Ibrutinib as an antitumor immunomodulator in patients with refractory chronic lymphocytic leukemia

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ABSTRACT
Ibrutinib has emerged as a promising therapy for patients with chronic lymphocytic leukemia (CLL) who are nonresponsive to standard therapies. The refractory state of monocytes and T-cell exhaustion in patients with CLL could explain the morbidity and mortality reported in these patients. We studied the effect of ibrutinib on the immune response of four relapsed patients with CLL during the first treatment cycle. We observed the ability to recover the standard response against bacterial stimulus in CD14+ cells, improving levels of phospho-Erk1/2 and antigen presentation. Meanwhile, ibrutinib drove Th1-selective adaptive immune response, which might help to decrease infectious complications. The potential effect of ibrutinib on CLL patient outcomes is worthy of further study, because infections could be reduced with the use of ibrutinib.

Introduction
Patients with chronic lymphocytic leukemia (CLL) are known to have a high risk of infections. The impaired immune response in these patients could help explain the associated morbidity and mortality. Indeed, patients with CLL are reportedly locked into a refractory state of the innate immune response in patients with CLL could explain the morbidity and mortality reported in these patients. We studied the effect of ibrutinib on the immune response of four relapsed patients with CLL during the first treatment cycle. We observed the ability to recover the standard response against bacterial stimulus in CD14+ cells, improving levels of phospho-Erk1/2 and antigen presentation. Meanwhile, ibrutinib drove Th1-selective adaptive immune response, which might help to decrease infectious complications. The potential effect of ibrutinib on CLL patient outcomes is worthy of further study, because infections could be reduced with the use of ibrutinib.

Results and discussion
Our previous work demonstrated that monocytes from patients with untreated CLL exhibited a refractory state in response to an endotoxin such as LPS. We therefore studied the inflammatory capacity of ibrutinib-treated patients. After LPS challenge, CLL monocytes produced significantly lower levels of TNFα, IL-1β, and IL-6 and enhanced levels of CCL2 in comparison with healthy controls; however, the therapy gradually reversed this phenotype (Fig. 1A). Additionally, phagocytic activity was high at baseline, similarly to that previously reported for a refractory state; it decreased during therapy, however, accompanied by low CD163 expression on the CD14+ cells (Fig. 1B). Levels of phospho-ERK1/2 proteins, crucial in the BTK pathway, and members of an NFκB signal transduction in CD14+ cells increased during treatment to reach levels similar to the controls (Fig. 1C). In contrast, the B cells showed an inhibition of phosphorylation after ibrutinib therapy (data not shown). Note that, due to the limited number of isolated CD14+ cells, this assay was performed on only two patients.

Collectively, our findings suggest that BTK is not essential for LPS-induced activation of the NFκB pathway. The activity of BTK-null monocytes from patients with X-linked agammaglobulinemia in response to LPS is reportedly not affected. In fact, ibrutinib inhibits NFκB signaling in B cells, offering a possible explanation for the various ERK phosphorylations.
between B and CD14+ cells.\textsuperscript{7} In agreement with these data, we have reported that the refractory state is a result of malignant B cell contact with monocytes; ibrutinib reduced malignant B cell counts (data not shown), which could explain the observed refractory state reduction.\textsuperscript{1}

To assess the functional capability of antigen presentation by CD14+ cells, we first analyzed the MHC II isotype HLA-DR, followed by an antigen presentation assay with tetanus toxin antigen. On the one hand, upregulation of HLA-DR was patent in LPS-stimulated CD14+ cells after therapy (Fig. 1D). On the other hand, CD4+ T-cell-specific proliferation was observed when T cells were co-cultured with CD14+ cells exposed to tetanus toxin (Fig. 1E). These data suggest that CD14+ cells from untreated patients exhibit a reduced ability to act as antigen-presenting cells.\textsuperscript{8} After therapy, however, CD14+ cells acquired an antigen-presenting ability, indicating that ibrutinib is a
possible immunomodulator that facilitates a switch to the immune adaptive response.

We also evaluated the reversion of T-cell exhaustion by analyzing their basal proliferation in T-cell subsets using a CFSE T-cell proliferation assay with PBMCs at 10 d and 30 d, stimulated with autologous tumor cells isolated at baseline. Our data showed enhanced proliferation of CD4\(^+\) and CD8\(^+\) cells at basal conditions and when they were exposed to their specific CLL tumor cells (Fig. 2A). Due to one of the patients not having enough circulating malignant B cells at baseline to perform this assay, Fig. 2A shows data from three patients. These results indicate an immunomodulation of the switch from the innate to the adaptive immune response, accompanied by the specific antitumor activity. We corroborated the tumor-specific cytotoxicity of PBMCs isolated from the patients (Fig. 2B).

A relapsed CLL patient cohort treated with ibrutinib has also been reported to show a correlation with a reduced number of viral infections during the first 6 mo of treatment.\(^4\) We explored the differentiation of T cells toward Th1 by analyzing the specific transcription factor T-bet, which is involved in Th1...

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Figure 2. Ibrutinib reduces T-cell exhaustion and PD-1/PD-L1 co-inhibitory axis in patients with CLL. T-cells were isolated from patients with CLL (n = 4) at baseline, 10 d and 30 d after treatment initiation. (A) T-cell CFSE proliferation at day 4 was evaluated in basal conditions and with autologous tumor cells (ratio 1:1). Proliferation percentages of CD4\(^+\) and CD8\(^+\) cells are shown, CLL (n = 3). (B) PBMC’s cytotoxic activity by europium-TDA release assay after 3 h of incubation with autologous tumor cells as target cells with specific ratios. Percentages of lysis are shown, CLL (n = 4). (C) Expression of T-bet on CD4\(^+\) cells. Percentage of distribution is shown, CLL (n = 4), HV (n = 5). (D) PD-1\(^+\) expression on CD4\(^+\) gated cells. Percentage of distribution is shown, CLL (n = 4), HV (n = 5). (E) PD-L1\(^+\) expression on CD4\(^+\) gated cells. Percentage of distribution is shown. *p < 0.05, **p < 0.01, ***p < 0.001, always compared with T = 0 (baseline), using the Mann–Whitney test.
development, between the innate and adaptive immune responses.9 We observed high T-bet expression on CD4+ T cells, suggesting an effect of ibrutinib on CD4+ T-cell differentiation (Fig. 2C). Note that ibrutinib has been shown to have an inhibitory role in ITK driving, a Th1-selective pressure in T lymphocytes, as demonstrated in CD4+ T cells pretreated with ibrutinib.10 In contrast, we did not observe differences in T-bet expression in CD8+ T cells (data not shown). The development of CD4+ and CD8+ T cells in ITK-deficient mice has been reported to be regulated by other pathways.11

T-cell exhaustion in patients with CLL is well explained by a T-cell dysfunction.2,12,13 This dysfunction has been associated with the expression of the programmed cell death protein 1 (PD-1) from patients with CLL,14 which co-inhibits the immune response.12 Our findings showed decreased PD-1 expression on CD4+ T cells after ibrutinib treatment (Fig. 2D). Accordingly, a novel study has shown that ibrutinib is able to reverse the exhaustion of CD8+ T lymphocytes through the decrease of PD1 expression, consequently enhancing the efficacy of T-cell chimeric antigen receptor engraftment.5 The PD-1 ligand (PD-L1) expression on the CD14+ subset also exerts immunosuppressive effects through binding to PD-1 on T cells, which negatively regulates T-cell activation.12 We observed a gradual decrease in PD-L1 expression on CD14+ cells (Fig. 2E) and on CD20+ cells (Fig. 2F).15

Despite the small ibrutinib-treated cohort studied, our brief report shows promising effects on the immune response after treatment initiation. Based on our findings, we can speculate that the innate immune response improves after the malignant B cells decrease in circulation. We have previously reported that monocytes in contact with malignant B cells induced a refractory state in nontreated CLL.1 On the other hand, the reduced PD1 expression on T cells during treatment could improve the adaptive immune response. Collectively, these data suggest that ibrutinib modulates the innate and adaptive immune response, showing a valuable immunomodulator effect, which needs further investigation in a large clinical trial. In addition, it is important to perform a complete study to verify role of ITK because some authors have suggested an ITK inhibition by ibrutinib.10

In summary, ibrutinib showed an effect in terms of decreasing the refractory state in monocytes and T-cell exhaustion accompanied by PD-1 and PD-L1. The reported data suggest that the antitumor activity of ibrutinib is not disrupted and that ibrutinib reduces the immunosuppressive status in patients with relapsed CLL. The highly active therapeutic effects of ibrutinib offer clinically promising outcomes for patients suffering from refractory CLL.

Materials and methods

Clinical trial design and patient population

Of 49 participants worldwide, 4 patients included in the phase I clinical trial PCI-32765LYM1003 were analyzed at our center. The minimum follow-up of the four patients with refractory CLL was 3 mo after ibrutinib initiation. In the first response assessment at 3 mo of treatment, patients 1, 2, and 3 achieved a partial response, according to the International Workshop on Chronic Lymphocytic Leukemia criteria. Patient 4 had stable disease.

Peripheral blood samples from the four patients with relapsed/refractory CLL were collected from December 2014 to December 2015 (see patient demographics, Table 1). The patients provided signed informed consent to participate in this biological study. The Institutional Review Board of La Paz University Hospital approved the study, which was conducted according to principles of Good Clinical Practice. All the four patients fulfilled the inclusion criteria to enroll in the PCI-32765LYM1003 clinical trial, thus, receiving demonstrated effective doses of ibrutinib. The median age was 70.5 ± 11.8 y. The median number of previous chemotherapy lines was 3. All the patients were refractory to the last salvage chemotherapy. Blood samples from all the patients were obtained at the following time points: baseline (before treatment), and at 10 d and 30 d after the initiation of ibrutinib therapy. Five sex- and age-matched control subjects were included; these healthy volunteers had no history of CLL or any other significant illness.

Flow cytometry and measurement of cytokines in peripheral blood monocytes by cytometric bead array

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using Ficoll-Histopaque Plus and were later stained with specific antibodies (CD14, CD163, HLA-DR, CD4+, CD8+, T-Bet, PD1, PD-L1, and CD20) from BD

Table 1. Characteristics and clinical status of ibrutinib initiation outcomes in patients with CLL. B2M: β-2 microglobulin limit of normal, 2 mg/L; LDH: lactate dehydrogenase, limit of normal (234 U/L). Previous lines correspond to number of previous chemotherapy lines.

| Patient | Patient 2 | Patient 3 | Patient 4 |
|---------|-----------|-----------|-----------|
| Age     | 74        | 72        | 54        | 82        |
| Sex     | Female    | Male      | Female    | Male      |
| Time from diagnosis | 6.4 y | 1.6 y | 4.2 y | 3.1 y |
| Binet stage | C      | C         | C         | Del11q   |
| Cytogenetics | +12     | Normal    | +12       | 515       |
| LDH     | 865       | 543       | 515       | 746       |
| B2M     | 2.81      | 4.71      | 5.16      | 6.36      |
| Previous lines | 4 (BR, R-Chl, ofatumumab, Obinutuzumab, Lidelalisib) | 2 (FCR, BR) | 3 (FCR, Bendamustine, Ofa-Benda, Obinutuzumab) | 5 (FCR, BR, aloSCT, DLI) |
| Leukocyte counts at inclusion cells × 10⁶/L of blood | 3.87 | 21.55 | 134.9 | 2.01 |
| Lymphocyte counts at inclusion cells × 10⁶/L of blood | 2.1 | 18.3 | 132.2 | 0.4 |
| Response at 3 mo of ibrutinib treatment | Partial response | Partial response | Partial response | Stable disease |

B2M, β-2 microglobulin limit of normal: 2 mg/L; LDH, lactate dehydrogenase, limit of normal (234 U/L). BR, Bendamustine + Rituximab; R-Chl, Rituximab + Chlorambucil; FCR, Fludarabine + Cyclophosphamide + Rituximab; Ofa-Benda, Ofatumumab + Bendamustine; aloSCT, allogeneic stem cell transplantation; DLI, donor lymphocyte infusion.
Immunoblot analysis

Expression of phospho-ERK1/2 was evaluated by western blot (BD FACSCalibur), and the collected data were analyzed using CellQuest Pro.

T-cell proliferation assays

T-cell proliferation was evaluated using a CFSE manufacturer’s protocol with PBMCs at 10 d and 30 d, stimulated with tetanus toxin (0.005 and 0.0025 U/mL) or autologous tumor cells isolated at baseline ratio (1:1) for 4 d. Cells were acquired by flow cytometry (BD FACSCalibur), and the collected data were analyzed using CellQuest Pro.

Cytotoxicity and phagocytic assay

PBMC cytotoxicity was monitored using a conventional 2 h europium-TDA release assay (PerkinElmer). The primary PBMCs were used as effector cells. The TDA-labeled tumor cell was used for target cells at effector-to-target cell (E:T) ratios of 8:1, 4:1, 2:1, and 1:1. Phagocytic ability was studied, following a previously described protocol.

Statistics

Statistical significance was calculated using the Mann–Whitney test. The differences were considered significant at p < 0.05 and the analyses were conducted using Prism 5.0 software (Graph Pad, USA).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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