Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells

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To examine the impact of tumors on the immune system, we compared global gene expression profiles of peripheral blood T cells from previously untreated patients with B cell chronic lymphocytic leukemia (CLL) with those from age-matched healthy donors. Although the cells analyzed were not part of the malignant clone, analysis revealed differentially expressed genes, mainly involved in cell differentiation in CD4 cells and defects in cytoskeleton formation, vesicle trafficking, and cytotoxicity in CD8 cells of the CLL patients. In coculture experiments using CLL cells and T cells from healthy allogeneic donors, similar defects developed in both CD4 and CD8 cells. These changes were induced only with direct contact and were not cytokine mediated. Identification of the specific pathways perturbed in the T cells of cancer-bearing patients will allow us to assess steps to repair these defects, which will likely be required to enhance antitumor immunity.

Introduction

Development of cancer is associated with immune suppression in the host, contributing to the failure to mount an effective immune response against the cancer cells (1). The mechanisms whereby specific T cell defects occur are not well understood but include production of immune-suppressive factors by cancer cells, direct tumor cell–T cell interactions, and induction of regulatory T cell subsets. Identification of the specific T cell defects that occur in cancer-bearing patients usually requires isolation of tumor-infiltrating lymphocytes, which limits the number of T cells that can be obtained for study. Tumor cells circulate in leukemia, so there is widespread interaction of cancer cells with T cells that can readily be sampled from peripheral blood. Specifically in B cell chronic lymphocytic leukemia (CLL), a number of well-characterized T cell defects have been described, and it is most likely that immunosuppression induced by the malignant B cells plays an important role in the induction of subsequent immune deficiency in this disease. CLL cells express high levels of immunomodulatory factors including TGF-β and IL-10 that suppress response to antigens, T cell activation, expansion, and effector function (2–5). FasL has been detected on a number of tumors, including CLL, and FasL-positive tumor cells can induce apoptosis in vitro (6, 7). T cells from patients with CLL have low levels of expression of CD80, CD86, and CD154 and are Th2-preponderant (8–11). We have observed functional T cell defects and increased expression of Th2-type chemokine receptors on T cells from patients with CLL compared with T cells of healthy donors (12). To examine the mechanisms of T cell defects in tumor-bearing patients, we analyzed the global gene expression profiles of highly purified CD4 and CD8 cells from peripheral blood from individuals with CLL compared with age-matched healthy donors. Similar defects requiring cell-cell contact were induced by coculture of healthy T cells with CLL cells. Therefore, contact with leukemic cells induces specific changes in both CD4 and CD8 T cells, resulting in functional impairment.

Nonstandard abbreviations used: CLL, chronic lymphocytic leukemia; cRNA, complementary RNA; siRNA, small interfering RNA.

Conflict of interest: The authors have declared that no conflict of interest exists.

Results

Gene expression profiling of CD4 and CD8 T cells from CLL patients and healthy donors. CD4 and CD8 cells were isolated from healthy donors and from previously untreated patients with B cell CLL, who were selected to represent the heterogeneity of this disease (Table 1). Global gene expression profiles were obtained and the microarray data analyzed using both unsupervised and supervised learning. Even though the cells being analyzed were not part of the malignant clone, in an unsupervised analysis, delineation of patients from healthy donors was possible in all cases using hierarchical clustering of CD8 T cells, and in the majority of cases using hierarchical clustering of CD4 T cells (see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI24176DS1).

In supervised analyses, there were no significant differences between gene expression profiles of CD4 or CD8 T cells from patients with CLL and gene expression profiles of CD4 or CD8 T cells from healthy donors, based on cell purity (less than 85% versus 85% or more), time from diagnosis (1–5 years versus 6–10 years), absolute white blood cell count (less than 20 mm^3 versus 20 mm^3 or more), stage of disease (0–I versus II–III), Ig heavy chain mutational status (mutated versus unmutated), or cytogenetic abnormalities (deletion 13q versus others). The majority of the contaminating cells in the T cell population were CD19 B cells.

Molecular defects in CD4 cells in tumor-bearing patients. By supervised analysis of CD4 cells, we identified 22 genes that had significantly increased expression and 68 genes that had significantly decreased expression ($P < 0.05$) in CD4 cells of CLL patients ($n = 22$) compared with healthy donors ($n = 12$) (Figure 1A). The differentially

Table 1
Patients' clinical disease characteristics

Sex		Age	Time from diagnosis	Rai stage of disease				Ig V_H mutation status		Cytogenetics by FISH				
F	M			Stage 0	Stage I	Stage II	Stage III	UM	M	Normal	Deletion 13q	Deletion 11q	Deletion 17p	Trisomy 12	T(5;14)
14	15	40–80 yr	1–10 yr	8	14	5	2	7	22	12	11	1	1	3	1

CD4 and CD8 cells were obtained from peripheral blood of patients with CLL. The patients were untreated and were chosen to represent the heterogeneity of this disease. Healthy donors were age matched. UM, unmutated; M, mutated.

genes is shown in Supplemental Table 1. The majority of the genes were involved in cell differentiation and proliferation, survival, cytoskeleton formation, and vesicle trafficking. For genes selected as representative of the defective pathways, changes in RNA expression were confirmed by real-time PCR and changes in protein expression by Western blot (Figure 2, A and B).

In the CD4 cells of CLL patients, there was decreased expression in a number of genes in the Ras-dependent JNK and p38 MAPK pathways. The JNK–p38 MAPK pathway plays major roles in CD4 T cell differentiation into Th1 or Th2 subsets (13–15). There was decreased gene expression in a number of components of this pathway, including the activator *MINK* (*MAP4K6*) (16); *GDI1* (17, 18), which serves as a negative regulator of small GTP-binding proteins in the Ras-dependent MAPK pathway in induction of NF-κB or actin cytoskeleton remodeling via the Arp2/3 complex; and *NFRKB*, which binds to several of the κB regulatory elements (17, 19, 20) (Figure 1B). There was also decreased expression of *PIK3CB*, a regulator of cell growth in response to various mitogenic stimuli through TCR/CD28, IL-1 receptor, G-protein coupled receptor, and members of the TNF receptor family (20, 21).

Differential expression of genes involved in cytoskeleton formation and vesicle trafficking in CD4 cells from CLL patients included decreases in *AAK1*, which plays a regulatory role in cell migration and clathrin-mediated endocytosis (22), and *AP3M2*, which facilitates budding of vesicles from the Golgi membrane and trafficking to lysosomes (23). There was increased expression, in CD4 cells from CLL patients, of *SPTBN1*; of *ARPC1*, which encodes an actin cytoskeleton–associated protein that plays a role in cell migration/motility or cytokine production/secretory functions by controlling actin polymerization; and of *ADIR* (Figure 1B).

Functionally, these changes would be expected to result in decreased Th1 differentiation, and we and others have previously demonstrated skewing of T cell responses to Th2 rather than Th1 differentiation in patients with CLL (12, 24).

Molecular defects in CD8 T cells in patients with CLL. By supervised analysis, a larger number of genes (n = 273) had deregulated expression in CD8 cells, including 105 genes that were downregulated and 168 genes upregulated in CD8 T cells from patients with CLL (n = 20) compared with healthy donors (n = 12) (P < 0.05) (Figure 3A). The differentially expressed genes were classified by their involvement in specific cellular pathways, and a number of representative genes of those pathways are listed in Supplemental Table 2. On analysis of these genes, the majority were involved in cytoskeleton formation, intracellular transportation, vesicle trafficking, or cellular secretion as well as cytotoxicity pathways in CD8 T cells (Figure 3B).

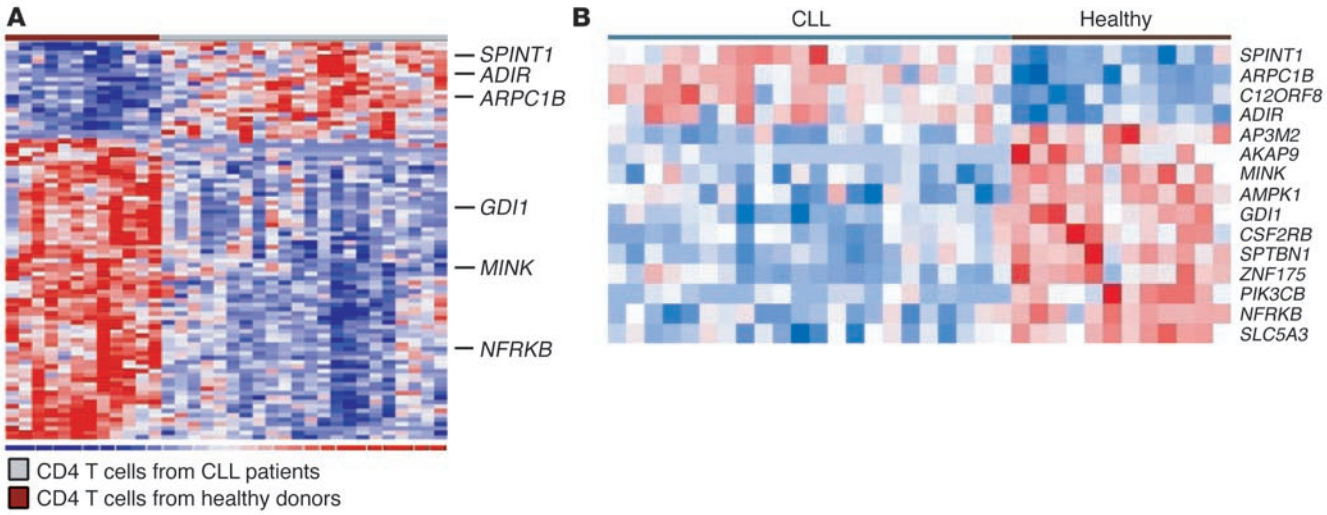

Figure 1
Differentially expressed genes in CD4 cells from patients with CLL compared with healthy donors. Dendrogram of differentially expressed genes by supervised analysis (P < 0.05). (**A**) CD4 cells from patients with CLL compared with healthy donors. Twenty-two genes were significantly

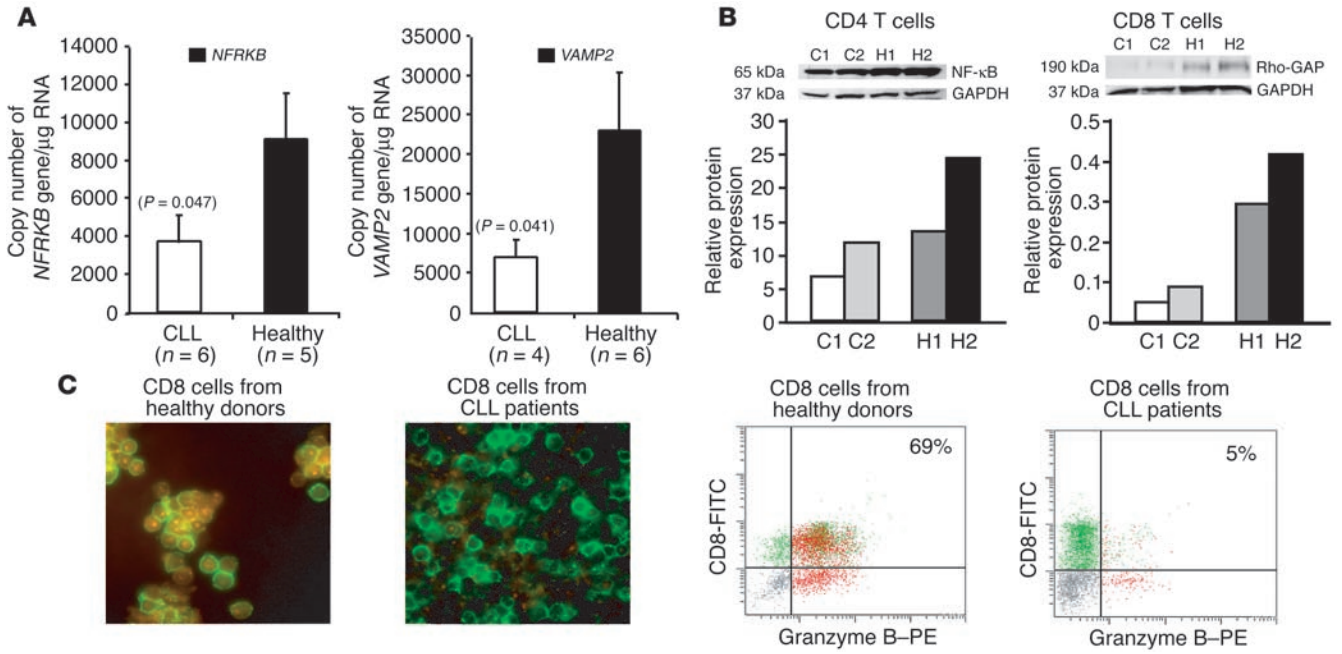

Figure 2
Validation of gene expression observed by microarray. (**A**) Concordant with data seen on microarray, by quantitative PCR there was decreased expression of *NFRKB* in CD4 cells and *VAMP2* in CD8 cells from CLL patients compared with healthy donors. The figure represents data from CD4 cells from 6 CLL patients and 5 healthy donors and CD8 cells from 4 CLL patients and 6 healthy donors. Statistical significance was assessed in a 2-tailed Student's *t* test. (**B**) Decreased expression of NF-κBp65 in CD4 cells and Rho-GAP p190 proteins in CD8 cells from CLL patients compared with healthy donors. The left 2 lanes represent protein expression in CD4 or CD8 cells from 2 CLL patients (C1 and C2), and the right 2 lanes represent 2 healthy donors (H1 and H2). The expression of proteins was normalized by GAPDH expression level and is shown as protein bands and densitometric intensity of each band. The figure is representative of 3 additional experiments performed on 6 different donors, all showing a similar pattern ($P < 0.05$). (**C**) Intracytoplasmic expression of the *GP1* gene product granzyme B, detected in CD8 cells from CLL patients and healthy donors by flow cytometry and fluorescent microscopy. To obtain at least 99% CD8 cell population, cells were purified using magnetically labeled negative cell-depletion antibodies. High expression of granzyme B in CD8 cells from healthy donors (CD8-FITC$^+$ granzyme B–PE$^+$, orange-brown) was observed compared with that in CD8 cells from CLL patients (CD8-FITC$^+$ granzyme B–PE$^-$, green). The figure is representative of experiments performed with 4 different patients and healthy donors ($P < 0.05$).

Impaired cytoskeleton formation, intracellular transportation, and cytotoxicity in CD8 T cells from CLL patients. We observed decreased expression of *ARAP3*, a Rho repressor gene that induces PI3K-dependent rearrangements in the cell cytoskeleton (25); *myosin IXB*, a GTPase-activating protein for the G protein Rho (26); *AP3M2*; *VAMP2*; *GPR57*; and *AKAP9*. There was increased expression of *CDC42*, *PIK4CB*, *RAB35*, *FLNA*, and *FMNL*, which associate with both Rac and profilin and regulate reorganization of the actin cytoskeleton in association with Rac (27, 28). Actin polymerization at the immune synapse is required for T cell activation and effector function, and T cell binding to APCs induces localized activation of CDC42 and WASP at the immune synapse (29, 30). There was increased expression of *ARPC1B*, required for the formation and stabilization of the immunological synapse at the interface between APCs and T lymphocytes (27–29). We also observed increased expression in *SPEC1*, which encodes a GTPase inhibitor protein that regulates *CDC42* function, and *NCK2*, which encodes an src homology domain–containing (SH2 and SH3 domain–containing) adaptor protein that couples receptor tyrosine phosphorylation to downstream effector molecules in cytoskeleton formation processes (31).

included *VAMP2*; *SCAMP1*, which encodes a carrier to the cell surface in post-Golgi recycling pathways during vesicular transport; *XAB2*, a Ras superfamily member involved in controlling a diverse set of essential cellular functions; and *GPR57*, a GTP-binding protein that activates JNK-, MAPK-, and p38-dependent pathways in the cytotoxic immune response (32). We observed increased expression in inhibitor genes including the Rab family members *RAB35*, *RAB22A*, the ral guanine nucleotide dissociation stimulator *RALGDS* that inhibits binding of Raf to Ras, and *RASGRP2*, an inhibitor of guanine nucleotide exchange factor. Also increased was *AP2B1*, an adaptin family member essential for the formation of adaptor complexes of clathrin-coded vesicles (31, 33). Adaptins interact with the cytoplasmic domains of membrane-spanning receptors in the course of their endocytic/exocytic transport. Likely as a consequence of these changes in structural proteins, we observed a decrease in cytotoxicity in CD8 cells of CLL patients compared with healthy donors (data not shown) and a decrease in granzyme B protein in CD8 T cells of CLL patients compared with healthy donors (Figure 2C). Of note, there was no decrease in granzyme B mRNA expression in the CD8 T cells in CLL patients, and we conclude that

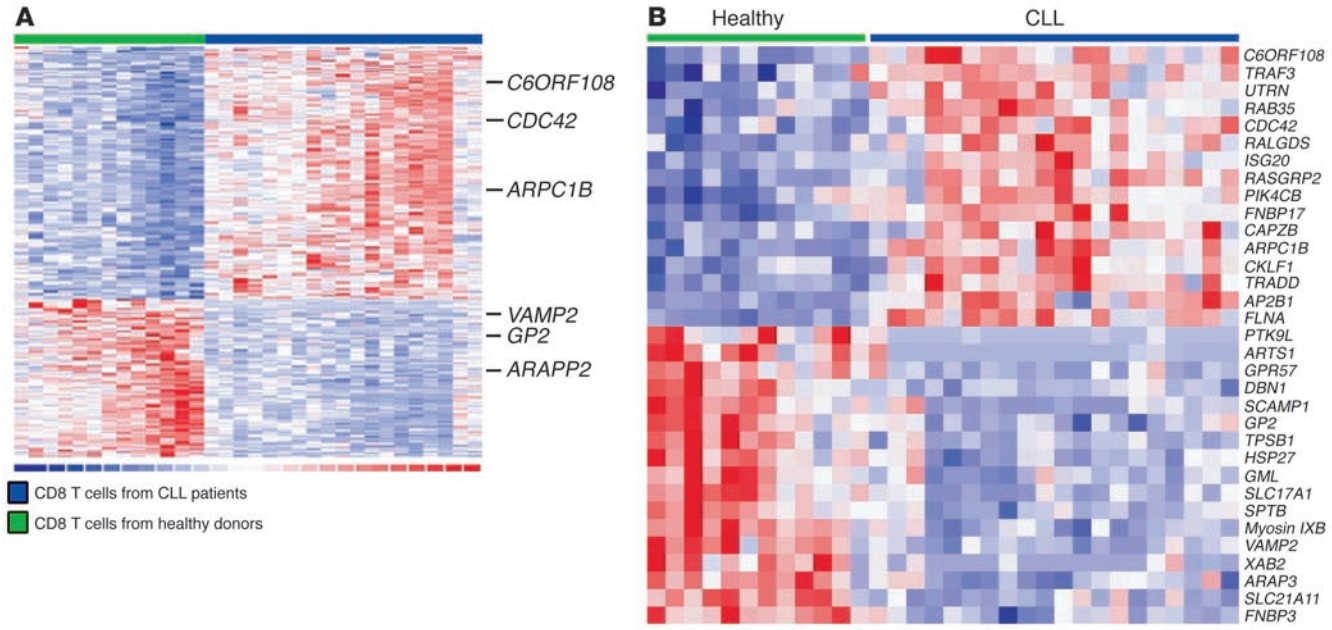

Figure 3
Differentially expressed genes in CD8 cells from patients with CLL. Dendrogram of differentially expressed genes by supervised analysis in CD8 cells from patients with CLL compared with healthy donors ($P < 0.05$). (**A**) One hundred sixty-eight genes were significantly increased (red) and 105 genes were significantly decreased (blue) in CD8 cells from CLL patients. (**B**) Dendrogram of differentially expressed genes involved in cytoskeleton formation, vesicle trafficking, and cytotoxicity pathways in CD8 cells. The dendrogram represents selected genes from **A**.

These changes would be expected to result in decreased cytotoxicity and effector function. We and others have previously demonstrated that such defects occur in the CD8 T cells in patients with CLL (12, 34, 35).

We therefore identified specific pathways with altered expression in CD4 and CD8 cells of CLL patients. From this we developed a representative protein expression panel using Western blot analysis and used this proteomic approach to assess whether CLL cells could induce similar changes in healthy allogeneic T cells and to elucidate the mechanism(s) whereby CLL cells could induce changes in these pathways, using cocultures of healthy T cells with CLL cells.

The CLL B cell–derived soluble factors induce alterations in chemokine and chemokine receptor expression but not cytoskeletal proteins in healthy T cells. CLL cells express cytokines known to inhibit T cell responses, including IL-10. We therefore hypothesized that release of these inhibitory cytokines would induce the changes in gene expression observed in healthy CD4 and CD8 cells. However, following culture of healthy CD4 or CD8 cells with sera from CLL patients or coculture of CLL cells or healthy B cells with healthy CD4 or CD8 cells in transwell culture plates, we did not observe changes in expression of cytoskeleton proteins or other genes that we have shown to be decreased in CD4 or CD8 cells in CLL patients (data not shown). The only defects shown to be induced by culture of healthy T cells with these soluble factors were altered expression of chemokines and chemokine receptors, including decreases in CXCR1, CXCR2, and CXCR4 and increases in CXCR3, CCR4, and CCR5 in CD4 T cells from healthy donors (Supplemental Figure 2). When IL-10 mRNA expression was inhibited by transient transfection of small interfering RNA (siRNA) targeting IL-10 (Supplemental Figure 3) [illegible] level of cytoskeletal proteins, but this blocked the changes in chemokine and chemokine receptor expression, suggesting that these alterations were indeed induced by IL-10 and not by other soluble factors (Supplemental Figure 2).

CLL B cells induce alteration in cytoskeleton formation and vesicle transportation pathways in T cells by cell-cell contact. Since soluble factors did not induce changes in healthy T cells, we cocultured CLL cells in direct contact with T cells from healthy donors and analyzed expression of proteins representative of the pathways found to be abnormal in the cancer-bearing patients. By 48 hours of culture of healthy donor CD4 T cells with tumor cells, we observed changes in protein expression patterns consistent with that seen in the CD4 cells of the CLL patients. Such changes included increased expression of Arp3 and decreased expression of NF-κBp65 and GDI1 (Figure 4A). Similarly, in CD8 cells, we observed changes in the expression pattern consistent with that observed by gene expression profiling, including decreased Rho-GAP and increased Arp3 and CDC42 protein (Figure 4B). Induction of these changes required cell-cell contact, and these changes were not observed after blockade of adhesion molecules using anti-CD54 and anti-CD11a mAbs (Figure 4C). These changes were not induced by coculture of allogeneic T cells with healthy B cells from the donors who were HLA matched to the CLL patients.

Discussion

Microarray-based expression profiling has been used most commonly to compare and contrast heterogeneous groups of human tumors to identify expression patterns associated with prognosis and to examine altered expression in tumor cells compared with [illegible]

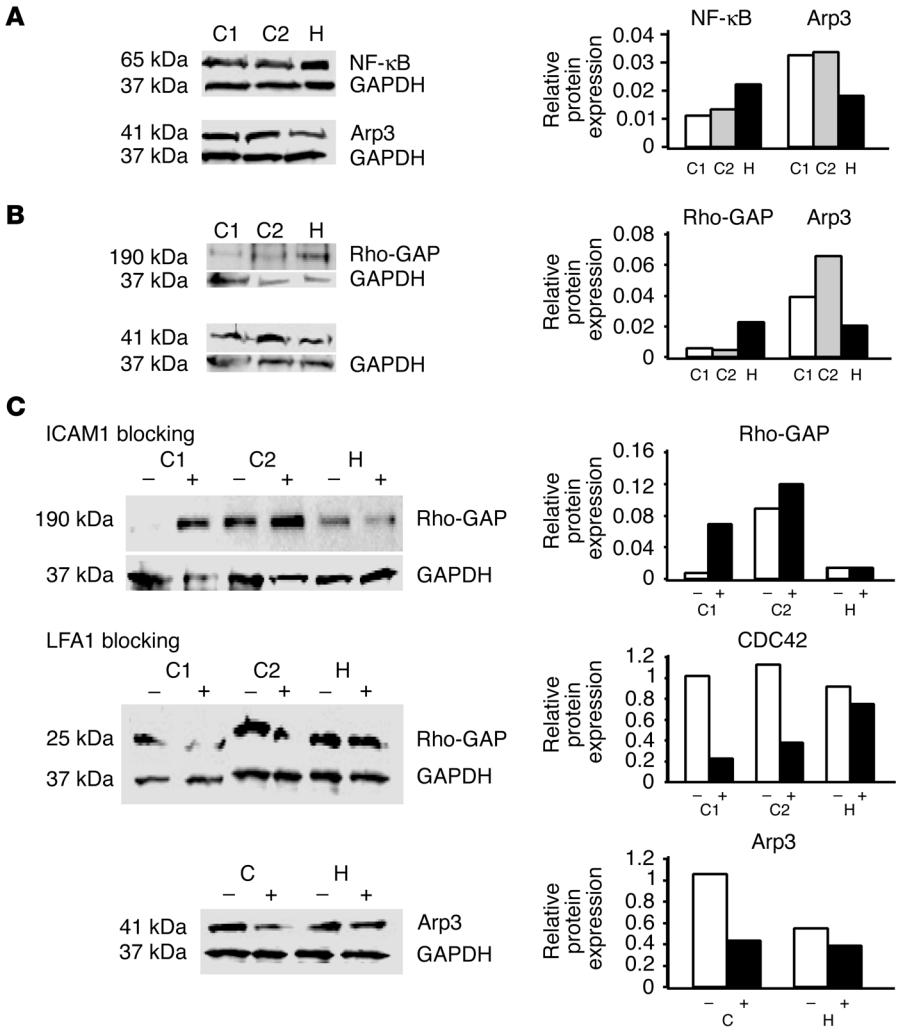

Figure 4
Impact of T cell–cancer cell contact on healthy T cells. Highly purified T cells from healthy donors were cocultured with B cells from CLL patients or from their HLA-matched healthy donors at a 2:1 (T/B cell) ratio for 48 hours. (**A**) Decrease in 65-kDa NF-κB and increase in 41-kDa Arp3 in healthy CD4 cells after contact with allogeneic CLL cells (C1 and C2) and healthy B cells (H). (**B**) Decrease in 190-kDa Rho-GAP and increase in 41-kDa Arp3 in healthy CD8 cells after contact with CLL cells (C1 and C2) and healthy allogeneic B cells (H). (**C**) Impact of CLL cell contact on cytoskeletal protein expression in allogeneic healthy T cells, confirmed by ICAM1 or LFA1 blocking. Highly purified CD4 or CD8 cells from healthy donors were cocultured with B cells from CLL patients (C1 and C2) or healthy donors (H) with or without blockade of the LFA1 or ICAM1 interaction. Expression of 190-kDa Rho-GAP in healthy CD8 cells was increased after blockade of the ICAM1 during CLL cell–T cell contact, and expression of 41-kDa Arp3 in CD4 T cells and 25-kDa CDC42 in healthy CD8 cells was decreased after blockade of the LFA1 or ICAM1. –, protein expression in nonblocked cells; +, protein expression in blocked cells. Protein expressions were normalized by GAPDH expression level and are shown as protein bands and densitometric intensity of each band. The figure is representative of 3 different experiments performed with 6 different patients with CLL and 6 different healthy donors showing a similar pattern ($P < 0.05$).

patients and demonstrate profound changes in gene expression of T cells in patients with CLL compared with healthy donors. Importantly, we demonstrate that these changes can be induced at the protein level in healthy T cells following short-term culture with direct contact with CLL cells.

Analysis of the differentially expressed genes in the T cells in CLL patients demonstrates a number of abnormalities in specific pathways. In CD4 cells, among the most marked changes observed were in the Ras-dependent JNK and p38 MAPK pathways (Figure 5). JNK and p38 MAPK pathways play a major role in regulating CD4 T cell differentiation into Th1 or Th2. JNK2 and p38 MAPKs mediate IFN-γ production and Th1 cell differentiation, and inhibition of p38 MAPK in dnp38 transgenic mice results in decreased IFN-γ production by Th1 cells (15, 36, 37). *ADIR* encodes a protein involved in protein processing in the endoplasmic reticulum and contains a putative IFN-responsive ATP-binding site involved in regulating expression of genes critical for antigen presentation and immune surveillance against viruses and tumor cells (38). Our data, demonstrating decreased expression in the p38 MAPK pathway activator genes such as *MINK*, *NFRKB*, and *PIK3CB*, are in keeping with our hypothesis

In CD8 cells, our findings are in keeping with the hypothesis that cell contact with CLL cells induces changes in gene expression in genes regulating cytoskeleton formation and vesicle trafficking (Figure 5), thereby resulting in the decreased cytotoxicity and effector function noted in this disease. The cytoskeleton is a cellular network of structural, adaptor, and signaling molecules that regulates most cellular functions during immune responses, including migration, extravasation, antigen recognition, activation, and phagocytosis. CD8 cytotoxic T lymphocytes mediate killing of cancer cells through polarized delivery of vesicles referred to as lytic lysosomes that contain apoptosis-inducing proteins including perforin and granzymes (39–41). Positioning of the secretory cleft and secretory lysosome polarization targeting cancer cells depend on cytoskeletal connections that regulate granule transport to the plasma membrane (40). The altered expression in regulator genes, including increased *RAB11B* and *RAB22A* and decreased *RAB35*, *VAMP2*, *SLC21A11*, and *SCAMP1*, indicated defects in vesicle formation and intracellular trafficking in CD8 cells in CLL patients. We observed decreased expression of *GP2* (41), and *TPSB1*, a gene encoding a tetrameric serine protease, concentrated and stored selectively in secretory granules (40, 42). In CD8 also we observed defects in the

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