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CHARACTERIZATION OF SPECIFIC OFF-TARGET EFFECTS OF BTK INHIBITORS ON INNATE ANTI-MOLD IMMUNITY IN PATIENTS WITH B-CELL NEOPLASMS: DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

Coordinatore del Corso di Dottorato:
Chiar.mo Prof. Michele De Luca

Relatore (tutor):
Chiar.mo Prof. Mario Luppi

Candidato:
Vincenzo Nasillo

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ABSTRACT

Invasive fungal infections (IFIs) represent a considerable cause of morbidity and mortality in patients with lymphoproliferative diseases. In the recent years, the introduction of new drugs targeting Bruton's tyrosine kinase (BTK) has allowed dramatic improvement in the prognosis of patients with chronic lymphocytic leukemia (CLL) and other B-cell neoplasms. Although these small molecules were initially considered less immunosuppressive than chemoimmunotherapy, an increasing number of reports have described the occurrence of unexpected opportunistic fungal infections, in particular invasive aspergillosis (IA). BTK represents a crucial molecule in the transmission of signaling cascade from several immune receptors, such as pattern recognition receptors (PRRs), CD11b/CD18, triggering receptor expressed on myeloid cells 1 (TREM-1) and Toll-like receptors (TLRs), which allow the recognition of fungi by the innate arm of the immunity. However, the immunopathogenetic mechanisms underlying the development of IFIs in patients treated with BTK inhibitors are not fully elucidated, and multiple pathways might be involved. Our in vitro study, based on different immunological functional assays, aims at characterizing the specific off-target effects of BTK inhibitors on anti-mold innate immune responses mediated by monocytes, macrophages and platelets, obtained from both CLL patients and healthy donors. Herein, we demonstrate that the pharmacological inhibition of BTK by two different compounds (ibrutinib and acalabrutinib) reduces signaling pathways activated by *Aspergillus fumigatus*, determining an exacerbation of an immunosuppressive signature, as well as a significant defect in the secretion of inflammatory cytokines, in macrophages and monocytes. We also observe a remarkable reduction in the phagocytic function of CLL-associated macrophages (*nurse-like* cells, NLCs) and CD14+ circulating monocytes. Moreover, we show, for the first time, that the exposure to BTK inhibitors impairs several immune functions of platelets in response to *Aspergillus fumigatus*, i.e., ability to adhere to conidia, activation (as indicated by the low levels of P-selectin expression) and direct killing activity. Consistently, hyphal damage test displays a striking reduction of the anti-hyphal lytic activity of monocytes, macrophages and platelets in the presence of BTK inhibitors, both in CLL patients and healthy donors. Overall, these effects concur to a failure in completely counteracting conidia germination. Our results reveal specific modifications in the innate anti-mold responses, induced by the inhibition of the BTK pathway in CLL patients, highlighting the relevance of this pleiotropic kinase as a "guardian" of the innate immunity.

ABSTRACT

Le infezioni fungine invasive (IFI) costituiscono una significativa causa di morbilità e mortalità nei pazienti con malattie linfoproliferative. Negli ultimi anni, l'introduzione degli inibitori della Bruton tirosin chinasi (BTK) ha permesso di migliorare drasticamente la prognosi dei pazienti con leucemia linfatica cronica (LLC) o con altre neoplasie delle cellule B mature. Sebbene queste molecole fossero inizialmente considerate meno immunosoppressive della classica chemio-immunoterapia, un numero crescente di segnalazioni ha descritto l'insorgenza di infezioni fungine opportunistiche inattese, in particolare l'Aspergillosi invasiva. La BTK rappresenta una molecola cruciale nella trasmissione della cascata del segnale da diversi recettori, come i *pattern recognition receptors* (PRRs), CD11b/CD18, *triggering receptor expressed on myeloid cells* 1 (TREM-1) e *Toll-like receptors* (TLRs), che permettono il riconoscimento dei funghi da parte del braccio innato dell'immunità. Tuttavia, i meccanismi immunopatogenetici alla base dello sviluppo di IFI in pazienti trattati con inibitori della BTK non sono ancora stati completamente delucidati. La nostra ricerca, basata su diversi saggi immunologici funzionali in vitro, mira a caratterizzare gli effetti specifici degli inibitori della BTK sulla risposta antifungina innata, mediata da monociti, macrofagi e piastrine, ottenuti da pazienti con LLC e volontari sani. Nel presente studio, l'inibizione della BTK mediante due diversi farmaci (ibrutinib e acalabrutinib) si associa a una ridotta trasmissione delle vie di segnale attivate da *Aspergillus fumigatus*, determinante un difetto significativo nella secrezione di citochine infiammatorie nei macrofagi e nei monociti. Si riscontra, inoltre, una notevole riduzione della funzione fagocitaria delle cellule macrofagiche associate alla LLC (dette *nurse-like cells*, NLCs), così come dei monociti circolanti CD14+. In aggiunta, per la prima volta, dimostriamo come l'esposizione agli inibitori della BTK comprometta diverse funzioni immunitarie delle piastrine in risposta ad *Aspergillus fumigatus*, quali l'attivazione (come indicato dai bassi livelli di espressione di P-selectina), la capacità di aderire ai conidi e l'attività di uccisione diretta. Coerentemente, il test di danno ifale (*hyphal damage test*) mostra una notevole riduzione dell'attività litica antifungina di monociti, macrofagi e piastrine in presenza di inibitori della BTK, sia nei pazienti che nei volontari sani.

Nel complesso questi effetti concorrono all'inefficacia nel contrastare completamente la germinazione dei conidi. I risultati del nostro studio rivelano pertanto specifiche modifiche nella risposta antifungina innata, indotte dall'inibizione della BTK in pazienti con LLC, dimostrando il ruolo di questa chinasi ubiquitaria come "guardiana" dell'immunità innata.

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BACKGROUND

BRUTON'S TYROSIN KINASE (BTK): ROLE IN BCR AND OTHER SIGNALING PATHWAYS

Bruton's tyrosine kinase (BTK) was originally identified in 1993 as a non-receptor protein tyrosine kinase that is defective in X-linked agammaglobulinemia (XLA) [1], an inherited immunodeficiency disease first described in 1952 by the pediatrician Ogden Carr Bruton [2]. XLA patients have very low numbers of circulating B cells, and serum antibodies are almost completely absent, due to an arrest in the B cell development at the transition from pro-B to pre-B cell stage [3]. Consequently, the clinical course of XLA is characterized by recurrent bacterial infections [4]. A milder phenotype of the disease is present in CBA/N mice, harboring a loss-of-function mutation in *BTK* [5,6]. These mice, known as *xid* (X-linked immunodeficiency) mice, show only minor defects in B cell development in the bone marrow, whilst the differentiation and survival of mature peripheral B cells is severely impaired [7–9]. A few months after the discovery of BTK, it was demonstrated that the stimulation of the B cell receptor (BCR) in mature B cells induces tyrosine phosphorylation of BTK and increases its kinase activity, thus placing BTK in the signal transduction pathway of BCR [10,11]. This receptor is expressed on the B cell surface and has the unique capacity to specifically recognize antigens due to hypervariable regions in the immunoglobulin heavy (IGH) and light (IGL) chains that together form the BCR [12]. BCR-stimulated B cells of Btk-deficient mice fail to progress into cell division, show a high susceptibility to apoptosis and have a limited induction of activation markers [13]. Furthermore, the XLA phenotype in humans can be explained by a parallel function of BTK in downstream signaling of the pre-BCR, an immature form of the BCR that performs functional IGH chain rearrangement by deposition of the IGHμ protein on the cell surface, thus mediating signals for survival, proliferation and cellular differentiation [14]. Loss of BTK function disrupts this pre-BCR checkpoint function. In patients with XLA, indeed, clonal expansion and developmental progression of IGHμ chain-expressing pre-B cells are abrogated [4,15].

BTK is also involved in many other signaling pathways in B cells, including chemokine receptors and Toll-like receptors (TLRs) (**Table 1**).

Interestingly, BTK is expressed in most B cell neoplasms, and small-molecule inhibitors of this kinase have shown excellent anti-tumor activity in both pre-clinical [16] and clinical studies [17,18]. In particular, the orally administered BTK inhibitor ibrutinib, which forms a covalent bond with a cysteine residue in the BTK active site, is approved for the treatment of patients with chronic lymphocytic leukemia (CLL) and other B cell malignancies [19].

Given the apparently B cell-restricted phenotype in XLA and *xid*, the role of BTK has been most extensively studied in the context of B cells. Nonetheless, BTK is expressed in all hematopoietic cells, except in T lymphocytes and plasma cells [20], and it is involved in multiple signaling pathways (**Table 2**).

Finally, since myeloid and other innate cells (particularly, the so-called *tumor-associated macrophages*, TAM) are important players in the tumor microenvironment (TME), actively contributing to cancer progression [21–25], there is a growing interest in the development of BTK inhibitors as anti-cancer drugs not only in B cell neoplasms, but also in other hematological malignancies and solid tumors as well [19,25,26].

BTK structure and activation

BTK belongs to the TEC family of non-receptor tyrosine kinases, which have been strongly conserved throughout evolution. Besides BTK, the TEC family comprises: tyrosine kinase expressed in hepatocellular carcinoma (TEC), interleukin-2-inducible T cell kinase (ITK), resting lymphocyte kinase (RLK) and bone marrow expressed kinase (BMX) [27]. BTK, TEC and ITK are quite similar and contain five different protein interaction domains. These domains include an amino terminal pleckstrin homology (PH) domain, a proline-rich TEC homology (TH) domain, SRC homology (SH) domains SH2 and SH3, as well as kinase domain with enzymatic activity (**Figure 1**) [27–29]. BTK is essentially cytoplasmic and is only transiently recruited to the membrane through interaction of its PH domain with phosphatidylinositol-3,4,5-triphosphate (PIP3), which is generated by phosphatidylinositol-3 kinase (PI3K) [29,30]. BTK activation occurs in two steps following its recruitment to the cell membrane. First, BTK is phosphorylated at position Y551 in the kinase domain by

spleen tyrosine kinase (SYK) or SRC family kinases [31]. Phosphorylation of BTK at Y551 promotes its catalytic activity and subsequently results in its autophosphorylation at position Y223 in the SH3 domain [32]. Phosphorylation at Y223 is thought to stabilize the active conformation and fully activate BTK kinase activity [32]. However, the functional importance of SH3 Y223 autophosphorylation remains unclear, because mutations of Y223 do not seem to affect the function of BTK during B cell development in vivo [33].

Almost 700 unique loss-of-function BTK mutations have so far been described in the mutation registry for XLA, including missense, nonsense and splicing mutations, as well as deletions and insertions [34].

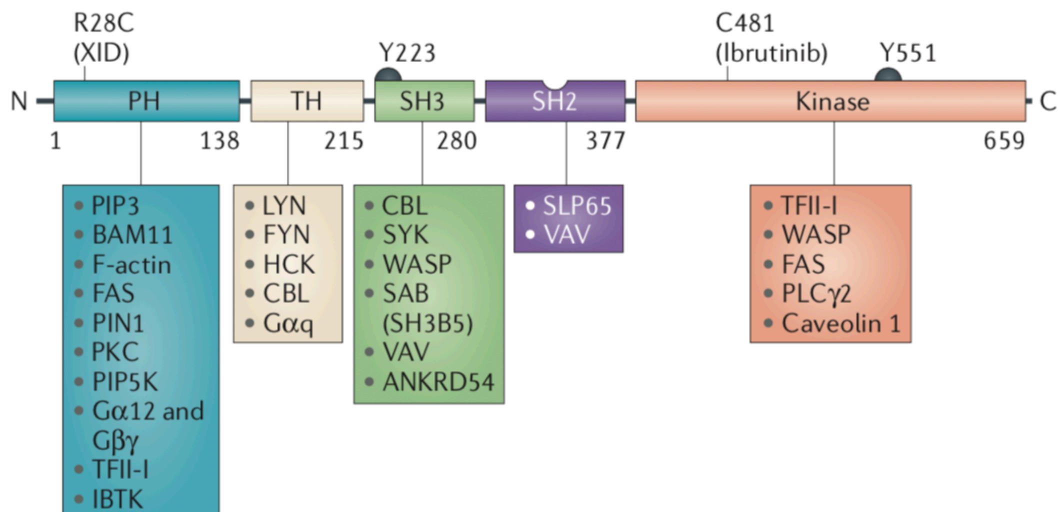

Figure 1. **Structure of BTK and related interactions.** BTK structure consists of five different domains. The position of the loss-of-function mutation that is present in X-linked immunodeficiency (*xid*) mice, the position of two tyrosine (Y) phosphorylation sites and the binding site of ibrutinib are shown. For each domain, interacting signaling molecules are shown. In addition, myeloid differentiation primary response 88 (MYD88), IL-1R-associated kinase (IRAK) and Toll/IL-1 receptor (TIR) domains of various Toll-like receptors (TLRs) have been shown to bind to BTK, but the interacting domain is unknown. ANKRD54, ankyrin repeat domain-containing protein 54; F-actin, filamentous actin; IBTK, inhibitor of BTK; PH, pleckstrin homology; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PIN1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; PKC, protein kinase C; PLCγ2, phospholipase Cγ2; SH, SRC homology; SLP65, SH2 domain-containing leukocyte protein of 65 kDa; SYK, spleen tyrosine kinase; TH, TEC homology; WASP, Wiskott–Aldrich syndrome protein. From Hendriks et al. *Nature Reviews Cancer* 2014 [29].

BTK in B cell receptor (BCR) signaling

BCR is essential for the survival of peripheral B cells [35]. In the absence of BTK, B cells have a high rate of apoptosis, which correlates with strongly reduced BCR-mediated induction of the anti-apoptotic protein Bcl-xL [36,37].

BCR cross-linking activates four families of non-receptor protein tyrosine kinases: phospholipase Cγ (PLCγ), mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway components and the serine/threonine kinase AKT (or protein kinase B, PKB) [31].

BCR has a very short cytoplasmic domain and cannot directly signal, but associates with the disulphide-linked Igα/Igβ (CD79a/CD79b) heterodimers. These transmembrane proteins contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domain (**Figure 2**) [29,38]. BCR engagement by antigen induces ITAM phosphorylation by Src-family protein tyrosine kinases, most probably LYN, thereby creating docking sites for SYK [39]. In addition, SYK and LYN also phosphorylate tyrosine residues in the cytoplasmic tail of the B-cell co-receptor CD19 and/or the adaptor protein B-cell PI3K adaptor (BCAP), which favors recruitment and activation of PI3K and the guanine nucleotide exchange factor VAV [40,41]. VAV further enhances enzymatic activity of PI3K through activation of RAC1, a member of Rho family of GTPases [42]. PI3K phosphorylates PIP2 to generate PIP3, a critical secondary messenger for activating downstream pathways. PIP3 interacts with the BTK PH-domain, resulting in its recruitment to the plasma membrane [43].

Furthermore, Ig-α contains a conserved non-ITAM tyrosine residue, Y204, which upon activation by SYK, recruits and phosphorylates the central B cell-linker molecule SH2-domain-containing leukocyte protein of 65 kDa (SLP65) [44]. Hence, the adaptor molecule Cbl-interacting protein of 85 kDa (CIN85) functions to oligomerize SLP65 and assembles intracellular signaling clusters for B cell activation [45]. SLP65 serves as a scaffold for various signaling molecules, including BTK and its substrate PLCγ2 [46]. In this micro-signalosome, BTK is activated through Y551 phosphorylation by LYN or SYK and subsequently at Y223 [31]. Fully activated BTK phosphorylates PLCγ2 which, in turn, hydrolyses PIP2 into inositol triphosphate (IP3) and diacylglycerol (DAG) [47]. IP3 regulates intracellular calcium levels and thereby activates nuclear factor of activated T cells (NFAT) transcription, via calcineurin and

calmodulin. DAG mediates activation of protein kinase Cβ (PKCβ), which induces activation of several members of the MAPK family, including extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and other MAPK targets, such as Jun N-terminal kinase (JNK), p38, and NF-κB pathway components [48].

Another important branching point is induced more upstream in the BCR signaling cascade: in addition to BTK, PIP3 also interacts with PH-domain of AKT, resulting in its recruitment to the plasma membrane [49]. Full activation of AKT requires phosphorylation at position T308, induced by 3-phosphoinositide-dependent protein kinase-1 (PDK1), and at S473, phosphorylated by mechanistic target of rapamycin (mTOR) complex 2 [49]. Fully activated AKT then returns to the cytoplasm to enable a pro- survival signaling program that involves NFAT, forkhead transcription factors (FOXOs) and NF-κB-mediated pathways. Of note, BTK positively regulates the phosphorylation of AKT [50]. TEC, which can partially compensate for BTK [51], may, on the other hand, limit the capacity of BTK to activate AKT [52]. Upon activation in germinal centers (GCs), B cells can perform IGH chain class switching; in this process, the IGH constant (C) region changes, whereas the variable (V) region remains the same. Intriguingly, in contrast to IgM, the IgG BCR contains a cytoplasmic domain of considerable length with an Ig tail tyrosine (ITT) motif, which amplifies the signal [53]. SYK is required for ITT phosphorylation followed by recruitment of BTK through the adapter protein Grb2, leading to enhancement of IgG BCR-induced calcium mobilization. This amplification loop is thought to represent a cell-intrinsic mechanism for rapid activation of class-switched memory B cells [30].

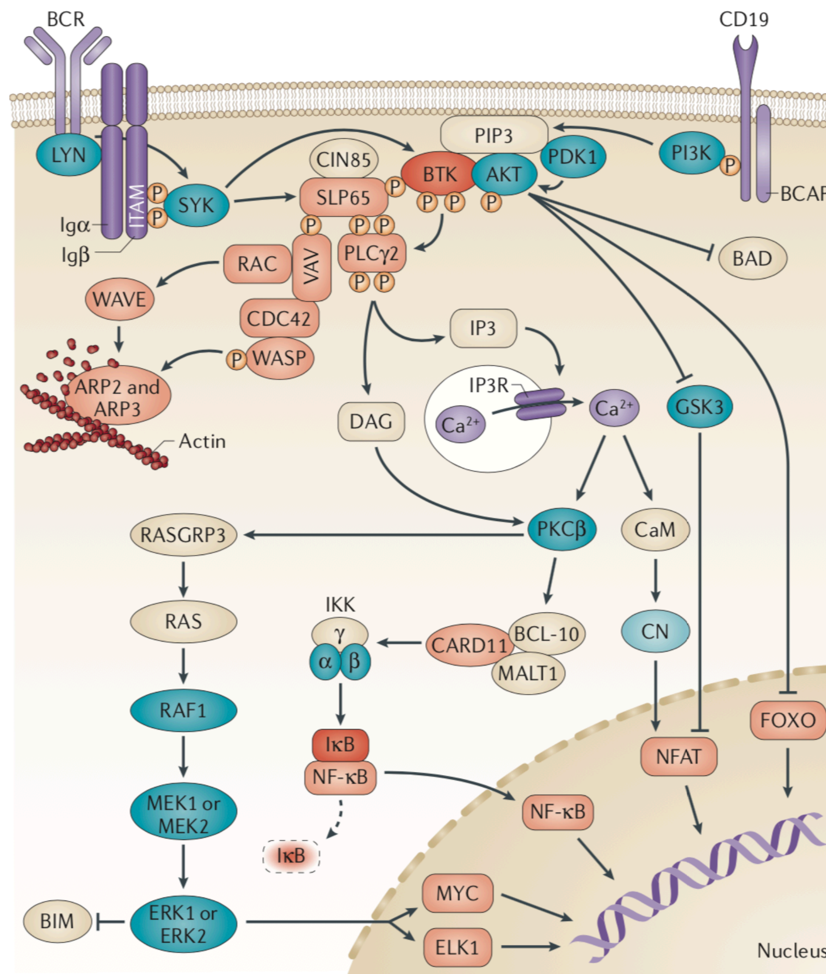

Figure 2. Signaling cascade showing important events downstream of BCR. Antigen engagement by the BCR leads to the formation of a micro-signalosome, whereby BTK activates four families of non-receptor protein tyrosine kinases that transduce key signaling events (see text for details). From Hendriks et al. *Nature Reviews Cancer* 2014 [29].

BTK in chemokine receptor signaling

The role of BTK in chemokine receptor pathways is crucial for B cell homeostasis, tissue homing and trafficking (**Figure 3a**). BTK is a key signaling molecule for the chemokine receptors CXCR4 and CXCR5 [54]. CXC-chemokine ligand 12 (CXCL12), which is highly expressed by stromal cells in the bone marrow and in GCs, induces BTK activation, probably by direct interactions between BTK and the CXCR4-linked heterotrimeric G protein subunits [54]. Both Gα and Gβγ subunits can directly bind to the PH domain and the adjacent TH domain of BTK [55,56]. Gβγ subunits might also bind to the catalytic domain [57]. Although Gβγ subunits stimulate membrane translocation, the membrane anchorage of BTK is dependent on PIP3 [57].

Since a reduction of CXCL12-controlled migration was found in B cells lacking LYN or SYK, these kinases were thought to activate BTK after CXCR4 ligation [54]. This was confirmed by the finding that CXCL12-induced phosphorylation of BTK at Y551 is reduced in the presence of a SYK inhibitor [58]. In agreement with the idea that BTK has a key role in chemokine receptor signaling, treatment of mantle cell lymphoma (MCL) and CLL cells with ibrutinib inhibited CXCL12-induced and CXCL13-induced phosphorylation of PLCγ2, ERK1, ERK2, JNK and AKT, as well as cell adhesion and migration, in vitro [58–60].

A role for BTK in chemokine signaling, in vivo, was first shown by adoptive transfer experiments with Btk-deficient B cells in mice, in which B cell homing to lymph nodes was particularly affected [54]. Moreover, treatment with ibrutinib frequently leads to an egress of malignant cells into the bloodstream [59,61,62], indicating that BTK function is essential for the homing of B cells into lymphoid tissues in humans.

BTK in tool-like receptor (TLR) signaling

A role for BTK in TLR signaling was initially demonstrated by the finding that the proliferation of Btk-deficient B cells is reduced in response to the TLR4 ligand bacterial lipopolysaccharide (LPS) [7]. Upon activation, most TLRs recruit the adaptor myeloid differentiation primary response 88 (MYD88). Exceptions to this include TLR3, a receptor that is specific for detecting viral double-stranded RNA. TLR3 uses TIR domain-containing adaptor protein inducing interferon-β (TRIF) and TLR4, which can signal both via a MYD88-dependent pathway (**Figure 3b**) and via a MYD88-independent pathway [63]. TLR signaling induces the downstream transcription factors NF-κB, activator protein 1 (AP1) and interferon regulatory factor 3 (IRF3), which, in B cells, results in upregulation of activation markers, proliferation, antibody secretion, class switch recombination and production of pro-inflammatory cytokines [63]. BTK can directly interact with cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domains of most TLRs, as well as with the downstream adaptors MYD88, TRIF and MYD88 adaptor-like protein (MAL or TIRAP), and IL-1R-associated kinase 1 (IRAK1) [30,64,65]. How B cells integrate adaptive BCR and innate TLR activation is still matter of research. Interestingly, TLR9 and BCR stimulation can synergistically induce the production of IL-6, whereby BTK is required for colocalization of TLR9 and BCR

in an autophagosome-like compartment [66]. Given that BCR signaling is initiated at the cell surface and continues to activate MAPK as it traffics to intracellular compartments [67], it is conceivable that in endosomes TLR and BCR signaling are interconnected by BTK [29].

BTK in Fc receptor signaling

BTK is involved in signaling of both activating and inhibitory Fc-receptors, whose balance regulates several myeloid cell processes, including activation, polarization and phagocytosis [30,68]. FcγRIIB is an inhibitory receptor that is exclusively expressed on B cells [69]. In contrast to the Igα/Igβ ITAM motifs, FcγRIIB has immune tyrosine inhibitory motifs (ITIMs) in its cytoplasmic domain (**Figure 3c**) [69]. Following activating Fc-receptor cross-linking, Src-kinases, SYK, PI3Kγ and BTK are activated. Conversely, inhibitory Fc-receptors (FcγRIIB), containing ITIM domains, recruit phosphatases and reduce BTK activation [30,69,70].

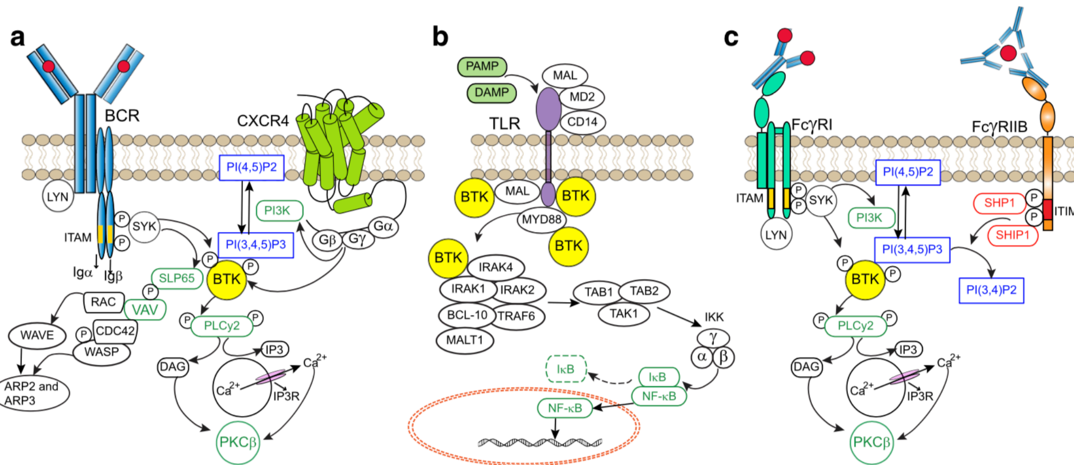

Figure 3. Role of BTK in signaling cascades downstream of (a) chemokine receptors, (b) Toll-like receptors and (c) Fcγ receptors. **a.** Chemokine receptors: upon chemokine binding to the extracellular domain Gα and Gβy subunits can independently activate PI3K, which results in activation of BTK, AKT and MAPK-dependent pathways. **b.** Toll-like receptors: upon ligand recognition, TLRs recruit different proteins such as TIR, MYD88, IRAK1 and TIRAP/MAL, all of which interact with BTK and induce downstream activation of NF-κB. **c**. Fc receptors: following FcγRI cross-linking, Src-kinases, SYK, PI3Kγ and BTK are activated. In contrast, inhibitory Fc-receptors (FcγRIIB, right side) containing ITIM domains, recruit phosphatases and reduce BTK activation. From Pal Singh et al. *Molecular Cancer* 2018 [30].

Table 1. The role of BTK in signaling pathways in B cells.

Via	Process involving BTK	References
BCR, TLR	Cytokine production (IL-6, TNF-α, IFN-γ, IL-10, IL-12)	[71,72]
BCR, BAFFR, TLR	Proliferation, differentiation and Ig production	[7]
BCR	Antigen presentation (via MHC-II) and co-stimulation (via CD86 and CD69)	[66]
BCR	Integrin-mediated adhesion of B cells to VCAM-1 and fibronectin	[73]
BAFFR	Homeostatic B cell survival	[74]
CXCR4, CXCR5, CCR7	Chemotaxis and homing in lymphoid organs	[54]
IL5-R	Proliferation and differentiation	[75–77]
FcγRI, FcγRIIB	Modulation of cell activation, shaping of B-cell repertoire and regulation of peripheral tolerance	[69,70]

BTK, Bruton's tyrosin kinase; BCR, B cell receptor; TLR, tool-like receptor; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; MHC, major histocompatibility complex; VCAM-1, vascular cell adhesion molecule 1; BAFFR, B-cell activating factor receptor; CCR, C-C chemokine receptor; CXCR, C-X-C chemokine receptor.

BTK in innate immune cells and related pathways

BTK is not expressed only in B cells, but it is found in many myeloid and other innate immune cells, encompassing monocytes, macrophages, neutrophils, platelets, natural killer (NK) cells, mast cells, dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs) [78]. Different groups demonstrated the involvement of BTK in the function, maturation and trafficking of innate cells, where it plays a pivotal role in the regulation of signal transduction through a plenty of cell pathways (**Table 2**). Notwithstanding, the role of BTK in innate immunity is far to be fully understood.

BTK is thought to be implicated in the maturation and function of granulocytes both in mice and humans, albeit conflicting data are reported in literature. In Btk-deficient mice, granulocytes exhibit maturation defect, inefficient granule function and impaired E-selectin-mediated recruitment to sites of inflammation [79,80]. Consistently, BTK seems to be involved in the maturation of neutrophils also in humans: XLA patients, who are often neutropenic, may present left-skewed maturation of the granulocytic lineage, with an increase of precursor cells (myelocyte/promyelocyte) stage [81–83].

However, these findings were not confirmed by other works, which suggested that BTK could be dispensable for human neutrophil functions [84,85].

BTK can be engaged by multiple TLRs expressed on human and mouse macrophages and DCs [64,86–90], although some studies, especially those involving XLA patients, were inconsistent about specific TLRs requiring BTK functions [84]. However, it's conceivable that the requirement for BTK functions is higher for BCR than TLR signaling; hence, a broad range of mutations can lead to the development of XLA, whereas only certain BTK mutations may cause a significant impairment of TLR signaling [78]. TLR-dependent BTK-activation promotes NF-κB and interferon-regulatory factor-dependent transcription of inflammatory cytokines and interferons (IFN) [64,88,90]. Accordingly, a downregulation of innate immune-related genes and an upregulation of oxidative phosphorylation and apoptosis-related genes was observed in XLA patients [91]. Importantly, BTK was found essential for the phagocytosis of infectious agents by mouse macrophages [92,93], while controversial findings were reported for human monocytes obtained from patients with XLA. In this context, indeed, data supporting a requirement for BTK activity in phagocytosis [91,94] are counteracted by data arguing for a redundant role of BTK in this process [84,85]. The high variability of naturally occurring BTK mutations, and their related phenotypes, likely accounted for such conflicting results.

In addition, it has recently been demonstrated that (i) BTK is critically required for NLP3-inflammasome-dependent IL-1β release from murine macrophages [95] and (ii) inflammasome activity is reduced in patients with XLA, possibly contributing to the immune impairment [78].

In line with the BTK functions in several innate pathways (particularly, TLRs), this kinase has been reported to be involved in the recognition of different infectious agents, such as *Listeria monocytogenes* [96] and *Aspergillus fumigatus (A. fumigatus)* [93].

The putative mechanisms underlying the correlation between the pharmacological inhibition of BTK and the onset of fungal infections will be discussed in detail below (see paragraph: "Response to fungal infection: focus on ibrutinib targets").

Table 2. The role of BTK in signaling pathways in innate cells.

Cell type	Via	Process involving BTK	References
Myeloid DC	TLR7, TLR9	IFN-β production	[90]
	TLR4	DC maturation and cytokine production	[97,98]
Plasmocytoid DC	TLR9	Cytokine production (IFN-α, TNF-α, IL-6), expression of CD40, CD69, CD86	[99]
Mast cell	FcεRI	Degranulation and cytokine production (IL-2, IL-3, IL-4, TNF-α, IL-6)	[100–102]
	FcγR	Degranulation and cytokine production (TNF-α, IL-8, MCP-1)	[103]
Basophil	FcεRI	Degranulation and cytokine production	[104–106]
Neutrophil	GM-CSFR, TLR	Maturation and function	[80]
	FcγR, TLR4	Degranulation, oxidative burst, pathogen engulfment and cytokine production	[107]
	FPR-1	fMLP-driven Mac-1-activation and infiltration into inflamed tissue	[108–110]
	CD162	E-selectin triggered activation of β2-integrin	[111,112]
	NLRP3	Inflammasome-mediated IL-1β secretion	[95]
	TREM-1	Degranulation, oxidative burst and L-selectin shedding	[113]
Monocyte	FCγR	Cytokine production	[103,114,115]
Macrophage	TLR2, TLR4	Microbicidal activity (through NO), cytokine production (TNF-α, IL-1β), M1 polarization	[116–118]
	FcγR	Cytokine production (TNFα, IL-6, IL-1β, MCP-1)	[103,119]
	CD40	Cytokine (IL-6, IL-8, TNF-α, IL-10) and NO production	[119,120]
	DDX41	IFN-type I response	[121]
	NLRP3	Inflammasome-mediated IL-1β secretion	[95]
	CSF1R	Survival	[122]
NK cell	TLR3	Activation	[123]
Platelet	Glycoprotein VI	Collagen and CD32 signaling	[124,125]
Osteoclast	RANK	Maturation and differentiation	[126,127]

BTK, Bruton's tyrosin kinase; DC, dendritic cell; TLR, tool-like receptor; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; FPR-1, Formyl Peptide Receptor 1; Mac-1, macrophage-1 antigen; TREM-1, triggering receptor expressed on myeloid cells 1; MCP-1, monocyte chemoattractant protein 1; NO, nitric oxide; CSF1R, colony stimulating factor 1 receptor; NK, natural killer; RANK, receptor activator of nuclear factor κ B.

TARGETING BTK IN B CELL NEOPLASMS: BIOLOGICAL AND CLINICAL ACTIVITY OF BTK INHIBITORS

Increasing evidence has revealed the critical role of BTK in both normal and neoplastic B cell, as well as in the crosstalk between the clonal cell population and the TME, thus suggesting that BTK could be harnessed as an attractive target for the treatment of B-cell malignancies [30]. In 1999, only a few years after the identification of BTK, the first rationally designed BTK small-molecule inhibitor (LFM-A13) clearly showed anti-leukemic activity in vitro [128]. Afterwards, more selective BTK inhibitors (BTKis) were developed, including the irreversible inhibitor ibrutinib, which induced objective clinical responses in dogs with spontaneous B cell lymphomas [16].

Ibrutinib is the first-in-class, orally bioavailable, covalent BTKi. Ibrutinib binds to the cysteine 481 (C481) residue of BTK and irreversibly blocks phosphorylation of downstream kinases in the BCR signaling pathway [16]. Since its discovery in 2007 [129], ibrutinib underwent rapid development. In November 2013, the first approval of ibrutinib by Food and Drug Administration (FDA) for the treatment of MCL ushered the era of BTKis [130]. Afterwards, ibrutinib was also approved for other B cell lymphoproliferative disorders, including CLL, Waldenström’s macroglobulinemia (WM) and marginal zone lymphoma (MZL) [30]. Moreover, ibrutinib is indicated as second-line treatment of steroid-refractory or steroid-resistant chronic graft versus host disease (cGVHD) [131]. In addition, very promising results have been reported in other B cell neoplasms, such as primary central nervous system lymphoma (PCNSL) [132] and activated B cell-like diffuse large B cell lymphoma (ABC-DLBCL) [133], as well as in autoimmune and inflammatory disorders [134,135].

Approved doses of ibrutinib (420 mg and 560 mg once daily) both allow to achieve a sustained and complete BTK occupancy (>95%) [136]. Despite a remarkable selectivity for BTK, ibrutinib also binds to other kinases with different affinities (**Table 3**). These unintended targets, all sharing with BTK a conserved cysteine residue in the ATP-binding site, encompass four TEC members (TEC, ITK, RLK and BMX), three EGFR family kinases (EGFR, HER2 and HER4) and three other kinases (JAK3, BLK and CSK) [137]. The inhibition of these kinases (“off-target” effects) is deemed to

contribute to anti-tumor activity of ibrutinib but it is also implicated in the development of toxicities [137].

In order to reduce the toxicity and improve the tolerability of BTKis, new covalent agents with more selective kinase inhibition profiles have been investigated.

Acalabrutinib is a covalent BTKi with less potent inhibition of TEC compared to ibrutinib and no EGFR or ITK inhibition [138]. Acalabrutinib is currently approved for the treatment of CLL [139–141] (both in Europe and in the United States) and MCL [142] (only in the United States).

Zanubrutinib has a more favorable pharmacokinetic profile and exhibits less off-target effects compared to ibrutinib [143]. The twice daily dosing of zanubrutinib achieves 8-fold higher plasma drug exposure than ibrutinib and a longer half-life than acalabrutinib, allowing an effective inhibition of newly synthesized BTK protein as well as preexisting BTK [143]. In 2019, the FDA granted accelerated approval of zanubrutinib for the treatment of relapsed/refractory MCL [144], whereas the drug is not currently approved in Europe. A phase I study showed higher BTK occupancy with twice daily (>95%) than once daily dosing (89%) of zanubrutinib in lymphoid tissue [143]. However, the same study demonstrated a homogeneously high BTK occupancy in blood and no difference in clinical outcome between once- and twice-daily dosing groups [143]. Therefore, both doses of zanubrutinib are being marketed. Notably, zanubrutinib was associated with a lower frequency and severity of bleeding and cardiovascular toxicities. The good safety profile, as well as the positive correlation between pharmacokinetics and BTK inhibition in lymphoid tissue, provide a theoretical basis for considering zanubrutinib a better covalent BTKi than others. However, further studies are needed to establish whether a more effective blockade of BTK translates into better clinical responses and, more in general, to determine the comparative safety and efficacy of different covalent BTKis.

Finally, non-covalent BTKis, not binding to C481, are now in the pipeline. These new upcoming compounds, designed to overcome resistance to covalent BTKis, may also have fewer side effects related to off-target inhibition of kinases other than BTK [145,146].

Table 3. Main features of covalent BTK inhibitors.

	Ibrutinib (PCI-32765)	**Acalabrutinib** (ACP-196)	**Zanubrutinib** (BGB-3111)	**Spebrutinib** (CC-292)	**Tirabrutinib** (ONO-4059)
Half-life	4-6 hours	1 hour	2-4 hours	1.9 hour	4-7 hours
IC_{50} (selectivity) BTK	0.5 nM (1)	5.1 nM (1)	1.8 nM (1)	9.2 nM (1)	6.8 nM (1)
IC_{50} (selectivity) ITK	10.7 nM (20)	>20000 nM (>1000)	3277 nM (>1000)	1050 nM (114)	>20000 nM (>1000)
IC_{50} (selectivity) TEC	78 nM (156)	93 nM (19)	1.9 nM (1)	8.4 nM (1)	48 nM (7)
IC_{50} (selectivity) EGFR	5.6 nM (10)	>1000 nM (>1000)	606 nM (336)	>20000 nM (>1000)	3020 nM (444)
Approved indications (FDA)	CLL, MCL, MZL, WM, cGVHD	CLL, MCL	MCL	Not approved	Not approved
Approved dose(s)	420 mg QD (CLL, WM, cGVHD) 560 mg QD (MCL, MZL)	100 mg BID	160 mg BID 320 mg BID	Not approved	Not approved
Comments	First-in-class BTK inhibitor	No ITK or EGFR inhibition	No ITK inhibition	Withdrawn from development due to lack of efficacy	Approved only in Japan for the treatment of PCNSL and WM

IC_{50}, half maximal inhibitory concentration; selectivity=$(IC_{50})_{kinase}/(IC_{50})_{BTK}$; nM, nanomolar; BTK, Bruton's tyrosin kinase; ITK, interleukin-2-inducible T cell kinase; TEC, tyrosine kinase expressed in hepatocellular carcinoma; EGFR, epidermal growth factor receptor; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; WM, Waldenström's macroglobulinemia, PCNSL, primary central nervous system lymphoma; cGVHD, chronic graft versus host disease; QD, once daily; BID, twice daily.

Targeting BTK in chronic lymphocytic leukemia (CLL)

CLL, the most common leukemia in the Western world, is characterized by the clonal expansion of mature B cells in the bone marrow, peripheral blood, spleen and lymph nodes [147]. The diagnosis of CLL requires the presence of ≥5000/μL circulating clonal B lymphocytes with a distinctive immunophenotype. In particular, CLL cells usually express CD5 and CD23, have low surface IgM expression and show a weaker expression of CD20, CD79b and CD81 compared to normal mature B cells [147]. Several genetic alterations with prognostic value and impact on treatment decisions have been described in CLL, including: deletions of the chromosomal regions 17p13 (containing the TP53 tumor suppressor gene), 11q23 (containing DNA damage checkpoint protein ATM) or 13q14 (miR-15a, miR-16-1), and trisomy of chromosome 12 [148]. In addition, more than 80% of cases harboring del(17p) also carry TP53 mutations in the remaining allele [149]. Furthermore, a significant proportion of CLL patients carries a TP53 mutation in the absence of a 17p deletion [150,151]. Of note, TP53 gene defects are associated with resistance to chemotherapy and a particularly dismal clinical outcome [147,152]. On the basis of somatic hypermutations (SHM) status of IGHV, CLL can be subclassified into *mutated CLL* (M-CLL) and *unmutated CLL* (U-CLL) [153]. M-CLL harbors a more favorable prognosis and is supposed to derive from post-GC B cells. The origin of U-CLL is less clear and several cellular origins have been postulated, including marginal zone B cells, CD5+ B cells, and regulatory B cells [153]. Although initial gene expression profiling (GEP) indicated that M-CLL and U-CLL were quite homogeneous and associated with memory B cells derived from T cell-dependent and T-cell independent responses, respectively [154], more recent reports have suggested distinct origins [155]. In particular, the study by Seifert et al. showed that U-CLL derives from unmutated mature CD5+ B cells, whereas M-CLL originates from a distinct, previously unrecognized, post-GC B cell subset with a CD5+/CD27+ surface phenotype [155].

Several findings support the relevance of chronic BCR-mediated signaling in CLL pathogenesis [156]. CLL cells exhibit anergic response to BCR ligation, thereby indicating chronic BCR internalization and signaling [157]. Since IgM–BCR responsiveness can be restored following in vitro culture, continuous antigen binding in lymphoid tissues is thought to be essential for the anergy of CLL cells [158].

Expansion of malignant CLL cells occurs in proliferation centers in lymph nodes, where antigens, chemokines, TLR ligands and T cell co-stimulation provide important signals for survival, proliferation and migration [159]. In CLL, the BCR repertoire is highly restricted (referred to as "stereotyped BCRs") [160–162], suggesting a role for antigenic selection in the initiation and/or progression of the disease. Antigens binding to clonal BCR comprise both specific self-antigens (such as non-muscle myosin IIA, vimentin, apoptotic cells, etc.) [163–165] and pathogen-derived antigens [166]. In contrast to this ligand-dependent BCR signaling, CLL cells were reported to display cell-autonomous calcium mobilization in the absence of exogenous ligands, by way of recognizing a single conserved BCR-internal epitope in the IGHV second framework region [167]. However, the two models (antigen-dependent and antigen-independent) of BCR signaling might not be mutually exclusive, as cell-autonomous signaling could be enhanced by an antigen-driven response.

In line with chronic BCR-mediated signaling, CLL cells exhibit constitutive activation of different BCR-associated kinases. BTK is essential for constitutively active pathways implicated in CLL cell survival, including AKT, ERK and NF-κB, both in patient cells and mouse models [30,60,168]. Parallelly, BTK is also critical for BCR- and chemokine-controlled integrin-mediated retention and/or homing of CLL cells in their microenvironment [58].

The active occupancy of the ATP-binding site of BTK by specific antagonists prevents the subsequent phosphorylation of BTK, PLCγ2, AKT and ERK, blocking the BCR signaling downstream of BTK. Therefore, after BTK inhibition, pathways involved in CLL cell survival and expansion, such as NF-κB pathway [169], as well as BAFF-R signaling [170], cannot be activated. Similarly, chemokine secretion, specifically CCL3 and CCL4, and adhesion of B cells, is inhibited [171]. The degree of BTK inhibition is traditionally measured by the assessment of target occupancy, which has been evaluated in phase I studies. Available data suggest that sustained target occupancy during acalabrutinib treatment is necessary for the down-regulation of pathogenic pathways and to hamper BTK re-synthesis in CLL patients [172].

Ibrutinib, the pioneer of BTKis, displays remarkable selectivity for BTK. However, its anti-tumor effect likely also relies on the indirect effect ("on/off-targets") on the TME [173], which plays a pivotal role in sustaining leukemic cell survival and growth in the

bone marrow and/or lymphoid tissues [171,174]. In this scenario, CLL cells are not innocent bystanders, but actively model and manipulate the surrounding microenvironment to their own. Among different players involved in this mutualistic crosstalk, *nurse-like cells* (NLCs), displaying features of leukemia-associated macrophages, deserve special mention, as they can preserve CLL cells' survival by creating a nurturing and permissive milieu [23]. The effect of the ibrutinib in the TME is further supported by the limited direct pro-apoptotic activity observed when purified CLL cells are incubated in vitro with ibrutinib [175], while apoptosis can be induced through mobilization of the leukemic cells from the lymph nodes in vivo [169,176]. Chemokine receptors and adhesion molecules expressed on CLL cells are involved in the leukemic cell homing to the lymphoid organs. As one prominent example, the CXCR4 chemokine receptor is highly expressed on the surface of CLL cells in the peripheral blood and mediates CLL cell chemotaxis and migration in response to CXCR4 ligand stromal cell-derived factor 1 (SDF-1α or CXCL12) produced by NLCs. BCR engagement on CLL cells induces increased expression of these molecules [177] and it is therefore not surprising that BTK inhibition by ibrutinib affects tumor cells-TME cells interactions, e.g., by impairing CXCR4 signaling and therefore inhibiting cellular adhesion [58,60]. The treatment of CLL cells with ibrutinib, in vitro, reduces cell survival and proliferation [60,178], blocks AKT and ERK phosphorylation [58,60] and abolishes vascular cell adhesion molecule 1 (VCAM-1)-mediated adhesion [58], as well as the expression of lymphocyte cytosolic protein 1 (LCP1), which is a filamentous actin (F-actin) crosslinking molecule essential for CXCL12-mediated migration [179]. The potential for disruption of co-stimulatory feedback in lymph node microenvironment was also shown by reduced CLL cell survival, proliferation and CCL3 and CCL4 production when CLL cells were co-cultured with NLCs [60]. In line with these findings, shortly after the start of ibrutinib treatment, a temporary increase in the absolute lymphocyte count, so-called "redistribution lymphocytosis", is observed in patients with CLL, reflecting the release of tumor cells from the lymphoid organs to the peripheral blood [61,62,176]. The downregulation of the CXCR4/CXCL12 axis [180] also leads to a decline in serum CCL3 and CCL4 levels [60].

Historically, chemoimmunotherapy has represented the cornerstone of CLL therapy. However, during the last decade new therapeutic agents, including those targeting

BTK, PI3K and BCL-2, have revolutionized the therapeutic landscape of CLL, by significantly improving all clinically relevant outcomes. In randomized studies, single-agent ibrutinib demonstrated superior progression-free survival (PFS) and overall survival (OS) compared to conventional single-agent chemotherapy or immunotherapy, both in treatment-naïve [181] and relapsed/refractory CLL patients [182]. In the first-line setting, BTKi-containing regimens outperformed doublet or triplet chemoimmunotherapy regimens, by improving PFS in four randomized studies [183–186] and even OS in one of the four cited studies [184]. Of relevance, high-risk patients, i.e. with relapsed/refractory or (del)17p/*TP53*-mutated disease, benefit the most from BTKis. Hence, ibrutinib is currently a standard of care for this group of patients [18]. Although overall response rates generally exceed 80%, in both treatment-naïve and relapsed/refractory patients, BTK inhibition alone is insufficient to eradicate CLL or achieve deep responses [187]. Treatment duration is indefinite, i.e. the patients continue treatment until disease progression or unacceptable toxicity occurs [188].

Side effects of BTK inhibitors

Adverse events of BTKis chiefly depend on off-target inhibition of other kinases endowed with a BTKi-biding cysteine in their catalytic domain. Therefore, safety profile of each BTKi is expected to directly correlate with its selectivity [137]. The long-term toxicity profile of ibrutinib is well characterized, based on 8 years of follow-up data since initial pivotal study. Most commonly observed side effects of ibrutinib include cardiac arrhythmias, hypertension, bleeding, infections, non-infectious pneumonitis, diarrhea and arthralgias [189]. Adverse events led to therapy halt in a significant portion of patients (9-23%) in clinical trials and the treatment discontinuation rates are even higher (23-49%) in the real-life practice [137].

Although second-generation compounds showed improved kinase selectivity for BTK, the toxicity profiles of individual BTKis are challenging to discern because of limited data, mostly deriving from non-randomized studies lacking of direct comparisons. Hence, results from ongoing randomized trials are eagerly awaited to determine the safety of second-generation BTKis in comparison with ibrutinib (NCT02477696, NCT03734016).

BTKis increase the risk of bleeding by inhibiting platelet aggregation and adhesion [190]. In normal conditions, subendothelial collagen provides a substrate for platelet adhesion and induces platelet activation. The platelets' response to collagen is mediated by two membrane proteins: integrin α2β1 and glycoprotein VI (GPVI). The former allows platelets to adhere to the subendothelial layer, while the latter is responsible for collagen-induced activation. The downstream signal ends with PLCγ2 activation and calcium mobilization. BTK and TEC take part to the transduction of the signal induced by GPVI/collagen interaction. Platelets from XLA patients display diminished aggregation, dense granule secretion and calcium mobilization in response to collagen and C-reactive protein [124]. Notwithstanding, XLA patients do not have an increased risk of bleeding [191]. As discussed above, ibrutinib inhibits both BTK and TEC, consequently leading to the impairment of collagen-induced activation. A murine study revealed that BTK and TEC are both required for physiological collagen-induced activation and aggregation of platelets [192], possibly providing an explanation for the hemorrhagic complications frequently observed in patients treated with ibrutinib and, to a lesser extent, also in patients treated with acalabrutinib and zanubrutinib. Compared with ibrutinib, acalabrutinib is less effective at inhibiting both GPVI signaling in vitro and collagen-mediated platelet aggregation ex vivo [193]. However, Bye et al. suggested that both BTK and TEC, albeit required for GPVI-mediated platelet aggregation, are redundant for platelet adhesion to collagen and thrombus formation [194]. In this view, the interference with the platelet functionality may occur through the inhibition of other kinases, such as SRC family kinases, targeted by ibrutinib but spared by more selective BTKis as acalabrutinib [194].

C-type lectin-like receptor 2 (CLEC-2) represents another receptor whose downstream signal transduction relies on BTK and TEC. Activation of CLEC-2 provides thrombus stabilization following platelets adhesion to damaged endothelium. A murine model showed a reduced CLEC-2-induced platelet aggregation when ibrutinib-treated platelets were stimulated with a selective CLEC-2 agonist [195,196]. In line with these findings, Nicolson et al. recently demonstrated that low concentrations of ibrutinib block CLEC-2-mediated activation of human platelets [197]. However, in clinical trials, most bleeding events were not severe, often presenting as bruises or petechiae,

whereas major hemorrhages are uncommon (2-5%) with either ibrutinib [198] or acalabrutinib [199].

Hypertension and atrial fibrillation are the two most common cardiovascular toxicities of BTKis. In a retrospective analysis of 562 patients treated with ibrutinib, new or worsening hypertension affected 78% of cases, occurred early in the treatment course, and was frequently associated with other cardiovascular complications, including atrial fibrillation [200]. It has been suggested that atrial fibrillation may be partially mediated by decreased PI3K-AKT signaling in cardiomyocytes due to BTK and TEC inhibition [201]. While there is no conclusive evidence demonstrating BTK expression in the heart, TEC has been detected in rat neonatal cardiomyocytes [202] and in adult mouse cardiac myocytes, where it is implicated in myocardial ischemia [203]. It was also shown that blocking HER2, another kinase targeted by ibrutinib, results in cardiomyocyte dysfunction and reduced heart contractile efficiency. In addition, the inhibition of C-terminal Src kinase (CSK) expressed in cardiac tissue has been proposed as a further off-target effect possibly contributing to the pathogenesis of ibrutinib-associated cardiovascular toxicities [204]. In support of this hypothesis, CSK knock-out mice, as well as ibrutinib-treated mice with wild-type CSK, developed atrial fibrillation, thus recapitulating observations from patients treated with ibrutinib. Of note, neither HER2 nor CSK are targeted by acalabrutinib, which only weakly binds to TEC. Such a difference in the selectivity spectrum probably accounts for the different incidences of atrial fibrillation registered with ibrutinib (7-13%) [200] and acalabrutinib (3-7%) [199].

Increased rates of infectious complications have also been reported in patients treated with ibrutinib. In a systematic review summarizing data from all prospective trials of ibrutinib in B cell neoplasms (covering 48 trial cohorts), infections of any grade occurred in 56% of patients receiving single-agent ibrutinib, while the rate of severe infectious events was as high as 26% [205]. In another publication analyzing findings from 378 patients with lymphoid cancers who received ibrutinib during a 5-year period, serious infections were reported in 11%, primarily during the first year of ibrutinib treatment [206]. Furthermore, in a multicentric retrospective study of CLL patients treated with ibrutinib in Italy, 32% of patients experienced at least one clinically relevant infectious event (defined as pneumonia, severe non-opportunistic infection or

opportunistic infection of any grade) [207]. More importantly, an increased risk for opportunistic infections in ibrutinib treated-patients was confirmed across different studies (incidence rate ranging 2-20%), with *Aspergillus* species being most frequently identified, as will be discussed in greater detail below (see paragraph: "Fungal infections in patients treated with ibrutinib: clinical data"). With regard to acalabrutinib, although definitive long-term data lack, the rate of infections varied in different preliminary reports, and only one case of fungal infection, after 2 months of therapy, was reported in phase I-II clinical trials [199].

Drug resistance

Patients exposed to long-term therapy with ibrutinib can eventually acquire resistance to covalent inhibitors, caused by the emergence of clones carrying mutations of *BTK* and/or *PLCG2*. *BTK* mutations can be detected either alone (~50% of BTKi-resistant cases) or concomitant to *PLCG2* mutations (additional 20-30%), whereas less than 10% of the patients harbor only *PLCG2* mutations. Approximately 20% of ibrutinib-resistant patients do not have detectable *BTK* or *PLCG2* mutations at progression [208]. Preexisting *BTK* or *PLCG2* mutations, causing primary resistance to BTKis, are uncommonly detected in CLL patients [209,210]. *BTK* mutations substitute the C481 residue with an alternative amino acid, most commonly serine, leading to the loss of a covalent bond between the drug and the kinase. Most *PLCG2* mutations identified to date affect the N-terminal SH2 domain with an autoinhibitory function [208]. Acquisition of BTK and PLCG2 mutations can occur any time in the clinical course [208]. Under selective pressure of BTK inhibition, CLL undergoes linear or branching evolution with the latter giving rise to a multiclonal disease [211]. BTKi-resistant CLL demonstrates an aggressive clinical course in the absence of effective salvage therapy [211]. Several approaches can be considered to prevent and treat the emergence of the resistance to BTKis, including multi-drug strategies combining agents with non-overlapping mechanisms or the use of non-covalent BTKis [212,213].

FUNGAL INFECTIONS IN PATIENTS TREATED WITH IBRUTINIB: CLINICAL DATA

Invasive fungal infections (IFIs) are opportunistic diseases that can occur in hematological malignancies in the presence of multiple predisposing factors. Acute myeloid leukemia (AML) and allogeneic hematopoietic stem cell transplantation (HSCT) are among the conditions with the highest rate of IFIs, with an incidence ranging from 5% to 20% in different epidemiological studies [214–216]. Lymphoproliferative disorders, in particular CLL, present some features that, at least in theory, could well correlate with a high risk of IFIs, similarly to other hematological malignancies. CLL is a disease of the elderly, being 70% of cases older than 65 years, often presenting multiple comorbidities. More importantly, CLL is characterized by variable degrees of immunodeficiency, accounting for high susceptibility to infections. Such immune impairment is principally inherent to the disease (related hypogammaglobulinemia, T-cell dysfunctions and neutropenia due to bone marrow infiltration) but can be further worsened by the effects of chemoimmunotherapy [217]. Nevertheless, chronic lymphoproliferative disorders are classically considered at low risk for IFIs [218,219]. In particular, before the introduction of ibrutinib, the risk of IFIs in CLL was deemed very low, with an estimated incidence of 0.1-0.4%, according to the SEIFEM-2004 IFI surveillance data [220].

In the recent years, new compounds targeting the BCR signaling pathway have successfully been introduced in the treatment algorithm of CLL and other chronic lymphoproliferative disorders [221]. These small molecules were initially considered less immunosuppressive than chemoimmunotherapy and were even reported, in some cases, to partially revert the immunosuppressive TME typical of CLL [173,222,223]. Patients treated with ibrutinib in the context of clinical trials showed moderate rate of infections, either comparable or inferior to the rate observed with chemoimmunotherapy [183,224]. Nevertheless, the risk of infection remains high even in CLL patients treated with chemo-free regimens [205,207,225].

An association between ibrutinib treatment and increased risk of mycotic infections was originally suggested by several case reports describing the occurrence of IFIs, in particular invasive aspergillosis (IA), in ibrutinib-treated patients (**Table 4**).

Ruchlemer et al. reported 35 cases (26 of whom CLL) of ibrutinib-associated IFIs (69% proven, 31% probable). The median number of prior therapies was 3 and 40% of patients were concomitantly treated with steroids. *A. fumigatus* was the most involved pathogen (63% of cases) and the main sites of infection were lung (69%), followed by central nervous system (CNS). The median duration of ibrutinib treatment before the onset of IFI was 45 days and, remarkably, most patients (79%) were not neutropenic in the month prior to initiation of treatment with ibrutinib [226]. Various other reports described cases of IA [227–234], mucormycosis [235–237] , cryptococcosis [238–241] and fusariosis [242], in patients with CLL and other B cell chronic neoplasms treated with ibrutinib. Moreover, a retrospective survey aimed at defining cases of IFI among centers of the French Innovative Leukemia Organization (FILO) CLL group identified 33 cases, comprising 30 CLL, 1 MCL and 2 WM. All but one received ibrutinib for relapsed/refractory disease. In this study, the majority of IFIs (90%) were IA, including 40.7% with CNS involvement. IFI was diagnosed after a median time from ibrutinib initiation of 3 months, and only 2 cases had a very late-onset of pulmonary aspergillosis (15 and 30 months) [243]. In a study collecting 494 CLL patients treated with ibrutinib in different hematologic centers in Italy, 14 cases of IFIs were reported among 24 cases of opportunistic infection, with an evident prevalence of IA (11/14 cases). Of note, IA was associated with SNC involvement in 3/11 patients (27%) and was the direct cause of death for 6/11 patients (55%) [207]. Furthermore, in 378 patients with lymphoid cancer (CLL and MCL) who received ibrutinib at Memorial Sloan Kettering Cancer Center, serious infections developed in 43 patients (11.4%), primarily during the first year of treatment, resulting in death in 6/43 patients (14%). Of these, IFIs occurred in 16 cases (37.2% of cases with serious infections, 4.2% of all patients), mainly without concomitant classic clinical risk factors for fungal infections (i.e., neutropenia, lymphopenia or use of corticosteroids). Patients with IFIs had an unusually high rate of CNS (49%) and multiorgan involvement (60%), accounting for very high mortality rate (69%) [206]. An unexpected high rate of IA was also reported in one trial conducted on patients affected by PCNSL, in which ibrutinib was administered in association with chemotherapy. Although very promising results were reported in this setting, an extremely high incidence of IA occurred (39% of patients) [244]. In addition, a single case of IA was also registered in a cohort of 13 relapsed/refractory

PCNSL patients treated with ibrutinib as single-agent [245]. Recently, patients with relapsed/refractory PCNSL treated with ibrutinib alone for 14 days and then up to 6 cycles of ibrutinib plus anthracycline-based chemotherapy achieved durable complete remission, but two deaths (15.4%) due to *A. fumigatus* infection occurred during ibrutinib window, suggesting that, in this setting, the use of fungal prophylaxis and cautious use of steroids may be necessary to reduce the incidence of IFIs [246].

An unexpected high incidence of *Pneumocystis jirovecii* pneumonia (PcP) was also reported in CLL patients receiving single-agent ibrutinib [247,248]. Five out of 96 patients (5.2%) developed PcP, none was on long-term steroids or other immunosuppressive agents and 4 were previously untreated, suggesting that ibrutinib per se may confer an increased risk of *Pneumocystis* infection. Clinical presentation of PcP ranged from asymptomatic to cough and mild dyspnea, with symptoms emerging after a median time of 6 months (2–24 months) from initiation of ibrutinib. In these patients CD4+ T-cell count was normal (>500/µL). Moreover, chest computed tomography scan revealed nontypical multifocal nodular infiltrates. All patients resolved the infection with early recognition and trimethoprim/sulfamethoxazole treatment, without intravenous therapy, adjunctive steroid treatment or mechanical ventilation [247]. More serious outcomes of PcP were presented by Lee et al. reviewing a series of PcP cases associated with the use of ibrutinib in patients affected by CLL, MCL and other B-cell neoplasms, submitted to the FDA Adverse Event Reporting System (FAERS). Among 13 patients with ibrutinib-associated PcP, 5 deaths were recorded [248]. The more favorable outcome of PcP in the former series is probably related to the closer clinical-laboratory monitoring for early detection of PcP in these patients, compared to the FAERS cases, possibly reinforcing the advice for a routine use of trimethoprim/sulfamethoxazole prophylaxis, especially in patients with additional risk factors of immunosuppression.

Although characterized by an impairment of immune cell functions due to immunosuppressive action of leukemic cells and further exacerbated by multiple lines of therapy, CLL population is classically considered at low risk of fungal infections [220]. The emergence of IFIs in several case reports and in some series of treated patients supports the notion that ibrutinib may act on the immunological mechanisms involved in fungal defense, thus increasing risk of fungal infections in treated patients.

Table 4. Case series of IA and other IFIs in patients treated with ibrutinib.

Reference	Number of cases (diagnosis)	IA n (%)	Other IFIs n (%)	ANC <500/μL n (%)	Median number of prior therapies	Median time after treatment (months)	Reported incidence
Ruchlemer et al. [226]	35 (26 CLL, 9 NHL)	22 (62%)	13 (38%)	5 (21%[a])	3	1.5	2.4%
Ghez et al. [243]	33 (30 CLL, 1 MCL, 2 WM)	27 (82%)	6 (12%)	2 (6%)	2	3	n.a.
Mauro et al. [207]	14 (CLL)	11 (79%)	3 (21%)	n.a.	n.a.	n.a.	2.8%
Varughese et al. [206]	16 (10 CLL, 6 NHL)	9 (56%)	7 (44%)	n.a.	2	3.5	4.2%
Rogers et al. [249]	17 (13 CLL, 4 NHL)	12 (71%)	5 (29%)	4 (29%[b])	3	4	2%
Frei et al. [250]	21 (CLL)	13 (62%)	8 (38%)	3 (16%)	1	4	2.5%
Fürstenau et al. [251]	11 (7 CLL, 4 NHL)	11 (100%)	0 (0%)	6 (67%[c])	2	1.64	n.a.
Holowka et al. [252]	5 (2 CLL, 3 MCL)	4 (80%)	1 (20%)	0 (0%)	1	3.5	2%
Lionakis et al. [244]	8[d] (PCNSL)	7 (88%)	1 (12%)	6[e]	2	1	39%
Teh et al. [253]	4 (CLL)	2 (50%)	2 (50%)	1 (25%)	4	5	12.1%
Jain et al. [254]	2 (CLL)	1 (50%)	1 (50%)	n.a.	2	2.1	1.5%
Baron et al. [255]	2 (1 CLL, 1 WM)	1 (50%)	1 (50%)	0 (0%)	2.5	1.5	n.a.
Gaye et al. [256]	2 (CLL)	2 (100%)	0 (0%)	1 (50%)	1	1.25	n.a.
Dunbar et al. [257]	2 (CLL)	2 (100%)	0 (0%)	0 (0%)	n.a.	n.a.	n.a.
Kaloyannidis et al. [258]	4 or 5[f] (cGVHD)	n.a.	n.a.	n.a.	2	n.a.	35-40%

IFIs, invasive fungal infections; IA, invasive aspergillosis; ANC, absolute neutrophil count; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin lymphoma; WM, Waldenström's macroglobulinemia; MCL, mantle cell lymphoma; PCNSL, primary central nervous system lymphoma; cGVHD, chronic graft versus host disease; n, number of patients; n.a., not available or not applicable; [a]calculated on 24 available patients; [b]calculated on 14 evaluable patients; [c]calculated on 9 evaluable patients; [d]2 cases of IA during ibrutinib alone, 5 cases of IA during ibrutinib + chemoimmunotherapy, 1 case of *Pneumocystis jirovecii* pneumonia during ibrutinib + chemoimmunotherapy; [e]neutropenia was not reported in the 2 cases who developed IA during the ibrutinib "window", while all patients (n=6) also treated with chemoimmunotherapy experienced at least one day of neutropenia (<500/μL); [f]mismatch between cases reported in the text (n=4) and table (n=5).

RESPONSE TO FUNGAL INFECTION: FOCUS ON IBRUTINIB TARGETS

The interactions between fungi and the host environment are complex and dynamic. The host immune system needs to recognize the pathogen to control fungal growth and prevent tissue invasion, whereas the mold requires nutrients and needs to adapt to the unfavorable environment by escaping immune recognition and counteracting host responses. Studies in mice have dissected the pathogenesis of aspergillosis and emphasized the role of antifungal innate and adaptive immunity. The cell wall of *A. fumigatus* is essential for fungal growth and resistance to the environmental stress. It contains the pathogen-associated molecular patterns (PAMPs) that are recognized by host pattern recognition receptors (PRRs) present on innate immune cells. In order to mount an efficient antifungal innate immune response, it is crucial for the host cells to recognize *A. fumigatus* and subsequently induce inflammatory networks, thus eventually enabling the clearance of the pathogen. Such immune responses result in direct phagocytosis and killing of the fungus, as well as cytokine signaling that regulates the activation of adaptive immune responses and epithelial host defenses. Alveolar macrophages and epithelial cells are the first to engage conidia in the lung through PRRs. Engagement of PRRs upregulates the induction of specific cytokines and chemokines and contribute to the maturation of DCs. Uptake of fungi by DCs induces DC maturation, which, in turn, promotes the differentiation of naïve T cells into effector T helper (Th) cells and T regulatory cells (Tregs). Th1 cells, producing IFN-γ, are protective and promote fungal clearance. Th17 cells are involved in neutrophil recruitment. Tregs produce IL-10, which acts as a homeostatic response to keep inflammation under control but ends up limiting the effectiveness of protective responses [259–261].

Innate immune cells in fungal infections

The importance of innate immunity in vivo has been confirmed by the discovery of rare inherited immune deficiencies (monogenic diseases), associated with sever pyogenic bacterial and/or muco-cutaneous fungal infections. Several molecular studies have identified major single nucleotide polymorphisms (SNPs)/haplotypes associated with susceptibility to fungal diseases and potentially influencing also disease outcome [262]. However, susceptibility to infections in the general population results from polymorphisms of several genes, each having small functional contributions (polygenic inheritance) and is influenced by the degree of general immunosuppression [262]. SNPs leading to long pentraxin-3 (PTX-3) deficiency, which hampers the normal alveolar expression of the protein and impairs the antifungal effector mechanisms of neutrophils, have been identified as strong predictor of IA in allogeneic HSCT recipients. This association was the sole to be validated in a large independent study and extended across different clinical settings, including AML [263]. A prospective, genetically-stratified, randomized, double-blind, event-driven multicentric trail is ongoing to assess the efficacy of posaconazole-based antifungal prophylaxis allocation strategies for AML patients under induction chemotherapy, being the allocation strategy based on the risk predicted by genotyping two SNPs of PTX-3.

Macrophages and neutrophils are the first line of defense against human fungal infections, acting mostly throughout phagocytosis and direct pathogen killing. Consistently, mice with depletion of neutrophils and monocytes are susceptible to IA [264]. In primary immunodeficiency, IA occurs almost exclusively in patients carrying genetic defects that impair the number and function of phagocytes, implying a pivotal role in fungal defense [265–267]. The prevalence of IA is particularly high in patients affected by chronic granulomatous disease (CGD) resulting from a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex defect in phagocytes that are unable to generate an efficient reactive oxygen species (ROS) production and killing of microorganisms [268].

The recognition of fungi by innate immune cells implies interaction of PAMPs (i.e., β-glucans, chitin and mannans) with multiple PRRs, including β-glucan receptor Dectin-1, the CD11b/CD18 (complement receptor 3, CR3), the triggering receptor expressed

on myeloid cells 1 (TREM-1) and TLRs. As above mentioned, BTK represents a crucial molecule in the transmission of signaling cascade from all these immune receptors [78]. Neutrophils are essential for eliminating *A. fumigatus*, especially in the early stages of infection, and neutropenia represents the primary risk factor for developing IA [259,269]. Neutrophil depletion in mice leads to higher mortality prior to or within 3 hours after exposition to *A. fumigatus* conidia, while neutrophil depletion at later stages of infection is associated with nearly normal survival rates [79]. Following inflammatory stimuli, neutrophils are recruited to sites of inflammation to exert the effector functions (**Figure 4**). BTK is required for E-selectin-mediated slow rolling of neutrophils on inflamed endothelial cells by activating β2-integrins through PLCγ2 and PI3Kγ pathways [111]. Accordingly, Btk-deficient mice show reduced neutrophil recruitment into inflamed tissue [270]. In addition, the selective reversible inhibition of BTK, by PRN473, dampens neutrophil influx via inhibition of adhesion receptor signaling pathways [110]. Recognition of *A. fumigatus* conidia by neutrophils relies on β-glucan identification by integrin CR3 which triggers a PI3K-dependent non-oxidative intracellular mechanism of killing involving lactoferrin-mediated sequestration of iron. When conidia escape from early killing and germinate, the extracellular destruction of *Aspergillus* hyphae needs opsonization and principally involves recognition via Fcγ receptors, signaling via SYK, BTK, PI3K, and PKC to trigger the production of toxic reactive oxygen metabolites by the NADPH oxidase and myeloperoxidase [271]. Neutrophils activate several mechanisms to control fungal infection, including release of granule proteins and ROS, phagocytosis of smaller conidia or formation and release of neutrophil extracellular DNA traps (NETs) for larger hyphae [272]. BTK-deficient neutrophils in mouse models show an impaired expression of granule proteins [80], ROS and nitric oxide in response to different inflammatory stimuli [270].

Macrophages eliminate pathogens by phagocytosis, a process of internalization followed by degradation. This process initiates by the recognition of specific PAMPs by several PRRs (**Figure 4**). One of the main PRRs involved in antifungal immunity is Dectin-1, a C-type lectin that interacts with fungal β-glucan. Following glucan recognition, the intracellular ITAM-like domain of Dectin-1 is phosphorylated, leading to the recruitment of SYK. Then, BTK and the guanine nucleotide exchange factor

Vav1 colocalize in the phagocytic cup. In particular, BTK seems involved in the formation of diacylglycerol (DAG), the recruitment of PKCε and the activation of calcineurin-NFAT signal [273]. The intracellular cascade generated by Dectin-1 drives actin polymerization and phagocytosis of fungi as well as cytokine release. Accordingly, BTK-deficient macrophages manifest defects in fungal phagocytosis and are unable to generate efficient bursts of ROS [270,274].

Several TLRs (TLR2, TLR4 and TLR9) are involved in response to fungal infection, and in particular to *A. fumigatus*, initiating signaling and inflammatory cytokine production upon exposure to fungal conidia spores and hyphae [275]. Absence or inactivation of TLRs leads to an increased risk of fungal infection. In particular, polymorphism Asp229Gly of TLR4 is associated with increase susceptibility to pulmonary aspergillosis and polymorphism T1237C in the promoter of TLR9 is associated with allergic bronchopulmonary aspergillosis [276,277]. TLR2 and TLR4 are membrane bound receptors recruiting TIR domain-containing adaptor proteins such as MYD88, which initiates signal transduction pathways that culminate in the activation of NF-κB, IRFs, or MAP kinases (**Figure 4**). TLR2 and TLR4 activation leads to BTK phosphorylation in macrophages, thus increasing NF-κB signaling as well as TNF-α and IL-1β expression upon stabilization of their mRNA throughout p38 MAPK [86]. Crucial activation of calcineurin-NFAT signal occurs via TLR9-dependent mechanism. Mechanistically, TLR9 is rapidly recruited to the *A. fumigatus*-containing phagosomes, then activating BTK which in turn activates PLCγ2. The PLCγ2 phosphorylation leads to calcium flux required for calcineurin-mediated NFAT translocation into the nucleus leading to TNF-α production. Inhibition of BTK reduces TNF-α response and NFAT activation [93].

Another mechanism implicated into immune response to fungal infections is the formation of inflammasome. NLRP3 inflammasome, a multiprotein complex comprising the NOD-like receptor NLRP3, the adaptor protein ASC and the proteolytic enzyme caspase-1, mediates host protection against *A. fumigatus* by cleavage of IL-1β into its bioactive form [278]. Ito et al. demonstrated that BTK is critically required for NLRP3 inflammasome-dependent IL-1β release from murine macrophages in a brain ischemia/reperfusion in vivo model [95]. However, further studies are necessary to

evaluate the direct role of BTK inhibition on the activation of NLRP3 inflammasome in response to fungal infection.

TREM-1 belongs to the immunoglobulin-like superfamily of receptors and is expressed on neutrophils and monocytes. TREM-1 is coupled to the ITAM-containing adaptor DAP12, leading to calcium mobilization and TNF-α and IL-8 secretion [279]. Some studies defined a role of TREM-1 in the immune response directed against *A. fumigatus* [280,281]. In myeloid cells, BTK is implicated in ITAM-mediated TREM-1/DAP12 signaling, being phosphorylated upon TREM-1 activation. After TREM-1 engagement, TNF-α production was reduced in peripheral blood mononuclear cells (PBMCs) from patients with XLA and in bone marrow-derived dendritic cells (BMDCs) from Btk-deficient mice [282].

DCs are antigen presenting cells (APCs) responsible for decoding the fungus-associated information and then for polarizing the immune response. After phagocytosis of conidia or hyphae, DCs migrate to the draining lymph nodes, maturate and produce different cytokines which induce selective priming of CD4+ T cells [283]. During pulmonary infections of *C. neoformans* and *A. fumigatus*, the activation of a protective Th1-type response relies on the presence of monocyte-derived DCs [284,285]. DCs recognize fungi directly through Dectin-2, dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) and mannose receptor (MR), inducing the production of inflammatory cytokines, encompassing IL-1, IL-12 and IL-23, but also IL-4 and IL-10 when in contact with fungal hyphae. It has been reported that DCs treated with ibrutinib and then stimulated with LPS show an increase capacity to promote the proliferation of CD4+ T lymphocytes and IL-17 production [98,286], but also an impairment of TLR9-mediated response [89].

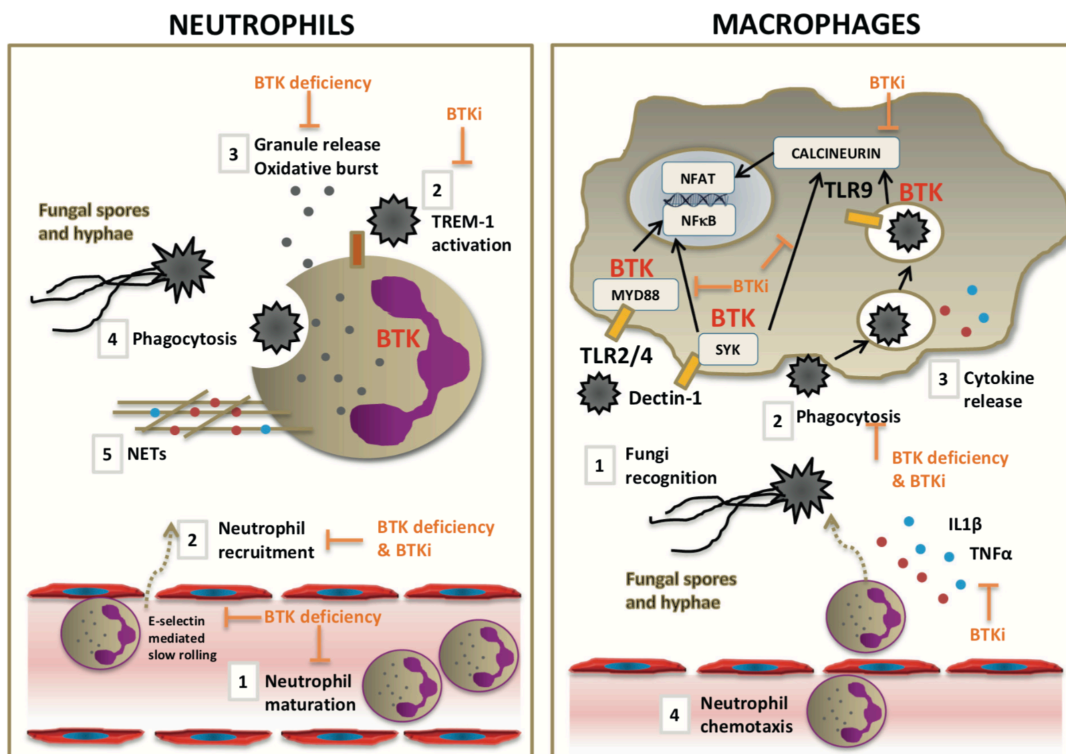

Figure 4. BTK is involved in immune cell response to fungal infection.
Neutrophils (left) are recruited from the bloodstream to sites of fungal inflammation by cytokines and activate several mechanisms to respond to fungal infection. BTK is required for the process of E-selectin-mediated slow rolling of neutrophils on inflamed endothelial cells. At site of infection, neutrophils release granule proteins, reactive oxygen species (ROS) or neutrophil extracellular DNA traps (NETs). BTK-deficient neutrophils are impaired in expression of granule proteins, reactive oxygen intermediates and nitric oxide. BTK is also involved in neutrophil recognition of fungi-associated molecular patterns, such as β-glucans, chitins and mannans (PAMPs). BTK inhibition disrupts the triggering receptor expressed on myeloid cells 1 (TREM-1)-mediated inflammatory response of neutrophils. Neutrophil maturation requires functional BTK for the expression of lineage-determining transcription factors C/EBPα and PU.1.
Macrophages (right) eliminate fungal spores and hyphae by phagocytosis. First, they recognize specific PAMPs by pattern recognition receptors (PRRs), such as Dectin-1 and Toll-like receptors (TLRs). BTK is implicated in multiple signaling cascades activated by PRRs by promoting nuclear localization of NF-κB and nuclear factor of activated T-cells (NFAT) transcription factors. In addition, macrophages release cytokines such as TNF-α and IL-1β, thus mediating neutrophil recruitment. BTK-deficient macrophages show defects in fungal phagocytosis, alteration of intracellular signal propagation after PAMPs-PRRs ligation and impaired cytokine release. From Maffei et al. *Blood Reviews* 2020 [287].

Platelets in fungal infections

Beyond their well-known functions in hemostasis and thrombosis, platelets play a crucial role in inflammatory and immune responses [288,289]. The relevance of this role has clearly emerged in the context of sepsis, where platelet count and activation correlate with disease outcome [290]. In particular, persistent thrombocytopenia is considered an independent risk factor for mortality in sepsis [291].

Platelets express several innate immune receptors, such as PRRs, TRLs and FcR, that allow the recognition of pathogens [292]. Further molecules, such as P-selectin (CD62P), GPIb (CD42b), GPIV, CD40L, and integrin αIIbβ3 enable the interactions between platelets and leukocytes [293]. This process leads neutrophils to produce ROS and form NETs [294]. In addition, platelets express soluble molecules, comprising chemokines (e.g., CCL3, CXCL4, CCL5 and CXCL7) and cytokines (e.g., IL-1β and TGF-β) [288]. These factors are present in platelet granules, but can also be synthesized de novo [289]. Such a broad armamentarium of immune receptors, adhesion molecules and soluble factors makes platelets full-fledged innate immune cells that can drive inflammation and mediate pathogen clearance by different mechanisms [288]. Besides direct pathogen elimination, platelets can bind to circulating pathogens via PRRs and interact with other cellular effectors of innate immunity, thereby activating and recruiting them to the infection site [295,296]. Once at the site of infection, neutrophils and monocytes initiate the process of immunothrombosis, which consists of the formation of intravascular fibrin and microthrombi in small vessels, whereby endothelial cells exposed to microorganisms adopt a pro-adhesive phenotype [297–299]. During immunothrombosis, neutrophils and monocytes release tissue factor (TF) and extracellular nucleosomes, and degrade endogenous anticoagulants, thereby facilitating activation of coagulation induced by inflammation [300]. In this process, platelets are not only critical in the thrombogenesis, but also contribute to the recruitment of leukocytes within the thrombus by adhesion molecules (P-selectins) and release of chemokines [296,301]. Moreover, platelets participate to NETs formation through the interaction of P-selectin with the P-selectin ligand on neutrophils [299]. NETs also induce a strong procoagulant response through several molecular processes, thus further contributing to immunothrombosis [302], whose physiological function is to avoid the pathogen spread, by confining it in the capillaries [301].

However, a deregulation of this process can result in a derangement of hemostasis, as observed in disseminated intravascular coagulation (DIC) or sepsis-induced coagulopathy (SIC) [303]. Platelets are also implicated in cell-mediated immune responses, as they can deliver pathogens to secondary lymphoid organs, thus promoting T cell immune response to pathogens [304].

Several studies have investigated the role of platelets in the control of fungal infections. The expression of CD62P, as well as of further molecules indicative of platelet activation (i.e., CD63, RANTES or CD40L), is found to be increased following platelet exposure to mature hyphae or conidia [305,306]. Intriguingly, Perkhofer et al. demonstrated that human platelets attenuate *Aspergillus* species in vitro through granule-dependent mechanisms [307]. The same group also showed that the combination of platelets and anidulafungin significantly reduces germination rate of *A. fumigatus*, alter hyphal elongation and downregulate the gene encoding for β-D-glucan, an essential component of the cell wall [308]. In addition, *A. fumigatus* antigens seem to affect platelet aggregation in vitro through the deposition of complement factors [309]. Further data suggest that platelets could trigger coagulopathy and activate neutrophils during *A. fumigatus* infection [310–312]. In line with these findings, thrombocytopenia constitutes both a risk factor for fungal infections in liver transplant recipients [313] and a predictor of outcome in neutropenia-related IA [314].

Since ibrutinib is known to dampen platelets' functions through inhibition of both BTK and TEC [192,315], it could be hypothesized that the increased risk of IFI in ibrutinib-treated patients might be partially due to thrombocyte impairment. Nonetheless, this hypothesis has not been explored so far.

Adaptive immune cells in fungal infections

Although severe and prolonged neutropenia is the most common risk factor for IFIs, these opportunistic infections also occur in immunocompromised hosts harboring impaired adaptive responses, due to quantitative and/or qualitative defects in lymphocytes, as observed in patients post allogeneic HSCT or, less commonly, in AIDS patients. Moreover, cryptococcosis and PcP are common in patients with inherited T-cell defects such as idiopathic CD4 lymphopenia and X-linked CD40 deficiency [83]. During fungal infection, the development of a specific adaptive immunity and the engagement of CD4+ T cells are key determinants of the outcome. When T cells encounter a peptide-loaded APC, the interaction between T cell receptor (TCR) in cooperation with co-receptors promotes the docking of zeta-associated protein of 70 kDa (ZAP-70) and activation of the linker for activated T cells (LAT). This adaptor protein recruits ITK, which phosphorylates PLCγ1 thus inducing NFAT nuclear translocation and activating NF-κB and MAPK signaling pathways. The relevance of ITK molecule for T cell function is well defined by the phenotype of ITK knockout mice, which exhibit profound defects in the development of CD4+ T cells [316]. In particular, CD4+ T cells fail to effectively differentiate to Th2 effector cells, compromising the ability to mount a protective response against pathogens such as *Listeria monocytogenes*. In addition, T cell migration to the lung and activation are impaired [317]. Moreover, ITK−/− cells fail to efficiently produce IL-17A, indicating a role of ITK in Th17 differentiation [318]. Of interest, severe viral and opportunistic infections including PcP were reported in humans with congenital ITK deficiency [319].

It is largely acknowledged that a Th1 response against fungi plays a protective action against the infection, while a Th2 response characterizes disease progression [320].

Th1 cytokines are mainly represented by IFN-γ, IL-1 and IL-12, while Th2 responses are associated with IL-4 and IL-10 production [321]. Protective T cells producing IFN-γ, IL-10, IL-4 and IL-17 have been described in patients with IFIs, and in particular in IA and mucorales infections [322,323]. Activated lymphocytes are able to bind both *A. fumigatus* conidia and hyphae, reducing fungal adherence rather than determining a direct fungal damage [324].

Protective T cells target predominantly aspergillus cell wall antigens, tend to increase during the infection and are associated with a better clinical outcome [325]. Through the production of IFN-γ, Th1 T cells are involved in the optimal activation of phagocytes at the site of fungal infection and promotion of B-cell production of opsonizing antifungal antibodies. It implies that the failure of T cells to properly deliver activating signals to effector innate immune cells may predispose to fungal infections. Engineered chimeric antigen receptor T-cells (CAR-T) modified to recognize β-glucan expressed on the cell wall of fungi are currently evaluated to defend immunocompromised patients against life-threating fungal infections, underscoring the relevance of understanding the regulation of T-cell response to fungal infection [326,327].

Conversely, Th2 cells dampen protective Th1 response and favor alternative pathway of macrophage activation (M2), thus are less efficient at controlling fungal growth. Moreover, Th2 cell response to fungi may be accompanied by a detrimental chronic inflammation and tissue remodeling [328].

In line with these findings, it has been reported that HSCT recipients with anti-*A. fumigatus* Th1 responses higher than Th2 responses have better survival outcomes, and that the adoptive transfer of IFN-γ producing Th1 cells, stimulated by *Aspergillus* antigens, may cure probable IA in haploidentical transplant patients [261]. However, Th2 response also exerts a protective effect against *C. neoformans* at early stage infection and against *P. jirovecii*. In addition, the ability of Th2 cells to recruit eosinophils at the site of infection contributes to *A. fumigatus* killing after exposure, suggesting that Th2 response may be in some cases beneficial for fungal clearance [260,329].

Th17 cells have important roles in the host response against fungal infections, playing a double role in IA. Th17 cells recruit neutrophils at the site of infection exerting a protective role against IA, but, on the other hand, they can promote a chronic inflammatory state with tissue damage without clearing the infection [330]. In patients with primary aspergillosis, *Aspergillus*-specific T cells from the lung showed a Th17 phenotype [331]. Multiple studies in both humans and mice indicate a role of Th17 cells in protection against candidiasis. IL-17 is quickly induced upon Candida infection and in turn modulates pro-inflammatory cytokines and antifungal proteins [332].

Thus, well-balanced Th1 and Th17-type immune responses against fungi are generally protective and facilitate phagocytic clearance through the release of IFN-γ, TNF-α, IL-17A, and IL-17F, but they have to be finely regulated to optimize fungal clearance, while minimizing tissue damage and recovery tissue homeostasis. Furthermore, Tregs, producing IL-10, have anti-inflammatory activity and down-regulate the protective Th1 immune response in the course of IA, enhancing a Th2 response [333].

Ibrutinib has multiple immunomodulatory effects on T cell populations, which have been recently reviewed by Mhibik and colleagues [334]. In T cells, ITK is highly expressed and can be blocked by ibrutinib in a BTK-independent manner. As above mentioned, ITK plays a pivotal role in T-cell maturation and differentiation into Th2 effector cells. By blocking ITK, ibrutinib has the potential to selectively decrease Th2 cell numbers causing Th1 skewing [335]. Consistent with this, several studies have reported the reduction of some Th2-related cytokines in ibrutinib-treated patients [336,337]. In a comprehensive study of ibrutinib effect on T cells CLL patients, ibrutinib was reported to increase the absolute number of CD4+ and CD8+ T cell populations, not showing the typical immunosuppressive features of exhausted T cells as indicated by the reduction in PD-1 and intracellular CTLA-4 expression [222]. Moreover, the number of stem memory T cells was increased, while Tregs showed a reduction in percentage among CD4+ population. Although not observing any signal of T cell polarization towards Th1 profile in CLL patients during ibrutinib treatment, a prompt improvement in the percentage of CD4+ cells secreting IL-17 (Th17) was observed. Overall, the study indicates that ibrutinib enhances the persistence/expansion of activated T cells, probably by sparing chronically stimulated T cells from activation-induced cell death, and concomitantly reduces the number of Tregs, increases Th17 CD4+ population and reverses the exhausted phenotype of T cell population [222].

All these data clearly indicate the presence of an immunomodulatory effect of ibrutinib on T cells, by allowing the reconstitution of effective functions throughout the decrease of Treg number and PD-1 expression on T cells, while promoting T cell survival and Th1 expansion.

Unlike ibrutinib, neither a modification of absolute number of T cells nor Th1/Th2 polarization was observed in patients treated with acalabrutinib in relapsed/refractory CLL [139,222], while a decrease of T cell counts to normal range after 6 months of

treatment was reported in high-risk treatment-naïve CLL setting [338]. Interestingly, acalabrutinib was found to decrease PD-1 and CTLA-4 expression on T cells and IFN-γ and TNF-α production by CD4+ cells in treated patients [222]. In Eμ-TCL1 CLL bearing mice, acalabrutinib reduced IL-4, while increasing IL-2 expression, cytotoxic T cell function and T cell synapse area [339]. Furthermore, an increase in Th17 cells number was observed in relapsed/refractory patients treated with acalabrutinib [222] or zanubrutinib [340]. Overall, these findings suggest that second-generation BTKis may influence T cell functions, independently to ITK and its downstream targets [341].

AIMS OF THE STUDY

Our in vitro study, based on different immunological functional assays, aims to characterize the specific off-target effects of BTK inhibitors (BTKis) on anti-mold innate immune response mediated by monocytes, macrophages and platelets, obtained from both patients with B-cell neoplasms and healthy donors.

In particular, we sought to investigate the biological effects and mechanisms mediated by two BTKis (namely, ibrutinib and acalabrutinib) on the monocyte/macrophage population in CLL patients, specifically focusing on the phagocytic activity and ability to counteract *A. fumigatus* growth. Moreover, we planned to determine how ibrutinib and acalabrutinib modify antifungal immune functions of platelets, aiming to dissect their involvement during *A. fumigatus* infection.

METHODS

Patients and samples

Blood samples from patients matching standard diagnostic criteria for CLL were obtained from the Hematology Unit of Modena Hospital, Italy, with a protocol approved by the local Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and used fresh or cryopreserved in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA), 50% fetal bovine serum (FBS), and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until use. To generate NLCs, PBMCs from CLL patients were cultured (107/mL) in RPMI-1640 medium with 10% FBS, 50 μg/mL gentamicin, 100 U/mL penicillin, 100 μg/mL streptomycin for 15 days. Fresh medium was added to the culture every 3 days. NLCs were generated as previously described [342] . NLCs were treated with ibrutinib or acalabrutinib (1 μM) or vehicle before evaluations.
For in vitro experiments on platelets, peripheral blood samples from healthy donors, BTK-inhibitor-naïve CLL patients and CLL patients under ibrutinib were centrifuged at 900 rpm for 15 minutes to obtain platelet-rich plasma (PRP). Before the evaluations, PRP was treated with ibrutinib or acalabrutinib (1 μM) or vehicle for 1 hour at 37 °C. For some experiment increasing concentrations (0.2 μM, 0.5 μM, 1 μM) of ibrutinib were used.

FITC-DEXTRAN uptake and confocal microscopy of nurse-like cells

For confocal microscopy experiments with NLCs, PBMCs from CLL patients were plated on glass coverslips in 24-wells plate in complete medium to generate NLCs as indicated. After 10 days, NLCs were treated with ibrutinib for 30 minutes and 1 hour, then the coverslips were transferred in new wells and incubated for 15 minutes at 37 °C in PBS 5% FCS with 1 mg/ml of FITC-DEXTRAN (Sigma). Then, coverslips were fixed (4% paraformaldehyde for 10 minutes at RT), permeabilized (0.1% saponin for 20 minutes at RT), blocked with goat serum (30 min at 4 °C) and incubated with anti-phalloidin-Alexa 568 (Invitrogen Life Technologies, 1:100, 1 hour at 4 °C) followed by secondary antibody Goat anti-Rabbit-Alexa 594 (Invitrogen Life Technologies,

1:300, 1 hour at 4 °C). Samples were counter-stained with DAPI and mounted in SlowFade Gold reagent (both from Invitrogen). Slides were analyzed using a TCS SP5 laser scanning confocal microscope equipped with 4 lasers (Leica Microsystems, Milan, Italy), images acquired with the LAS AF software and processed with Adobe Photoshop (Adobe Systems, San Jose, CA). Pixel intensity analyses were performed using the ImageJ (downloadable at http://rsbweb.nih.gov/ij/) and the LAS Application Suite (Leica Microsystems) software. Mean pixel intensity was calculated by defining a region of interest (ROI) and measuring green fluorescence pixel intensity. Results are expressed as fold change compared to untreated control.

MTT assay and viability

NLCs activation was monitored using a yellow tetrazolium MTT assay (Trevigen, Gaithersburg, MD, USA). In this assay, dehydrogenases expressed by metabolically active cells convert MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dyphenyltetrazolium bromide) into intracellular purple formazan. NLCs were cultured into a 96-well plate for 12 days and then treated with ibrutinib for 24 hours. Cells were then incubated with MTT at 37 °C for 24 hours, followed by a 4h-incubation with 100 µL detergent reagent. Absorbance readings were performed at 570 nm in a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). NLCs viability were tested by Annexin V-PI staining and analyzed by flow cytometry.

Flow cytometry

To determine the abundance of NLC surface markers, after 10 days the floating cells were removed by washes and the adherent cells were treated with ibrutinib for 1 hour or 24 hours, then detaching NLCs with PBS/EDTA solution. Cells were stained with the following antibodies and corresponding isotype controls: APC-conjugated CD14, CD163 (both BD Biosciences Pharmingen, San Jose, CA, USA), PE-conjugated CD11b CBRM 1/5 (the activated epitope of CD11b MAC-1) (eBioscience), pBTK Tyr551 (GeneTex, USA), pBTK Tyr223 (Novus Biological, Littleton CO, USA). Events were acquired using a FACSCalibur (Becton Dickinson, San José, CA, USA) or FACSAria cytometers and then analyzed by FlowJo Software (Tree Star, Ashland,

OR, USA). In all the experiments, an isotype control sample for each condition was acquired to exclude autofluorescence background.

Analysis of gene expression profile

CLL cells were carefully washed off and adherent NLCs were treated over-night with 1µM ibrutinib or vehicle. NLCs were lysed to obtain RNA samples. Total RNA was extracted by using RNeasy Mini kit Plus (QIAGEN). Large-scale gene expression profiling (GEP) was performed by hybridizing RNA on 4X44K Whole Human Genome Microarray (Agilent Technologies). Fluorescence data were analyzed with Feature Extraction Software v10.5 (Agilent Technologies). Supervised analysis based on paired t-test with multiple testing correction (Benjamini Hochberg FDR) were performed by using Gene Spring GX v11.5 (Agilent) software. Genes were defined as differentially expressed between ibrutinib-treated vs. vehicle-treated group at a significant level of $p<0.05$ and with a fold change cut off ± 2. Gene Ontology Tool (http://www.geneontology.org/) was used to classify genes in functional categories. GO offers a comprehensive analysis from molecular level to larger pathways deepening and understanding the gene functions. Data have been deposited in NCBIs Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/, GSE142292).

Real time PCR

RNA was extracted with the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). RNA (100 ng) was reverse transcribed using Transcription High fidelity cDNA Synthesis kit (Roche Applied Science, Penzeberg, Germany). All samples were analyzed in real time on LightCycler 480v.2 (Roche) in duplicate). Amplification of the sequence of interest was normalized to a housekeeping reference gene (Glyceraldehyde 3-phosphate dehydrogenase, GAPDH) and compared to a calibrator sample (Universal Human Reference RNA; Stratagene, Cedar Creek, TX). Primers are listed in **Table 5**.

Human Magnetic Luminex Screening Assay

NLCs were treated overnight with ibrutinib or DMSO and the following day conditioned media were collected, centrifuged to pellet residual cells and store at -80°C. Amount of CCL3, CCL4, CCL18, CCL22, CXCL10, CXCL9, CXCL12,

CXCL13, IL-12, IL-2, IL-8 were measured in duplicate in conditioned medium by laboratory service through luminex screening assay (Labospace, Italy).

XTT assay

NLCs were cultured in 96 well plate in triplicate and then treated with ibrutinib or DMSO. *A. fumigatus* conidia (2×10^3 per well) were plated over NLCs or alone (positive control) and incubated at 37 °C for 36 hours to allow germination.
For experiments on platelets, *A. fumigatus* conidia were incubated for 16 hours to produce hyphae, with ibrutinib-treated, acalabrutinib-treated or vehicle-treated platelets, at platelets to conidia (effector to target, E:T) ratio of 100:1. Conidia alone were used as positive control. Each experimental condition was performed in triplicate. Then, NLCs or platelets were lysed hypotonically with H_2O and hyphae were incubated for 1 hour at 37 °C with 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxyanilide sodium salt (XTT; Sigma) plus coenzyme Q0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) for colorimetric measurement of hyphal metabolic activity. Absorbance was determined at 450 nm using an enzyme-linked immuno-sorbent assay plate reader, and antifungal activity was calculated as the percentage of hyphal damage according to the equation: $[1-(X/C)]*100$, were X is optical density of test wells and C is optical density of control wells with hyphae only.

Nurse-like cells phagocytosis assay

Phagocytosis was inspected by using CytoSelectTM 96-Well Phagocytosis assay (Cell Biolabs, San Diego, CA, USA) according to manufacturer's instructions. NLCs were generated from CLL patients and then treated with ibrutinib for 1 hour. After incubation, zymosan particles were added to cells for 1 hour, then NLCs were fixed and external zymosan particles were blocked. After permeabilization, zymosan particles engulfed by NLCs were measured by colorimetric detection.

CD14+ monocytes phagocytosis assay

PBMCs isolated from CLL patients or healthy donors were suspended in culture medium either in presence or absence of ibrutinib for 1 hour. Zymosan A FITC-fluorescent BioParticles (Molecular probes, Eugene, Oregon) were added and

incubated with cells at 37°C for 1 hour. Then, PBMCs were stained with CD14 APC and CD11b PE Abs. To distinguish the cells which have phagocytosed these from those simply binding the beads at the surface, a short incubation with trypan blue, followed by a wash with PBS, quenched surface FITC fluorescence. Analysis was performed by flow cytometry gating CD14+/CD11b+ cells and analyzing the mean fluorescence intensity in the positive zymosan population.

Cytokine secretion assay (CSA)

NLCs or PBMCs isolated from CLL patients or healthy donors were treated with ibrutinib or acalabrutinib for 24 hours and stimulated with germinated boiled killed *A. fumigatus* inactivated conidia or zymosan and analyzed using CSA for TNF-α according to manufacturer's instructions (CSA Detection kit; Miltenyi Biotec). Cells were immunostained with TNF-α catch reagent and incubated for 2 hours at 37 °C to allow cytokine secretion. After washes, cells were labeled with TNF-α Detection antibody conjugated to PE and CD14 APC Ab. An isotype control sample for each condition was acquired to exclude autofluorescence background.

Immunoblotting

NLCs were pretreated with ibrutinib or acalabrutinib overnight following to stimulation with $2x10^5$/ml germinated boiled killed *A. fumigatus* inactivated conidia for 2 hours or 50 μg/ml zymosan for 1 hour. Proteins (80 μg/lane) were electrophoresed on 4% to 20% SDS-polyacrylamide gradient gels (Biorad laboratories, Hercules, CA, USA). Membranes were immunoblotted with primary antibodies (listed in **Table 6**) and incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:50000; GE Healthcare, Uppsala, Sweden) for 1 hour and developed using HRP conjugates Western Bright Sirius (Advasta, Menlo Park, CA, USA). Images were acquired and analyzed using Image Lab Software v.3.0 (Biorad Laboratories).

In vivo analysis

PBMCs from CLL patients under ibrutinib therapy were collected and stored in liquid nitrogen pre and after 3 months of treatment. Viability of CD14+ cells was measured by Annexin V-PI analysis. Secretion of TNF-α was analyzed by CSA. Phagocytosis

was tested using zymosan A FITC-fluorescent BioParticles gating by flow cytometry CD14+/CD11b+ cells.

Conidia-platelets adherence assay

Aspergillus conidia-platelet adherence was determined by a previously described spectrophotometric method [305]. In short, treated or untreated platelets were incubated with *A. fumigatus* conidia at E:T ratio of 100:1 for 30 min at 37°C, and centrifuged at 500 rpm for 5 min at 4°C. Platelets alone and conidia alone were used as controls. The 700 nm optical density (OD_{700}) of supernatant was determined spectrophotometrically, and the percentage of platelet adherence was calculated by using the following formula: 1—OD reaction supernatant/0.5 × (OD conidia supernatant + OD platelet supernatant) × 100.

P-selectin expression assay

PRP treated with ibrutinib, acalabrutinib or vehicle were labeled with anti-CD42b and anti-CD62P antibody. CD42b is a membrane glycoprotein constitutively expressed on platelet surface, useful to identify PLT population, while CD62P is marker of activated platelets. Labeled platelets were then incubated with heat-inactivated *A. fumigatus* swollen conidia, at a E:T ratio of 100:1. After 30, 60, 90, 150, 210 and 270 minutes, samples were acquired and analyzed on a BD Accury C6 flow cytometry. Analysis was performed gating CD42b+ cells and analyzing the mean fluorescence intensity in the positive CD62P population. Results were reported as percentages of CD62b expression normalized on DMSO-treated non-stimulated platelets.

Statistical analyses

Data were analyzed using SPSS version 20.0 (SPSS, Chicago, IL, USA). In some experiments, results were normalized on control (100%) (vehicle-treated samples). Normalization was performed by dividing the value of a particular sample treated with ibrutinib to the value of the corresponding sample treated with vehicle (DMSO). P values were calculated by Student t test (*$p<0.05$, **$p<0.01$). Data are presented as mean and standard error of the mean (SEM) is depicted as error bars.

Table 5. Primers used in real-time PCR.

Gene	Forward primer	Reverse primer
CD163	5'-GAAGATGCTGGCGTGACAT-3'	5'-GCTGCCTCCACCTCTAAGTC-3'
IL-10	5'-CATAAATTAGAGGTCTCCAAAATCG-3'	5'-AAGGGGCTGGGTCAGCTAT-3'
MRC1	5'-CACCATCGAGGAATTGGACT-3'	5'-ACAATTCGTCATTTGGCTCA-3'
CCL18	5'-ATGGCCCTCTGCTCCTGT-3'	5'-AATCTGCCAGGAGGTATAGACG-3'
IL-1	5'- GGTTGAGTTTAAGCCAATCCA-3'	5'- TGCTGACCTAGGCTTGATGA-3'
TNF-α	5'-CAGCCTCTTCTCCTTCCTGAT-3'	5'-GCCAGAGGGCTGATTAGAGA-3'
IL-1β	5'-TACCTGTCCTGCGTGTTGAA-3'	5'-TCTTTGGGTAATTTTTGGGATCT-3'

Commercially available primers (TaqMan Gene Expression Assays; Life Technologies) used were: Hs00237184_m1 (NAMPT).

Table 6. Antibodies used in immunoblotting.

Antibody	Manifacturer
Anti-phospho-BTK Tyr^{551} rabbit	GeneTex, Irvine, CA
Anti-phospho-BTK Tyr^{223} rabbit	Cell Signaling Tech, Beverly, MA
Anti-Akt rabbit	Cell Signaling Tech, Beverly, MA
Anti-phospho-Akt rabbit	Cell Signaling Tech, Beverly, MA
Anti-phospho-BTK rabbit	Cell Signaling Tech, Beverly, MA
Anti-phospho-IκBα	Cell Signaling tech, Beverly, MA
Anti-IκBα	Cell Signaling Tech, Beverly, MA
Anti-phospho-STAT1 mouse	ECM Biosciences, Versailles, KY
Anti-STAT1 mouse	Cell Signaling Tech, Beverly, MA
Anti-β-actin mouse	Abcam, Cambridge, UK

RESULTS

BTK protein is expressed and targeted in nurse-like cells by ibrutinib

Since the expression of BTK is not restricted to B cells, but it is also present in myeloid cells like monocytes/macrophages, we sought to determine the expression profile and the activation status of BTK in NLCs. CLL cells were completely removed with several washes and after 1 hour of incubation with ibrutinib NLCs were collected. The purity of NLC preparations was assessed by phase-contrast microscopy and by western blot with CD19 antibody (data not shown). As shown in **Figure 5**, NLCs revealed the expression of BTK with two different sites of phosphorylation (Tyr^{551} and Tyr^{223}). We asked whether ibrutinib would interfere with BTK activation in these cells. NLCs were treated with ibrutinib 1 μM for 1 hour and BTK phosphorylation was determined. Ibrutinib reduced the level of phosphorylated BTK at both sites Tyr^{551} and Tyr^{223} as determined by western blot (**Figure 5A** and **Figure 5B**, n=5). This result was also confirmed by flow cytometry (**Figure 5C**) and immunofluorescence microscopy (**Figure 5D**). Moreover, treatment with ibrutinib affected the activation of BTK-downstream signaling pathways in NLCs as the PI3K and MAPK pathways leading to decreased AKT and ERK1/2 phosphorylation. These results imply that ibrutinib, targeting BTK in NLCs, may modify the biological functions of NLCs. As consequence, in the following experiments we explore the effects of BTK inhibition on pivotal mechanisms of macrophage functionality such as phagocytic capacity and M1 vs M2 polarization.

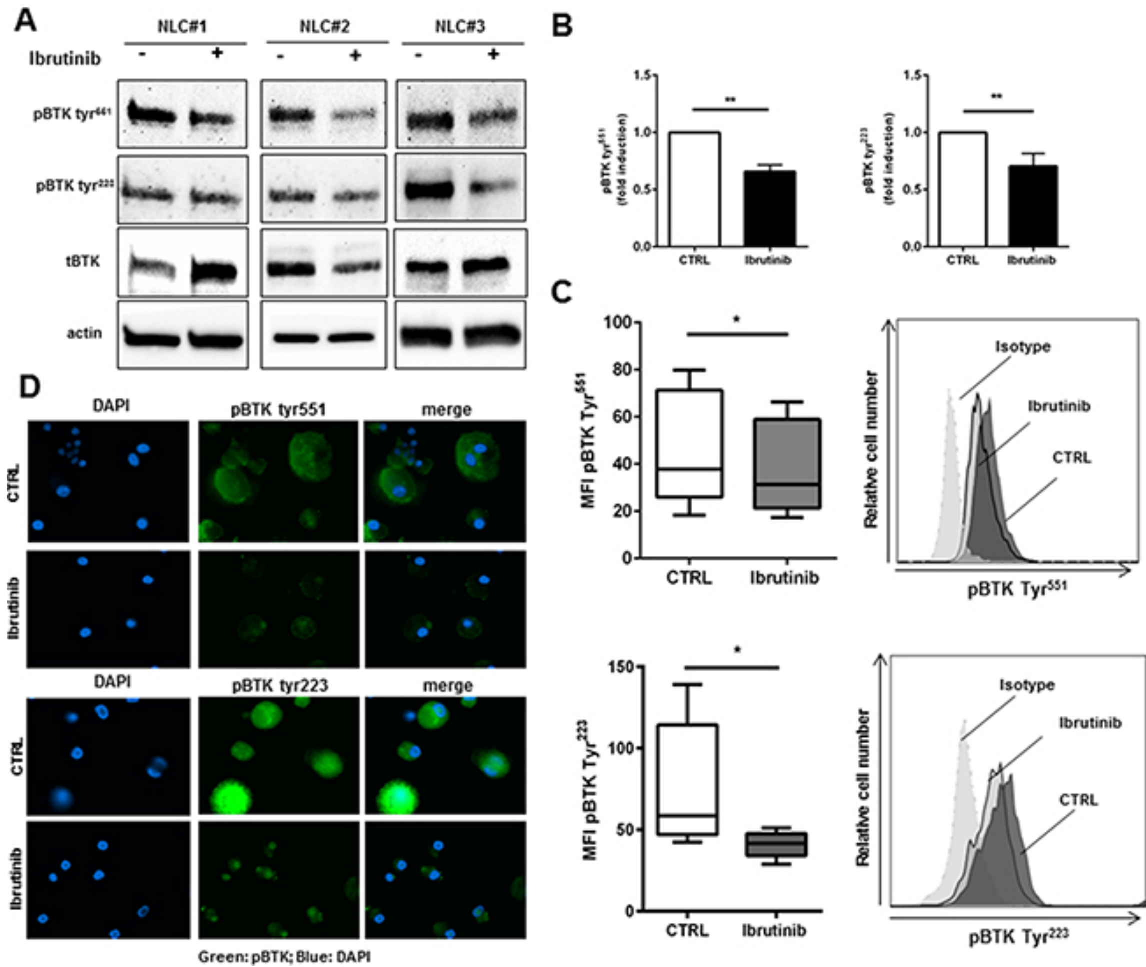

Figure 5. Ibrutinib specifically targets BTK in NLCs. NLCs (n=5) were treated or not with ibrutinib 1 μM for 1 hour. Then, cell lysates were analyzed by immunoblotting using anti-phospho-BTK Tyr^{551}, Tyr^{223}, total BTK and anti-actin antibodies. Three representative samples are depicted in panel **A**. In panel **B** bar diagram represents densitometric quantification of bands relative to phospho-BTK Tyr^{551} and phospho-BTK Tyr^{223} normalized on β-actin. Data are presented as mean ± SEM of 5 different NLC samples (**p<0.01). NLCs were treated with ibrutinib for 1h before assessing expression of phospho-BTK Tyr^{551} and Tyr^{223} by flow cytometry (**C**) and immunofluorescence microscopy (**D**) (n=5, *p< 0.05).

Ibrutinib affects phagocytic activity of nurse-like cells

We first observed that after ibrutinib treatment the activation status and viability of NLCs was not affected (**Figure 6A**) and the morphology of NLCs was preserved. In macrophages, BTK is involved in cytoskeleton remodeling and is required for optimal phagocytosis in the process of ingestion and phagosomes formation [343]. In line with these evidences, we investigated the ability of NLCs to engulf and ingest particles after treatment with ibrutinib. We treated NLCs with ibrutinib for 30 minutes or 1 hour and uptake of FITC-dextran particles was quantified by confocal microscopy. Ibrutinib decreased NLC phagocytic activity compared to control (n=6, $p<0.01$ for both time-points, **Figure 6B**). One of the main events accompanying phagocytic cup formation is the activation of MAC-1 (CD11b/CD18) [343]. Consistently, we also found an impairment of MAC-1 (CD11b/CD18) mean expression from 19% (±1%) to 13% (±1%) (n=6, $P< 0.05$, **Figure 6C**).

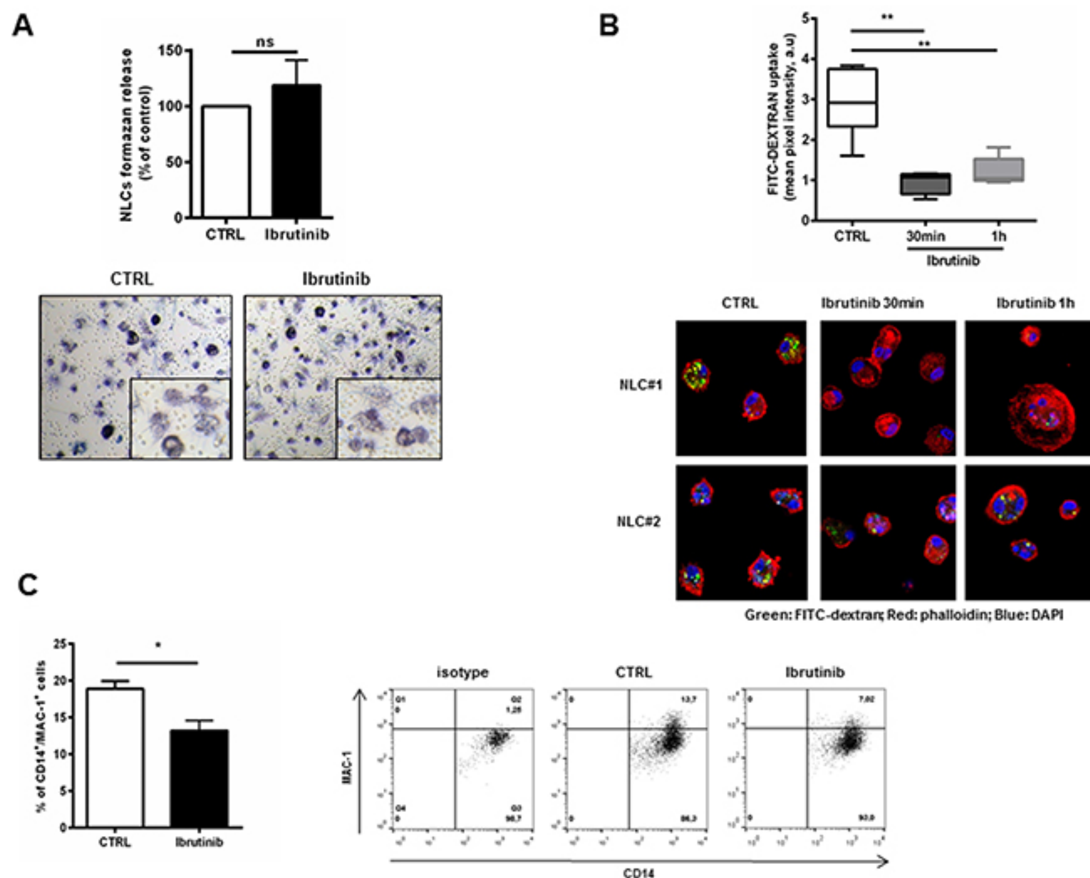

Figure 6. Ibrutinib impairs the phagocytic activity of NLCs. A. Bar diagram represents the formazan release by metabolically active NLCs treated with ibrutinib 1 µM for 24 hours compared to untreated control (n=6, *p*=not significant). In the bottom panels, representative phase-contrast micrographs demonstrate NLC activation (as insoluble formazan precipitate) after treatment with ibrutinib. **B.** Box-plots summarize FITC-Dextran uptake by NLCs treated or not with ibrutinib 1 µM relative to 6 independent experiments (**p<0.01). In the bottom panels, two representative NLC samples show confocal staining of NLCs with FITC-dextran, phalloidin and DAPI. **C.** Bar diagram shows the percentage of positive CD14+ NLCs stained for MAC-1 Ab or isotype control (n=6, p<0.05). Contour plots show a representative sample.

Ibrutinib enhances the immunosuppressive features of nurse-like cells

BTK is involved in macrophages lineage commitment to inflammatory profile. Several evidences have shown that NLCs are closely associated to TAM with peculiar M2-skewed properties. We sought to determine whether treatment with ibrutinib further stimulates the expression of M2 polarization markers. After 24 hours of treatment, the transcriptional signature of NLCs exposed to ibrutinib showed the induction of M2 markers CD163 ($p<0.01$), IL10 ($P<0.05$), MRC1 (CD206) ($p<0.01$), CCL18 ($p<0.01$) and PD-L1 ($p<0.05$) compared to control (n=8) and the concomitant down-regulation of M1 macrophages markers IL-1, TNF-α and IL-2 ($p<0.01$ for all) (**Figure 7A**). These data were confirmed by an induction of the surface expression levels of M2 polarization markers CD163 and CD206 compared to untreated controls (n=7, $p<0.01$ for all) (**Figure 7B** and **7C**). Of interest, we also detected the induction of NAMPT, known to enhance the immunosuppressive profile of NLCs (**Figure 7A** and **7D**).

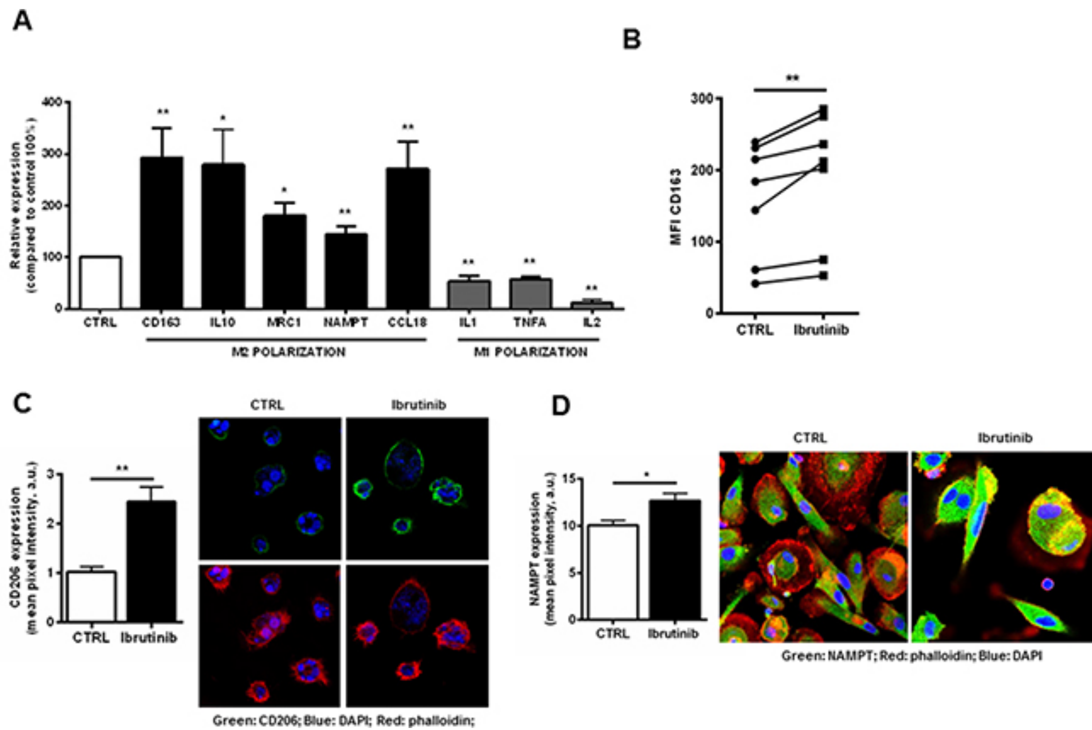

Figure 7. Exposure of NLCs to ibrutinib intensifies the expression of genes involved in M2 polarization. A. NLCs from 8 CLL patients were exposed to ibrutinib or vehicle (DMSO) for 24 hours. Transcriptional levels of CD163, IL-10, MRC1, NAMPT, CCL18 and PD-L1 (M2 polarization) and IL-1, TNFα, IL-2 (M1 polarization) were measured by quantitative reverse-transcription PCR (n=8, *p<0.05, **p<0.01). **B.** Diagrams show CD163 fluorescence intensity of NLCs treated with ibrutinib for 24 hours. Values of untreated and treated samples (n=7) are connected by lines (p<0.01). **C.** Bar diagram shows cumulative analysis of CD206 pixel intensity scoring at least 10 different cells for 3 different samples (p<0.01). On the right one representative sample stained for CD206, DAPI and phalloidin. **D.** NAMPT expression on NLCs, treated with ibrutinib 1 µM for the indicated time, was evaluated by confocal microscopy (n=6) using anti-NAMPT and secondary Alexa-488-anti-rabbit antibodies. Phalloidin and DAPI were used to counterstain. Graph shows cumulative data of green fluorescence pixel intensity (n=6, p<0.05).

Ibrutinib modifies gene expression profile of nurse-like cells

To examine the molecular modifications induced by ibrutinib treatment, a large-scale gene expression profiling (GEP) was performed on NLCs generated from CLL patients after 15 days of culture. Treatment with ibrutinib did not affect NLC viability and morphology (data not shown). Genes were defined as differentially expressed between ibrutinib-treated vs. vehicle-treated group at a significant level of $p<0.05$ and with a fold change cut off ± 2. The supervised analysis identified 566 differentially expressed genes, 409 down-regulated and 157 up-regulated by treatment (**Figure 8A**). Among down-regulated genes, the most represented gene ontology categories were related to immune system process, inflammatory response, immune response, cytokine activity, implying the ability of ibrutinib to modify the expression of genes implicated in immune function of NLCs (**Figure 8B**). In particular, the down-regulated profile included several genes belonging to tumor necrosis factor receptor family (TNF, TRAF1, TNFSF15, TNFRSF12A, TRAF4, TNFSF14, TNFRSF9) and interleukin 1 (IL1R2, IL1RN, IL1B) (**Figure 8C**, n=10, $p<0.01$). IL-1β is a potent pro-inflammatory cytokine that together with TNF-α plays a crucial role in host responses to infection by bacteria, virus, parasites. TNF-α is a master regulator of inflammatory cytokine production triggering other molecules as IL-8, CCL3, CCL4, CCL2, MMP, ROS. On this line, we found a down-regulation of several chemokines, i.e. CCL1, CCL3, CCL7, CXCL13, CCL22, CCL4 and CSF-1, and metallopeptidases. We detected an increased expression of CXCL12 (**Figure 8C**, n=10, $p<0.01$) that has a critical role in monocytes extravasation, enhances the expression of CD14 and CD163 and induces the secretion of angiogenic factors as VEGF and CCL1. Moreover, CXCL12 resulted up-regulated during inflammatory processes and cancer. In NLCs, ibrutinib impaired the expression of CSF-1, macrophage colony-stimulating factor, that activates in vivo and in vitro the anti-bacterial and antifungal activities of macrophages increasing the phagocytic activity and the production of reactive oxygen intermediates. In addition, CSF-1 renders macrophages responsive to a secondary signal that triggers their immunological functions mediated by secretion of molecules as TNF-α. We analyzed the conditioned medium of NLCs after treatment with ibrutinib. We detected a significant decrease in chemokines CCL3, CCL4, CCL22, CXCL9, CXCL12, CXCL13 and CCL2 and in interleukins IL12, IL2 and IL8 (**Figure 8D**, n=17, *$p<0.05$, **$p<0.01$). Extremely

variable levels of CXCL10 secretion were measured with about half NLC samples showing a huge increase, whereas others experiencing a decrease in its release. No modulation of the Th1 attractor CXCL9 and the naïve T cell attractor CXCL18 was detected (**Figure 8D**).

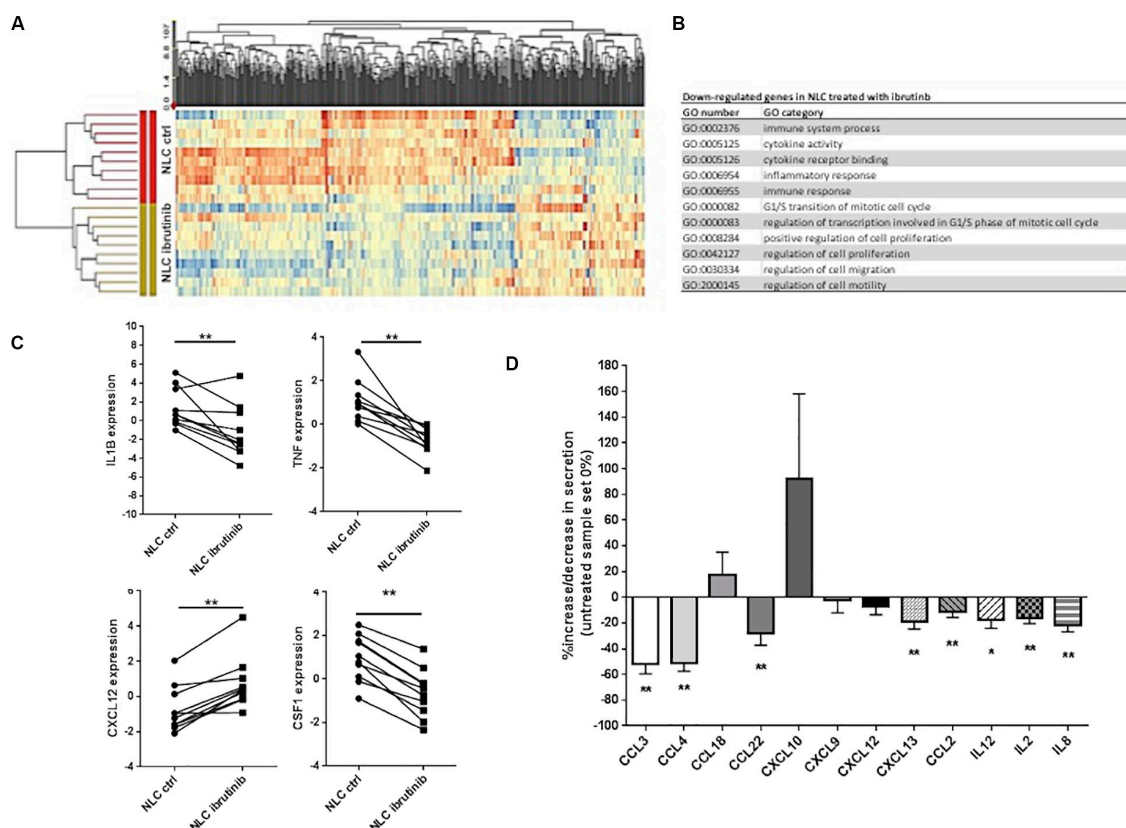

Figure 8. Ibrutinib modifies NLC gene expression profile. NLCs were generated from PBMCs of 10 CLL patients after 15 days of culture. Then, CLL cells were carefully washed off by vigorously pipetting and adherent NLCs were treated over-night with 1 μM ibrutinib or vehicle (DMSO). **A.** Heat map depicts differentially expressed genes between NLCs treated or not with ibrutinib. Genes were defined as differentially expressed between ibrutinib-treated vs. vehicle-treated group at a significant level of $p<0.05$ and with a fold change cut off ±2. The supervised analysis identified 566 differentially expressed genes, 409 down-regulated and 157 up-regulated by treatment. **B.** Among down-regulated genes, the most represented GO categories were related to immune system process, inflammatory response, immune response, cytokine activity, implying the ability of ibrutinib to modify the expression of genes implicated in immune function of NLCs. **C.** The down-regulated profile included several genes belonging to interleukin 1 (IL-1β) and tumor necrosis factor receptor family (TNF-α). Moreover, we found the down-regulation of several chemokine CXCL12 and CSF1. Values of untreated and treated samples (n=10) are connected by lines (**$p< 0.01$). **D.** Bar diagram depicts the level of chemokines and interleukins secreted by NLCs either treated or not with ibrutinib. Secretion was measured on NLC supernatant by ELISA (n=20, *$p<0.05$, **$p<0.01$).

BTK inhibition affects the release of pro-inflammatory cytokines by monocyte/macrophage population during fungal infection

A. fumigatus induces the release of pro-inflammatory cytokines important for host defense as TNF-α and IL-1β. Considering that ibrutinib down-modulated TNF-α and IL-1β related genes in NLCs, we focused our attention on their production. The expression of both TNF-α and IL-1β was accentuated by *A. fumigatus* stimulation and affected by blocking BTK (**Figure 9A**, n=8, $*p<0.05$, $**p<0.01$). In addition, stimulation with zymosan intensified the expression of both TNF-α and IL-1β that was significantly limited by treatment with ibrutinib (**Figure 9B**, n=7, $*p<0.05$, $**p<0.01$). Concordantly, secretion of TNF-α by NLCs was strongly induced by pulsing the cells with *A. fumigatus* conidia ($p<0.01$) and was significantly reduced by ibrutinib (**Figure 9C**, n=6). To decipher the importance of BTK in NLCs during a fungal infection, we inspected the effect of acalabrutinib, a more specific BTK inhibitor. Treatment of NLCs with acalabrutinib affected the expression of TNF-α and IL-1β either without stimulus or in presence of *A. fumigatus* (**Figure 9D**, n=7, $*p<0.05$, $**p<0.01$).

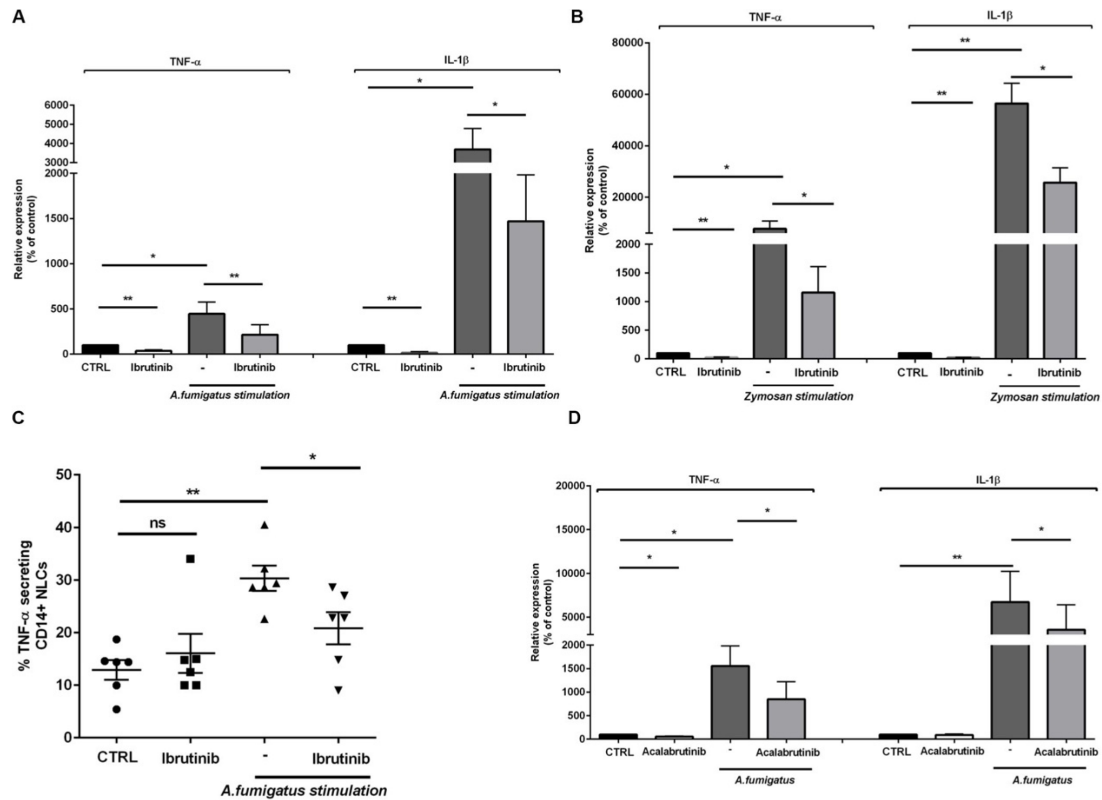

Figure 9. Ibrutinib alters the inflammatory profile of macrophage population. NLCs were treated overnight with ibrutinib and then stimulated with *A. fumigatus* conidia or zymosan. **A.** Bar diagrams show the expression level of TNF-α and IL-1β in NLCs measured by real-time PCR. As shown ibrutinib decreased the amount of TNF-α and IL-1β in presence or not of *A. fumigatus* stimulation (n=8, *p<0.05, **p<0.01). **B.** Ibrutinib was able to reduce the expression of both TNF-α and IL-1β in NLCs with or without zymosan stimulation (n=8, *p<0.05, **p<0.01). **C.** Dot plot diagram depicts the ability of NLCs to secrete TNF-α after an overnight incubation with ibrutinib and then stimulated or not with *A. fumigatus* stimulation, measured by CSA. As shown, the level of TNF-α production after treatment with ibrutinib either in presence of *A. fumigatus* stimulation was significantly decreased (n=6, *p<0.05, **p<0.01).
D. NLCs were treated overnight with acalabrutinib and then stimulated with *A. fumigatus* conidia. Bar diagrams show the relative expression of TNF-α and IL-1β in NLCs after treatment either in presence or not of stimulation (n=6, *p<0.05, **p<0.01).

Then, we moved toward analyzing the CD14+ circulating counterpart in CLL patients. Inhibition of BTK in monocytes by ibrutinib and acalabrutinib interfered with TNF-α secretion either in presence or absence of zymosan particles (**Figure 10A** and **10B**, n=6, *$p<0.05$, **$p<0.01$). Circulating CD14+ monocytes isolated from healthy donor were tested during treatment either with ibrutinib or acalabrutinib and then stimulated with zymosan. Reduced levels of TNF-α secretion were detected after inhibition of BTK either in presence or absence of any stimulus. Monocytes responded to the addition of zymosan with high TNF-α secretion that was significantly counteracted by both ibrutinib and acalabrutinib (**Figure 10C**, n=7, *$p<0.05$, **$p<0.01$).

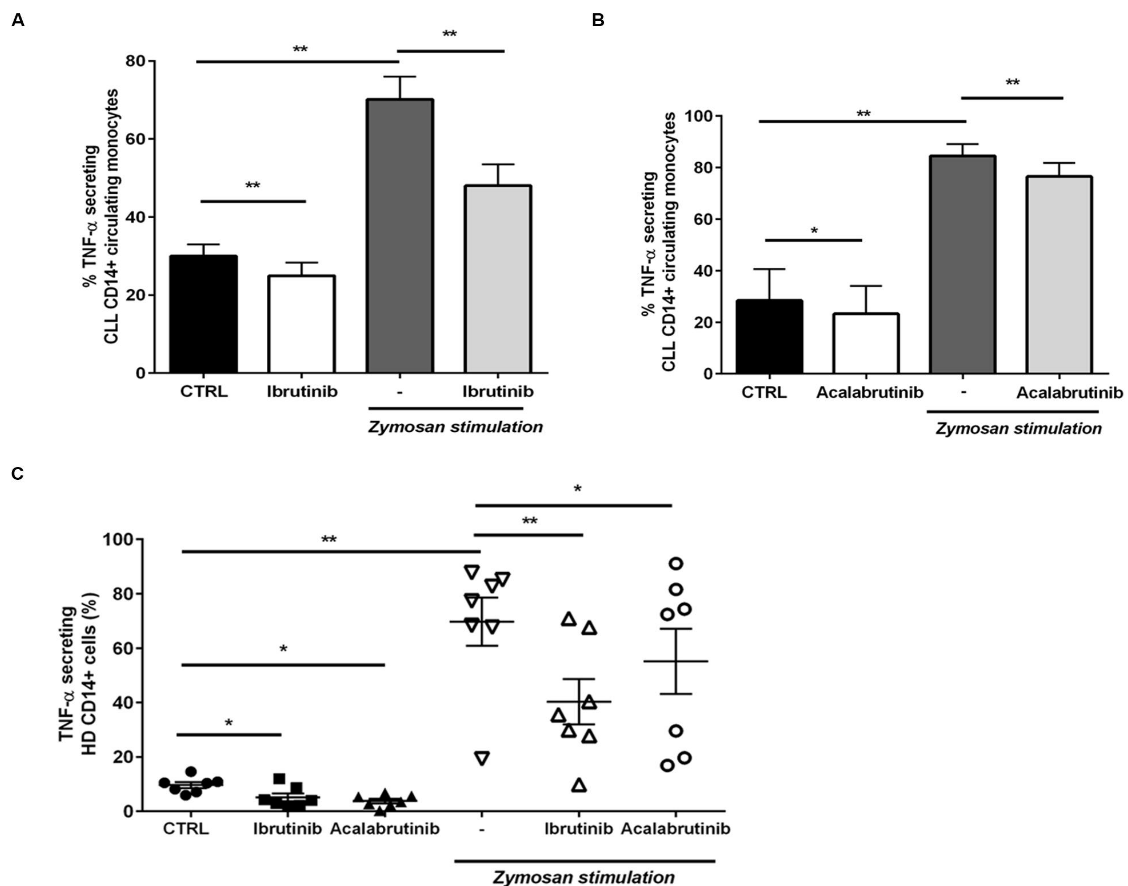

Figure 10. BTK inhibition modifies CD14+ circulating monocytes in CLL patients and healthy donor. **A.** CD14+ monocytes were pre-treated with ibrutinib for 1 hour and stimulated with zymosan. Bar diagrams show the secretion of TNF-α by CD14+ circulating population in CLL patients measured by CSA (n=6, *$p<0.05$, **$p<0.01$). **B.** CD14+ monocytes were pre-treated with acalabrutinib for 1 hour and stimulated with zymosan. Bar diagrams show the secretion of TNF-α by CD14+ circulating population in CLL patients measured by CSA (n=8, **$p<0.01$). **C.** Dot plots show the amount of TNF-α secretion in CD14+ monocytes in healthy volunteers. Ibrutinib and acalabrutinib strongly affected the level of TNF-α either in presence or absence of zymosan stimulation (n=7, *$p<0.05$, **$p<0.01$).

Ibrutinib hampers response of nurse-like cells during *A. fumigatus* infection

A crucial step of *A. fumigatus* infection spreading is germination, i.e. the transition from resting conidia to invasive growth. Since ibrutinib accentuated an immunosuppressive profile of NLCs, we determined the effect of the drug on NLC response to *A. fumigatus* infection. Firstly, we demonstrated the ability of NLCs to induce damage in *A. fumigatus* hypha germination counteracting the fungal metabolic activity. Treatment with ibrutinib determined a reduction of this capability leading to a residual growth of *A. fumigatus* hyphae (**Figure 11A**, n=5, $**p<0.01$). In **Figure 11B**, two representative microphotographs show germination of *A. fumigatus* onto NLC culture. BTK is required for optimal FcγR-mediated phagocytosis in primary peritoneal macrophages and ibrutinib decreased dextran phagocytosis impairing MAC-1 (CD11b/CD18) expression. In accordance, we tested NLCs treated with ibrutinib for their ability to phagocyte zymosan particles. As shown in **Figure 11C**, ibrutinib decreased the engulfment activity in 11 different NLC samples ($*p<0.05$). To confirm this result, we analyzed the phagocytic function of CD14+ monocytes either in CLL patients and in healthy donors. As shown in **Figures 11D** and **11E**, ibrutinib significantly interfered with phagocytosis of zymosan both in CLL patients and in healthy donor samples. A mean reduction of 40% in CLL monocytes (n=7, $**p<0.01$) and of 27% in healthy donor monocytes (n=9, $**p<0.01$) was detected. Overall, our findings indicate that NLC-mediated immune response is markedly inhibited by ibrutinib during *A. fumigatus* infection.

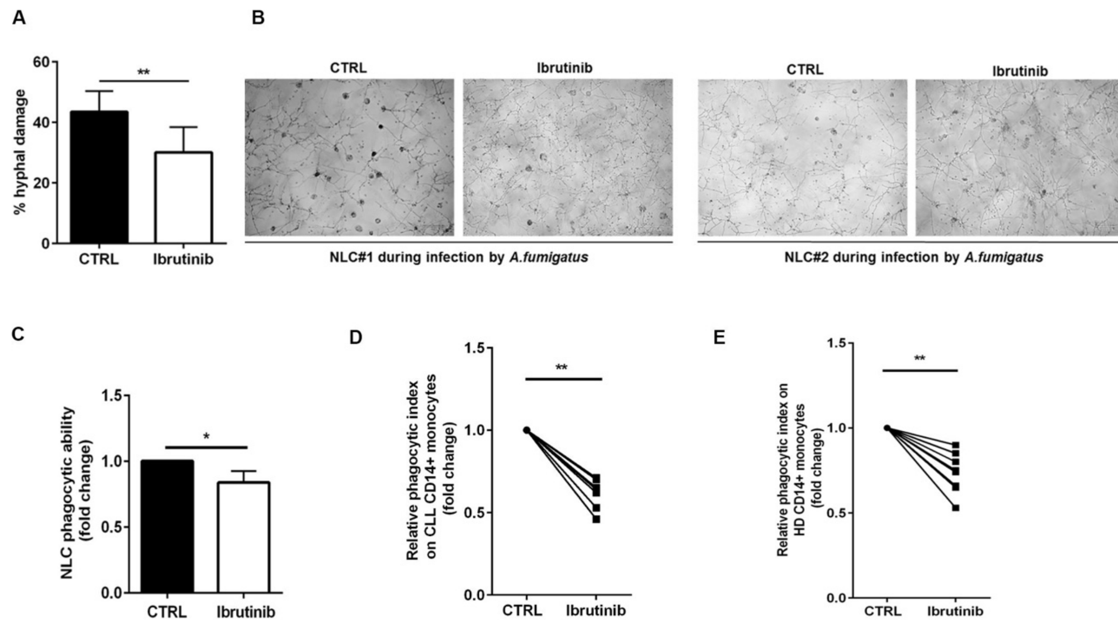

Figure 11. Ibrutinib affects the antifungal activities of NLCs. A. Bar diagram shows the percentage of hyphal damage induced by NLCs either treated or not with ibrutinib (n=5, $**p<0.01$). **B.** Photomicrographs show the growth of *A. fumigatus* onto NLCs previously treated overnight with ibrutinib in two representative CLL patients. **C.** NLCs obtained from eight CLL patients were treated or not with ibrutinib overnight, followed by phagocytosis assay. Bar diagram depicts the reduction in phagocytic ability expressed in fold change induced by ibrutinib in NLCs ($*p<0.05$). **D.** Circulating CD14+ CLL monocytes were pre-treated with ibrutinib for 1 hour and then stimulated with zymosan particles labeled with a red dye. The phagocytic activity of monocytes was measured by flow cytometry gating CD11b+ CD14+ population. Ibrutinib significantly affected the ability of monocytes to engulf zymosan particles (n=7, $*p<0.01$). **E.** CD14+ monocytes of healthy donors were pre-treated with ibrutinib for 1 hour and then stimulated with zymosan particles labeled with a red dye. The phagocytic activity of monocytes was measured by flow cytometry (n=9, $**p<0.01$). Lines show the reduction in engulfment ability induced by ibrutinib.

Signaling pathways are impaired by BTK inhibition in nurse-like cells during fungal infection

In macrophages, BTK drives secretion of inflammatory cytokines upon TLR, NLRP3 and TREM-1 stimulation. On this line, we asked if inhibition of BTK through ibrutinib and acalabrutinib was able to counteract the activation of inflammatory signaling pathways by swollen *A. fumigatus* conidia or zymosan stimulation. Ibrutinib blocking BTK phosphorylation significantly interfered with STAT1 and IκBα phosphorylation during conidia stimulation (**Figure 12A**, n=4). Accordingly, during zymosan stimulation ibrutinib decreased the level of IκBα and AKT phosphorylation (**Figure 12B**, n=4). We also tested the effects of acalabrutinib on NLCs during zymosan stimulation detecting a significant inhibition in IκBα and AKT phosphorylation (**Figure 12C**, n=4).

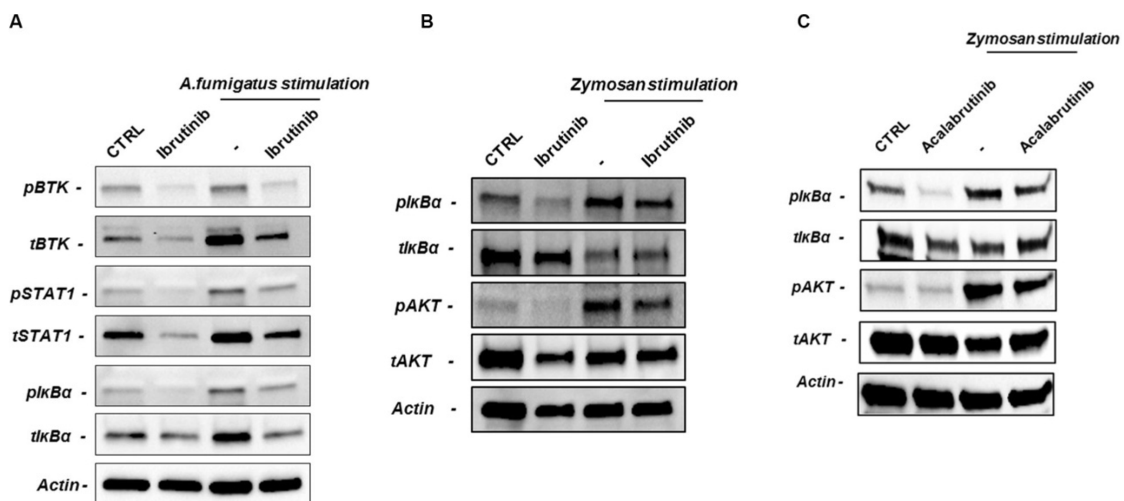

Figure 12. BTK inhibition impairs NLC–mediated response against *A. fumigatus* and zymosan. NLCs were treated overnight with ibrutinib or acalabrutinib and then stimulated with *A. fumigatus* conidia for 2 hours or with zymosan for 1 hour. **A.** Blots show the signaling pathways affected by ibrutinib during *A. fumigatus* stimulation. Ibrutinib efficiently inhibited phosphorylation of BTK, STAT1 and IκBα during conidia stimulation (n=4). **B.** Ibrutinib affects phosphorylation of AKT and IκBα during zymosan stimulation (n=4). **C.** NLCs were treated with acalabrutinib overnight and then stimulated with zymosan for 1 hour. Blots show a reduction of IκBα and AKT either in presence or absence of zymosan stimulation (n=4).

Monocytes show an impairment of immunomodulatory features during treatment with ibrutinib in CLL patients

Different studies have demonstrated insurgence of early-onset IA and other fungal infections in patients treated with ibrutinib. Given this occurrence of infections, we planned to analyzed blood samples isolated from CLL patients during treatment with ibrutinib comparing the CD14+ monocytic population before treatment and after 3 months. Firstly, we measured the viability of CD14+ circulating cells observing no difference between the 2 time-points (**Figure 13A**, n=14, *p*=not significant). Then, we focused the attention on the immunological properties of monocytes before and during treatment with ibrutinib. We analyzed the ability of monocytes to release TNF-α. As shown in **Figure 13B**, at basal level in a total of 12 CLL patients we monitored a significant decreased of TNF-α secretion after 3 months of treatment ($*p<0.05$) with a mean secretion of 14.7% (±2.2%) pre-treatment that was reduced to 11.8% (±1.7%) during the first 3 months of therapy. Among the CLL samples, 5 of 12 experienced an important drop in TNF-α secretion during treatment, instead we detected a slight increase in just 2 samples. We examined the ability of CD14+ cells to engulf zymosan particles. As reported in **Figure 13C**, we found a significant reduction of phagocytosis ranging from a decrease of 60% to a 2% (n=13, $*p<0.05$) with just 2 CLL patients showing an opposite trend with an increase of 6% and 11% respectively. Altogether, these results reported for the first time the biological effects induced in the monocytic population of CLL patients during treatment with ibrutinib, highlighting how inhibition of BTK affects its inflammatory profile that may compromise response to fungi infection.

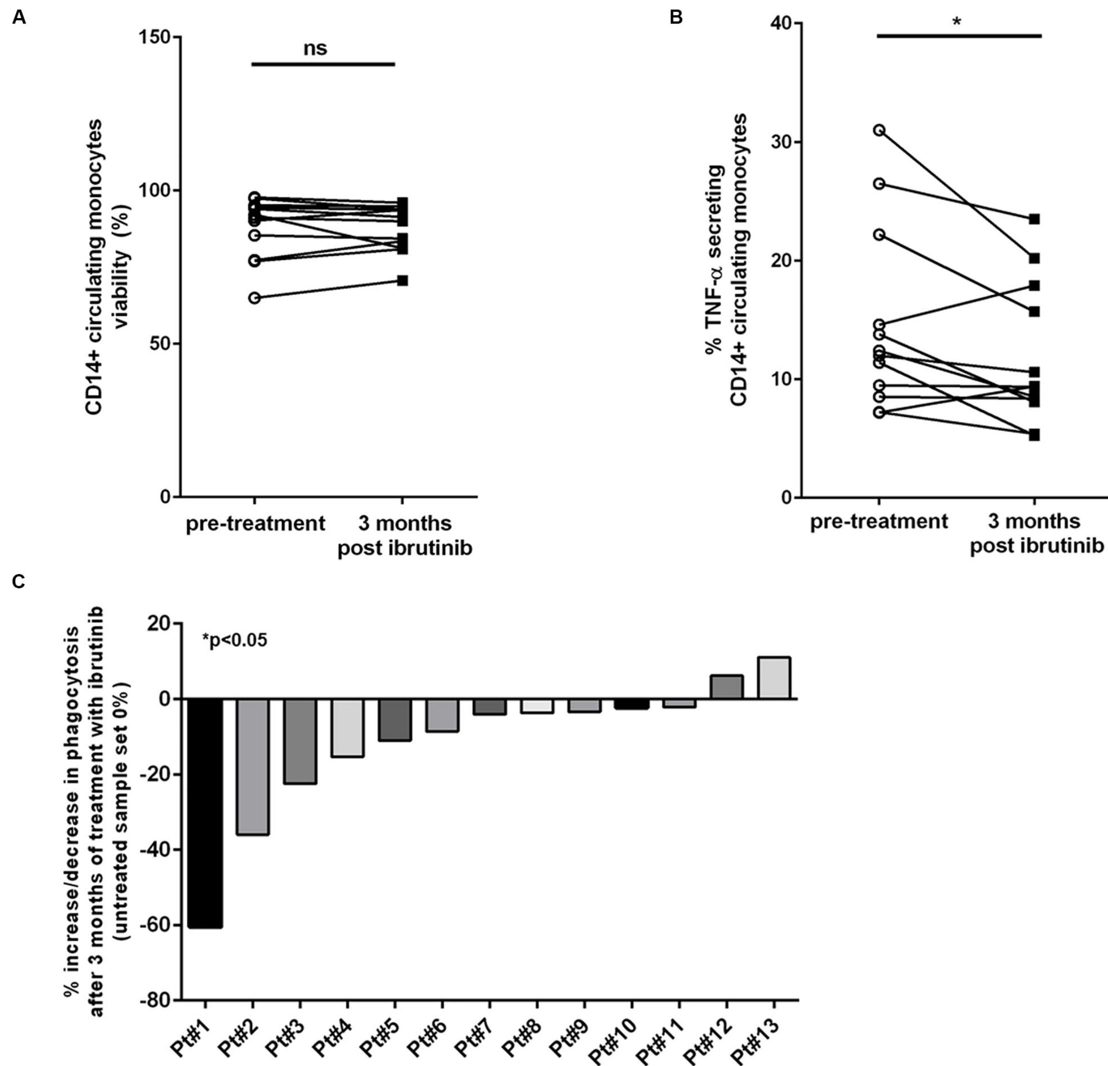

Figure 13. Circulating CD14+ monocytes are altered during ibrutinib treatment in CLL patients. Blood samples isolated from CLL patients, pre-treatment and after 3 months of treatment with ibrutinib were analyzed. **A.** Diagram shows the viability of CD14+ monocyte population. Ibrutinib did not affect the survival of monocytic population (n=14, p=not significant). **B.** Secretion of TNF-α was measured in 12 CLL patients. Diagram shows a significant reduction of TNF-α levels after 3 months of treatment with ibrutinib (n=12, *$p<0.05$). **C.** The phagocytic activity was measured in 13 CLL samples. Bar diagram shows the percentage of increase or reduction in phagocytic activity after 3 months of treatment with ibrutinib compared with pre-treated sample for each patient (n=13, *$p<0.05$).

Ibrutinib inhibits the ability of platelets to adhere to conidia

The ability of platelets to adhere to conidia was assessed in 3 healthy subjects and 3 CLL patients by spectrophotometric methods. Our data confirmed the ability of platelets to adhere to conidia in both healthy subjects and CLL patients, with mean adhesion rates of 56.6% (±3.7%) and 46% (±3%), respectively. Treatment with ibrutinib, resulted in a significant reduction in adhesion ability, in a dose-dependent manner. Specifically, in healthy subjects, mean values of 47% (±6.2%), 34.6% (±2.5%) and 17% (±3%) were found in the presence of ibrutinib at 0.2 1μM, 0.5 1μM and 1 μM, respectively. In patients with CLL, the adhesion rate decreased to 40% (±2.5%), 23% (±2.9%), and 1% (±1.3%), in the presence of ibrutinib 0.2 to 0.5 and 1μM, respectively **(Figure 14)**.

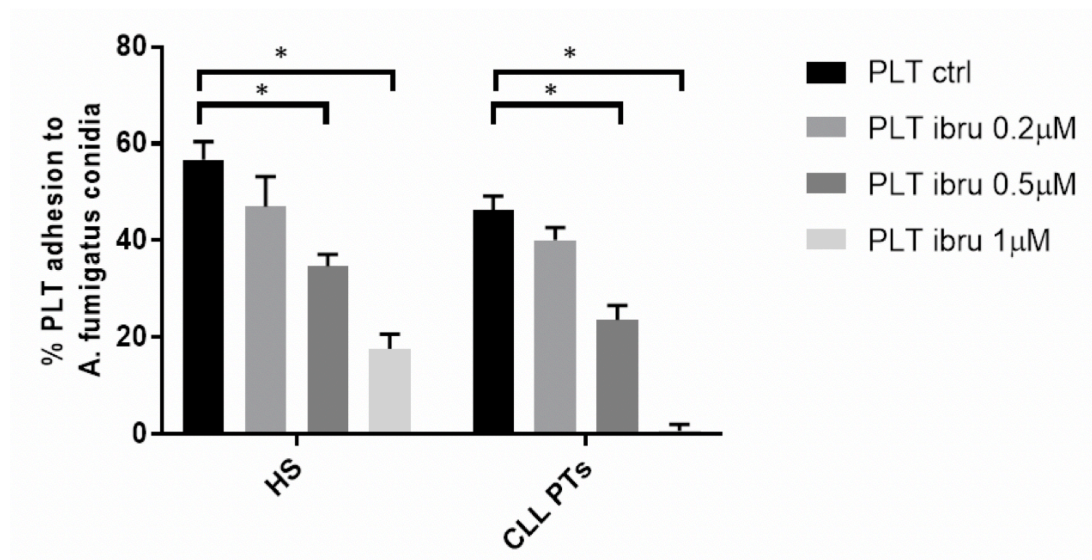

Figure 14. Ibrutinib inhibits platelet adhesion to conidia. Platelets, treated or not with 3 different concentration of ibrutinib (0.2-0.5 and 1 μM), were incubated with *A. fumigatus* conidia at an effector to target (E:T) ratio of 100:1 for 30 minutes at 37°C and then centrifuged at low intensity. Then the OD_{700} of the supernatant was determined by spectrophotometer, and the percentage of platelet adhesion was calculated in relation to the OD_{700} of platelets and conidia alone (*p<0.05). HS, healthy subjects; PLT, platelets; PTs, patients; ctrl, control; ibru, ibrutinib.

Ibrutinib and acalabrutinib reduce P-selectin expression on platelets in response to *A. fumigatus* conidia

To assess whether BTK inhibition affects *A. fumigatus*-induced platelet activation, surface expression of CD62P (P-selectin), a marker of alpha granule secretion, was evaluated by flow cytometry at baseline and following stimulation with *A. fumigatus* conidia. We first performed in vitro experiments on platelet-rich plasma (PRP) from 6 healthy donors and 6 BTK-inhibitor-naïve CLL patients.

Platelet interaction with conidia strongly induced P-selectin expression on platelet surface, both in healthy volunteers (mean CD62P expression 144%, range 107-184%) and CLL patients (mean 153%, range 126-185%), with different exposure time according to patient individual variability, ranging from 90 to 270 minutes. Of note, P-selectin exposure induced by conidia was significantly reduced in the presence of both ibrutinib and acalabrutinib. In detail, in ibrutinib-treated platelets, mean CD62P expression was 86% and 79%, in healthy donors and CLL patients, respectively. Similarly, in acalabrutinib-treated platelets mean CD62P expression was 91% and 85%, in healthy donors and CLL patients respectively. Basal CD62P expression was also statistically significant reduced in ibrutinib- and acalabrutinib-treated non-stimulated platelets compared to controls, in both groups (**Figure 15**).

In contrast, response to 10 μM thrombin receptor agonist peptide (TRAP) was not altered (data not shown).

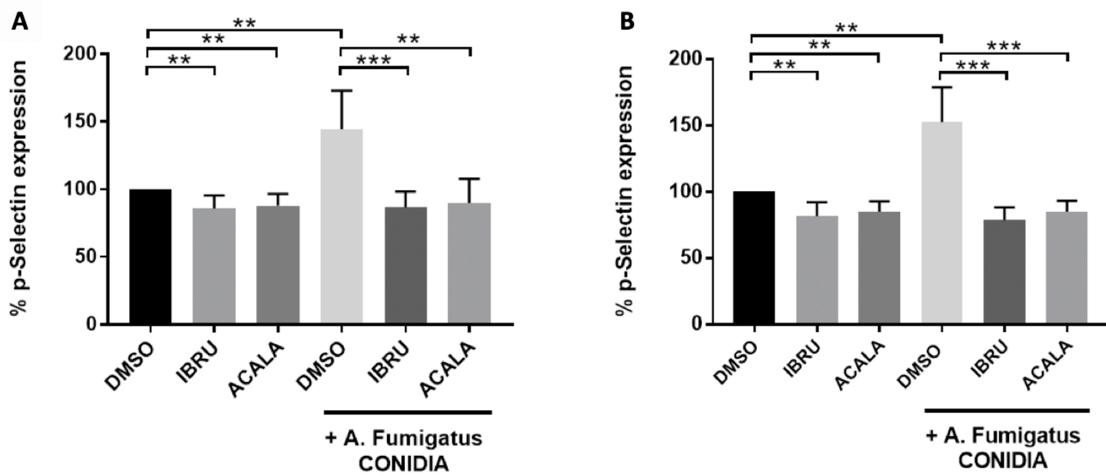

Figure 15. In vitro effects of ibrutinib and acalabrutinib on platelets degranulation in response to *A. fumigatus* conidia. Platelet-rich plasma (PRP) from healthy volunteers (**A**) and BTK-inhibitor naïve CLL patients (**B**) was treated with 1 μM ibrutinib (IBRU), acalabrutinib (ACALA) or vehicle (DMSO) for 1 hour at 37°C and stimulated or not with *A. fumigatus* conidia at effector:target ratio of 100:1 for 30, 60, 90, 120, 180 or 270 minutes. Platelets activation was detected by flow cytometry, labelling samples with PE-conjugated anti-CD42b antibody, a surface marker constitutively expressed on platelets, and with an FITC-conjugated anti-CD62P/P-selectin antibody, a marker of alpha granule secretion. Results were reported as percentages of CD62b expression normalized on DMSO-treated non-stimulated platelets (**p<0.01; ***p<0.001).

Ibrutinib and acalabrutinib hamper platelet-mediated hyphal damage

To further explore the effects of BTK-inhibition on antifungal activity, we measured hyphal damage induced by ibrutinib, acalabrutinib or vehicle-treated platelets, performing a colorimetric assay with XTT. Firstly, we confirmed the ability of platelets to impair *A. fumigatus* hyphal germination and elongation, by counteracting the fungal metabolic activity. Indeed, vehicle-treated platelets from healthy donors and CLL patients induced 46% (range 28-58%) and 44% (range 30-58%) mean hyphal damage, respectively. Moreover, we found that ibrutinib- and acalabrutinib-treated platelets showed a statistically significant reduced capacity to induce hyphal damage compared with vehicle-treated platelets. In particular, ibrutinib-treated platelets induced 34% (range 17-50%) and 33% (range 12-51%) mean hyphal damage in healthy volunteers and CLL patients, respectively. Similarly, acalabrutinib-treated platelets induced 38% (range 24-55%) and 34% (range 17-51%) mean hyphal damage, in healthy donors and CLL patients, respectively (**Figure 16**).

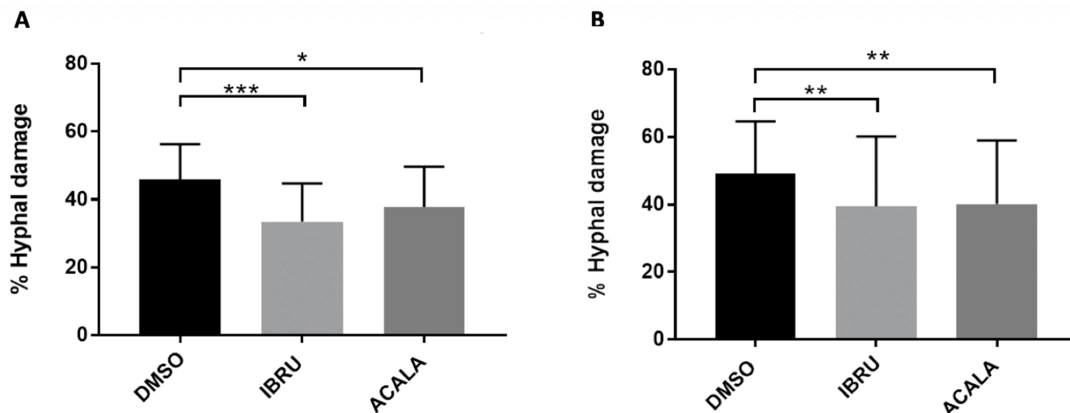

Figure 16. In vitro effects of ibrutinib and acalabrutinib on platelet-mediated hyphal damage. Platelet-rich plasma (PRP) from healthy volunteers (**A**) and BTK-inhibitor naïve CLL patients (**B**) was treated with 1 μM ibrutinib (IBRU), acalabrutinib (ACALA) or vehicle (DMSO) for 1 hour at 37 °C. *A. fumigatus* conidia were incubated for 16 hours at 37 °C in RPMI medium plus 1% sodium pyruvate to produce hyphae with or without platelets, at a platelets to conidia ratio of 100:1. For measurement of hyphal metabolic activity, XTT salt plus 40 μg/ml coenzyme Q was added. Absorbance was determined at 450 nm using an enzyme-linked immuno-sorbent assay plate reader, and antifungal activity was calculated as the percentage of hyphal damage equal to (1-X/C)*100, where X is the optical density of test well and C is the optical density of control wells with hyphae only (*p<0.05; **p<0.01; ***p<0.001).

Platelet-mediated antifungal activity decreases during ibrutinib treatment in CLL patients

Next, to verify the clinical relevance of these findings, we evaluated PLT-mediated antifungal activity in 6 CLL patients before receiving ibrutinib and after 1, 3 and 6 moths during the course of treatment. In these patients, before treatment, P-selectin expression on platelet surface was induced in response to *A. fumigatus* conidia (mean CD62P expression 160%, range 120-224%). In contrast, after 1, 3 and 6 months of therapy, *A. fumigatus*-induced P-selectin mean expression was significantly reduced: 124% (range 97-137%) at month +1, 107% (range 80-114%) at month +3, and 99% (range 87-111%) at month +6 (**Figure 17**).

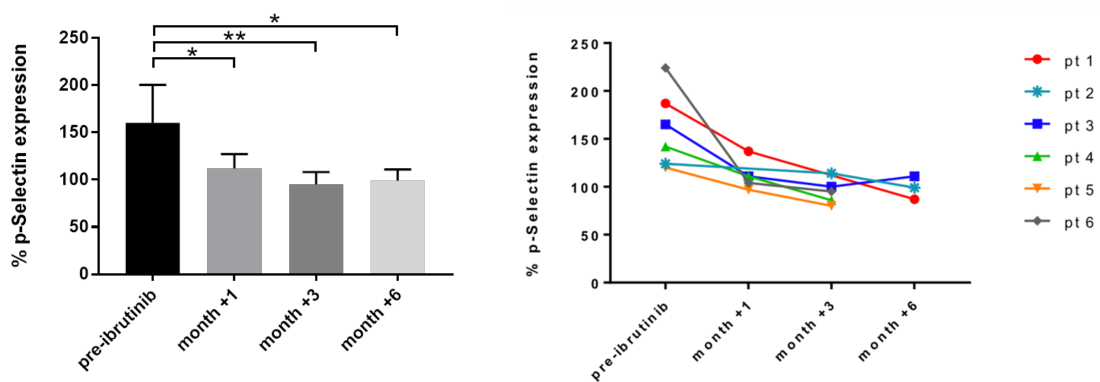

Figure 17. Platelet-mediated antifungal activity in CLL patients under ibrutinib. Bar (left) and line graph (right) showing P-selectin expression in samples collected before starting ibrutinib and during the course of treatment (*$p<0.05$; **$p<0.01$).

DISCUSSION

BTK inhibitors (BTKis) pioneered the major shift of therapeutic approaches for chronic lymphocytic leukemia (CLL) from chemoimmunotherapy to targeted therapy. The introduction of BTKis has allowed dramatic improvement in the prognosis of patients with CLL and other B cell neoplasms, particularly for those harboring high-risk features. Although these small molecules were initially considered less immunosuppressive than chemoimmunotherapy, an increasing number of reports have described the occurrence of infectious complications, especially in ibrutinib-treated patients, with an unexpectedly high incidence of opportunistic invasive fungal infections (IFIs), mainly invasive aspergillosis (IA). Peculiarly, cases of IA occurring under ibrutinib are characterized by an early onset (usually within the first 6 months of treatment) and high rate of CNS involvement [287,344]. Of note, patients with chronic lymphoid neoplasms are usually considered at low risk of IA [220], and the risk is even lower in patients with X-linked agammaglobulinemia (XLA) [345]. As a matter of fact, mechanisms behind the emergence of IA in patients receiving ibrutinib remain elusive. Several lines of evidence suggest that the increased risk for IFIs cannot strictly be attributed to the effects of ibrutinib on the humoral immunity, raising the possibility of off-target effects as major contributors [346,347].

Ibrutinib is an irreversible BTKi affecting pathways downstream of BCR in malignant B lymphocytes, but also has potent effects upon the normal cells of the immune system, thus exerting an immunomodulatory effect [348]. BTK represents a crucial molecule in the transmission of signaling cascade from several immune receptors, such as PRRs, CD11b/CD18, TREM-1 and TLRs, that allow the recognition of fungi by the innate arm of the immunity. Relevant to this, macrophages and neutrophils, whose functions are essential for an efficient antifungal immune response, simultaneously express BTK and TEC, both inhibited by ibrutinib [78]. However, although the involvement of BTK in several innate pathways has been acknowledged for years [78], much remains to be learned about the specific functions of BTK within the innate immune system and their potential clinical impact. In particular, the immunopathogenetic mechanisms underlying the susceptibility of IFIs in patients treated with BTKis are not fully

elucidated and multiple cell pathways are likely to be implicated. Moreover, by considering the antifungal properties of platelets and the well-known anti-platelet effects of ibrutinib, it could also be argued that a defective thrombocyte-mediated immune response may contribute to the increased risk of IFIs in ibrutinib-treated patients. Nonetheless, this suggestive hypothesis has never been investigated so far.

In order to characterize the specific off-target effects of BTKis on anti-mold innate immune response mediated by monocytes, macrophages and platelets, we performed a broad functional in vitro analysis on samples from both CLL patients and healthy donors.

In the present study, we describe how BTK inhibition compromises an adequate inflammatory response by innate cells in CLL patients during *A. fumigatus* infection. Ibrutinib targets BTK expressed in CLL-associated macrophages (also known as *nurse-like cells*, NLCs), exacerbating their immunosuppressive profile through polarization toward M2-type macrophages, showing impaired phagocytic activity [343]. Then, we explored the potential effects of ibrutinib to influence the ability of NLCs to effectively hinder the growth of *A. fumigatus*. Conflicting results of ibrutinib effects on phagocytic ability have been reported so far. Several studies have demonstrated the involvement of BTK in FcγR-mediated phagocytosis in macrophages [92]. Phagocytosis of *A. fumigatus* conidia activates TLR9 recruitment to the phagosome and, through BTK, induces PLCγ and calcineurin-mediated NFAT nuclear translocation [349]. Recently, Bercusson et al. reported an impairment in hampering the fungal growth, without a significant difference in phagocytic activity of monocyte-derived macrophages treated with ibrutinib [350]. In addition, a reduction of FcγR-mediated cytokine production, but not phagocytic ability, was observed in circulating monocytes [115]. Conversely, BTK-deficient macrophages show defects in fungal phagocytosis, and TLR-mediated phagocytosis of tumor cells seems to be impaired by ibrutinib treatment of macrophages, implying a relevant role of BTK in the phagocytic activity of innate immune cells [274,351]. These findings were also confirmed in the neutrophil population, where ibrutinib-induced reduction of *A. fumigatus* engulfment and killing was described both in vitro and in vivo [352].

Similarly, we detected an impairment of phagocytic activity in NLCs together with a significant reduction of MAC-1 (CD11b/CD18) activation, essential for phagocytic cup

formation [343]. By extending these observations, here we also demonstrate that ibrutinib affects the ability of NLCs to counteract *A. fumigatus* conidia germination. To confirm this result, we also tested the circulating CD14+ monocyte population isolated from both CLL patients and healthy donors, likewise detecting a significant decrease of phagocytosis upon ibrutinib treatment. The fungal cell wall contains polysaccharides and lipid moieties that activate an immune response with a strong production of cytokines, including TNF-α, IL-1β, IL-6, IL-8. The classic PRRs for *A. fumigatus* include β-glucan receptor Dectin-1, CD11b/CD18 (also known as complement receptor 3, CR3), TREM-1 and TLRs. Stimulation of NLCs with either *A. fumigatus* conidia or zymosan induces the phosphorylation of BTK, which in turn activates downstream cascade with STAT1, IκBα and AKT. Herein, we show the impairment of inflammatory pathways caused by ibrutinib, despite stimulation with either *A. fumigatus* conidia or zymosan. As described by Cervantes-Gomez et al., ibrutinib therapy leads to a decrease in phosphorylated and total BTK protein in CLL cells, and a decline in BTK total protein was observed in circulating leukemic cells after four weeks of treatment [353]. In our experimental setting, exposure to ibrutinib determines a reduction in the activation of these pathways on CLL-derived macrophages, thus confirming the results of a previous work on human macrophages isolated from healthy donors [350]. Since PRR engagement can determine phagocytosis, macrophage activation, and strong induction of pro-inflammatory responses, we planned to determine the inflammatory profile of NLCs and CLL monocytes during stimulation with *A. fumigatus* and zymosan. Our data from gene expression profile (GEP) show that ibrutinib intensely forces an immunosuppressive profile in NLCs, with relevant impairment in the expression of genes related to TNF and IL-1 families. Notably, the expression and secretion of TNF-α and IL-1β in NLCs were strongly affected by ibrutinib when reproducing fungal infection in vitro. Confirmation of a reduction in TNF-α secretion ability was also found in CD14+ circulating monocytes, both in CLL patients and healthy donors.

Similarly, acalabrutinb, a second-generation BTKi, more selective than ibrutinib, induced a reduction of TNF-α in both NLCs and CD14+ circulating monocytes. This suppressive effect was also retained upon stimulation with fungal antigens.

Thereafter, in order to investigate the biological modifications induced in vivo by ibrutinib, we analyzed circulating monocytes of CLL patients before and after three months of treatment with ibrutinib. In our cohort of CLL patients, we confirm our in vitro data, in particular: (i) ibrutinib confers an exacerbation of some immunosuppressive features in monocytes; (ii) basal secretion of TNF-α by circulating monocytes is effectively reduced after initiating ibrutinib therapy; (iii) the phagocytosis ability is hindered during treatment with ibrutinib. These results indicate that such modifications may compromise an efficient inflammatory response during fungal infection.

Besides neutrophils and monocyte/macrophage populations, NK cells and platelets (both expressing BTK) represent two further relevant cell types involved in the host innate immune responses. Interestingly, Flinsenberg et al. have recently reported that ibrutinib, but not zanubrutinib, significantly suppresses NK-cell cytotoxicity in MCL patients, most likely through an off-target inhibition of ITK (rather than BTK) [354].

In our work, we demonstrate, for the first time, that the exposure to BTKis impairs different immune functions of platelets. Our original data show that in vitro inhibition of BTK, by either ibrutinib or acalabrutinib, suppresses platelet-mediated antifungal activities, with a reduction of platelet to conidia adhesion, conidia-mediated P-selectin expression and platelet-induced hyphal damage, thereby supporting the hypothesis, never tested so far, that ibrutinib may favor the development of IFIs also through a detrimental effect on platelets.

The lesson learned from IFIs, unexpectedly arising in patients treated with ibrutinib, made physicians aware of the potential clinical consequences deriving from the pharmacologic inhibition of a pleiotropic kinase, such as BTK, as well as from the "off-target" effects related to this (not so) targeted drug. Relevant to this, ibrutinib, but not other more selective BTKis, has been proposed as a new predisposing factor for IFIs and incorporated as a novel host factor for the definition of probable invasive pulmonary mold disease by the European Organization for Research and Treatment of Cancers and the Mycoses Study Group [355]. Acalabrutinib and zanubrutinib have not yet been associated with an increased risk for IFIs and have already been shown to have a differential impact on both innate and adaptive immunity compared with ibrutinib.

However, prospective studies are warranted to precisely assess the risk of IFIs occurring in patients treated with ibrutinib and, importantly, with other BTKis.
Moving from "bench" to "bedside" and back again, our study reveals specific modifications in innate immune responses mediated by multiple cell types, induced by ibrutinib and acalabrutinib, both in CLL patients and healthy donors, shedding light on the relationship between IFIs and treatment with BTKis and highlighting the biological and clinical relevance of BTK as a "guardian" of the innate immunity.
Further studies are required to disclose whether the multifunctional impairment in innate immunity is strictly caused by the inhibition of BTK outside the BCR (i.e., on unwanted cellular targets expressing BTK) or, more likely, is also mediated by unselective molecular inhibition of other kinases.
Moreover, it should be noted that the exposure to ibrutinib is probably not sufficient, by itself, to the development of IFIs and additional host-related factors, both inherited and acquired, as well as environmental factors, are likely implicated. Moving from this observation, the development of novel score systems, including polymorphism analysis of candidate genes (i.e., PTX3, TLR4, etc.) [262] is warranted to refine individual risk stratification of patients, possibly guiding the indications to the antifungal prophylaxis.
In perspective, new immunological tools and specific inflammatory biomarkers, able to explore both T and innate cells, could usefully be implemented in clinical practice, hopefully allowing to identify and monitor "protective vs permissive" immune profiles in patient at risk to develop IFIs.

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