The Bruton tyrosine kinase inhibitor ibrutinib abrogates triggering receptor on myeloid cells 1-mediated neutrophil activation

Recurrent infections are common complications in patients with non-Hodgkin lymphomas, for example, chronic lymphocytic leukemia (CLL). The secondary immune defect as the underlying cause of frequent infections is in part due to hypoimmunoglobulinemia or diminished T- and B-cell responses suppressing protective immunity.[1] In addition, such recurrent infections may also be treatment related, for example, by cytotoxic agents causing neutropenia, but also by other mechanisms, such as the late onset neutropenia associated with rituximab after achieving complete remission.[2] In this context, targeting the Bruton tyrosine kinase (BTK), a critical non-receptor tyrosin kinase in B-cell development and activation, is a novel treatment approach apparently avoiding the cytotoxic side effects of established regimens.[3] While inherited genetic defects in the Btk gene

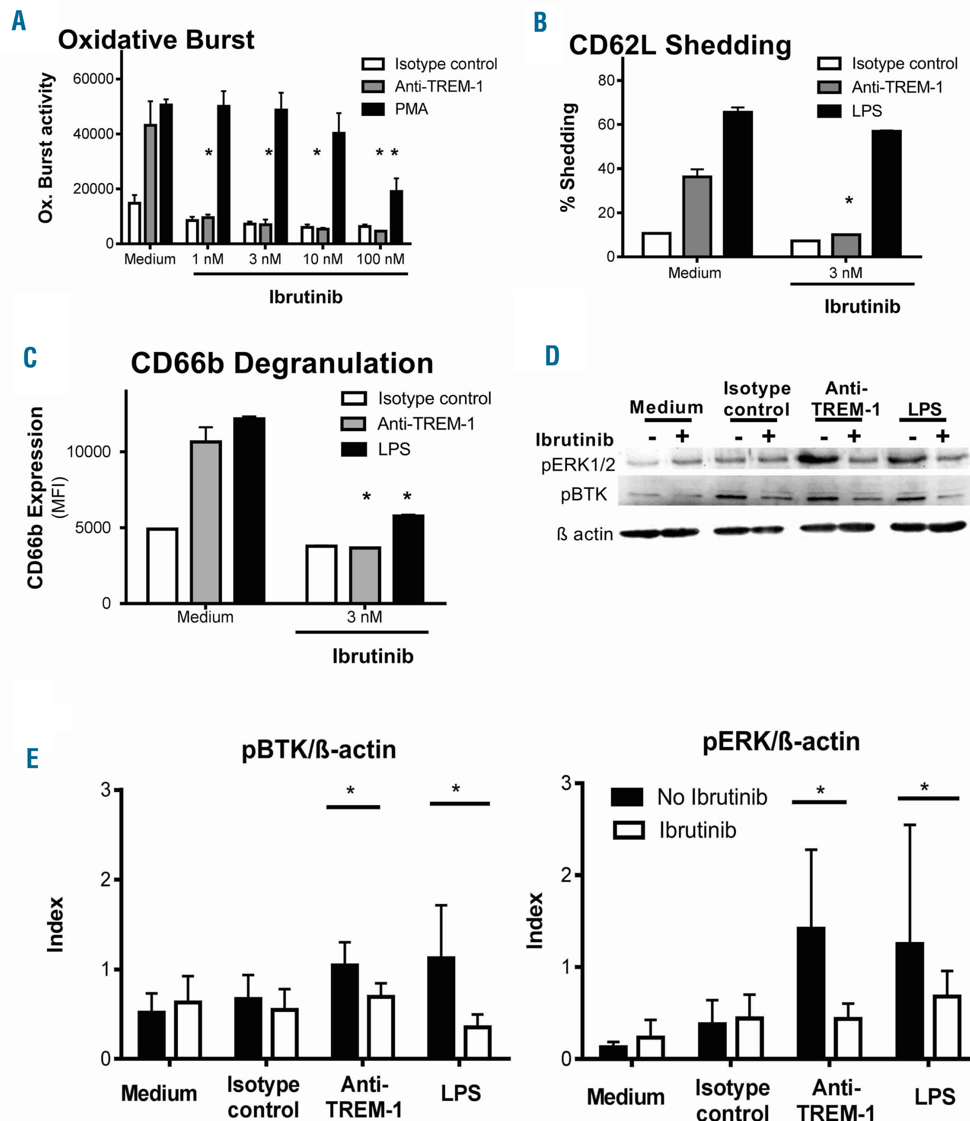

Figure 1. Ibrutinib abrogates oxidative burst activity, CD66b degranulation and CD62L shedding in TREM-1 activated human neutrophils. (A) Human polymorphonuclear neutrophils (PMNs) were stimulated with an isotype-matched control mAb, anti-TREM-1 or phorbolester (PMA) in the presence or absence of titrated amounts of ibrutinib (see *Online Supplementary Appendix* for details). Oxidative burst activity (mean±standard deviation, assayed in triplicate wells with purified PMNs; cumulated data of n=8 individual healthy donors) of human PMNs after 120 minutes (min) was analyzed using dichloro-dihydro-fluorescein diacetate (DCFH-DA). (B and C) Human PMNs were stimulated with an isotype-matched control mAb, anti-TREM-1 or lipopolysaccharide (LPS, 1 μg/mL). (B) CD62L shedding rate (cumulated data of n=10 individual healthy donors) and (C) expression of CD66b [mean fluorescence intensity (MFI); cumulated data of n=11 individual healthy donors] in the presence or absence of ibrutinib (3 nM) were determined by flow cytometry after 60 min. (D) Western blot analysis for total or phospho-ERK1/2 (pERK1/2), total or phospho-BTK (pBTK) and β actin of PMN lysates after stimulation with medium, isotype control, anti-TREM-1 or LPS with or without ibrutinib (3 nM), as indicated. (E) Densitometric analysis of Western blot analyses for pERK/β actin (right) or pBTK/β actin (left) ratios, respectively. Data shown are mean±standard deviation from cumulated data of n=3 individual experiments with healthy donors. *P<0.05 by Kruskal-Wallis and Dunn *post hoc* test compared to the corresponding control without inhibitor (medium).

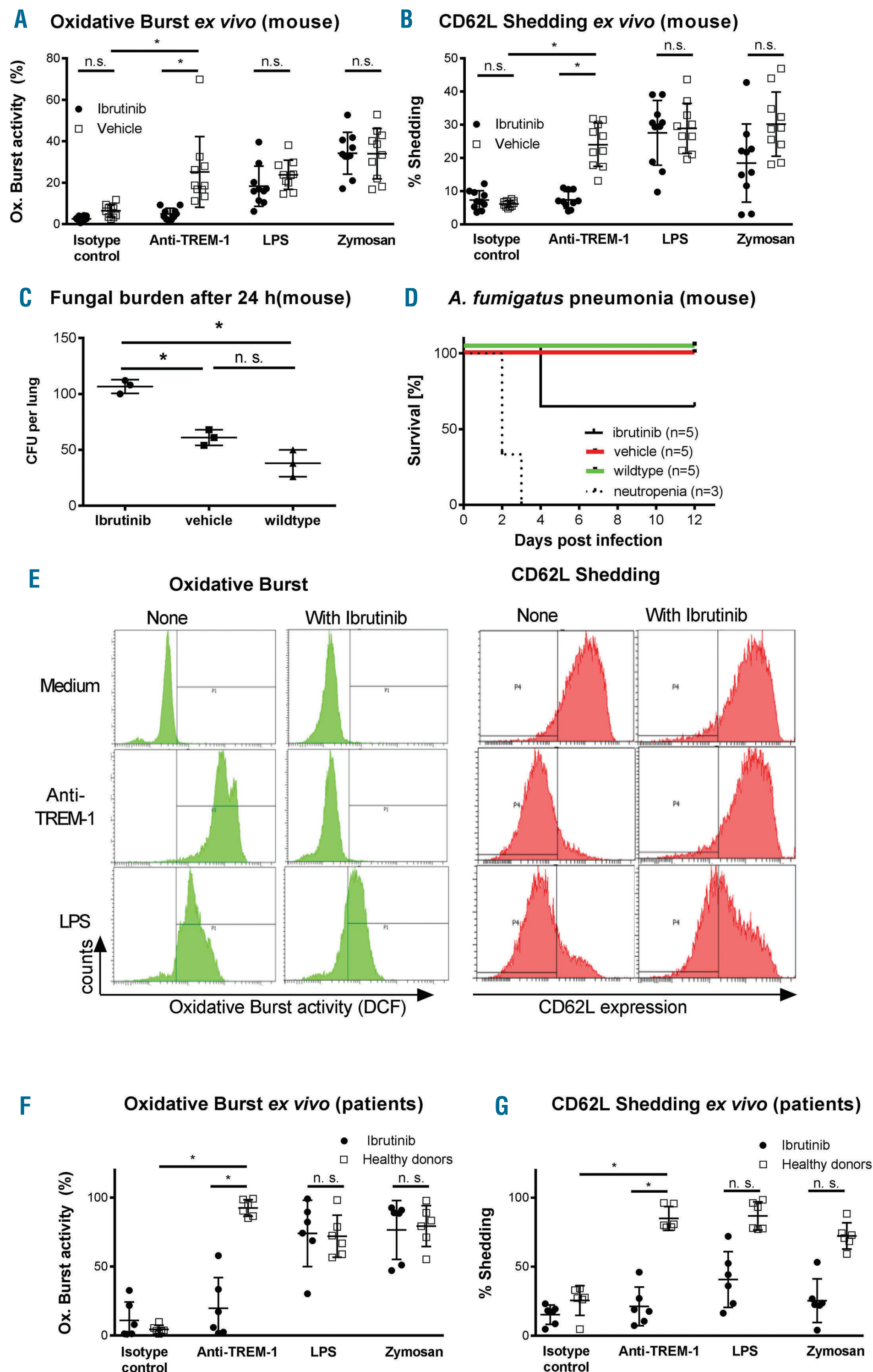

Figure 2. Ibrutinib suppresses fungal clearance in mice and TREM-1-mediated neutrophil activation in mice and humans. C57BL/6 mice (n=10 per group) were treated either with intraperitoneal (I.p.) ibrutinib (6 mg/kg) or vehicle for three days. Bone marrow-derived PMN incubated with isotype control, anti-TREM-1, LPS (1 μg/mL) or zymosan (10 μg/mL). (A) Oxidative burst activity and (B) CD62L shedding were determined by flow cytometry. (C and D) Mice (n=8 per group) were treated either with ibrutinib (6 mg/kg) or vehicle for three days and subsequently infected i.t. with *A. fumigatus* (10^7 conidia per animal). (C) At 24 hours post infection, 3 mice per group were sacrificed, and fungal burden was assessed by plating serial dilutions of lung homogenates on AMM agar plates and enumerating colony-forming units (CFU). (D) Survival of *A. fumigatus* challenge in the indicated groups. Neutropenic mice (n=3) were treated with anti-Gr1 (150 μg i.p. on Day -1). (E) Polymorphonuclear neutrophils (PMNs) from a 74-year old female patient with recurrent mantle cell lymphoma were analyzed for oxidative burst activity (green) and CD62L shedding (red) upon TREM-1 or TLR4 ligation (with LPS) before the initiation of ibrutinib (none, left) and after six weeks on ibrutinib (560 mg QD) (with ibrutinib, right). (F and G) Heparinized blood from healthy volunteers or lymphoma patients treated with ibrutinib without any clinical evidence of infection (see *Online Supplementary Appendix* for details) was used. PMN of ibrutinib-treated patients (n=6) or healthy donors (n=6) were analyzed after indicated stimulations (60 min. at 37°C) for (F) oxidative burst activity and (G) CD62L shedding. *Significant difference by Kruskal-Wallis and Dunn *post hoc* test.

cause X-linked agammaglobulinemia (XLA), inhibition by the small compound ibrutinib down-regulates B-cell receptor signaling by suppressing B-cell receptor overstimulation and subsequently B-cell activation in normal and malignant B cells.[4] Clinical studies showing the safety and efficacy of ibrutinib have led to approval of the drug by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of CLL, relapsed or refractory mantle cell lymphoma, and Waldenström's macroglobulinemia.[5] Nevertheless, infections still remain a major cause of complications in patients receiving ibrutinib,[1] even though the risk of infections decreases over time under treatment.

Importantly, BTK is not only relevant in B cells, but also in innate immune cells such as polymorphonuclear neutrophils (PMN) and macrophages.[6] In particular, BTK is involved in Triggering receptor expressed on myeloid cells 1 (TREM-1; CD354) signaling in monocytic cells.[7] TREM-1 is an activating receptor of the immunoglobulin superfamily on monocytes/macrophages and PMNs, and regulates innate immune responses. This has been demonstrated in particular in microbial infections and sepsis,[8] but also in other inflammatory conditions such as peripheral artery disease[9] or inflammatory bowel disease.[10]

For signal transduction, TREM-1 associates with the transmembrane adapter protein DAP-12 leading to downstream activation of mitogen-activated protein kinases (MAPK) such as p38MAPK, extracellular regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K). Activation of TREM-1 occurs in concert with Toll-like receptors (TLR) and NOD-like receptors (NLR) leading to synergistic cell activation and the amplification of inflammatory responses.[11] With respect to TREM-1 specific activation, BTK is also required for the activation of monocytic cells.[7] This prompted us to ask whether the inhibition of BTK also impairs the TREM-1-mediated inflammatory responses by PMNs.

To assess whether ibrutinib affects TREM-1-mediated PMN activation, we stimulated purified PMNs from healthy donors with TREM-1 specific or control monoclonal antibodies (mAbs) in the presence of ibrutinib and analyzed the oxidative burst (*Online Supplementary Appendix*). To exclude cytotoxic effects of the inhibitor, we included stimulations with phorbolester (PMA) as an activator of protein kinase C that strongly induces oxidative burst (Figure 1A). The PMA-induced oxidative burst was only suppressed at high concentrations of ibrutinib (100 nM). In contrast, TREM-1 ligation induced a comparable oxidative burst activity that was completely abrogated even at low concentrations of ibrutinib (ie. 3 nM) indicating that ibrutinib specifically inhibits the TREM-1-induced oxidative burst.

To confirm this, we activated PMNs *via* TREM-1 and analyzed CD62L shedding in the presence of ibrutinib. As a positive control, we used the TLR4 agonist lipopolysaccharide (LPS), observing a strong increase in CD62L shedding upon TLR4 or TREM-1 ligation, but not with the control mAb (Figure 1B). Again ibrutinib completely abrogated TREM-1, but not LPS-induced CD62L shedding, further supporting the notion that ibrutinib is a specific inhibitor of TREM-1-mediated PMN activation. In addition, we assessed the degranulation of secondary granules using the upregulation of CD66b surface expression, once again demonstrating the complete abrogation of CD66b upregulation upon TREM-1 ligation (Figure 1C). Interestingly, we observed a partial inhibition of CD66b degranulation upon TLR4 ligation in the presence of ibrutinib, indicating that this inhibitor also affects TLR4-mediated PMN activation to some extent.

Next, we stimulated PMNs *via* TREM-1 and performed western blot analyses for ERK1/2, a well known downstream kinase of TREM-1, as previously shown by us and others.[11] Ligation of TLR4 or TREM-1 both mediated ERK1/2 phosphorylation (Figure 1D and Figure 1E, right panel, as quantitative analysis), while there was no detectable signal in the isotype-matched control or medium alone. Consistent with the previous results, PMNs stimulated in the presence of ibrutinib displayed a nearly complete suppression of phospho-ERK1/2 upon TREM-1 ligation. In contrast, the LPS-induced phospho-ERK1/2 signal remained nearly unaffected indicating that ibrutinib impairs TREM-1-mediated PMN activation by specifically inhibiting the TREM-1 downstream signaling cascade. In addition, we detected an increased phosphorylation of BTK upon TREM-1 or TLR4 ligation. In line with our previous results, BTK phosphorylation was diminished in the presence of ibrutinib, confirming the specificity of ibrutinib for BTK also in PMNs (Figure 1D and Figure 1E, left panel, as quantitative analysis). Interestingly, the activation of BTK was not only abrogated upon TREM-1, but also upon TLR4 ligation, indicating a role for BTK for both receptors. However, there are apparently distinct functional roles of BTK and signaling cascades of TREM-1 *versus* TLR4. Nevertheless, our results obtained in primary PMNs are consistent with Omsby *et al*. who reported similar results for a monocytic cell line[7] and also identify BTK as a crucial signaling molecule in the TREM-1 signaling cascade to regulate TREM-1-dependent cell activation in general.

To verify the relevance of these results *in vivo*, healthy mice were treated with the BTK inhibitor ibrutinib or vehicle for three days. Subsequently, we stimulated PMNs with anti-TREM-1, LPS or zymosan (as another TLR agonist triggering TLR2/6) *ex vivo*. PMNs from vehicle-treated mice displayed a robust induction of the oxidative burst (Figure 2A) and shedding of CD62L (Figure 2B) upon ligation of TREM-1, TLR4 or TLR2/6. In contrast, the oxidative burst and CD62L shedding upon TREM-1 ligation were completely inhibited in PMNs from mice pre-treated with ibrutinib, while there was no significant impairment of the TLR-induced oxidative burst or in CD62L shedding, indicating that our previous results obtained *in vitro* also hold true *in vivo*. In agreement with this, when challenged with *A. fumigatus* conidia, ibrutinib-treated mice had a higher fungal burden in lungs compared to the vehicle-treated controls (Figure 2C). While the *A. fumigatus* challenge was lethal in neutropenic mice (after depletion with anti-Gr-1, clone RB6 8C5, see broken line in Figure 2D), ibrutinib treatment (black line) resulted in an impaired survival compared to vehicle (red line) treated or untreated (wild-type; green line) controls (Figure 2D). These data suggest that ibrutinib treatment *in vivo* affects PMN activation leading to impaired TREM-1-dependent immune responses, eg. in fungal infections where TREM-1 is important.[8]

Finally, we were interested in further evaluating this effect of ibrutinib on PMN activation in humans and analyzed PMN effector functions as above in a 74-year old female patient with recurrent mantle cell lymphoma before and post six weeks of treatment with ibrutinib. In this patient, before treatment, the TREM-1- and TLR4-mediated oxidative burst (green) and shedding of CD62L (red) were intact (left), indicated by the increased signal in green fluorescent DCF and reduced CD62L expression (Figure 2E). In contrast, after six weeks of ibrutinib treatment (right), the TREM-1 induced oxidative burst and shedding of CD62L was completely abrogated, while the

TLR4 induced activation was mainly retained. For confirmation of these results, we analyzed another 6 patients with recurrent B-cell non-Hodgkin lymphomas receiving ibrutinib (see *Online Supplementary Appendix* for patients' characteristics) and compared this to the PMN functionality of 6 healthy donors. The oxidative burst upon LPS or zymosan treatment was comparable between healthy donors and patients treated with ibrutinib (Figure 2F and G). Interestingly, CD62L shedding was somewhat reduced in patients under treatment with ibrutinib, even though the difference was not significant. This is in agreement with our western blot analysis which revealed a reduced BTK phosphorylation also upon TLR4 ligation in the presence of ibrutinib (Figure 1D). Therefore, this might reflect the fact that BTK is also involved in the TLR and Dectin-1 signaling pathways.[12,13] However, in contrast to this, the TREM-1-mediated oxidative burst and CD62L shedding were strongly suppressed in PMNs from ibrutinib-treated patients, while TREM-1 ligation in PMNs from healthy donors was unaffected in both effector assays. Our data should be interpreted with caution as to the clinical significance of these findings since we have not controlled for patient individual variability of the assays, having analyzed PMN functions pre and post dosing of ibrutinib only in one patient (Figure 2E). This is a limitation of the presented data that can only be overcome within a prospective clinical trial. Nevertheless, these data suggest that inhibition of BTK by ibrutinib suppresses TREM-1-mediated PMN functionality, potentially in a clinically relevant way. Ibrutinib-mediated impaired PMN functionality may contribute to the increased rate of infections in patients treated with ibrutinib beyond the impairment of B-cell responses.[1] An additional aspect that deserves attention is the question as to whether ibrutinib possibly favors the generation of particular PMN subsets with suppressive functional phenotypes that are increasingly being recognized.[14] However, this needs to be addressed in future studies.

Previous reports have focused on the influence of ibrutinib on anti-tumor responses.[15,16] This included impaired antibody-dependent cytotoxicity by PMNs and natural killer cells and variable relevance for this *in vivo*. In our data, we now provide the first evidence for a specific mechanism involving TREM-1 as an important inflammatory receptor that may be involved in the frequently observed infectious complications in patients receiving ibrutinib. We also suggest that ibrutinib may be a novel tool to modulate pathological TREM-1-driven immune responses, such as sepsis,[8] inflammatory bowel disease,[10] peripheral artery disease,[9] or psoriasis.[17]

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