Disruption of in vivo Chronic Lymphocytic Leukemia Tumor–Microenvironment Interactions by Ibrutinib – Findings from an Investigator-Initiated Phase II Study

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Abstract

Purpose: Chronic lymphocytic leukemia (CLL) cells depend on microenvironmental interactions for proliferation and survival that are at least partially mediated through B-cell receptor (BCR) signaling. Ibrutinib, a Bruton tyrosine kinase inhibitor, disrupts BCR signaling and leads to the egress of tumor cells from the microenvironment. Although the on-target effects on CLL cells are well defined, the impact on the microenvironment is less well studied. We therefore sought to characterize the in vivo effects of ibrutinib on the tumor microenvironment.

Experimental Design: Patients received single-agent ibrutinib on an investigator-initiated phase II trial. Serial blood and tissue samples were collected pretreatment and during treatment. Changes in cytokine levels, cellular subsets, and microenvironmental interactions were assessed.

Results: Serum levels of key chemokines and inflammatory cytokines decreased significantly in patients on ibrutinib. Furthermore, ibrutinib treatment decreased circulating tumor cells and overall T-cell numbers. Most notably, a reduced frequency of the Th17 subset of CD4+ T cells was observed concurrent with reduced expression of activation markers and PD-1 on T cells. Consistent with direct inhibition of T cells, ibrutinib inhibited Th17 differentiation of murine CD4+ T cells in vitro. Finally, in the bone marrow microenvironment, we found that ibrutinib disaggregated the interactions of macrophages and CLL cells, inhibited secretion of CXCL13, and decreased the chemotraction of CLL cells.

Conclusions: In conjunction with inhibition of BCR signaling, these changes in the tumor microenvironment likely contribute to the antitumor activity of ibrutinib and may impact the efficacy of immunotherapeutic strategies in patients with CLL. Clin Cancer Res; 22(7); 1572–82. ©2015 AACR.

See related commentary by Bachireddy and Wu, p. 1547

Introduction

Chronic lymphocytic leukemia (CLL) cells depend on interactions with the microenvironment to such a degree that they may be considered “addicted to the host.” The lack of a defined oncogenic driver, thus the absence of “oncogene addiction,” and the apparent importance of B-cell receptor (BCR) signaling emphasize the importance of tumor–microenvironment interactions in the pathogenesis of CLL (1, 2). Without support from the microenvironment, modeled by culturing CLL cells together with monocyte-derived nurse-like cells (NLC) or on bone marrow stroma, CLL cells die in culture (3). Even in in vivo models, such as in the NSG CLL xenograft model, CLL cells depend on a functional microenvironment, in particular the presence of T cells (2, 4). Our group has previously shown that CLL cells in the lymph nodes have activated BCR and NF-κB signaling, resulting in their activation and proliferation. In contrast, circulating cells in the blood tend to be resting and quiescent (1). The recent clinical success of targeting kinases essential for BCR signaling with small-molecule inhibitors underscores the importance of this pathway. The Bruton tyrosine kinase (BTK) inhibitor ibrutinib and the PI3K inhibitor idelalisib have been shown to extend survival of patients with relapsed or refractory CLL and have obtained regulatory approval (5, 6). Notably, both ibrutinib and idelalisib, to varying degrees, also affect a number of signaling pathways and cell types that play a role in the tissue microenvironment (7).
Translational Relevance

The introduction of ibrutinib and other agents targeting the B-cell receptor (BCR) pathway has significantly improved the prognosis for patients with CLL. In addition to directly targeting the tumor cells, BCR-directed kinase inhibitors disrupt tumor–microenvironment interactions that are critical for CLL progression. Given that understanding changes in the tumor microenvironment is essential for discerning possible synergistic and antagonistic effects of future combination regimens, we sought to characterize the in vivo changes in the microenvironment during ibrutinib treatment. On ibrutinib, a global reduction of chemokine receptors and inflammatory cytokines was observed in patient serum and bone marrow supernatant. Furthermore, we found distinct changes in overall T-cell numbers, T-cell subsets, T-cell activation and pseudoexhaustion. In addition, interactions between tumor cells and macrophages decreased on treatment. These microenvironmental changes provide the basis for the design of future combination therapies that may target several pathways in both the CLL cell and the tumor microenvironment.

The interactions between CLL cells and microenvironmental cells are bidirectional. CLL cells secrete cytokines that attract accessory cells, such as macrophages and T cells, and modulate their antitumor activity (8–12). For instance, T cells from CLL patients have been shown to be in a pseudoexhausted state (13). In addition, subset changes in T cells have also been reported in patients treated with ibrutinib, BCR and NF-κB signaling pathways and inhibited proliferation (22), and, at least for persistent leukemic disease, does not predict inferior durability of response (27). However, continued therapy with ibrutinib is required, and some patients, especially those with adverse disease features may eventually develop resistance to ibrutinib (28).

Although on-target effects of ibrutinib on tumor cells are well characterized, the effects on the host microenvironment are less investigated. In vitro, ibrutinib inhibits the secretion of cytokines from activated T cells and through inhibition of ITK has been reported to exert a Th1-selective pressure in T lymphocytes (21, 29). Subsequently, mice with deleted ITK were shown to lack differentiation of Th17 T cells, as identified by intracellular IL17A (30). Thus, ibrutinib may affect T-cell subsets indirectly through alteration of the tumor cells and by direct inhibition of T-cell pathways. More recently, ibrutinib has been shown to interfere with Fc-receptor signaling, which reduced initiation of phagocytosis and NK-cell activation by rituximab-bound CLL cells in vitro (31, 32). Despite the data on ibrutinib-mediated effects in different cell systems in vitro, little is known about how ibrutinib, directly or indirectly, affects the composition of the tumor microenvironment in vivo. As the microenvironment is critical in the pathogenesis of CLL, studies of the in vivo effects of ibrutinib on the microenvironment are needed. We thus set forth to describe the impact of ibrutinib on the cells that shape the tumor microenvironment in patients with CLL enrolled on an investigator-initiated phase II trial of ibrutinib monotherapy (26).

Materials and Methods

Patients and clinical data

We report correlative analyses on 80 CLL patients treated on an investigator-initiated phase II study of single-agent ibrutinib (NCT01500733). Six additional patients on the study are not included in this analysis due to incomplete data. For most experiments, only a subgroup of the patients is represented due to availability of samples. The study was approved by the local ethics committee, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Briefly, both treatment-naïve and relapsed/refractory patients with either TP53 aberrations or age ≥65 years were eligible (Table 1) and were treated with ibrutinib 420 mg orally once daily until disease progression or the occurrence of intolerable side effects. Clinical baseline data and laboratory results were retrieved from electronic records at the Clinical Center, NIH (Bethesda, MD).

Bone marrow specimens

Bone marrow biopsies performed at prespecified time points for assessment of clinical response during treatment were evaluated by two independent hematopathologists. Immunohistochemistry (IHC) was performed for CD79a, CD3, and CD68 as part of standard pathology work up. The percentage of CD79a- and CD3-positive cells was recorded and the degree of CD68-positive macrophage extensions in contact with CLL cells were semiquantitatively assessed on a scale from 0 (no extensions) to 4 (max number of extensions). Microphotographs were performed with an Olympus BX51 microscope and Olympus DP72 camera (Olympus) at original enlargements as indicated in figures.

gress from the tissue sites, and persistent disease in blood, lymph nodes, and bone marrow is typically demonstrable even after years of single-agent therapy (23, 26, 27). This residual disease appears to be quiescent, with downregulation of intracellular signaling pathways and inhibited proliferation (22), and, at least for persistent leukemic disease, does not predict inferior durability of response (27). However, continued therapy with ibrutinib is required, and some patients, especially those with adverse disease features may eventually develop resistance to ibrutinib (28).

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Flow cytometry

Peripheral blood mononuclear cells (PBMC) were prepared by density-gradient centrifugation (Lymphocyte Separation Media; ICN Biomedicals). Viably frozen cell suspensions were analyzed on an LSRFortessa or a FACScanCanto II flow cytometer (BD Biosciences). Cell populations were gated on forward scatter and side scatter. For T-cell analyses, additional CD3, CD4, and CD8 gating was used with a minimum of 50,000 CD4+ T cell events being collected. For analysis of proliferation, intracellular staining for Ki67 was performed. In addition, surface staining for the activation markers HLA-DR and CD39 as well as PD-1 was performed (all antibodies from BD Biosciences). Cell populations were identified as previously described and expressed as percentages of the parent population (33). The human Th17 subset of CD4+ T cells was identified as CXCR3-negative and CCR6-positive (34).

Cytokine and chemokine measurements in serum and bone marrow supernatants

For cytokine measurements, serum samples and bone marrow supernatants were kept at –80°C until analysis. Serum samples were analyzed by the HCYT MAG-60K-PX38 (Millipore) assay and bone marrow samples by a HCYP MAG-62K custom designed assay (Millipore) according to instructions from the manufacturer with a Luminex 200 instrument (Luminex) for determination of individual cytokine concentrations. For serum samples, 36 cytokines were analyzed (all included in Supplementary Table S1 for completeness); only the 10 chemokines and 10 inflammatory cytokines are discussed in details.

In vitro murine Th17 differentiation

Murine CD4+ T cells were differentiated as previously described (30). Briefly, naïve CD4+ T cells from C57BL/6 mice were cocultured at a ratio of 1:5 with mitomycin-treated T-depleted splenocytes as antigen-presenting cells with 1 μg/ml anti-CD3 plus 3 μg/ml anti-CD28 and 20 ng/ml IL-6 (PeproTech), 5 ng/ml TGFβ1 (R&D Systems), 10 μg/ml of each anti-IL4, anti-IFNγ, and anti-IL12 antibodies (Bio X Cell). Ibrutinib (Selleckchem) was added to the culture medium at the indicated concentrations during the whole differentiation period. Murine Th17 cells were identified by intracellular staining for IL17A (eBioscience; ref. 30).

NLC cocultures

NLC were allowed to grow out from fresh CLL PBMCs for 2 weeks as previously described (3). Nonadherent cells were harvested and replated on autologous NLC at desired concentration. Ibrutinib at 1 μmol/L or control media were added. Viability of CLL and NLC cells was assessed with the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies) according to the manufacturer’s protocol.

Migration assays

PBMCs collected pretreatment were evaluated for their ability to migrate towards patient-matched bone marrow supernatant collected either pretreatment or on ibrutinib. Chemotaxis assays across polycarbonate Transwell inserts were performed as previously described (35). Pretreatment CLL PBMC cells (106) in AIM-V medium (Gibco) were transferred into the top chambers of Transwell culture insert (Corning Incorporated) with a diameter of 6.5 mm and a pore size of 5 μm. Filters were then placed onto wells containing medium (control) or a mixture of 50% medium and 50% bone marrow supernatant collected either pretreatment or after 8 weeks on ibrutinib. CLL PBMCs were allowed to migrate for 3 hours at 37°C. Migrated cells in the bottom chamber were then collected and counted in triplicates using AccuCount blank particles (Spherotech) to quantify the absolute number of input and migrated B and T cells stained with CD19 and CD3 as previously described (2). The migration index was determined by subtracting the medium control percentage migration from the chemoattractant percentage migration and normalizing.

Statistical analysis

Statistical comparisons were performed as Student paired t test except for cytokine levels, where Wilcoxon matched-pairs signed-rank test was used due to nonparametric data distribution, and dose–response effect, where two-way ANOVA was used (GraphPad Prism v. 6, GraphPad Software).

Results

Ibrutinib decreases serum levels of multiple inflammatory cytokines and chemokines

In this study, we focused on in vivo changes in tumor–microenvironment interactions during treatment of CLL with ibrutinib. We have previously reported the clinical results and correlative analyses from our investigator-initiated phase II single-agent ibrutinib trial, including the on-target effects of BTK inhibition in CLL cells (22, 23, 26). Of 86 patients enrolled, 80 are included in this analysis. In six patients, data or samples for analysis were not available due mostly to early discontinuation of study in these patients. Of the 80 patients included, 50 (62.5%) received ibrutinib as first-line therapy, and 47 (58.8%) had a TP53 aberration (either deletion of chromosomal arm 17p or TP53 mutation). For most experiments, only a subset of patients is represented due to availability of samples. The baseline characteristics of the patients are provided in Table 1.

The chemokines CCL3 and CCL4 are upregulated by BCR signaling in CLL cells (1, 36), and an on-target effect of ibrutinib is the rapid inhibition of expression and secretion of both chemokines (22, 37). In addition, Herman and colleagues previously showed that ibrutinib inhibits cytokine secretion from T cells in vitro (29). Here, we systematically measured an array of cytokines.
Ibrutinib alters the expression of inflammatory cytokines and chemokines in serum from patients with CLL. A, percent changes in the evaluated chemokines after eight weeks on ibrutinib treatment compared with pretreatment levels. B, percent changes in evaluated inflammatory cytokines after eight weeks on ibrutinib treatment compared with pretreatment levels. In both panels, median with interquartile range is shown. See Supplementary Table S1 for a complete list of tested cytokines/chemokines and changes over time. Statistical analyses were by Wilcoxon matched-pairs signed-rank test. Asterisks indicate degree of statistical significance compared with pretreatment levels: *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001.

Ibrutinib decreases cell numbers, activation, proliferation, and pseudoehaustion of T cells

At the time of treatment initiation, most patients had elevated circulating T-cell numbers (CD3⁺; Fig. 2A). Upon ibrutinib treatment, a significant decrease in the number of circulating T cells was observed at 24 weeks with normalization of T-cell counts in most patients by 48 weeks (Fig. 2A; P < 0.0001). A significant decrease in T-cell counts was seen for treatment naïve as well as relapsed or refractory patients, and the initial T-cell counts were in the same range for these two patient groups (Fig. 2B). A similar decrease in T-cell numbers was seen in the bone marrow (Fig. 2C; P < 0.05). The reduction in T-cell numbers paralleled the progressive decrease in CD19⁺ tumor cells in circulation (Supplementary Fig. S2A) and in the bone marrow (Supplementary Figs. S2B and S2C). We next evaluated changes in CD4⁺ and CD8⁺ T-cell populations. For most patients, both CD4⁺ and CD8⁺ T-cell numbers were elevated at baseline and normalized upon ibrutinib treatment (Figs. 2D and E; P < 0.0001). Interestingly, we noted that patients with an unmutated IGHV displayed a significantly larger reduction in all T-cell populations (data not shown). The CD4/CD8 ratio in peripheral blood remained stable over the course of ibrutinib treatment (Supplementary Fig. S3A). Similarly, the CD4/CD8 ratio in the bone marrow remained constant on ibrutinib (Supplementary Fig. S3B-D).

It has previously been shown that CLL cells cause chronic activation of T cells leading to their exhaustion (13). Therefore, we next evaluated whether ibrutinib affects activation and pseudoehaustion of T cells. The proportion of CD4⁺ T cells that expressed HLA-DR or CD39, two commonly used activation markers, decreased on ibrutinib treatment (Fig. 2F; n = 10, P < 0.01). The proportion of CD8⁺ cells expressing HLA-DR was also decreased during treatment (Fig. 2F; n = 10, P < 0.01). Furthermore, proliferation of activated T cells (HLA-DR⁺), as indicated by the expression of Ki67, decreased as early as eight weeks on treatment (Fig. 2G; n = 8, P = 0.02). Finally, as a measure of pseudoehaustion, we evaluated the expression of PD-1 on T cells (13). In vivo, PD-1 expression on T cells decreased with time on treatment, with a significant decrease as early as at four weeks and a further decrease by eight weeks of treatment (Fig. 2H; n = 10, P < 0.01). Taken together, T-cell activation, proliferation, and pseudoehaustion decreased during treatment with ibrutinib.

Ibrutinib treatment reduces Th17 T cells and inhibits in vitro differentiation of Th17 cells

Dubovsky and colleagues reported that ibrutinib irreversibly inhibits ITK and drives a Th1-selective pressure in T lymphocytes (21). Furthermore, ITK knockout mice show a striking decrease in Th17 differentiation (30). Whether ibrutinib affects Th17 differentiation has not been reported. We therefore evaluated the effect
Figure 2. Ibrutinib decreases T-cell numbers and T-cell activation and reverses pseudoexhaustion. A, absolute CD3+ T-cell counts in the peripheral blood. The normal range (615–2348/μL) is indicated by the right-sided gray bar. Only patients (n = 78) with data available both pretreatment (PRE) and at 24 weeks (24W) were included; of these, 47 had reached 48 weeks of treatment (48W). (Continued on the following page.)
Ibrutinib Disrupts CLL Tumor-Microenvironment Interactions

Figure 3.
Ibrutinib treatment leads to a reduction in the Th17 T-cell subset and inhibits in vitro differentiation of murine Th17 cells. A, percentage of Th17 cells (CXCR3\(^{\text{neg}}\) and CCR6\(^{\text{+}}\)) of total CD4\(^{\text{+}}\) T cells pretreatment (PRE) and after 24 weeks of ibrutinib treatment (24W), \(n = 10\). Each symbol represents a patient; line represents mean. B, influence of ibrutinib on Th17 differentiation in vivo and reduces the number of Th17 cells in CLL patients in vivo through ITK-dependent and/or independent pathways.

Ibrutinib treatment disrupts macrophage–CLL interaction in bone marrow

Tumor-associated macrophages are recognized as important components of the tumor-supporting microenvironment. Monocyte-derived NLCs that grow out from CLL peripheral blood samples in culture support the survival of CLL cells in vitro (18). This prosurvival effect of NLCs, and by extension of

of ibrutinib on Th17 cells in the blood and found a mean decrease of 22% in the frequency of CD4\(^{+}\) Th17 cells (CXCR3\(^{\text{neg}}\) and CCR6\(^{\text{+}}\); ref. 34) after 24 weeks of ibrutinib treatment (Fig. 3A; \(n = 10\), \(P < 0.04\)). Consistently, reduced proliferation of Th17 cells, as reflected in a mean decrease in Ki67-positive cells of 25%, was detected in the blood already at eight weeks from treatment start (Fig. 3B; \(n = 8\), \(P < 0.05\)). Next, we chose a well-established Th17 differentiation assay using murine CD4\(^{+}\) T cells upon in vitro ibrutinib. Each symbol represents an individual experiment; line represents mean. Drug effect assessed by two-way ANOVA analysis (\(P = 0.005\)); statistical analyses (A and B) were determined by Student paired \(t\) test.
macrophages, appears to be dependent on direct cell–cell contact. Cell–cell contact between CLL cells and macrophages in the bone marrow has been reported and is thought to play a pathogenic role (12, 17). Notably, the CLL cell–derived cytokines CCL3 and CCL4 are potent chemoattractants that could recruit macrophages to CLL infiltrates in the bone marrow. As these chemokines rapidly decrease on ibrutinib, as shown here and reported by others (2, 37), we sought to assess changes in the interactions between CLL cells and macrophages during treatment.

We evaluated bone marrow specimens for macrophages by IHC. As previously reported, macrophages (CD68+) were interspersed among CLL cells in the bone marrow (12) and remained in this distribution on ibrutinib treatment (Fig. 4A and B). However, the dotted appearance of CD68+ cellular extensions in close proximity to CLL cells disappeared during ibrutinib treatment (Fig. 4A and B, insets; schematically depicted in Fig. 4C). These findings were semiquantitatively graded on serial bone marrow biopsies during ibrutinib treatment (Supplementary Figs. S4A–S4E for grading key). A significant decrease was seen over time (Fig. 4D; P < 0.001). To further understand the in vivo disruption of CLL–macrophage interactions in the bone marrow, we utilized an in vitro system of CLL cells cocultured with autologous NLCs (3, 18). The viability of CLL cells but not NLCs was significantly reduced with the in vitro addition of ibrutinib to this coculture system (Fig. 4E; n = 5, P < 0.001). Thus, ibrutinib disrupts the tight association of CLL cells and macrophages in vivo and inhibits the supportive effect of NLCs in vitro without directly affecting the survival of the microenvironmental cells.

Ibrutinib decreases CXCL13 and CLL cell migration toward bone marrow supernatant

We next evaluated changes in chemokines secreted by accessory cells in the supernatant of bone marrow aspirates pretreatment and on ibrutinib. Of all the tested chemokines (Supplementary Table S2), CXCL13, secreted predominantly by macrophages (41), showed the most significant and sustained decrease in the bone marrow supernatant obtained from patients on ibrutinib.
compared with matching pretreatment samples (Fig. 5A; n = 11, P < .01). IL16 levels were also significantly decreased on ibrutinib, while CXCL12 levels increased in a time-dependent manner. The latter perhaps reflects the decrease of tumor infiltration and consequently less internalization of the chemokine by CLL cells. Supplementary Figure S5A–SSD shows the absolute levels pretreatment, at eight weeks, and at 24 weeks on ibrutinib for the evaluated chemokines. To evaluate the impact of the chemokine content in bone marrow on CLL cells, we analyzed the migration of CLL cells collected pretreatment towards autologous bone marrow supernatant collected either at baseline or after eight weeks of ibrutinib treatment. For all patients evaluated, a decreased migratory response was seen for bone marrow supernatant collected during ibrutinib treatment as compared with paired baseline samples (Fig. 5B; n = 8, P = 0.007).

**Discussion**

CLL cells critically depend on signals from the tissue microenvironment, while simultaneously altering their surrounding microenvironment, through both the secretion of cytokines/chemokines and direct cell–cell interactions. These effects create a microenvironment favorable to tumor growth by coopting macrophages and T cells and inhibiting antitumor immune responses (8, 12). Consequently, targeting tumor–microenvironment interactions is a promising area for therapeutic intervention, yet few agents are available to specifically target such pathways. With more widespread use of BCR inhibitors, it is becoming clear that these agents, directly and/or indirectly, affect not only the tumor cells but also more broadly impair the immune microenvironment. This may have important consequences for putative combination therapies and the safety of long-term administration of such agents (21, 31, 32, 42). Here we aimed to characterize the extent of these changes by systematically assessing the effect of ibrutinib on (i) cytokines and chemokines secreted in the peripheral blood and bone marrow, (ii) the subset distribution and activation state of T cells, and (iii) the cellular composition of the bone marrow microenvironment in patients with CLL (summarized in Fig. 6).

Notably, the levels of most chemokines and inflammatory cytokines decreased during treatment with ibrutinib. Several of the chemokines having reduced serum levels in patients treated with ibrutinib, including IL8, CCL3, CCL4, and CCL22, are secreted by CLL cells in response to, among others, BCR and CD40 signaling (10, 37). Thus, the decrease in the serum levels of these chemokines is considered an on-target effect of BCR inhibitors (22). Other chemokines that decreased upon ibrutinib treatment may be primarily secreted by cells in the microenvironment, including CXCL10, the levels of which correlate with time to the treatment of disease, and CCL2, which enhances the viability of CLL cells in vitro (15, 16). The decrease in chemokine secretion from host cells in the tissue microenvironment may be a consequence of the treatment-induced changes in the CLL cells. For example, the serum levels of IL8 and IL10, which are secreted by monocytes exposed to CLL-conditioned medium in vitro, decreased during treatment (11). However, ibrutinib may also inhibit secretion of cytokines through a direct effect on immune cells other than CLL cells, as exemplified by the decreased secretion of TNFα and IL10 from activated T cells treated with ibrutinib in vitro (29). Furthermore, some effects on the microenvironment may be due to inhibition of kinases other than BTK, in particular ITK, and possibly TEC by ibrutinib (20, 21, 43). Other BTK inhibitors in preclinical and clinical development, for example, CC-292 and ACP-196, may more selectively inhibit BTK and thus be expected to have a partially different impact on microenvironmental cells.

Chronic activation of T cells decreased during ibrutinib treatment as demonstrated by reduced expression of the activation markers HLA-DR and CD39 and a decrease in T-cell derived cytokines such as TNFα and IL10. T-cell inactivation and cytokine changes during ibrutinib treatment are significant because cancer in general, and CLL in particular, is associated with a chronic inflammatory drive (44). A decrease of PD-1 surface expression was also demonstrated that, along with decreased TNFα levels, indicates the potential normalization of the T-cell exhaustion previously described as a hallmark of CLL (13). Notably, ibrutinib can also alter the composition of T-cell subsets by exerting a Th1-selective pressure as described previously (21) and inhibit differentiation of the Th17 T-cell subset as shown here. The latter effect is in line with data from ITK knockout mice in which Th17 differentiation is impaired (30). We confirmed that ibrutinib inhibits Th17 differentiation in vitro, choosing a murine system
because of the more established nature of the assay. The observed shifts in T-cell subsets, based on direct and indirect effects, and the decrease in chronic T-cell activation could have clinical implications as they may contribute to the reported efficacy of ibrutinib in graft-versus-host disease (45). In addition, these T-cell effects could enhance T-cell–mediated antitumor responses, especially in conjunction with intratumoral CpG injection or PD-1 blockade (42, 46). Furthermore, a skewing of T cells towards Th17 subset differentiation in CLL may reflect disruption of a protective immune response and increased autoimmunity (14), the T-cell changes described here may indicate a normalized T-cell state. Macrophages play a pivotal role for CLL cells in the microenvironment. This is exemplified by the ability of macrophage-like NLCs to extend survival of CLL cells in vitro cultures (3, 18) and the importance of macrophages for the development of leukemia in the TCL-1 mouse model (47). CD68+ macrophages are found in CLL infiltrates in the bone marrow and are thought to be recruited by CLL cell–derived cytokines such as CCL3 and CCL4 (12, 17). The reduction in macrophage pseudopodia interacting with CLL cells despite the persistence of both cell types in the bone marrow, supports the conclusion that ibrutinib disrupts tumor–microenvironmental interactions rather than the viability of the microenvironmental cells. Consistently, in vitro CLL cell death increased with ibrutinib treatment even in the presence of NLCs, while NLC viability was unaffected. As macrophage differentiation is reported to be dependent on both BTK and TEC kinases (48), it is likely that disruption of macrophage/NLC support of CLL cells is, at least in part, mediated through direct effects of ibrutinib. In addition to changes in cell–cell contact, levels of macrophage-derived CXCL13, also known as B-cell–attracting chemokine 1 (41), decreased in the supernatant from bone marrow aspirates upon ibrutinib treatment. CXCL13 has been reported to exert chemoattractant effects on CLL cells through the CXCR5 chemokine receptor (8, 49). Consistent with these observations, migration of CLL cells towards bone marrow supernatant collected during ibrutinib therapy decreased compared with pretreatment samples. Thus, ibrutinib disrupts direct cell–cell interactions as well as secretion of soluble factors from macrophages. CLL cells shape their microenvironment in ways that support tumor growth and inhibit antitumor T-cell responses into a more resting state.
concurrent inhibition of microenvironmental support. For example, changes in tumor-associated macrophages in vitro and the inhibitory effects in coculture experiments with NLCs in vitro (37) suggest that ibrutinib may be sufficient to abrogate the supporting role of macrophages in the pathogenesis of CLL. On the other hand, the effects exerted by ibrutinib may have a major impact on other therapies. For example, cellular effector functions of mAbs are inhibited by ibrutinib (31, 32), raising the question whether such combinations are worthwhile. In contrast, ibrutinib may improve T-cell function and could thereby enhance immunotherapeutic strategies such as chimeric antigen receptor T-cell therapy, bispecific antibodies, or immune checkpoint inhibitors. Although further studies are necessary to detail exactly how ibrutinib modulates the microenvironment, our data provide an overview of changes occurring in the tumor microenvironment that can inform the rationale design of combination therapy. The implications of these findings, especially on duration or extent of response, should be evaluated in future studies.

Disclosure of Potential Conflicts of Interest

C.U. Niemann is a consultant/advisory board member for Gilead, Janssen, and Roche. A. Wiestner reports receiving commercial research grants from and is a consultant/advisory board member for Pharmacyclics. No potential conflicts of interest were disclosed by the other authors.

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Clinical Cancer Research

Disruption of in vivo Chronic Lymphocytic Leukemia Tumor – Microenvironment Interactions by Ibrutinib – Findings from an Investigator-Initiated Phase II Study

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