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Chronic Lymphocytic Leukemia Cells Express CD38 in Response to Th1 Cell–Derived IFN-γ by a T-bet–Dependent Mechanism

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Chronic lymphocytic leukemia (CLL) is a B cell malignancy associated with increased levels of inflammatory cytokines. Similarly, expression of CD38 on CLL cells correlates with CLL cell survival and proliferation, but the mechanisms that regulate CD38 expression and inflammatory cytokines remain unclear. We have recently demonstrated that patients have CLL-specific Th cells that support CLL proliferation. In this article, we show that CLL cells attract such Th cells, thereby establishing an Ag-dependent collaboration. Blocking experiments performed in vitro as well as in vivo, using a xenograft model, revealed that secretion of IFN-γ was a major mechanism by which CLL-specific Th cells increased CD38 on CLL cells. The expression of the transcription factor T-bet in peripheral blood CLL cells significantly correlated with CD38 expression, and transient transfection of CLL cells with T-bet resulted in T-betCD38hi cells. Finally, chromatin immunoprecipitation experiments revealed that T-bet can bind to regulatory regions of the CD38 gene. These data suggest that CLL cells attract CLL-specific Th cells and initiate a positive feedback loop with upregulation of T-bet, CD38, and type 1 chemokines allowing further recruitment of Th cells and increased type 1 cytokine secretion. This insight provides a cellular and molecular mechanism that links the inflammatory signature observed in CLL pathogenesis with CD38 expression and aggressive disease and suggests that targeting the IFN-γ/IFN-γR/JAK/STAT5/bet/CD38 pathway could play a role in the therapy of CLL.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CLL, chronic lymphocytic leukemia; qRT-PCR, quantitative RT-PCR.

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IFN-γR expression on CLL cells correlated with disease severity (12–14). Similarly, IFN-γ conferred resistance to apoptosis and increased CLL cell motility (12, 13). Thus, many effects of IFN-γ overlap with those described for CD38, although the molecular mechanisms remain unclear. IFN-γ is a type 1 cytokine that can stimulate macrophages to secrete TNF-α, another proinflammatory cytokine that is increased in CLL patients and has antiapoptotic effects on CLL cells (14).

The transcription factor T-bet is the key regulator in Th1 cell polarization and type 1 immune responses (15) and has also been detected in CLL cells (11, 16). In murine B cells, T-bet plays a role in B cell migration (17) and is essential for class switch to IgG2a and for IFN-γ production (15, 18, 19). The transcription factor is crucial for the development of the proinflammatory B effector 1 cells, a B cell subset that can secrete IFN-γ and IL-12 (20). T-bet was also found in precursor B cell lymphoblastic leukemia/lymphoma, marginal zone lymphoma, and hairy cell leukemia, but not in mantle cell leukemia, follicular lymphoma, diffuse large B cell lymphoma, and Burkitt lymphoma (16).

In this article, we describe how CLL cells attract autologous CLL-specific Th1-like cells by secretion of type 1 chemokines. CLL cells and Th cells then establish a collaboration that involves IFN-γ and T-bet and results in increased CD38 expression on CLL cells.

Materials and Methods

Patient material and normal controls

Patients diagnosed with CLL (Supplemental Table I) were recruited after informed consent at the outpatient clinic, Department of Hematology, Oslo University Hospital, Norway. The Regional Ethics Committee for Medical and Health Research Ethics approved the project. Blood samples were collected before initiation of treatment except for patients 124 and 135; see Supplemental Table I for more information.

Cell isolation

Mononuclear cells were isolated from blood or fine-needle aspirates of CLL-affected lymph nodes of CLL patients using Lymphoprep (STEMCELL Technologies). CD4+ T cells were purified using the Dynabeads Flow-Comp Human CD4 Kit (Dynal; Invitrogen). CLL cells were isolated using the Dynabeads CD19 panB Kit (Dynal; Invitrogen). Purity of the cells was tested by flow cytometry and was ≥96%.

Generation of CLL-specific T cell lines

CD4+ Th cells derived from blood or lymph nodes of CLL patients were stimulated with irradiated (20 Gy) autologous CLL cells in RPMI 1640 + Glutamax-1 (Life Technologies; Invitrogen) in the presence of heat-inactivated autologous serum. After 2 d, the Th cells were supplemented with 20 U/ml IL-2 (Roche). On day 10, the Th cells were rested for 2 d by withdrawal of IL-2 and then restimulated with irradiated autologous CLL cells in the presence of 10% endothelin- and mycoplasma-tested heat-inactivated FCS (Biokom AG). Th cells from lines in generations 5–7 were used.

Model of Ag-dependent Th1 cell–CLL cell collaboration and microarray experiments

To study Ag-dependent Th1 cell–CLL cell interaction, we use a human Th1 cell clone (T18) specific for an epitope in the mouse Ig κ-chain, when presented on the MHC class II haplotype (HLA-DRB1*0401) (11). This Th1 clone is not related to CLL but allows functional studies of Ag-dependent cognate interaction with Th cells. In the model system, mouse κ+ Abs against BCR light chains were used to target the Ag to DR0401+ CLL cells and study genetic chances in CLL cells upon Ag-specific interaction with Th cells. CLL cells were incubated with T18 cells, with or without Ag; 32–45 × 10^6 CLL PBMCs were incubated overnight, with or without 2 μg/ml mouse κ+ IgG anti-κ (Diatet; A85B) and anti-κ Abs (Diatet; 4C2), as described in Ref. 11. The PBMCs from individual patients were then cultured in the presence or absence of 12.5 × 10^6 T18 cells. On day 3, CLL cells were purified by negative selection, using CD3 and CD14 Dynabeads (Invitrogen). The purity of CD19+CD4+ cells for gene expression profiling, as assessed by flow cytometry, was 93–99%, median 98%. CD4+ T cell contamination was low (0.03–0.6%, median 0.05%). A total of 1500 ng biotin-labeled cRNA was used to hybridize onto Illumina HumanWG-6 v3 Expression BeadChips. Gene expression profiling was performed as reported (11). Analysis was performed using Genespring GX v11 software (Agilent, Santa Clara, CA). The microarray data series has been deposited in the Gene Expression Omnibus under accession number GSE48268 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48268).

Migration assays

CLL cells were cocultured with CD40L-transfected or untransfected mouse NIH/3T3 fibroblasts (kind gift of Dr. Janne Bornhorst, Institute of Clinical Medicine, University of Oslo, Oslo, Norway). Alternatively, CLL cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (2 μM; Sigma-Aldrich) for 4 h and then washed to remove PMA and ionomycin. As a negative control, CLL cells were stimulated in the presence of GolgiStop (4 μM/ml; BD). Supernatants were harvested after 24 h and transferred to the lower wells of Transwell plates with 5-μm pores (Corning). A total of 200,000 autologous Th cells from CLL-specific Th cell lines or freshly isolated autologous CD4+ cells were placed in the corresponding upper well. After 3 h, the lower and upper wells were analyzed by flow cytometry, and the percentage of migrated cells was determined. The Δ migration was calculated by subtracting the spontaneous migration (toward medium without supplements). This was 5% in Fig. 1C.

Transient transfections

Cryopreserved CLL cells were thawed and incubated at 37°C for 2 h. CLL cell transfections were performed with the Nucleofector Kit for Human B Cells (Lonza Group), using program U13 and 1 μg pCMV6-T-bet or pCMV6-entry negative control (both Origene). The pmaxGFP (Lonza) plasmid was used to optimize conditions and time points. Expression of GFP, T-bet, and CD38 was verified by quantitative RT-PCR (qRT-PCR) and flow cytometry after 24 h and 48 h. The efficiency of transfection was highest after 24 h, with median of 23% GFP+ CLL cells (range, 13–38%).

Cytokines and other cell culture reagents

Cytokines from PeproTech were as follows: IFN-γ (50 ng/ml), IL-4 (25 ng/ml), and IL-6 (20 ng/ml). Cytokines from Immuntools were as follows: IL-1β (10 ng/ml), IL-5 (10 ng/ml), TNF-α (10 ng/ml), CCL3 (10 ng/ml), CCL5 (100 ng/ml), and CXCL10 (50 ng/ml). Other reagents were as follows: CpG (1 μg/ml; Invivogen), anti–IFN-γ-R Abs (GIR-208, 10 μg/ml; BD), IgG1 isotype controls (10 μg/ml; BD), and eBioscience, and Ruxolitinib (SelleckChem).

Flow cytometry analysis

Abs used for surface stainings. BioLegend: CCR5 (HEK/1/85a) and CXC3R3 (TG1/CXCR3); eBioscience: CD44-A88K (OKT4), CD4-PerCP- (RPA-T4), CD5 (L17F12), CD19 (HIB19), CD38 (HIT2), and IFN-γR (GIR-208); with corresponding isotype controls. Stainings for T-bet were performed using the FoxP3 Staining Buffer Set (eBioscience) and anti–T-bet–PerCP Abs (eBioscB10; eBioscience) or isotype control IgG1k.

Intracellular cytokine and chemokine staining. For intracellular staining of IFN-γ, CCL3 and CCL4, CLL cells or Th cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (2 μM; Sigma-Aldrich) for 6 h in the presence of GolgiStop (4 μM/ml; BD) for the final 4 h. The staining was performed using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD). The following Abs were used: anti–IFN-γ–eBioscience (45B3), CCL3/MIP-1α (R&D Systems; 95342), and CCL4/MIP-1β (R&D Systems; 24006).

Analysis of BrdU incorporation. CLL cell proliferation was analyzed using the BrdU Flow Kit (BD) and an Alexa 488–labeled anti-BrdU Ab (BD; clone 3D4) according to the protocol. Samples were acquired using FACSCalibur or LSR II flow cytometers (BD Pharmingen), and data were analyzed using FlowJo software (www.flowjo.com; Tree Star). CLL cells were analyzed by gating on CD19+CD5+CD4+ cells; Th cells were analyzed by gating on CD19+CD5+ CD4+ cells.

Determination of cytokines by cytometric bead array

A total of 1 × 10^5 Th cells from CLL-specific lines were cultured for 24 h in 250 μl RPMI 1640 with FCS in the presence of PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (2 μM; Sigma-Aldrich). CLL cells were isolated using the Dynabeads CD19 Pan B Kit (Dynal; Invitrogen). Purity, verified by flow cytometry, was ≥98%. Contaminating CD4+ T cells were ≤0.5%. A total of 6 × 10^5 CLL cells were cultured for 24 h in 1 ml RPMI 1640 with FCS, either alone or together with CD40L transfected or untransfected mouse NIH/3T3 fibroblasts. Supernatants were analyzed with the Bio-Plex system, using the Bio-Plex Pro Human Cytokine 17-Plex Kit or 27-Plex Kit (all from Bio-Rad).

ELISA

A total of 1 × 10^5 Th cells from CLL-specific lines were cultured for 24 h in 250 μl RPMI with FCS in the presence of PMA (50 ng/ml; Sigma-
Western blot

CLL cells were isolated using MACS CD19 microbeads (Miltenyi Biotec). Purity, verified by flow cytometry, was ≥98%. A total of 4 × 10^6 CLL cells were cultured for 48 h in 1 ml RPMI 1640 with FCS and stimuli, as indicated. Nuclear extracts were prepared as follows: All buffers contained EDTA-free complete protease inhibitor (Sigma-Aldrich). Cells were resuspended in 400 μl buffer A (10 mM KCl; 1 mM DTT; 10 mM HEPES, pH 7.9; and 1 mM EDTA). After 15 min of incubation, 25 μl 10% Nonidet P-40 (Sigma-Aldrich) was added and the tube was vortexed vigorously for 30 s, followed by a centrifugation step (1 min, maximum speed, 4˚C). The translucent nuclear pellet was washed once with 1 ml buffer A, resuspended in 50 μl buffer B (20 mM HEPES, pH 7.9; and 1 mM EDTA), and incubated for 15 min at 4˚C on a roller. Nuclear debris and genomic DNA were removed by centrifugation (5 min, maximum speed, 4˚C). The supernatant was diluted one third with buffer D (20 mM HEPES, pH 7.9; 1.5 mM MgCl2; 0.2 mM EDTA; 1 mM DTT; and 0.1% Nonidet P-40). Total protein concentration was determined using a NanoDrop 1000 system (Thermo Scientific). Samples were loaded next to a protein-mass ladder (Fermentas) on a NuPAGE 4–12% bis-Tris gel (Invitrogen Life Technologies). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). To confirm equal sample loading and transfer, the membranes were cut horizontally at ∼45 kDa, and the high molecular mass half of the membrane was incubated with a mouse anti-human T-bet Ab (eBioscience; 4B10, 1:250) in PBS-Tween 20% with 3% milk. After blocking, the membranes were cut horizontally at ∼45 kDa, and the high molecular mass half of the membrane was incubated with a mouse anti-human PCNA Ab (Santa Cruz Biotechnology; PC10, 1:1000) in PBS-Tween 20% with 3% milk overnight at 4˚C. The low molecular mass half of the membrane was incubated with a mouse anti-human T-bet Ab (eBioscience; 4B10, 1:250) in PBS-Tween 20% with 3% milk overnight at 4˚C. The blots were developed using anti-mouse IgG1-HRP (Southern Biotech; 1:3000) 1 h at room temperature and developing solution ChemiGlow (ProteinSimple), followed by visualization with an LAS 1000 camera (Fujifilm).
Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed with a 7900 Fast Real-Time PCR System (Thermo Fisher Scientific). TaqMan Master Mix and primers and probes specific for CD38 and the housekeeping gene HMBS were from Applied Biosystems. The data were analyzed with SDS2.3 software (Applied Biosystems), and HMBS was used to normalize Ct values. Fold increase in 4D and 4G was calculated using the following equation: fold increase = 2^{\Delta\Delta Ct}, where \Delta\Delta Ct equals \Delta Ct(T-bet–transfected) − \Delta Ct(empty vector–transfected).

Chromatin immunoprecipitation analyses

Chromatin immunoprecipitation (ChIP) assays were performed as described (21), using purified CD19+ CLL cells. Protein–DNA complexes were cross-linked with formaldehyde at a final concentration of 1.42% for 15 min. Formaldehyde was quenched with 125 mM glycine for 5 min, and cells were harvested. Cells were lysed with immunoprecipitation buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.5; 5 mM EDTA; Nonidet P-40, 0.5% v/v) containing protease inhibitors (Sigma-Aldrich). The chromatin was sheared by sonication and incubated with anti-human T-bet Ab (eBio-science; 4B10, 10 mg/ml) or the corresponding IgG1 isotype controls overnight at 4°C. As a positive control, an anti-human RNA polymerase II Ab (Santa Cruz Biotechnology) was used. The input samples were precipitated by adding 10% 3 M NaOAc and 300% EtOH (100%) and incubating overnight at –80°C. The cleared chromatin was then incubated with protein A or protein G Sepharose beads (GE Healthcare), and the DNA was isolated with a 10% (w/v) Chelex 100 resin (Bio-Rad). Samples were treated with proteinase K (Sigma-Aldrich) at 55°C for 30 min. The proteinase K was then inactivated by boiling the samples for 10 min. qRT-PCR was performed as described above, with primers and probes from Applied Biosystems. A total of 1% of the input was used for PCR reactions, and IP samples were normalized to the input using the following equation: \Delta Ct = Ct(IP) − Ct(input*6.64). \Delta\Delta Ct values were derived by subtracting \Delta Ct values of isotype-treated samples from \Delta Ct values of IP samples. Fold enrichment equals 2^{\Delta\Delta Ct}.

In vivo experiments.

Mouse experiments were approved by the National Committee for Animal Experiments (Oslo, Norway) and performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ JAX) mice (The Jackson Laboratory) were conditioned with 25 mg/kg busulfan (Busilvex; Pierre Fabre Pharma) i.p. 24 h prior to xenotransplantation. Mice were injected i.v. with 4.1 × 10^7 CLL PBMCs with or without 2.4 × 10^7 autologous CLL-specific Th cells (patient CLL114). Engraftment of human cells was tested by blood sampling. After 70 d, mice were injected daily with anti–IFN-\gamma Abs (R&D Systems; GIR208, 1 mg per mouse i.p.) or isotype control Abs for 7 d. BrdU was administered during the final 3 d i.p. (1 mg/d) and in the drinking water (0.8 mg/ml). Finally, the mice were euthanized, and bone marrow cells were analyzed by flow cytometry.

Statistics

Data sets were analyzed using repeated measures ANOVA, linear regression, the Wilcoxon signed rank test, and the Student t test, as indicated.
Results

CLL cells promote migration of CLL-specific Th cells through secretion of Th1-attracting chemokines

Our previous results (11) suggest that CLL cells are expanded in patients by interactions with their CLL-specific Th cell partners. Such an Ag-dependent collaboration is dependent upon the ability of CLL cells to attract CLL-specific Th cells to their proliferation site. It has previously been described that CLL cells cocultured with macrophages express the type 1 chemokines CCL3 (MIP-1α) and CCL4 (MIP-1β) (22). We confirmed these findings and extended the results to lymph node–derived CLL cells (Fig. 1A, Supplemental Fig. 2A, 2B). In addition, we found that CLL cells also expressed CXCL10 (IP-10) and CCL5 (RANTES) (Fig. 1A). Chemokine expression was increased after CD40 ligation. The detected chemokines are ligands for CCR5 (CCL3, CCL4, CCL5) and CXCR3 (CXCL10), receptors that are expressed by CLL-specific Th cells (Fig. 1B). Consistently, CLL-specific Th cells, as well as polyclonal blood Th cells, from patients migrated toward supernatants from CLL cell cultures (Fig. 1C, Supplemental Fig. 2C). Migration was boosted when supernatants from CD40L- or PMA/ionomycin-stimulated CLL cells were used. Thus, CLL cells could attract their CLL-specific Th cell partners; the migration was enhanced when CLL cells were activated by CD40L costimulation.

Ag-dependent interaction between CLL cells and Th1 cells results in a positive feedback loop

In addition to the chemokines, CLL cells secreted IL-12/70, IFN-γ, IL-1β, IL-6, and TNF-α (Supplemental Fig. 2D). Secretion was enhanced by CD40L stimulation. To investigate the response to Th1 cells, we used a model system of Ag-dependent collaboration with Th1 cells (see Materials and Methods). CLL cells responded by increasing transcription of Th1-associated chemokines, type 1 cytokines (IFNG, TNF, IL1A, IL1B, IL6, IL12B), IFN-γ receptors (IFNGR1, IFNGR2) as well as signaling components (JAK1, STAT1), proteasome subunits (PSMB9, PSMB10), STAT1 pathway activators (CIITA), and key negative feedback components of the IFN-γ pathway (SOCS1, SOCS3), but did not upregulate other cytokines such as IFNβ1, TGFB1, IL2, IL10, IL4, and IL13 (Fig. 1D and data not shown). The median contamination of Th cells was low (0.05%; see Materials and Methods), and Th cell–derived gene expression signals such as TCR genes were not increased (TCRA; Fig. 1D and data not shown). The data also suggested some unspecific, bystander effects, as CCL3, CCL2, and TNF were upregulated in CLL cells in the absence of T cell Ag, similar to that described secondary to CLL interaction with macrophages (22). In summary, CLL cells responded rigorously to cognate interaction with T cells and expressed markers resembling those found in type 1 inflammatory B cells (Be1 cells).

IFN-γ upregulates the expression of CD38

The expression of the CD38 gene was also upregulated (Fig. 1D). Because CLL-specific Th cells derived from blood as well as from lymph node biopsy samples produce high amounts of IFN-γ (Ref. 11 and Supplemental Fig. 2E–G), we proceeded to investigate whether IFN-γ was responsible for the increased CD38 expression on CLL cells upon contact with specific Th cells. Indeed, provision of IFN-γ increased the expression of cell surface CD38 (Fig. 2A). As previously reported for IFN-α (23), this upregulation was most pronounced in patients with >30% CD38+ cells, that is, in CD38-positive patients (Supplemental Fig. 3A). We found no correlation between IFN-γR expression and CD38 expression (Supplemental Fig. 3B). Other Th cell–derived cytokines did not change CD38 levels (Fig. 2B, Supplemental Fig. 3C). IFN-γ signals via JAK1/JAK2 kinases. Accordingly, the upregulation of CD38 in the presence of IFN-γ was significantly and incrementally inhibited by the JAK1/JAK2 inhibitor ruxolitinib (Fig. 2C, 2D). These results demonstrate that upregulation of the biomarker CD38 is directly related to IFN-γ. To test the importance of IFN-γ in physiologic CLL cell–Th cell collaborations, IFN-γR blocking Abs were introduced into autologous CLL-specific Th cell–CLL cell collaboration cultures. Anti–IFN-γR blocking Abs abolished CD38 upregulation on CLL cells (Fig. 3A). These results were further confirmed in xenograft experiments, in which autologous pairs of CLL cells and Th cells were introduced into NSG mice. In these in vivo experiments, blocking IFN-γ signaling not only inhibited CD38 upregulation but also significantly inhibited CLL cell proliferation (Fig. 3B). In summary, the IFN-γ signaling pathway was responsible for increased CD38 expression upon contact with autologous Th cells in vitro and in vivo, which is in agreement with previous findings in which CD38hiK67+ cells were juxtaposed to Th cells in pseudofollicles (6).

The transcription factor T-bet is expressed in CLL cells and mediates CD38 upregulation

IFN-γ exerts many of its effects via the transcription factor T-bet (15, 18, 19, 24, 25). Others (16) and we (11) have found that CLL

FIGURE 3. IFN-γR blocking reduces CD38 expression in vitro and in vivo. (A) CLL cells were cultured alone or together with autologous, CLL-specific Th cells. The IFN-γ pathway was blocked by addition of anti–IFN-γR Abs and compared with cultures without blocking (isotype controls). After 48 h, CD38 expression was analyzed by flow cytometry. (B) NSG mice were injected with CLL cells and CLL-specific Th cells from patient 114. After 70 d of expansion, mice were treated with blocking anti–IFN-γR Abs (lower panel) or isotype controls (top panel) for 7 d. CLL cells in the bone marrow were analyzed for BrdU incorporation and CD38 expression by flow cytometry. Shown are analyses of four and five mice, respectively. Significance was analyzed by the Student t test (p = 0.006 for CD38 and p = 0.008 for BrdU).
cells express T-bet. Basal T-bet expression in CLL cells could be increased by stimulating the cells with IFN-γ (Fig. 4A). Accordingly, xenografted mice treated with anti–IFN-γR blocking Abs expressed lower T-bet levels than did control mice (Fig. 4B). Because IFN-γ promoted the expression of both T-bet and CD38, we hypothesized that T-bet could be the transcriptional regulator that mediated IFN-γ–induced CD38 upregulation. This conjecture was supported by significant correlation between the expression levels of CD38 and T-bet on CLL cells from patients’ blood (Fig. 4C). To address whether T-bet can promote CD38 expression in CLL cells, we transiently transfected CLL cells with T-bet. T-bet overexpression led to an increase in CD38 on

FIGURE 4. T-bet expression by CLL cells is enhanced by IFN-γ and mediates CD38 upregulation. (A) CLL cells were stimulated for 72 h with or without IFN-γ. Shown is expression of T-bet and PCNA (sample loading control). (B) NSG mice were injected with CLL cells and CLL-specific Th cells from patient 114. After 70 d of expansion, mice were treated with blocking anti–IFN-γR Abs (lower panel) or isotype controls (top panel) for 7 d. CLL cells in the bone marrow were analyzed for T-bet expression by flow cytometry. Shown are analyses of four and five mice, respectively. Statistical significance was analyzed by the Mann–Whitney U test (p = 0.016). (C) Ex vivo expression of T-bet and CD38 was analyzed by flow cytometry. Shown are mean fluorescence intensities (MFI) of T-bet versus CD38 normalized (ratio) to the corresponding isotype controls of patients 103, 109, 110, 121, 124, 125, 128, 132, 135, and 136. R² and p values were determined using linear regression. (D) CLL cells were transfected with T-bet (pCMV6-T-bet) or an empty vector (pCMV6-entry). Expression of T-bet and CD38 was analyzed by qRT-PCR after 24 h and 48 h. Depicted is the fold increase in mRNA expression of T-bet-transfected cells compared with empty vector–transfected cells. Shown is one of three independent experiments; mean and SEM represent data from three patients (122, 132, and 135). (E) CLL cells were transfected with T-bet or an empty vector. Expression of T-bet and CD38 was analyzed by flow cytometry after 24 h. The T-bethiCD38hi population present in T-bet–transfected samples is highlighted. Three representative flow cytometry analyses are shown (n = 6). (F) Summary of transfection experiments (patients 107, 114, 122, 125, 128, and 132). The filled circles represent MFI of T-bet (left panel) and CD38 (right panel) in T-bet–transfected compared with empty vector–transfected cells (normalized to the corresponding isotype controls). Average increase was 53% for T-bet and 38% for CD38. The p values were analyzed using the Wilcoxon signed rank test and were 0.031 for both T-bet and CD38. (G) CLL cells were transfected with T-bet or an empty vector in the presence of blocking anti–IFN-γR Abs or isotype controls. Expression of CD38 mRNA was analyzed by qRT-PCR after 24 h. Depicted is the fold increase in mRNA expression of T-bet–transfected cells compared with empty vector–transfected cells. Shown is one of three independent experiments; mean and SEM represent data from three patients (CLL 122, 132, and 135).
both mRNA and protein levels and to the appearance of a T-bet^{hi} CD38^{hi} population (Fig. 4D–F), demonstrating that T-bet can act as a positive regulator of CD38 expression.

**T-bet binds to the CD38 regulatory region**

Overexpression of T-bet upregulated CD38 levels even in the presence of IFN-γR blocking Abs (Fig. 4G), suggesting that induction of IFN-γ production was not the main mechanism by which T-bet increased CD38 levels. To investigate whether T-bet might regulate CD38 expression in a direct manner, we screened the 5′ end of intron 1 of the CD38 gene for T-bet consensus sequences. This region plays a central role in transcriptional regulation of CD38, as reviewed (8). We found three putative T-bet binding sites (Fig. 5A). To analyze whether T-bet binds to the CD38 gene in vivo, we overexpressed T-bet in CLL cells and performed ChIP assays (Fig. 5B). Binding sites 2 and 3 were consistently occupied by T-bet in transfected CLL cells, whereas enrichment of binding site 1 was less pronounced. Similarly, when CLL cells were treated with IFN-γ, which enhances T-bet expression, T-bet binding to consensus sequence 2 and 3 was observed (Fig. 5C). Binding site 2 was enriched even in untreated/untransfected cells, suggesting constitutive T-bet binding to this site.

Together, these results indicate that T-bet binds to the regulatory region of the CD38 gene to regulate its transcription.

**Discussion**

In this article, we demonstrate that CLL cells upregulate CD38 in response to Th1 cell–derived IFN-γ in an IFN-γ–, JAK1/JAK2–, and T-bet–dependent manner. CLL cells secrete chemokines that attract CLL-specific Th1-like cells, thereby enabling collaboration between CLL cells and their Th1 cell partners. Once established, positive feedback with upregulation of type 1 cytokines and inflammatory chemokines can reinforce the collaboration between CLL cells and their Th cell partners in the CLL microenvironment in vivo (as illustrated in Supplemental Fig. 1).

Previously, CD38, IFN-γ, and T-bet have all been linked to survival, migration, and activation in normal B cells or in CLL cells (3, 4, 6–8, 12, 13, 15, 17–20). Although each of the factors may also have independent roles, the current results link these observations to a common pathway. As CD38, as well as T-bet, is expressed in a wide range of cells, including epithelial cells, smooth muscle cells, innate immune cells, and other hematopoietic cells (26), it is likely that this IFN-γ–dependent pathway may be of direct relevance beyond the B cell field.

A large number of studies have pointed to the importance of the microenvironment in CLL expansion within CLL pseudofollicles. Stromal cells, vascular endothelial cells, monocyte-derived nurse-like cells, as well as Th cells, are thought to support migration, survival, and proliferation of CLL cells (6, 11, 22, 27, 28). The interaction of CLL cells with surrounding cells seems to involve both chemokines and cytokines (12–14). The current results link many of these factors into a coherent picture.

It is well established that CLL is associated with inflammatory phenomena and autoimmune disease, such as autoimmune hemolytic anemia and thrombocytopenia as well as exaggerated inflammatory response to minor insults (29, 30). The inflammatory phenomena can be monitored clinically as increased type 1 cytokines and chemokines (TNF-α, IL-1, IL-6, CCL3, and IFN-γ) and inflammatory markers (β2 microglobulin, C-reactive protein, and elevated sedimentation rate) (17). In molecular terms, the central orchestrator of type 1 immune responses is the IFN-γ–responsive transcription factor T-bet, found in our study both in CLL cells and in their CLL-specific Th1 cell partners. It is likely that the direct consequence of T-bet expression is a positive feedback including type 1 cytokines (macrophage activation and direct antiapoptotic effects on CLL cells), chemokines (recruitment of Th1 cells and monocytes), and expression of the prosurvival factor CD38 (Supplemental Fig. 1). The secretion of inflammatory chemokines and cytokines and partnership with IFN-γ–secreting Th cells, as well as T-bet expression, are reminiscent of the B effector 1 subset of B cells (31), for which an autocrine feedback loop has been described: B cells primed by Th1 cells are activated to secrete IFN-γ in an IFN-γR– and T-bet–dependent manner, imprinting a type 1 phenotype on the B cells. Such B effector 1 cells secrete TNF-α, IL-1β, IL-6, IL-12, IFN-γ, and IL-10 (31).

**FIGURE 5.** T-bet binds to the CD38 gene regulatory region. (A) Schematic representation of the 5′ end of the CD38 gene intron 1 and nucleotide sequences of the T-bet consensus sequence and the three putative binding sites. Core nucleotides are underlined. (B and C) Binding of T-bet to the CD38 gene was assessed by ChIP. Shown is fold enrichment compared with isotype-precipitated samples, normalized to the input. Primers for the Ifng promoter (Ifng P) served as positive control, and primers targeting an intergenic region served as negative control. Shown are mean and SEM from three independent experiments, each of which was performed with cells from a different patient (122, 128, and 132). (B) ChIP analysis of T-bet– or empty vector–transfected CLL cells. (C) ChIP analysis of IFN-γ–treated and untreated CLL cells.
In terms of chemokines, it was demonstrated that activated CLL cells produced CCL3 and CCL4 (22). In normal B cells, T-bet regulates the expression of both CCL3 and CCL4 (32). This function of T-bet may also be relevant in CLL cells, as increased levels of CCL3 were found to correlate with disease severity (33). We found that CLL cells secrete CCL3, CCL4, and, in addition, CCL5 and CXCL10, matching the chemokine receptors (CCR5, CXCR3) on CLL-specific Th-like cells and attracting the Th cells in migration assays. The expression of these Th1 cell attracting chemokines was increased by Th1 cells in an Ag-dependent manner.

It has been suggested that cells of the monocyte lineage play an integral part in the CLL microenvironment (22, 34). It is possible that the Th1 cell–CLL cell partnership is consolidated by macrophages. In this regard, it is relevant that CLL cells can secrete proinflammatory type 1 cytokines, such as IL-12 (35) and TNF-α, and express TNF-α receptors (14). Moreover, CLL cells secrete IL-1α, IL-1β (36), and IFN-γ (37). Together with the cytokines secreted by Th1 cells, these factors could set the stage for IFN-γ–stimulated, TNF-α–secreting macrophages. It should be noted that CLL cells can also express IL-10, as also seen in our work with CD40L stimulation, but not with Th1 stimulation. The difference may be related to other Th cell factors that modulate CD40 signals, such as the Th1 cytokine IFN-γ, which is known to inhibit IL-10 production (38). Even so, the role of IL-10 in the biology of CLL pseudofollicles deserves attention and is likely to be complex. For example, IL-10 can serve as a growth factor for human B cells, can promote B cell responses, is part of the type 1 inflammatory B cytokine profile, yet may dampen inflammatory and cytokotoxic responses and induce CLL cell apoptosis (31, 39, 40).

The current results are of clinical interest because inhibiting the IFN-γ pathway may play a role in the future therapy of CLL. In this study, we impeded activation and proliferation of CLL cells in vivo with an anti–IFN-γ mAb and constrained the activation in vitro with the JAK1/2 inhibitor ruxolitinib. The current results suggest that interfering with the IFN-γ/JAK1, 2/T-bet/CD38 axis may be a promising strategy, with potential antiproliferative and proapoptotic effects. Of interest, two independent phase I/II or phase II trials with ruxolitinib for CLL patients have been started in 2014.

The effects of IFN-γ and IFN-α on CD38 expression in both B cells and CLL cells have been reported previously, but the mechanisms have remained undefined (41, 42). In this article, we identify two T-bet binding sites in the 5′ end of intron 1 of the CD38 gene. This region has emerged as a key regulatory region, containing a CpG island, potential binding sites for the transcription factors Sp1 and E47, as well as a retinoic acid response element responsible for the upregulation of CD38 in response to all-trans retinoic acid (8). Of note, the region also contains a single nucleotide polymorphism (184C > G, rs6449182) that is associated with poor prognosis and Richter syndrome transformation in CLL patients (9). Because IFN-α also triggers T-bet expression (43), it is likely that T-bet also controls IFN-α–induced CD38 expression.

CD38 can bind CD31 (PECAM-1); crosslinking of CD38 with agonistic Abs or its natural ligand CD31 could promote proliferation and survival of CLL cells (8). Moreover, CD38 is also an ectoenzyme that catalyzes the conversion of NAD into cADPR as well as conversion of NADP into NAADP. Both products are Ca2+-mobilizing messengers important for cell signaling (26). However, it is currently unclear what specific signals in CLL cells could be related to Ca2+ signaling and active egress from the lymphoid tissue.

CLL develops gradually through premalignant phases as monoclonal B cell lymphocytosis, a stage characterized by the same BCR as emergent CLL (44). Although T-bet expression has yet to be determined in MBL cells, earlier studies have found increased inflammatory cytokines in such patients (45). CLL cells as well as autoreactive B cells in autoimmune disease share anergic features, including BCR downregulation, deranged signaling pathways, and tendency for apoptosis (11, 46, 47). It has been suggested that both B cell types require Th cell help to negate apoptosis (5, 11, 48). In this respect, it is predicted that MBL B cells expressing autoreactive BCR would quickly undergo apoptosis in the absence of Th cell help. The availability of Th cell help, however, could allow MBL B cells to progress to CLL. Such chronic partnership and establishment of a progressively dominant T-bet–driven type 1 response could explain the associated inflammatory features described above.

Disclosures
The authors have no financial conflicts of interest.

References


