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Chronic Lymphocytic Leukemia Cells Are Activated and Proliferate in Response to Specific T Helper Cells

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SUMMARY

There is increasing interest in the chronic lymphocytic leukemia (CLL) microenvironment and the mechanisms that may promote CLL cell survival and proliferation. A role for T helper (Th) cells has been suggested, but current evidence is only circumstantial. Here we show that CLL patients had memory Th cells that were specific for endogenous CLL antigens. These Th cells activated autologous CLL cell proliferation in vitro and in human → mouse xenograft experiments. Moreover, CLL cells were efficient antigen-presenting cells that could endocytose and process complex proteins through antigen uptake pathways, including the B cell receptor. Activation of CLL cells by Th cells was contact and CD40L dependent. The results suggest that CLL is driven by ongoing immune responses related to Th cell–CLL cell interaction. We propose that Th cells support malignant B cells and that they could be targeted in the treatment of CLL.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a lymphoid malignancy characterized by the accumulation of mature clonal $CD5^+$ B cells derived from proliferation within localized pseudofollicles in the bone marrow and secondary lymphoid organs (Burger et al., 2009). Studies show that the stromal microenvironment can play a role in supporting CLL cell survival via antiapoptotic effects. CLL cells are thought to interact with the endothelia, stroma, and monocyte-derived nurse-like cells in a process involving cytokines as well as chemokines (Burger et al., 2009). However, it remains to be demonstrated which specific cellular and molecular mechanisms support the proliferation of CLL cells.

The B cell receptor (BCR) of B cells delivers a low-level, tonic signal in the absence of antigen. CLL cells may express polyreactive/crossreactive/autoreactive BCR that can support a level of BCR signaling above this tonic signaling (Stevenson et al., 2011; Dühren-von Minden et al., 2012). Moreover, a subgroup of patients with mutated immunoglobulin H (IgH) chain (of the VH7-3 family) have CLL cells with specificity for β-(1,6)-glucan cell wall constituents of commensal yeast and filamentous fungi (Hoogeboom et al., 2013). CLL cells have an aberrant phenotype that includes IgM downregulation, reduced responsiveness to BCR ligands, reduced expression levels of CD21, above-baseline intracellular Ca^{2+}, and activation of BCR pathway kinases, as well as negative feedback regulation, including SHP-1 activation (Caligaris-Cappio, 1996; Dühren-von Minden et al., 2012; Tibaldi et al., 2011). In fact, CLL cells have been likened to anergic autoreactive B cells (Caligaris-Cappio, 1996) that are somehow aberrantly rescued by the tumor microenvironment (Muzio et al., 2008; Stevenson et al., 2011).

The anergic B cell subset shares these BCR-dependent features, including high intracellular Ca^{2+} as well as ERK-phosphorylation and feedback regulation (Goodnow et al., 1988; Yarkoni et al., 2010). Importantly, it was shown that anergic B cells could be rescued from apoptosis by T helper (Th) cells, allowing normal Th cell–B cell collaboration and autoantibody production (Fulcher et al., 1996). These results raise the question as to whether Th cells could play a similar role in CLL.

T cells, mostly of the $CD4^+$ Th cell type but also of the $CD8^+$ cytotoxic T lymphocyte (CTL) type, are known to infiltrate pseudofollicles of CLL (Pizzolo et al., 1983). We previously showed in a mouse model that Th cells specific for a B cell lymphoma marker, BCR V regions (idiotype [Id]), can chronically activate B cells, resulting in the development and maintenance of B cell lymphomas (Zangani et al., 2007). These findings suggested that Th cells might play a supportive role in B cell malignancies in humans.

Here, we hypothesize that proliferation of CLL cells in patients may be driven by cognate interactions with Th cells, during which CLL cells present antigen to antigen-specific Th cells. Such an antigen could be derived from an extracellular source, or it could be produced by the CLL cell itself (endogenous Ag).

To examine this hypothesis, we must resolve three central questions: (1) Are CLL cells in the resting state able to endocytose, process, and present antigen efficiently to Th cells? (2) If so, can stimulated Th cells reciprocate and support the activation and proliferation of CLL cells? (3) If the answer to the first two questions is yes, what antigen is involved in such a cognate interaction?

Before turning to Th cells from CLL patients, we first investigated the antigen-presenting cell (APC) function of resting CLL cells by targeting antigen to various antigen uptake pathways. Utilizing an antigen-specific Th cell clone (not related to CLL), we demonstrated that resting CLL cells were able to efficiently endocytose, process, and present complex protein antigens on major histocompatibility complex (MHC) class II. This model system allowed quantitative and well-controlled analysis of cognate Th cell–CLL cell interactions. Reciprocally, the CLL cells were receptive to help from this antigen-specific Th cell clone. In the second part of the study, we extended these experiments to Th cells from individual CLL patients. We generated Th cell lines for each patient by stimulating Th cells with the patients' own CLL cells. We found that such patient Th cells had specificity for endogenous CLL cell antigen, including epitopes within the BCR. These autologous Th cells supported CLL cell activation and proliferation in vitro as well as in vivo in the bone marrow niche of Nod Scid gamma (NSG) mice.

RESULTS

CLL Cells Can Endocytose Complex Antigen and Are Efficient APCs for Specific CD4$^+$ Th Cells

If Th cell–CLL cell interactions play a role in expanding CLL cells in patients, resting CLL cells must have the ability to efficiently activate Th cells. Before investigating CLL cell interactions with autologous Th cells (see below), we first studied the antigen-presentation capacity of CLL cells by utilizing a human Th cell clone that is specific for a nonamer peptide derived from the constant region of mouse kappa (Ms Cκ) light chains presented on histocompatibility leukocyte antigen (HLA)-DRB1*0401 (Schjetne et al., 2002). This Th cell clone is not related to CLL, but it allowed functional studies of the antigen presentation of CLL cells and antigen-dependent cognate interaction with Th cells. In this model system, DR0401$^+$ CLL cells (8/28 patients; Table S1) endocytosed antigen (Ms Cκ$^+$IgG) via Ms Cκ$^+$IgG targeted to cell-surface receptors in the endocytic pathway (see Figure 1A for the experimental strategy). As for relevant targets, we found that CLL cells expressed endocytic receptors such as Fc receptors (CD32 and CD23) and BCRs, although the number of BCRs present was less than 10% of that found on normal B cells (Figures 1 and S1).

With a 90% reduction in BCR on CLL cells (Figure 1B), the utility of the BCR for internalization of antigen may be questioned. In fact, delivery of antigen to the BCR of CLL cells resulted in a surprisingly potent stimulation of Th cells: with one exception (CLL110, of the eight CLL tested), APC function was comparable to that of normal B cells (Figure 1B). CLL cells were also efficient in uptake, processing, and presentation when Ms Cκ$^+$IgG antigen was targeted to conventional antigen uptake pathways (CD32 and CD23), but not to markers absent on the CLL cells, such as CD16 or inappropriate light chains (Figures 1C and S1). CLL cells lacking the DR0401 restriction element (CLL105, CLL112, CLL113, CLL114, CLL117, CLL124, and CLL109) were incapable of stimulating Th cells (Figures 1C and S1). Pretreatment of CLL cells with demethylated CpG DNA, which increases the surface levels of costimulatory and HLA class II molecules on CLL cells (Decker et al., 2000), did not significantly improve APC function (Figure S1). This demonstrated a pre-existing APC efficiency of resting CLL cells. The APC function of purified CLL cells was equivalent to that of unpurified CLL cells from peripheral blood mononuclear cells (PBMCs), as CLL cells accounted for >98% of the MHC class II$^+$ cells in the PBMCs (Figure S1). The Th cell stimulation obtained with targeted antigen was similar or superior to that induced by the Th cell mitogen phytohemagglutinin (data not shown). In summary, CLL cells were found to have an intact ability to endocytose, process, and present antigen to CD4$^+$ Th cells, comparable to that of normal B cells.

Antigen-Specific Th Cells Induce CLL Cell Activation

Because the CLL cells were efficient antigen-dependent activators of Th cells, we turned our attention to the reciprocal activation and analyzed whether CLL cells could be stimulated by antigen-activated Th cells (see Figure 2A for the experimental strategy). DR0401$^+$ CLL cells responded significantly in a Th cell- and antigen-dependent manner by becoming activated blasts, upregulating activation markers (CD69 and CD38), HLA-DR and costimulatory receptors (CD86), adhesion molecules (CD54), receptors for Th cell help (CD40), and receptors for Th cell cytokines (CD25), Figures 2B–2D and S2. CLL cells did not lose HLA-DR expression and resembled plasma blasts (Figure 2D). CD27 and CD275 (ICOS-ligand) levels were reduced, consistent with activation-induced shedding. Targeting antigen to the BCR yielded CLL cell responses similar to those obtained with delivery via CD32 (Figures 2D and S2). CLL cells lacking DR0401 did not activate T18 in the presence of antigen and therefore received no help from T18 cells. Thus, CLL cells from four DR0401-negative patients did not show induction of the activation markers shown in Figures 2D and S2.

Antigen-Driven Th Cell–CLL Collaboration Can Support Secretion of CLL-Derived Monoclonal Ig

Some CLL patients have low levels of CLL-derived monoclonal Ig in the serum. CLL cells that collaborated with Th cells in an antigen- and Th cell-dependent manner increased both surface and intracellular levels of Ig (Figure 2E). Moreover, T cell-activated CLL cells secreted IgM, detected as IgM with the appropriate L chain (either κ or λ). We found very little IgM with the inappropriate L chain, demonstrating a negligible contribution of normal B cells (Figure 2F). The levels of secretion varied markedly between the CLL cells (20- to 40-fold), indicating an intrinsic variation in the ability of CLL cells to secrete Ig.

Th Cells Induce CLL Cell Proliferation in an Antigen- and Contact-Dependent Manner

In addition to the activation described above, CLL cells proliferated when they collaborated with Th cells in an antigen- and cell-contact-dependent manner, a response that could be inhibited

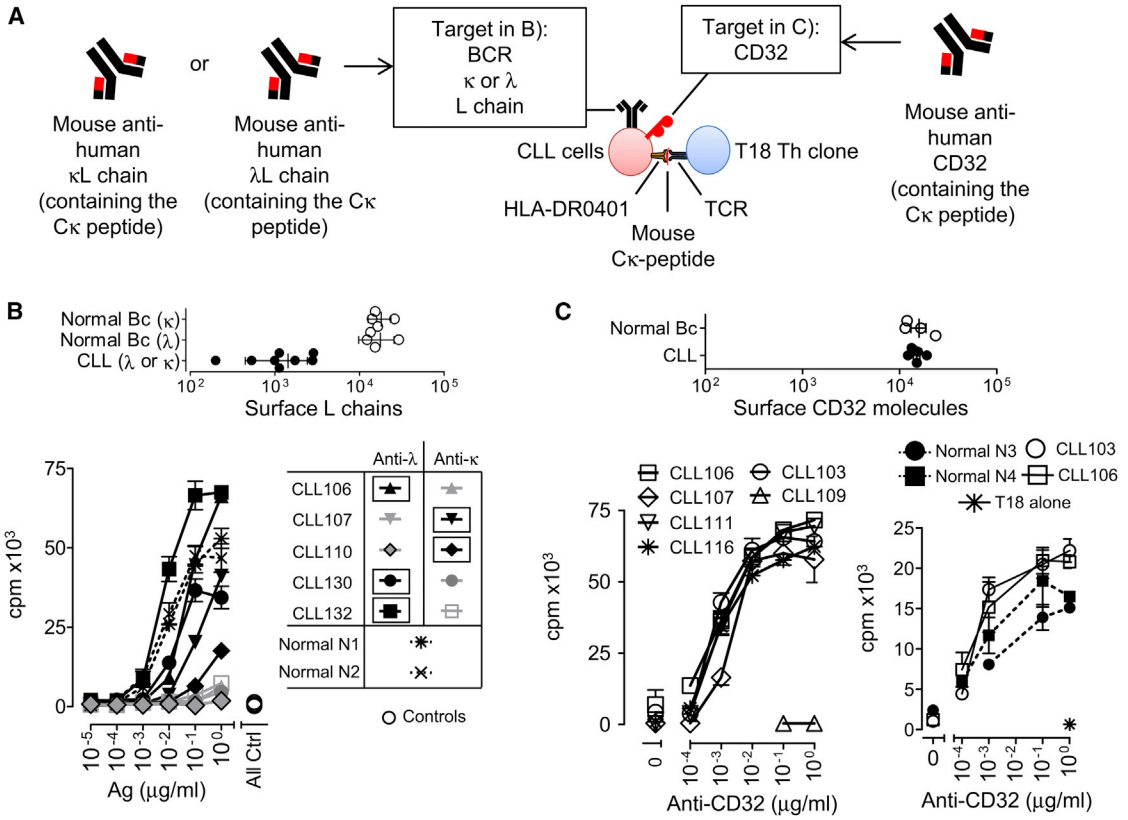

Figure 1. Analysis of the Efficiency of CLL Cells to Endocytose, Process, and Present Antigen to Th Cells

(A) CLL cells were tested for their ability to internalize, process, and present complex protein antigen in the form of mouse anti-human mAbs. Mouse antibodies expressing κ L chains all harbor a peptide determinant in the Cκ of the L chain. After processing, this Cκ peptide can be presented on HLA DRβ*0401 and recognized by the Cκ-peptide/DR0401-specific T18 Th cell clone. Left: Testing mouse anti-human κ (Ms κ^+IgG anti-Hu) versus mouse anti-human λ (Ms κ^+IgG anti-Hu λ), corresponding to experiments in B). Right: Testing mouse anti-human CD32 (Ms κ^+IgG anti-Hu CD32), corresponding to experiments in C).

(B and C) Proliferation of MsCκ/DR0401-specific T18 cells (^{3}HTdR incorporation) in response to presentation of Ms κ^+Ig by DR0401$^+$-CLL cells.

(B) Top: numbers of BCRs (detected by L-chain stain) on normal B cells compared with CLL cells. Bottom: CLL cells present antigen endocytosed via BCRs (Ms κ^+IgG anti-Hu κ or Ms κ^+IgG anti-Hu λ) with efficiency comparable to that of normal B cells. The L chain isotype (κ or λ) expressed on patient CLL cells is indicated by the black symbol (boxed symbols in legend table); nonexpressed L chain isotypes are gray. Controls (CLL alone, Th cells alone, Th cells alone with antigens, and CLL cells with Th cells but no antigen) are indicated by overlapping open circles.

(C) Top: numbers of CD32 molecules on normal B cells versus CLL cells. Bottom left: Th cell proliferation in response to antigen (Ms κ^+IgG anti-Hu CD32) targeted to CD32 on DR0401$^+$ CLL cells compared with control (CLL109, DR0401$^-$). Bottom right: Th cell proliferation in response to antigen (Ms κ^+IgG anti-Hu CD32) delivered via CD32 on DR0401$^+$ CLL cells (open symbols) and normal DR0401$^+$ B cells (filled symbols). Error bars: SD.

See also Figure S1 and Table S1.

by anti-CD40L (Figures 3 and S3). Blastoid CLL cells underwent DNA replication (bromodeoxyuridine [BrdU] incorporation) in response to Th cells and antigen (Figure 3A). However, in about half of the patients, in spite of activation and blastogenesis, the proliferation was minor. In these patients, CLL cells only proliferated in a Th cell- and antigen-dependent manner when they were provided with exogenous interleukin-2 (IL-2), TLR9 ligands (CpG2006), or both (Figure S3). Such in vitro cofactor-dependent CLL cells nevertheless proliferated in response to help in vivo (see below).

Polyclonal Patient Th Cells Can Induce the Activation and Proliferation of CLL Cells

The CLL responses described above were obtained with a single Th cell clone. To test whether such CLL cell responses were a general feature of interactions with activated Th cells, we generated alloreactive T cell lines from patients and healthy volunteers. Both patient and control polyclonal alloreactive T cells supported activation and proliferation of CLL cells in a manner similar to that described above (Figure S4). In these allo-driven T cell–CLL cell collaboration assays, we also included CLL cells from six DR0401$^-$ patients in the analysis, and found that these CLL cells were activated similarly to CLL cells from the eight DR0401$^+$ patients tested as described above. These experiments demonstrated that Th cells from patients were not functionally impaired and could support antigen-dependent CLL activation.

Autologous CLL-Specific Th Cell Lines Can Support CLL Cell Activation

The above results suggested that immune responses involving CLL cells, Th cells, and either exogenous antigens or

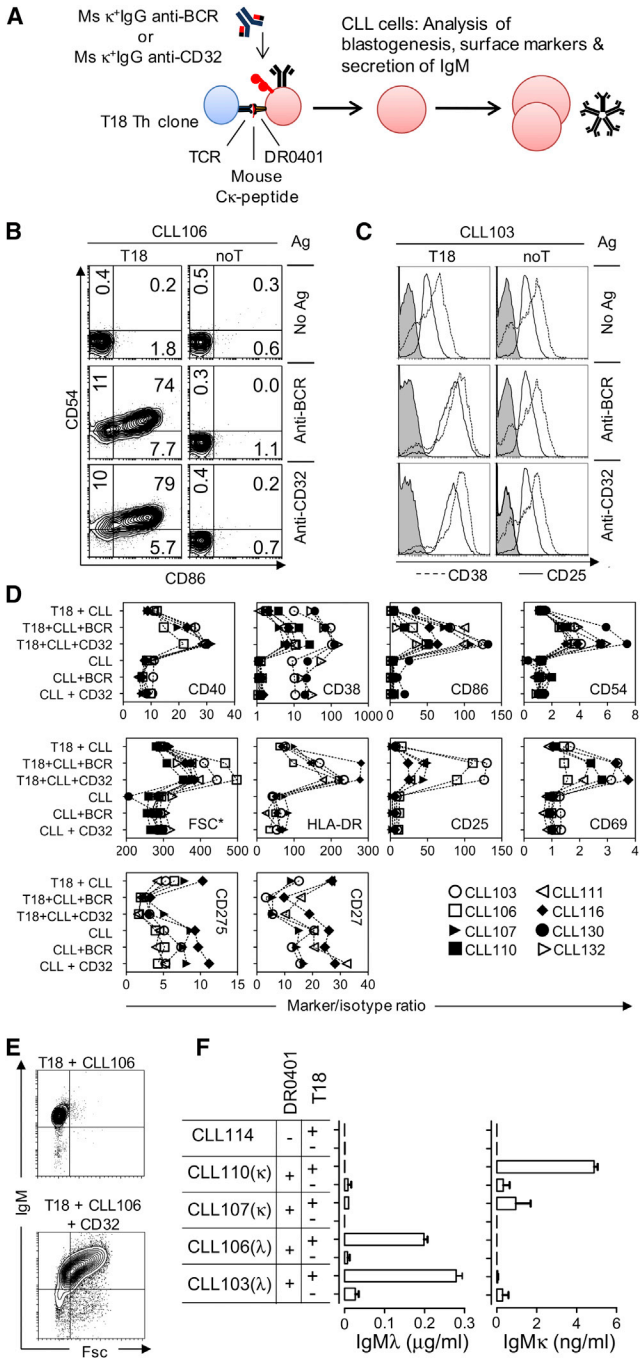

Figure 2. Analysis of CLL Cell Activation and Antigen-Driven CLL Cell–Th Cell Collaboration

(A–F) Analysis of the outcome of the antigen-dependent Th cell activation of CLL cells. Assays as shown in Figure 1 were performed on CLL cells with the appropriate HLA-DR0401. CLL cells were analyzed for expression of surface markers (B–D) or cell size and production of monoclonal IgM (E and F).

(B–F) PBMCs from patients were incubated in the presence or absence of T18 and antigen (Ms κ+IgG anti-HuCD32 or Ms κ+IgG anti-BCR [a mixture of both Ms κ+IgG anti-Hu κ and Ms κ+IgG anti-λ]) for 96 hr.

(B) Contour plot of DR0401+ CLL106 CLL cells (CD5+19+ gate) showing CD86 versus CD54 surface expression in the presence or absence of T18 and antigen.

endogenous CLL antigens could support CLL cell growth in pseudofollicles. We therefore turned our attention to the Th cells in patients (see Figure 4A for the experimental strategy). In fact, proliferating Th cells could be observed directly in unfractionated PBMCs, but not in the absence of CLL cells (Figures 4B and 4C). These Th cells were derived from CD45RO+ (memory) Th cells (Figure 4C). The results demonstrated that patients had a subset of Th cells that were stimulated directly by the patients' own CLL cells. Th cells were restimulated every 10 days by autologous CLL cells to generate CLL-specific Th cell lines (Figures 4A and 4D). After three 10-day stimulation cycles, the CLL-specific Th cell lines proliferated in response to either autologous CLL cells or autologous CLL cell lysate presented by APCs from HLA class II matched donors (Figures 4A, 4E, and 4F). Moreover, such CLL-specific Th cells stimulated HLA class II-restricted CLL cell activation and proliferation in a manner equivalent to that described above, including dependency upon the CD40/CD154 pathway (Figure 4G). Further, the CLL-specific Th cells stimulated the activation of CLL cells at a level comparable to that seen with the T18 Th cell clone or allo-specific Th cells. CLL cells upregulated the activation markers BCR, CD54, CD25, CD40, CD38, and HLA-DR, but not CD138 (Figures 4H, 4I, S3, and S4). Finally, we tested Th cells for responses toward epitopes within the CLL BCR. HLA-matched donor cells presenting monoclonal antibody (mAb) derived from CLL hybridomas activated autologous Th cells, demonstrating a significantly increased Th cell specificity for this endogenous CLL antigen (Figure 4J). We therefore conclude that effector Th cells specific for endogenous CLL antigens can exist in CLL patients and that they can support CLL cell growth and differentiation.

Autologous CLL-Specific Th Cell Lines Can Support CLL Cell Activation and Proliferation In Vivo

It was previously described that autologous Th cells can support CLL cells in NSG mice, presumably through activation of expanding xenoreactive Th cells with accompanying bystander activation of CLL cells. In such mice, CLL cells do not expand without Th cells (Bagnara et al., 2011). We conditioned mice with busulfan (see Experimental Procedures) before injecting (1) patient PBMCs or (2) PBMCs and CLL-specific Th cells (Figure 5A). In the latter group, the proliferation of both CLL cells and Th cells was dramatically and significantly increased in the bone

(C) Gated CD5+19+ CLL103 cells (DR0401+) in histograms showing surface expression of CD25 and CD38 versus isotypic control (gray).

(D) Surface markers on CLL cells of DR0401+ patients stimulated as listed on the y axis. +BCR or +CD32 indicates that Ms κ+IgG antigen was delivered to the BCR or CD32. Staining intensity relative to background is shown on the x axis (see Experimental Procedures). Note that in one graph, the mean forward scatter (FSC*) is plotted. See also Figure S2 for examples of the flow-cytometry plots used for data generation.

(E) IgM staining of permeabilized cells (extracellular + intracellular IgM expression) versus FSC in DR0401+ CLL106 cells cocultured with T18 in the presence or absence of antigen (Ms κ+anti-HuCD32).

(F) Secretion of IgM by activated CLL cells. Purified CD5+ CLL cells were preincubated with antigen (see Experimental Procedures) and mixed with T18 cells as indicated. Secreted IgMλ and IgMκ were measured by ELISA. The L chain expression (κ/λ) of the various CLL cells is indicated in parentheses. n = 4; error bars: SD is shown.

See also Table S1.

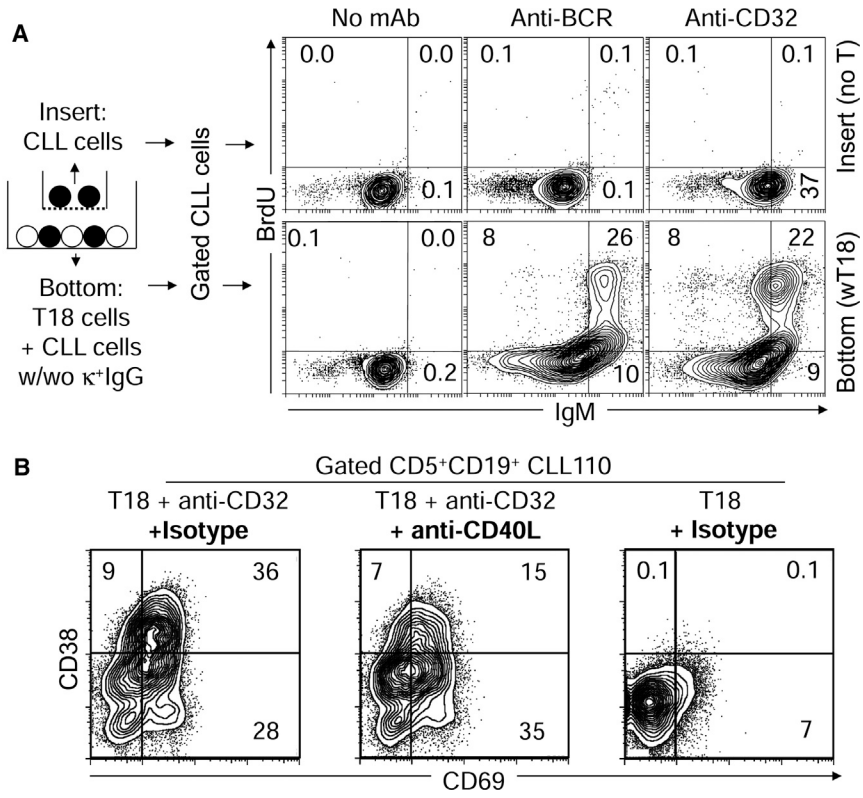

Figure 3. Analysis of Reciprocal Th Cell Stimulation of CLL Cells

(A and B) Activation of CLL cells in response to collaboration with antigen-specific T18 cells.

(A) IgM (intra- and extracellular expression) versus BrdU staining on $CD5^+CD19^+$ gated CLL106 cells from Transwells (see Experimental Procedures) as indicated in the presence or absence of antigen (Ms κ^+IgG anti-Hu λ or Ms κ^+IgG anti-CD32).

(B) Ms κ^+IgG anti-CD32 Ag/Th-cell-dependent CLL cell activation in the presence of anti-CD40L or isotype. Expression of CD38 versus CD69 is shown.

See also Figure S3 and Table S1.

marrow, demonstrating that autologous pairs of CLL cells and CLL-specific Th cells could support the proliferation of each other in this niche (Figures 5B and 5C). It is notable that CLL cells from the patient shown in Figure 5 belonged to the group of patients whose Th cells supported the activation and blastogenesis of CLL cells, but CLL cells required cofactors for marked proliferation in vitro (Figure S3). Because cofactors were not provided in these in vivo experiments, the results suggest that stromal factors help support the T cell–CLL cell expansion in vivo.

Autologous CLL-Specific Th Cell Lines Have a Th1-Like Phenotype

Activated autologous Th cells secreted interferon γ (IFN-γ; Figure 6A; data not shown) and had surface markers indicating a Th1-like phenotype. The Th cells expressed the IFN-γ-associated transcription factor T-Bet and were $CXCR3^+$, $CCR5^{-/+}$, $CCR6^-$, CRTh2 $(CD294)^-$, $CXCR5^-$, $ICOS^-$, IL-21^-, PD-$1(CD279)^{-/low}$, and Bcl-6^- (data not shown). Reciprocally, we confirmed that CLL cells express the T-Bet transcription factor (Figure 6B), as described previously in analyses of CLL in pseudofollicles (Dorfman et al., 2004).

CLL Cells that Collaborate with Antigen-Specific Th Cells Undergo Marked Changes in Gene Expression

To further study the CLL cell–Th cell collaboration and CLL cell activation, we utilized a well-defined model system and T18 cell clone. The gene expression profiles of purified CLL cells from T-B collaboration assays (antigen: Ms $C\kappa^+$IgG anti-λ/κ) were analyzed (Figure 7). The CLL cells had undergone significant changes in the expression of 1,604 genes (730 upregulated and 874 downregulated; Figure 7), including significant upregulation of mitosis-related genes (Table S2). Gene set enrichment analysis (GSEA; http://www.broadinstitute.org/gsea/) indicated significant similarity to cancer-related GSEA modules, including gene sets that are expressed in large cell lymphomas but are repressed in CLL cells (Table S3). Similar results were obtained using the Global Cancer Map tool (Broad Institute) and Oncomine analysis tool (Figure 7E; Table S4). Further, genes involved in negative regulation of apoptosis were significantly upregulated, whereas apoptosis-inducing genes were downregulated (Tables S5 and S6). To reveal potential signaling pathways responsible for the altered gene expression, we further tested the upregulated genes for potential transcription factor binding sites (Table S7) and found a significant association with transcription factors such as members of the NF-κB1 pathway. The gene expression profiles of CLL cells within pseudofollicles in lymph nodes were described in a recent study (Herishanu et al., 2011), and we found that 52 of the upregulated genes described herein overlap with the 133 overexpressed lymph node genes reported in that study (Herishanu et al., 2011; Table S8).

DISCUSSION

Previous studies have reported a high frequency of Th cells in CLL pseudofollicles (Pizzolo et al., 1983; Ghia et al., 2002; Patten et al., 2008). In this work, using a model system and a Th cell clone not related to CLL, we found that CLL cells could efficiently present complex protein antigen to Th cells, provided that antigen was delivered to the conventional antigen uptake pathways, including the BCR as well as Fc receptors. Subsequent to antigen presentation by resting CLL cells, Th cells reciprocally induced CLL cell proliferation and differentiation, including upregulation of costimulatory receptors and activation markers on CLL cells, blastogenesis, secretion of Ig, and altered gene expression profile, but not loss of HLA-DR expression. Turning to patients and their Th cells, we found that the CLL patients

harbored Th cells that were stimulated by endogenous antigens presented by CLL cells, and that a fraction of such Th cells were specific for CLL cell antibody. These CLL-specific Th cells in CLL patients had a Th1-like phenotype and could support significantly increased CLL proliferation in the bone marrow of NSG mice. Taken together, the results suggest that CLL is a disease driven by immune responses via a process in which Th cells engage CLL cells in response to exogenous antigen (which may be efficiently internalized by the BCR and other uptake pathways), or Th cells respond to endogenous CLL cell antigen presented on the CLL cells' own HLA class II molecules, as demonstrated here.

With regard to the mechanism for antigen-driven Th cell–CLL cell collaboration, the central facets are similar to those of normal Th cell–B cell collaboration, i.e., dependency upon (1) presentation of antigen on HLA class II, and (2) cell-cell contact, including the CD40/CD40L pathway (Grewal and Flavell, 1998). Some studies have described CLL cells as poor APCs in mixed leukocyte reactions prior to any activation (Halper et al., 1979; Ranheim and Kipps, 1993), whereas others demonstrated efficient antigen-dependent stimulation (Yasukawa et al., 1988; Hall et al., 2005). Here, we found that CLL cells are equally efficient as normal B cells in presenting antigen to memory Th cells. After cognate Th cell activation by the CLL cells, Th cells helped CLL cells in a contact- and CD40-dependent manner. As a result, the CLL cells upregulated costimulatory and adhesion molecules, as well as HLA-DR, that could augment antigen presentation. These findings extend previous studies that demonstrated a noncognate, bystander activation of CLL cells in vitro and in vivo (Tretter et al., 1998; Patten et al., 2005; Bagnara et al., 2011).

Other aspects of the antigen-driven Th cell–CLL cell interaction did not conform to the norm: (1) CLL cells became activated, proliferating Ig-secreting blasts, but did not downregulate HLA/costimulatory molecules or express the plasma cell marker CD138. Interestingly, it has been shown that polyclonally activated Th cells induce blastogenesis but do not downregulate the aberrantly expressed survival factor lymphocyte enhancer binding factor 1 (LEF-1), in contrast to CpG/cytokine-stimulated blasts that have reduced LEF-1, allowing apoptosis (Gutierrez et al., 2011). (2) CLL cells did not require additional BCR signals to undergo this activation, as antigen delivered to non-BCR endocytosis receptors was sufficient (see discussion below). (3) CLL cells could present endogenously derived antigen to Th cells, and such Th cells were readily detectable in patients. In line with the third point, it was previously shown that patients may have Th cells specific for antigens such as Id, but the significance and function of such Th cells have not been determined (Rezvany et al., 2000).

It has been speculated that CLL cells represent the malignant counterparts of anergic B cells, a subset destined to apoptose due to chronic BCR stimulation (Caligaris-Cappio, 1996; see Introduction). Concerning the role of BCR ligation in experiments such as those conducted here, a level of BCR signaling may already be ongoing in CLL cells, indicating an ''anergic signaling phenotype'' (Chiorazzi and Efremov, 2013; Stevenson et al., 2011), although it has been suggested that this signaling may be balanced by the tuning and establishment of a continuum of functional anergy as seen in mice studies (Zikherman et al., 2012). In any case, additional ligation by fluid-phase BCR mAbs produced little change in gene expression. By contrast, after antigen-dependent activation by Th cells, the CLL cells dramatically changed gene expression and expressed 52 of 133 of the genes found to be upregulated in CLL cells in lymph nodes of patients (Herishanu et al., 2011). In terms of signaling, CD40 ligation rapidly activates the canonical NF-κB1 pathway through members of TNFR-associated factor (TRAF) family of cytoplasmic proteins, causing RelA-dependent gene expression, in addition to slower noncanonical NF-κB2 activation (RelB) (Bishop et al., 2007). However, the BCR and CD40 share multiple components, such as the protein tyrosine kinases Syk, Lyn, Btk, and Akt, as well as the PI-3 kinase and phospholipase Cγ2 (Faris et al., 1994; Ren et al., 1994; Ying et al., 2011; Bishop et al., 2007). Hence, in line with the above discussion about anergic B cells, we suggest that the BCR pathway (constitutively triggered) and CD40 pathway (CD40L on Th cells) converge (Ying et al., 2011) to allow CLL cell activation.

As regards Th cells, important previous findings have special relevance to the current work. First, mitogenic stimulation of CLL cells has been described to have a Th cell component, because the presence of T cells augments the activation of CLL cells (Robèrt, 1979). Second, allogeneic Th cell help was sufficient for generation of blasts secreting IgM (Fu et al., 1979). Third, Th cells were found juxtaposed to activated proliferating CLL blasts in pseudofollicles (Ghia et al., 2002; Patten et al., 2008). Fourth, such Th cells expressed CD40L (CD154), indicating recent activation (Ghia et al., 2002) as well as a potential to stimulate CLL cells via CD40. The crucial outstanding question is, why and how are such Th cells activated, and what antigen do they recognize? There are three distinct but not mutually exclusive possibilities: (1) Th cells could be specific for antigens derived from a hitherto unrecognized pathogen, or respond to yeast or fungal antigens internalized by anti-β-(1,6)-glucan expressing CLL cells (Hoogeboom et al., 2013); (2) Th cells could be specific for autoantigens presented by the CLL cell (Hall et al., 2005); and (3) Th cells could be specific for secretory pathway autoantigens (Weiss and Bogen, 1991; Rezvany et al., 2000) that are endogenously expressed in the CLL. In the current work, we find that Th cells are specific for proteins in CLL lysates, and that some of the Th cells in such polyclonal lines are specific for the CLL mAb.

We found that the autologous CLL-specific Th cells were IFN-γ secreting Th1-like cells, as was previously reported (Gitelson et al., 2003). Moreover, similar results were very recently found in preliminary studies of Th cells from lymph nodes with CLL cell involvement (S.B., A.P.R., A.O., K.M.T., G.E.T., B.B., and L.A.M., unpublished data). How does this finding compare to what is known about the microenvironment in pseudofollicles? First, CLL cells secrete CCL3 and CCL4 chemokines that attract Th1 cells (Burger et al., 2009). Second, CLL patients have increased IFN-γ in sera, and IFN-γ can inhibit the apoptosis of CLL cells (Buschle et al., 1993). Third, CLL cells are positive for the IFN-γ-associated transcription factor T-Bet (Dorfman et al., 2004), as also confirmed herein. Thus, the results suggest that IFN-γ may play an important role in the survival of CLL cells.

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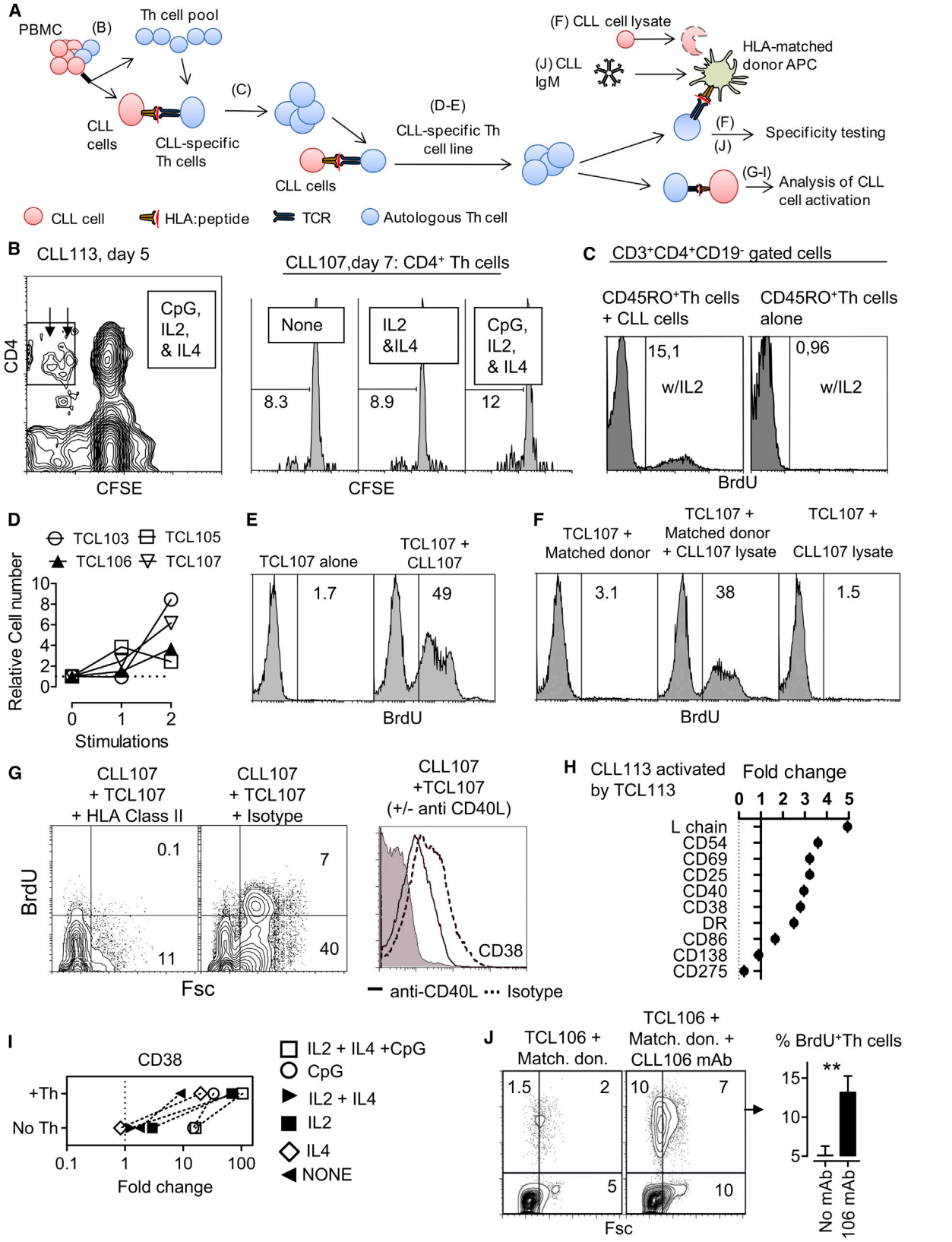

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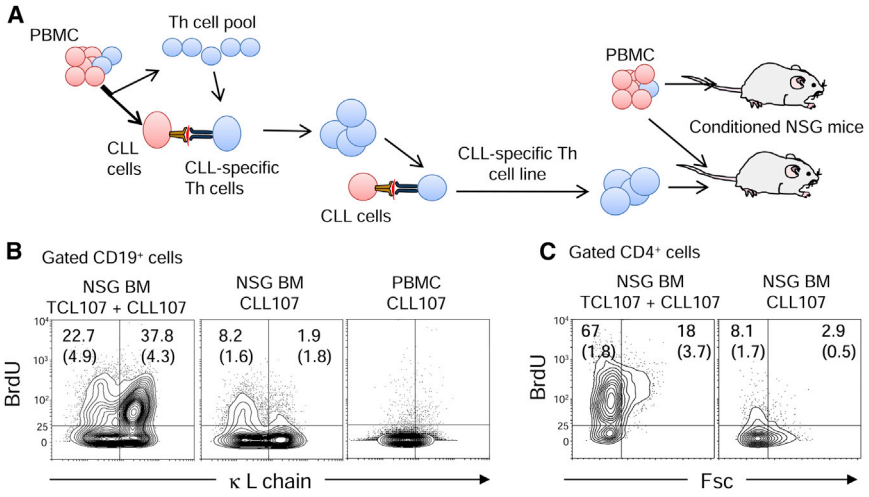

Figure 5. Analysis of Th Cell Collaboration with CLL Cells In Vivo

(A) Autologous CLL-specific Th cells were generated. PBMC CLL cells were injected into NSG mice alone or together with autologous CLL-specific Th.

(B) Stimulation of CLL107 cells by TCL107 in the bone marrow (BM) of NSG mice. κ L chain expression versus in vivo BrdU incorporation of gated $CD19^+$ CLL cells is shown (quadrants: mean [SD]; n = 4, $p < 0.0004$). Right: A priori expression of κ L chains, and control (background) anti-BrdU staining of CLL cells.

(C) Stimulation of TCL107 by CLL107 cells in the BM of NSG mice. Proliferation (BrdU) versus cell size (FSC) of gated $CD4^+$ cells is shown. Left: mice receiving TCL107; right: mice receiving only PBMC, PBMC $CD4^+$ Th cells are shown (quadrants: mean (SD); n = 4; $BrdU^+$ cells, $p < 0.0001$). See also Table S1.

CLL-specific Th cells could be readily demonstrated in patients and responded with proliferation and cytokine secretion to the presentation of endogenous CLL cell antigen. Previously, T cells have been thought to be functionally deficient (Görgün et al., 2005). Even so, a relatively high-frequency Th cell repertoire has been demonstrated against CLL cells or defined endogenous antigen (Rezvany et al., 2000; Kokhaei et al., 2007; Gitelson et al., 2003). Moreover, CLL cells have been implicated in the stimulation of autoreactive Th cells in CLL patients suffering from autoimmune hemolytic anemia, suggesting that CLL cells play a role in breaking the tolerance of Th cells in such patients (Hall et al., 2005). As concerns $CD8^+$ T cells, patients have CTLs of low frequency that respond to CLL idiotypic peptide (Trojan et al., 2000). However, there is increasing evidence of exhaustion in an expanded subset of CTLs that may have been chronically stimulated by CLL cells directed toward an unknown antigen (Riches et al., 2013).

The current work extends previous results that suggested that Th cells may play a role in the pathogenesis and expansion of CLL cells (Ghia et al., 2002; Patten et al., 2005, 2008; Tretter et al., 1998; Bagnara et al., 2011). Our findings that (1) CLL cells are functionally normal APCs, (2) patients harbor a subset of CLL-specific Th cells, and (3) these Th cells support CLL cell

Figure 4. Analysis of Interaction between Autologous CLL-Specific Th Cells and CLL Cells

(A) Outline of the experimental strategy. PBMCs were investigated for Th cells undergoing cell divisions ex vivo in the presence of CLL cells (B). Sorted memory Th cells were tested for response toward autologous CLL cells (C). Th cells were stimulated by CLL cells to make CLL-specific Th cell lines (D). Th cell lines were tested for responsiveness toward CLL cells and HLA-matched donor APCs presenting CLL cell lysates (F). Th cell lines were tested for their ability to stimulate autologous CLL cells (G–I). Th cells were tested for responsiveness toward CLL-derived IgM presented by HLA-matched donor APC (J).

(B) Carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMCs from patients CLL113 and CLL107 were cultured for 5 and 7 days, respectively, in media with or without supplements as indicated. Left: CD4 versus CFSE contour plot. Box and arrows: $CFSE^{Low}$ proliferating Th cells. Right: CFSE expression of gated $CD4^+CD19^-$ cells. The percentage of CFSE-decayed cells (marker) is indicated.

(C) BrdU incorporation of sorted $CD4^+CD45RO^+$ $CD45RA^-$ T cells from the blood of patient CLL113 in response to autologous CLL cells (left) or alone (right).

(D) Establishment of CLL-specific Th cell lines (TCL). $CD4^+$ Th cells were purified from patient PBMCs and stimulated by autologous CLL cells in the presence of IL-2 in medium with autologous serum as the protein supplement. After 10-day cycles, the cells were counted and restimulated. Relative Th cell numbers are shown.

(E and F) Responses of gated $CD4^+CD19^-$ Th cells.

(E) Proliferation of TCL107 cells (Th cells alone [left] or in the presence of autologous CLL107 [right]).

(F) TCL107 is specific for a CLL107 antigen. TCL107 was cultured with or without PBMCs from an HLA-DR, DQ, and DP matched healthy donor in the absence or presence of lysate of freeze-thawed CLL107 cells as indicated.

(G–I) Responses of gated $CD5^+CD19^+$ CLL cells are shown.

(G) Stimulation of CLL107 by TCL107 is inhibited by HLA class II or CD40L (CD154) mAb. Left: $CD19^+$ CLL107 cells were purified and cultured in the presence of CpG, IL-2, and TCL107 in the presence of anti-pan HLA class II or the appropriate isotype control. FSC versus BrdU incorporation in CLL cells on day 4 is shown. Right: CLL107 cells were cultured in the presence of TCL107 and anti-CD40L or an appropriate isotype control. The CD38 expression of CLL cells is shown for each of these two conditions. Gray, isotypic control for CD38 FACS staining.

(H) Stimulation of CLL113 CLL cells by autologous CLL113-specific TCL113 Th cells for 72 hr. Fold change of markers on gated CLL cells (Th-cell-activated CLL cells versus CLL cells alone).

(I) Analysis of CLL105 CLL cell response (CD38 upregulation) to the autologous CLL105-specific Th cell line TCL105 in the presence of cytokines or CpG2006 (see Experimental Procedures).

(J) Stimulation of TCL106 by HLA-matched donor APCs presenting purified IgM from CLL106 hybridoma (CLL106 mAb). TCL106 was cultured with donor APC in the absence or presence of CLL106 mAb (as indicated). FSC versus BrdU incorporation is shown. Right bar graph: TCL106 responsiveness to presentation of CLL106 mAb in six separate experiments. Error bars indicate SD.

See also Figure S4 and Table S1.

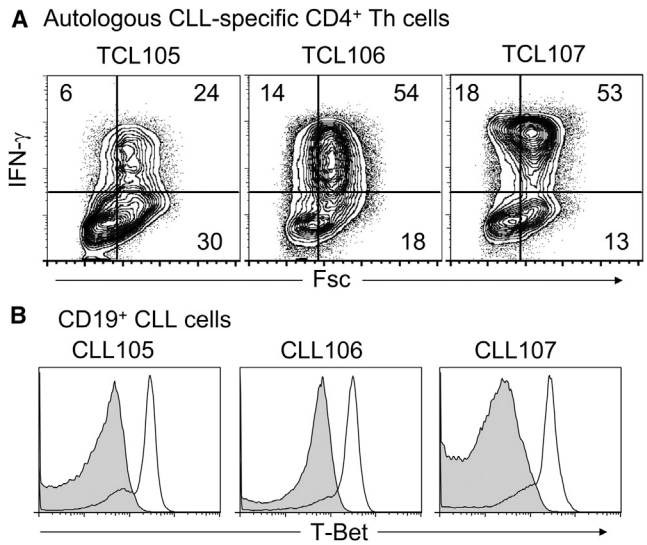

Figure 6. Analysis of IFN-γ Secretion by Autologous CLL-Specific Th Cells and T-Bet Expression in CLL Cells

(A) IFN-γ secretion by activated autologous CLL-specific T cell lines (IFN-γ versus FSC).

(B) Expression of T-Bet in CLL cells (T-Bet [line] versus isotype [filled histogram]).

See also Table S1.

activation and expansion suggest that Th cells are important drivers in this disease. Further work is required to define the fine specificity of the CLL-specific Th cell subset in patients and to extend such analyses to the microenvironment of the lymph node.

EXPERIMENTAL PROCEDURES

Patient Material and Normal Controls

Patients who had been diagnosed with CLL (see Table S1) were recruited at the hematological outpatient clinic at Oslo University Hospital, Rikshospitalet, Norway, after they provided informed consent. The regional ethics committee approved the project.

Th Cell and CLL Cell Isolation

$CD4^+$ Th cells were purified from patient or control PBMCs using the Dynabeads FlowComp Human CD4 kit (Dynal; Invitrogen) according to the product manual. CLL cells were positively selected using MACS beads (Miltenyi Biotec) by selection via rituximab (anti-CD20, devoid of Ms Cκ-antigen), anti-CD19, or anti-CD5 as indicated. In the antibody secretion assay (Figure 2F), CLL cells were positively selected (and concomitantly loaded with antigen) with Ms $C\kappa^+$ biotinylated $CD5^+$ mAb/streptavidin-MACS beads.

Culture of $CD4^+$ Th Cells and Generation of Th Cell Lines

Autologous CLL-specific Th cells were stimulated by autologous irradiated CLL cells in culture media supplemented by autologous serum, and thereafter using pooled human serum from healthy donors. The Th cell lines and the T18 clone were stimulated in 10-day cycles by irradiated APCs, with provision of 20 U/ml recombinant human IL-2 (rhIL-2; Roche). Alloreactive $CD4^+$ Th cell lines were generated by stimulating purified $CD4^+$ cells with 20 Gy irradiated mixed allogenic PBMCs from more than five CLL patients (see Extended Experimental Procedures for details).

Th Cell Proliferation Assays and Th Cell–CLL Cell Collaboration Assays

Th cells (5×10^4/well) were tested for proliferation (^{3}H-thymidine incorporation) in 96-well plates in response to irradiated CLL cells or PMBCs (2×10^5/well) and antigen as indicated. Alternatively, cocultured cells were tested for BrdU incorporation (see Extended Experimental Procedures for details). CpG ODN2006 (1–2 μg/ml; Invivogen) and rhIL-2 (Roche) were included in some assays as indicated. Pathway inhibition was performed with HLA DR, DP, or DQ mAb (Tu39; Becton Dickinson), or CD154 mAb (24-31; eBioscience).

Antigens in T18 Assays

Antigen was delivered to the processing pathway of CLL cells utilizing Ms $C\kappa^+$ mAbs targeting the following: Hu λ L chain (4C2), Hu κ L chain (A8B5) (both L chain mAbs with the same isotype [IgG1]), and IgM (1030) from Diatec; CD16 (CB16) and CD23 (EBVCS2) from eBioscience; and CD32 (3D3) and CD81 (VB070) from Becton Dickinson. In the assays shown in Figures 1A and 1B, cells from normal $DR0401^+$ donors N1 and N2 received a mixture of Ms κ^+IgG anti-Hu λ and Ms κ^+IgG anti-Hu κ to allow uptake and presentation by both λ- and κ-positive B cells. In the assays shown in Figure 2F, $CD5^+$ CLL cells were purified with MACS utilizing Ms κ^+anti-HuCD5 mAb. In these assays, sorted, antigen-loaded CLL cells (Ms κ^+anti-HuCD5 plus additional Ms κ^+anti-HuCD23) were incubated with T18 cells.

Surface Antigen Quantification

Quantification of surface antigen was performed using mAbs in the T18 assays described above and the bead-based Cellquant Calibrator kit (BioCytex) according to the manufacturer's guidelines.

Fluorescence-Activated Cell Sorting Analysis

Fluorochrome-labeled mAbs with the indicated specificities or isotype controls were used for fluorescence-activated cell sorting (FACS) analysis of surface expression and proliferation on the indicated days. See the Extended Experimental Procedures for an extensive list of mAbs. In Figures 2 and 4, the changes in staining intensity are shown as the ratio of signal with the mAb specific for the marker compared with the isotype control (ratio = $MFI_{marker}/MFI_{isotype}$).

In Vivo Experiments

Mice experiments were approved by the National Committee for Animal Experiments (Oslo, Norway). NSG mice (The Jackson Laboratory) were conditioned with busulfan (Busilvex; Pierre Fabre Pharma) and injected i.v. with PBMCs or PBMCs and CLL-specific Th cells. Mice were analyzed on day 42; see the Extended Experimental Procedures for details.

Microarray Experiments

CLL cells with or without 2 μg/ml of mouse κ^+ κ and λ IgG antibodies were incubated with T18 Th cells. On day 3, CLL cells were purified (see Extended Experimental Procedures). The purity of $CD19^+CD4^-$ cells for gene expression profiling as assessed by flow cytometry was 93%–99%, median 98%. $CD4^+$ cell contamination was low (0.00%–0.6%, median 0.05%). For details on RNA purification, data acquisition, and analysis see the Extended Experimental Procedures.

Statistics

One-way ANOVA with the Bonferroni multiple-comparisons test and Student's t test was performed as indicated.

ACCESSION NUMBERS

The microarray data series has been deposited in the Gene Expression Omnibus under accession number GSE48268 (see http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48268).

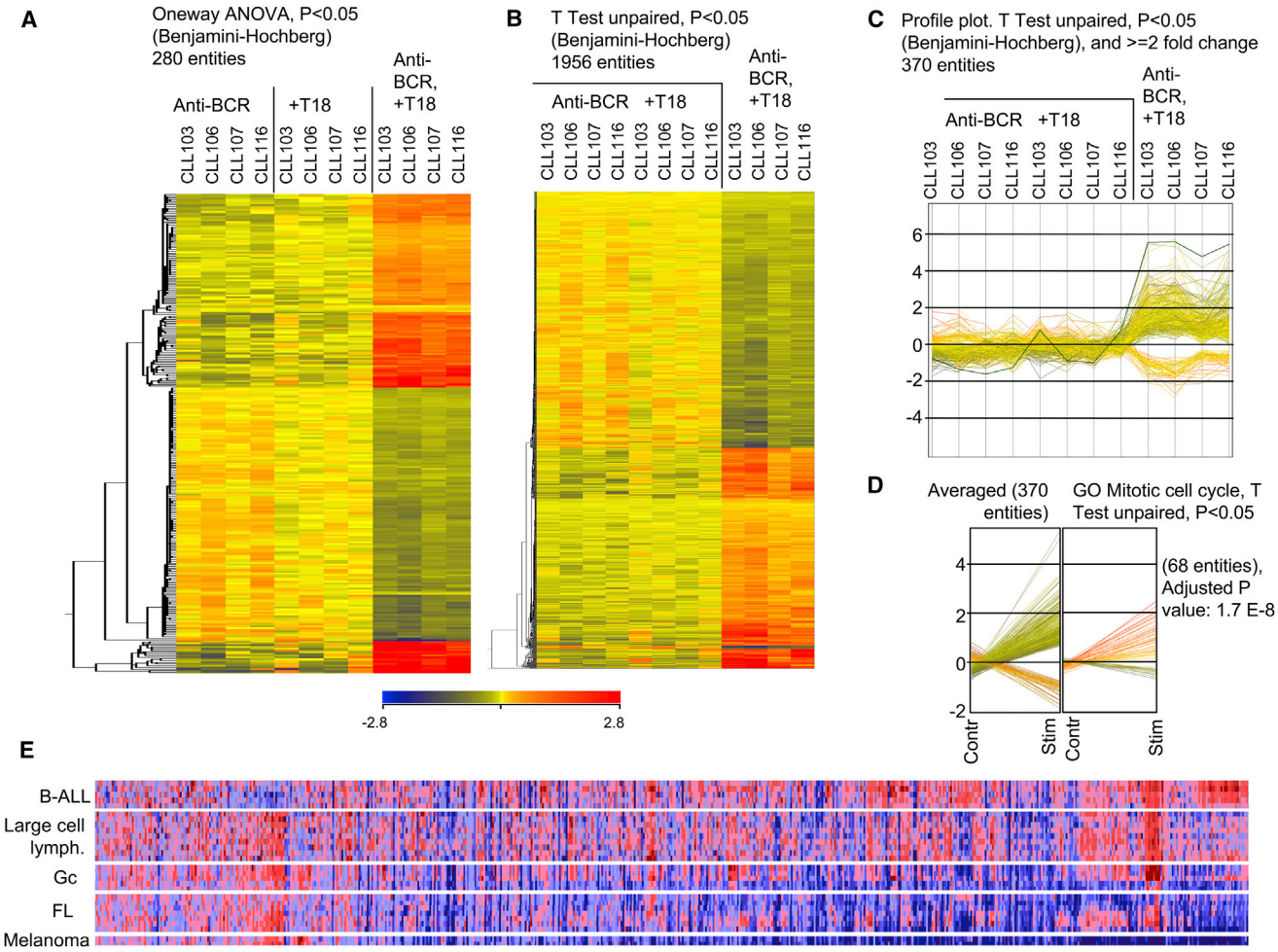

Figure 7. Analysis of Gene-Expression Profiles of CLL Cells Activated by T18 Cells and Antigen as Compared with Controls

(A–E) CLL cells were cultured with or without the T18 Th cell clone for 3 days in the presence or absence of Ms κ^{+}IgG anti-BCR.

(A) Unsupervised hierarchical clustering (hierarchical entity tree, Euclidean, centroid) of significantly changed gene expression after one-way ANOVA (Benjamini-Hochberg, asymptotic p value computation, $p < 0.05$) comparing each of the two control groups (CLL + Th; CLL + anti-BCR) with the activated group (CLL + Th + anti-BCR). The hierarchical entity tree presents 280 probe-defined entities that represented 244 unique Entrez gene Id and Hugo terms.

(B) Unsupervised hierarchical clustering (Euclidean, centroid) of significantly different gene expression after unpaired t test (Benjamini-Hochberg corrected, asymptotic p value computation, $p < 0.05$) comparing controls with the test group, i.e., Th cell–CLL cell collaboration (CLL + Th + anti-BCR). The hierarchical entity tree represents 1,956 probe-defined entities, 1,604 of which were unique Entrez gene Id and Hugo terms.

(C) Profile plot of data from (B) after unpaired t test (Benjamini-Hochberg, $p < 0.05$), including only genes with >2-fold change.

(D) Left: Profile plot as in (C), all entities, averaged data (controls versus CLL + Th + anti-BCR stimulated cells). Right: GO-term mitotic cell cycle from data as in (B) (unpaired t test, Benjamini-Hochberg, $p < 0.05$).

(E) Comparison of upregulated genes in Th cell/antigen-activated CLL with expression in the Global Cancer Map (Broad Institute; 686 of 730 genes were recognized by the application). Red, expressed; blue, repressed (gene expression profiles from the Global Cancer Map as published in Ramaswamy et al., 2001). See also Tables S1, S2, S3, S4, S5, S6, S7, and S8.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.07.011.

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WEB RESOURCES

The URLs for data presented herein are as follows:

DAVID, http://david.abcc.ncifcrf.gov
FlowJo, http://www.flowjo.com
Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48268
GSEA, http://www.broadinstitute.org/gsea/
Oncomine, http://www.oncomine.com
oPOSSUM, http://www.cisreg.ca/cgi-bin/oPOSSUM/opossum

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