell differentiation in the thymus.[39] Several studies have described aberrations in BMP signaling pathways in solid tumors, suggesting that survival and expansion of the CD3⁻CD4⁺ T-cell clone in L-HES could be in part the result of a switch from negative TGF-β regulation to positive BMP signaling, and the processes involved are currently under investigation.

The sequential analysis of P1's clinical evolution revealed that almost one-third of the genes whose expression changed in association with the development of T lymphoma were already abnormally expressed in L-HES patients during chronic disease. The majority of these genes were not altered in response to in vitro activation, further suggesting that they reflect inherent changes in CD3⁻CD4⁺ T-cell biology associated with transformation and deregulated growth. These genes include progressively deregulated oncogenes, transcription factors, and signaling genes. Together with the genes that were newly altered in P1's T lymphoma cells, this relatively small number of genes identifies critical players in chronic and malignant L-HES. Good examples are the 3 genes, *RBBP8, MAP3K8*, and *PTPRN2*, whose expression increased stepwise CD3⁻CD4⁺ T cells, first in chronic disease and then in association with T lymphoma. Furthermore, their increased expression paralleled decreases in microRNAs predicted to target them, and our preliminary miRNA transfection experiments lend credence to the functional consequences. For example, the MAP3K8 oncogene is a member of the serine/threonine protein kinase family that was identified by its transforming activity. Activated MAP3K8 induces the ERK1/2, JNK, NF-κB, and p38MAPK pathways, and a study has shown that it is constitutively activated in HTLV-I-transformed human CD4+ T-cell lines.[59] The *MAP3K8* gene therefore illustrates a gene deregulation (increased expression) detected in our patient cohort during chronic disease, which was further augmented in L-HES–associated T lymphoma and identified as a potential target of microRNAs shown to be downmodulated in the patients' cells.

Our objective in this study was to provide a global assessment of gene expression changes characteristic of the CD3⁻CD4⁺ T cells during chronic and malignant L-HES as a means of

identifying the deregulated pathways that underlie their abnormal persistence and expansion in vivo. These data reveal important gene expression changes in receptors whose altered expression may contribute to the $CD3^-CD4^+$ T cell–modified responses to environmental stimuli as well as deviations in homeostatic growth control pathways whose perturbations may favor outgrowth of the abnormal T-cell clone. Preliminary functional experiments confirmed that the aberrant pathways identified in the $CD3^-CD4^+$ T cells warrant further in-depth exploration, and several specifically deregulated genes point to potential new drug targets and diagnostic markers.

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Authorship

Contribution: M.R. and K.W.-G. designed the research; M.R. and M.L. performed the research; M.R., M.L., C. Sibille, F.R., M.G., and K.W.-G. analyzed and interpreted the data; C. Sotiriou contributed vital analytical tools; C.G. and B.H.-K. performed the statistical analysis of microarray data; and M.R., F.R., and K.W.-G. wrote the manuscript.

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References

1. Roufosse F, Cogan E, Goldman M. Lymphocytic variant hypereosinophilic syndromes. *Immunol Allergy Clin North Am.* 2007;27(3):389-413.
2. Roufosse F, Schandene L, Sibille C, et al. Clonal Th2 lymphocytes in patients with the idiopathic hypereosinophilic syndrome. *Br J Haematol.* 2000;109(3):540-548.
3. Kitano K, Ichikawa N, Mahbub B, et al. Eosinophilia associated with proliferation of CD(3+)4-(8-) alpha beta+ T cells with chromosome 16 anomalies. *Br J Haematol.* 1996;92(2):315-317.
4. Bank I, Amariglio N, Reshef A, et al. The hypereosinophilic syndrome associated with CD4+CD3− helper type 2 (Th2) lymphocytes. *Leuk Lymphoma.* 2001;142(1):123-133.
5. Simon HU, Plotz SG, Dummer R, Blaser K. Abnormal clones of T cells producing interleukin-5 in idiopathic eosinophilia [see comments]. *N Engl J Med.* 1999;341(15):1112-1120.
6. Brugnoni D, Airo P, Rossi G, et al. A case of hypereosinophilic syndrome is associated with the expansion of a CD3-CD4+ T-cell population able to secrete large amounts of interleukin-5. *Blood.* 1996;87(4):1416-1422.
7. O'Shea JJ, Jaffe ES, Lane HC, MacDermott RP, Fauci AS. Peripheral T cell lymphoma presenting as hypereosinophilia with vasculitis: clinical, pathologic, and immunologic features. *Am J Med.* 1987;82(3):539-545.
8. Ravoet M, Sibille C, Roufosse F, et al. 6q- is an early and persistent chromosomal aberration in CD3−CD4+ T-cell clones associated with the lymphocytic variant of hypereosinophilic syndrome. *Haematologica.* 2005;90(6):753-765.
9. Roumier AS, Grardel N, Lai JL, et al. Hypereosinophilia with abnormal T cells, trisomy 7 and elevated TARC serum level. *Haematologica.* 2003; 88(7):ECR24.
10. Schandene L, Roufosse F, de Lavareille A, et al. Interferon alpha prevents spontaneous apoptosis of clonal Th2 cells associated with chronic hypereosinophilia. *Blood.* 2000;96(13):4285-4292.
11. Cools J, DeAngelo DJ, Gotlib J, et al. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med.* 2003;348(13):1201-1214.
12. Roufosse F, Schandene L, Sibille C, et al. T-cell receptor-independent activation of clonal Th2 cells associated with chronic hypereosinophilia. *Blood.* 1999;94(3):994-1002.
13. Zhang L, Wang L, Ravindranathan A, Miles MF. A new algorithm for analysis of oligonucleotide arrays: application to expression profiling in mouse brain regions. *J Mol Biol.* 2002;317(2):225-235.
14. Kennedy RE, Kerns RT, Kong X, Archer KJ, Miles MF. SScore: an R package for detecting differential gene expression without gene expression summaries. *Bioinformatics.* 2006;22(10):1272-1274.
15. Willard-Gallo KE, Badran BM, Ravoet M, et al. Defective CD3gamma gene transcription is associated with NFATc2 overexpression in the lymphocytic variant of hypereosinophilic syndrome. *Exp Hematol.* 2005;33(10):1147-1159.
16. de Lavareille A, Roufosse F, Schandene L, Stordeur P, Cogan E, Goldman M. Clonal Th2 cells associated with chronic hypereosinophilia: TARC-induced CCR4 down-regulation in vivo. *Eur J Immunol.* 2001;31(4):1037-1046.
17. Cogan E, Schandene L, Crusiaux A, Cochaux P, Velu T, Goldman M. Brief report: clonal proliferation of type 2 helper T cells in a man with the hypereosinophilic syndrome [see comments]. *N Engl J Med.* 1994;330(8):535-538.
18. Hamalainen H, Zhou H, Chou W, Hashizume H, Heller R, Lahesmaa R. Distinct gene expression profiles of human type 1 and type 2 T helper cells. *Genome Biol.* 2001;2(7):RESEARCH0022.1-0022.11.
19. Wang YH, Angkasekwinai P, Lu N, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC activated Th2 memory cells. *J Exp Med.* 2007; 204(8):1837-1847.
20. Caruso R, Stolfi C, Sarra M, et al. Inhibition of monocyte-derived inflammatory cytokines by IL-25 occurs via a p38 Map kinase-dependent induction of SOCS-3. *Blood.* 2009;113(15):3512-3519.
21. Longhi MP, Harris CL, Morgan BP, Gallimore A. Holding T cells in check: a new role for complement regulators? *Trends Immunol.* 2006;27(2): 102-108.
22. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* 1995; 3(5):541-547.
23. Owaki T, Asakawa M, Fukai F, Mizuguchi J, Yoshimoto T. IL-27 induces Th1 differentiation via p38 MAPK/T-beta- and intercellular adhesion molecule-1/LFA-1/ERK1/2-dependent pathways. *J Immunol.* 2006;177(11):7579-7587.
24. Oliver-Vila I, Saborit-Villarroya I, Engel P, Martin M. The leukocyte receptor CD84 inhibits FcvarepsilonRI-mediated signaling through homophilic interaction in transfected RBL-2H3 cells. *Mol Immunol.* 2008;45(8):2138-2149.
25. Ryan EJ, Marshall AJ, Magaletti D, et al. Dendritic cell-associated lectin-1: a novel dendritic cell-associated, C-type lectin-like molecule enhances T cell secretion of IL-4. *J Immunol.* 2002;169(10): 5638-5648.
26. Oh KI, Kim BK, Ban YL, et al. CD99 activates T cells via a costimulatory function that promotes raft association of TCR complex and tyrosine phosphorylation of TCR zeta. *Exp Mol Med.* 2007;39(2):176-184.
27. Kim N, Luster AD. Regulation of immune cells by eicosanoid receptors. *Sci World J.* 2007;7:1307-1328.
28. Prinz I, Gregoire C, Mollenkopf H, et al. The type 1 cysteinyl leukotriene receptor triggers calcium influx and chemotaxis in mouse alpha beta- and gamma delta effector T cells. *J Immunol.* 2005; 175(2):713-719.
29. Cosmi L, Annunziato F, Galli MIG, Maggi RME, Nagata K, Romagnani S. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur J Immunol.* 2000;30(10):2972-2979.
30. De Fanis U, Mori F, Kurnat RJ, et al. GATA3 up-regulation associated with surface expression of CD294/CRTH2: a unique feature of human Th cells. *Blood.* 2007;109(10):4343-4350.
31. Roufosse F. Clonal expansion of helper type 2 lymphocytes as a cause of the hypereosinophilic syndrome: clinical and immunological aspects [doctoral thesis]. Université Libre de Bruxelles, 2001.
32. Freishtat RJ, Mitchell LW, Ghimbovschi SD, Meyers SB, Hoffman EP. NKG2A and CD56 are coexpressed on activated TH2 but not TH1 lymphocytes. *Hum Immunol.* 2005;66(12):1223-1234.
33. van Doorn R, Dijkman R, Vermeer MH, et al. Aberrant expression of the tyrosine kinase receptor EphA4 and the transcription factor twist in Sezary

syndrome identified by gene expression analysis. *Cancer Res.* 2004;64(16):5578-5586.

34. Gordon KJ, Blobe GC. Role of transforming growth factor-[beta] superfamily signaling pathways in human disease. *Biochim Biophys Acta.* 2008;1782(4):197-228.
35. Classen S, Zander T, Eggle D, et al. Human resting CD4+ T cells are constitutively inhibited by TGFbeta under steady-state conditions. *J Immunol.* 2007;178(11):6931-6940.
36. Kadin ME, Cavaille-Coll MW, Gertz R, Massague J, Cheifetz S, George D. Loss of receptors for transforming growth factor beta in human T-cell malignancies. *Proc Natl Acad Sci U S A.* 1994; 91(13):6002-6006.
37. Jelinek DF, Tschumper RC, Stolovitzky GA, et al. Identification of a global gene expression signature of B-chronic lymphocytic leukemia. *Mol Cancer Res.* 2003;1(5):346-361.
38. Bolkenius U, Hahn D, Gressner AM, Breitkopf K, Dooley S, Wickert L. Glucocorticoids decrease the bioavailability of TGF-beta which leads to a reduced TGF-beta signaling in hepatic stellate cells. *Biochem Biophys Res Commun.* 2004; 325(4):1264-1270.
39. Hager-Theodorides AL, Outram SV, Shah DK, et al. Bone morphogenetic protein 2/4 signaling regulates early thymocyte differentiation. *J Immunol.* 2002;169(10):5496-5504.
40. Kitisin K, Saha T, Blake T, et al. Tgf-Beta signaling in development. *Sci STKE.* 2007;399:cm1.
41. Blyth K, Vaillant F, Hanlon L, et al. Runx2 and MYC collaborate in lymphoma development by suppressing apoptotic and growth arrest pathways in vivo. *Cancer Res.* 2006;66(4):2195-2201.
42. Lee KB, Jeon JH, Choi I, Kwon OY, Yu K, You KH. Clusterin, a novel modulator of TGF-beta signaling, is involved in Smad2/3 stability. *Biochem Biophys Res Commun.* 2008;366(4):905-909.
43. Knoops L, Renauld JC. IL-9 and its receptor: from signal transduction to tumorigenesis. *Growth Factors.* 2004;22(4):207-215.
44. Marelli-Berg FM, Cannella L, Dazzi F, Mirenda V. The highway code of T cell trafficking. *J Pathol.* 2008;214(2):179-189.
45. Hung CH, Chu YT, Hua YM, et al. Effects of formoterol and salmeterol on the production of Th1- and Th2-related chemokines by monocytes and bronchial epithelial cells. *Eur Respir J.* 2008; 31(6):1313-1321.
46. Pals ST, de Gorter DJ, Spaargaren M. Lymphoma dissemination: the other face of lymphocyte homing. *Blood.* 2007;110(9):3102-3111.
47. Kanellopoulou C, Monticelli S. A role for microRNAs in the development of the immune system and in the pathogenesis of cancer. *Semin Cancer Biol.* 2008;18(2):79-88.
48. Shannan B, Seifert M, Boothman DA, Tilgen W, Reichrath J. Clusterin and DNA repair: a new function in cancer for a key player in apoptosis and cell cycle control. *J Mol Histol.* 2006;37(5): 183-188.
49. Tavazoie SF, Alarcon C, Oskarsson T, et al. Endogenous human microRNAs that suppress breast cancer metastasis. *Nature.* 2008;451(7175):147-152.
50. Calin GA, Pekarsky Y, Croce CM. The role of microRNA and other non-coding RNA in the pathogenesis of chronic lymphocytic leukemia. *Best Pract Res Clin Haematol.* 2007;20(3):425-437.
51. Christoforidou AV, Papadaki HA, Margioris AN, Eliopoulos GD, Tsatsanis C. Expression of the Tpl2/Cot oncogene in human T-cell neoplasias. *Mol Cancer.* 2004;3(1):34.
52. Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev.* 2007;21(5):578-589.
53. Li QJ, Chau J, Ebert PJ, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell.* 2007;129(1):147-161.
54. Ringrose A, Zhou Y, Pang E, et al. Evidence for an oncogenic role of AHI-1 in Sezary syndrome, a leukemic variant of human cutaneous T-cell lymphomas. *Leukemia.* 2006;20(9):1593-1601.
55. Tili E, Michaille JJ, Cimino A, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-α stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol.* 2007;179(8):5082-5089.
56. Vaklavas C, Tefferi A, Butterfield J, et al. "Idiopathic" eosinophilia with an occult T-cell clone: prevalence and clinical course. *Leuk Res.* 2007; 31(5):691-694.
57. Bergua JM, Prieto-Pliego E, Roman-Barbera A, et al. Resolution of left and right ventricular thrombosis secondary to hypereosinophilic syndrome (lymphoproliferative variant) with reduced intensity conditioning allogenic stem cell transplantation. *Ann Hematol.* 2008;87(11):937-938.
58. Ogawa K, Funaba M, Tsujimoto M. A dual role of activin A in regulating immunoglobulin production of B cells. *J Leukoc Biol.* 2008;83(6):1451-1458.
59. Babu G, Waterfield M, Chang M, Wu X, Sun SC. Deregulated activation of oncoprotein kinase Tpl2/Cot in HTLV-I-transformed T cells. *J Biol Chem.* 2006;281(20):14041-14047.