Effect of the BTK inhibitor ibrutinib on macrophage- and γδ T cell-mediated response against Mycobacterium tuberculosis

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The Bruton’s tyrosine kinase (BTK) inhibitor ibrutinib is approved by the Food and Drug Administration for its use as first-line treatment in chronic lymphocytic leukemia (CLL). Despite its efficacy, patients treated with ibrutinib rarely achieve complete responses and usually remain under treatment until progression. Considering the inherent high susceptibility of CLL patients to infections, a better understanding of ibrutinib effects on the immune system might help to estimate the risk of infectious complications on treated patients. Besides its effects on leukemic B cells, ibrutinib also affects functions on T cells, natural killer cells, and macrophages1–3. Macrophages are central players of the innate immune response against fungi, extracellular bacteria, and in particular against the intracellular bacteria Mycobacterium tuberculosis (Mtb). The World Health Organization (WHO) estimates that one-third of the world’s population is infected with Mtb, the causing agent of tuberculosis, a severe infection that kills near 1.3 million people per year and the leading cause of death from a single infectious agent (Global TB report, WHO, 2017). Notably, the incidence rate of tuberculosis kills near 1.3 million people per year and the leading cause of death from a single infectious agent (Global TB report, WHO, 2017). Notably, the incidence rate of tuberculosis incidence are up to 14 times higher than in USA and other developed countries. Given the relevance of macrophages in Mtb immune response, we here evaluated the in vitro effects of ibrutinib on this cell compartment. Additionally, we studied its effects on γδ T cells, another innate immune component reported to be involved in Mtb response5. Strikingly, we found that ibrutinib affects macrophage’s phenotype and the response of both macrophages and γδ T cells to Mtb.

Macrophages were differentiated from human monocytes with macrophage colony-stimulating factor (M-CSF), then pre-treated with ibrutinib for 30 min, and afterward exposed to irradiated Mtb for 24 h. We found that clinically relevant doses of ibrutinib (0.03–0.3 µM) significantly reduced the release of tumor necrosis factor (TNF)-α (Fig. 1a and Supplementary Fig. 1a), while interleukin (IL)-10 and IL-8 secretion was only affected at 3 µM, which is a concentration higher than the one reported in the plasma of treated patients (Fig. 1b, c). Importantly, macrophage viability was not affected by ibrutinib (Supplementary Fig. 1b). Inhibition of TNF-α secretion was associated with a diminished phosphorylation of the p65 subunit of the transcription factor nuclear factor (NF)-κB (Fig. 1d), a key regulator of cytokine production in macrophages. Given the relevance of Toll-like receptor (TLR) 2 and TLR4 in Mtb recognition by macrophages, we evaluated the effect of ibrutinib on TNF-α secretion in response to lipopolysaccharide (LPS), a TLR4 ligand, and Pam3CSK4, a TLR2 ligand. Results in Fig. 1e show that ibrutinib impaired TNF-α secretion induced by...
Fig. 1 Ibrutinib impairs macrophage-mediated response against *Mycobacterium tuberculosis*. Macrophages were obtained by culturing monocytes from healthy donors (HD) or CLL patients for 5 days in RPMI 10% FCS in the presence of M-CSF (50 ng/ml). a–c HD-macrophages were stimulated with irradiated *Mtb* (MOI equivalent to 2) in the presence or absence of ibrutinib (Ibru) and after 24 h TNF-α, IL-8, and IL-10 secretion was measured by ELISA in culture supernatants. Bars represent mean ± SEM of cytokine concentration in control (ct.) cultures (white bars) or *Mtb*-stimulated cultures (gray bars). *n* = 10, *p* < 0.05, Kruskal–Wallis test, followed by Dunn’s multiple comparisons test. d Phosphorylation of p65 (p-p65) was evaluated by western blot in HD macrophages after 15 min of stimulation with *Mtb* in the presence or absence of ibrutinib. Bands on the immunoblots were quantified using the ImageJ software (NIH Image). Results are shown as the mean ± SEM of the ratio p-p65/β-actin in arbitrary units (A.U.). *n* = 6, *p* < 0.05, Friedman test, followed by Dunn’s multiple comparisons test. e HD macrophages were stimulated with LPS (100 ng/ml) or Pam3CSK4 (100 ng/ml) in the presence or absence of ibrutinib for 24 h and TNF-α production was measured by ELISA. *n* = 10, *p* < 0.05, Kruskal–Wallis test, followed by Dunn’s multiple comparisons test. f CLL macrophages were stimulated with irradiated *Mtb* (MOI equivalent to 2), LPS (100 ng/ml), or Pam3CSK4 (100 ng/ml) in the presence or absence of ibrutinib for 24 h and TNF-α production was measured by ELISA in culture supernatants. *n* = 7, *p* < 0.05, Kruskal–Wallis test, followed by Dunn’s multiple comparisons test.
these ligands. Of note, the inhibition in response to TLR2, but not to TLR4, stimulation was observed even at low concentrations of ibrutinib (30 nM) (Supplementary Fig. 1c, d), suggesting a differential involvement of BTK, or other ibrutinib targets, in TLR2 and TLR4 signaling pathways. Inhibition of TNF-α secretion in response to Mtb, Pam3CSK4, and LPS was also observed in ibrutinib-treated monocyte-derived macrophages from CLL patients (Fig. 1f). Clinical characteristics of CLL patients included in this study are presented in Supplementary Table 1.

Then, given that macrophages with different polarization profiles have different abilities to promote an efficient immune response against Mtb, the M1 pro-inflammatory profile being more effective than the M2 anti-inflammatory profile, we aimed to evaluate the effect of ibrutinib on macrophage polarization in interferon (IFN)-γ-induced M1, IL4-induced M2, and IL-10-induced M2 profiles. M1 and M2 polarization was confirmed by analyzing the expression of CD16, CD14, CD163, CD206, CD86, and HLA-DR by flow cytometry (Supplementary Fig. 2). We found that ibrutinib impaired M1 polarization as shown by the upregulation of M2-associated markers CD16, CD14, CD163, and CD206 and by downregulation of the M1-associated markers CD86 and HLA-DR (Fig. 2a, b). The expression of these markers was not modified by ibrutinib on IL-4- and IL-10-induced M2 macrophages (Supplementary Fig. 3). These results are in line with those recently published by Fiocari et al. showing that ibrutinib promotes an M2 phenotype in nurse-like cells from CLL patients. Impairment in M1 polarization, induced upon ibrutinib exposure, was not associated with a decrease in signal transducer and activator of transcription factor (STAT) 1 phosphorylation (Supplementary Fig. 4). Interestingly, we found that ibrutinib enhanced macrophage three-dimensional migration in Matrigel (Fig. 2c), a feature of macrophage with an M2 profile. We also found that M1 macrophages polarized in the presence of ibrutinib showed a decreased secretion of TNF-α and an increased secretion of IL-10 compared to control M1 macrophages, resembling the cytokine profile associated with M2 macrophages (Fig. 2d). M1 and M2 macrophages also differ in their glucose metabolic pathways. During M1 polarization, macrophages activate the aerobic glycolytic pathway, increasing glucose uptake and lactate production, while M2 macrophages preferentially use the oxidative metabolism to obtain energy. Importantly, this switch towards aerobic glycolysis seems to be necessary for an effective differentiation into the M1 profile. We found that ibrutinib treatment of M1 macrophages reduced both glucose consumption and lactate production (Fig. 2e). The impairment in M1 polarization, and the reduction in glucose consumption and lactate production induced by ibrutinib were also confirmed in macrophages from CLL patients (Supplementary Fig. 5). We also evaluated whether ibrutinib affects the functionality of polarized macrophages and found that ibrutinib decreased TNF-α production in response to Mtb and increased migration in matrigel in M1 macrophages (Supplementary Fig. 6). Taken together, these results showed that ibrutinib affects M1 polarization of macrophage and their function, which could have detrimental consequences on the immune response to Mtb in patients treated with ibrutinib.

Then, since treatment of macrophages with ibrutinib during M1 polarization increased CD206 expression (Fig. 2a), a receptor involved in Mtb phagocytosis, we evaluated whether macrophage phagocytosis and/or the intracellular growth of Mtb was affected in this situation. As shown in Fig. 2f, M1 macrophages polarized in the presence of ibrutinib showed a slight increase in Mtb uptake, while the intracellular growth of the bacteria was not modified (Supplementary Fig. 7). On the other hand, when we compared the effect of ibrutinib on the intracellular growth of already infected M1 macrophages, we found that ibrutinib impaired their killing capacity as shown by the increase in the bacillary load (Fig. 2g, h).

T cells bearing the γδ T cell receptor also participate in the innate host defense against Mtb. γδ T cells are found in Mtb-induced lesions in humans, they release IFN-γ in response to Mtb, a cytokine associated with protective immunity, and are cytotoxic against Mtb-infected macrophages resulting in killing of intracellular bacilli. Therefore, we evaluated the effect of ibrutinib on human γδ T cells stimulated with Mtb and found a significant decrease in the expression of the activation marker CD69 and in the secretion of IFN-γ (Fig. 2i, j), suggesting that γδ T cell response to Mtb might also be compromised in ibrutinib-treated patients.

Results presented here show that ibrutinib affects macrophages and γδ T cells, which are important players in an effective immune response to Mtb. In particular, we observed that ibrutinib, used at doses found in the plasma of treated patients (0.03–0.3 µM), impairs immune mechanisms that contribute to the control of Mtb infection such as TNF-α secretion by macrophages, M1 polarization, Mtb intracellular growth in M1 macrophages, and IFN-γ secretion by γδ T cells. Importantly, the ability of the host to limit Mtb at the site of infection depends on the formation and maintenance of an effective granuloma structure. In this context, TNF-α production by activated macrophages plays a key role in Mtb control by promoting granuloma formation. In fact, patients treated with TNF-α-blocking agents have an increased risk of tuberculosis and the assessment of latent infection is recommended before starting such treatment. Our results showing that ibrutinib significantly decrease TNF-α secretion in response to Mtb suggests that this
Fig. 2 (See legend on next page.)
mechanism might be compromised in ibrutinib-treated patients. Although ibrutinib treatment in CLL is associated with lower rates of infections compared to the standard chemotherapy regimen (Fludarabine, Cyclophosphamide, and Rituximab), major infections, particularly those of the respiratory tract, are still significant, reported in about one-third of ibrutinib-treated patients. The risk of infections appears to be highest during the first 6 months of treatment, and a subsequent improvement in patient’s cellular immunity has been suggested by the observation that ibrutinib favors a Th1 polarization and an increase in the T cell repertoire. Also, recovery from a refractory state of monocytes and T cells, by the downregulation in the T cell repertory, is shown in the right panel. Representative images from the top and inside of matrigel are shown.

In this report, patients who developed PCP did not have a low CD4+ T cell count, which is considered the primary risk factor for PCP. Interestingly, alveolar macrophages, TLR2 signaling, and TNF-α secretion are involved in an effective immune response against P. jirovecii. Given that TLR2 is a receptor also involved in the recognition of motifs present in several fungi, our finding that ibrutinib impairs macrophages’ response through TLR2 might explain, at least in part, the increased susceptibility of ibrutinib-treated patients to PCP and also the presence of other opportunistic fungal infections.

Although further studies are needed to confirm the relevance of our observations in vivo and their impact in clinical practice, we consider that these results represent a warning especially in countries with a high incidence of tuberculosis.
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Conflict of interest
R.G. receives a Non-clinical Investigator Initiated Study (IS) grant from Janssen; F.R.B. and R.G. receive compensation as speakers from Janssen. R.G. receives compensation as speaker and as a consultant from Roche and Bristol Myers Squibb. F.R.B. receives compensation as member of Advisory Board from Abbvie. The other authors declare that they have no conflict of interest.

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