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

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Materials and Methods

Patients and Materials

This study was conducted according to the Declaration of Helsinki and approved by the local ethics committee, Landesärztekammer Rhineland-Palatine, Approval no. 837.224.10 (7233). After obtaining written informed consent, heparinized blood from healthy volunteers or patients (median age 68 years; range 60-81) treated with ibrutinib lacking clinical evidence of infection was used. Diagnosis included DLCBL (n=1), CLL (n=3) and MCL (n=2) with at least three prior lines of treatment (range 3 to 7). Mouse studies were approved by the local authorities (Landesuntersuchungsamt Rhineland-Palatine, Approval no. 23 177-07 / G 16-1-020). The following Abs were used: anti-human TREM-1 (clone 6B1, IgG1, from Becton Dickinson or anti-mouse TREM-1 from R&D Systems, Wiesbaden, Germany), an isotype matched control mAb (clone 4C9, in-house), β-actin, BTK, phosphor-BTK, ERK1/2 and phospho-ERK1/2 (all from Cell-Signaling Technology, Danvers, MA, USA), CD11b PE and CD62L APC (BioLegend, San Diego, CA, USA), CD66b FITC (Beckman Coulter, Krefeld, Germany).

PMN purification and activation

Human PMNs were isolated from heparinized blood by dextran sedimentation and histopaque density centrifugation as previously described [1]. Purity of human PMNs was assessed by flow cytometry using CD66b and CD11b and was > 98%.

PMNs from C57BL/6 mice (6 to 8 weeks) were isolated from femoral bone marrow cells and enriched for PMNs by magnetic cell sorting (MACS) using biotin labeled Ly-6G/C specific antibodies (clone RB6-8C5) and streptavidin beads (Miltenyi, Bergisch Gladbach, Germany) as previously described [1]. Purity of the cells was assessed by flow cytometry using Ly-6G and CD11b. In general, > 92 % of cells were Ly-6G^{+} CD11b^{+}.

Mouse or human PMNs were preincubated at 37°C for 30 min. with ibrutinib or vehicle (DMSO) as indicated (BIOZOL, Eiching, Germany). Cells were stimulated with lipopolysaccharide (LPS), zymosan or phorbolester (PMA) (all from Sigma-Aldrich, Taufkirchen, Germany). For the ligation of TREM-1, anti-TREM-1 or an isotype matched control monoclonal antibody (each at 10 µg/ml) was coated on 96-well flat- bottom plates for functional assays or on 12-well plates for Western blot analyses (both from Greiner Bio One, Frickenhausen, Germany).

Oxidative burst activity

For the assessment of the oxidative burst activity, dichloro-dihydro-fluorescein diacetate (DCFH-DA, Sigma, Taufkirchen, Germany) was added to the cells at 8.3 nM. Human PMNs were incubated for 180 min. at 37°C in a fluorescence reader (SpectraFluor 4 or Genios, Crailsheim, Germany) detecting green fluorescent DCF in 5 min. intervals. Oxidative burst activity of stimulated cells was obtained by

subtraction of the background fluorescence of labeled cells incubated in medium alone at the corresponding time points.

For the *ex vivo* oxidative burst activity of human or mouse PMNs, purified cells were stimulated as indicated in the presence of DCFH-DA (8.4 nM) and harvested after 60 min. at 37°C for analysis by flow ctometry.

CD66b degranulation and CD62L shedding

PMNs were stimulated as indicated above in 96-well plates in triplicate wells and harvested after 60 min. at 37°C. Subsequently, the cells were washed twice with PBS / 1% BSA, and labelled with fluorochrome conjugated mAbs against CD66b, CD11b and CD62L. Flow cytometric analyses were done on a LSR II (Becton Dickinson). Expression of CD66b was as mean fluorescence intensity (MFI). Shedding rate was calculated as follows gating the rate of CD62L positive events (on CD66b and CD11b positive PMNs): Shedding rate = (100 - % CD62L positive events)/100.

Western Blot analysis

After stimulation in the absence or presence of the indicated inhibitor for 30 min., PMNs were harvested and washed twice with Cells were lysed with a modified urea buffer (7 M urea, 2 M thiourea, 5 mM DTT, 2% CHAPS, l0 mM PMSF, 0.5 mM Na-orthovanadat, 5mM NaF and complete protease inhibitor cocktail). Protein content was quantified by the method of Bradford. Lysates were resolved to 12% SDS PAGE transferred onto a PVDF membrane (Merck Millipore, Billerica, Mass., USA) and probed with the appropriate antibodies and secondary antibodies conjugated with

horseradish peroxidase. Western blots were developped by the ECL detection system (Pierce, Bonn, Germany) [2]. Quantification of Western blot band was performed by Quantity One Software (Biorad, Munich, Germany).

Fungal strains and culture conditions

The *A. fumigatus* strain ATCC 46645 was kindly provided by M. Gunzer (Molecular Immunology, University of Duisburg-Essen, Germany) and cultured in *Aspergillus* minimal medium (AMM) with 1 % (w/v) glucose as carbon source as described previously [1]. Briefly, conidia were incubated on AMM agar plates for four days at 37°C and 5 % CO_2. Resulting spores were removed with sterile water containing a small amount of glass pearls. The obtained spore suspension was filtered twice through a sterile 40 µm nylon mesh and stored at 4 °C in sterile water until required.

Mouse model of IPA

Mice received 10^7 *A. fumigatus* conidia i.t. as described previously [3]. Briefly, a 22 G indwelling venous catheter (B. Braun AG, Melsungen, Germany, Vasofix Braunüle) was used for tracheal intubation of anesthetized animals, 100 µl spore-suspension was added and inhaled without further help. For enhancing fungal distribution, mice were ventilated mechanically for two minutes using an animal respirator (MiniVent, Hugo Sachs, March-Hugstetten, Germany, 250 breaths/min, 300 µl/breath). After infection, severity of systemic infection was examined daily and overall survival were monitored for 14 days [3]. Where indicated, PMN depletion was induced by i.p. injection of anti-Gr-1 antibody (150 µg, clone RB6-8C5) one day before infection (indicated as neutropenia in Fig. 2D).

Fungal load in the lungs in vivo

To characterize *in vivo* fungal burden, lungs of sacrificed mice were dissected 24 h after infection [3]. Lungs were homogenized mechanically and serial dilutions were plated on sabouraud-4 %-glucose agar. After 48 h at 37°C and 5 % CO_2, colony-forming units (CFU) were enumerated.

Statistical analyses

All graphing and statistical analyses were performed using GraphPad Prism V5.0a (Graphpad, San Diego, CA, USA). For all analyses, a value of $p < 0.05$ was considered significant. For multiple group comparisons, Kruskal-Wallis test and Dunn post hoc test were used as indicated.

References

1. Kapp K, Prufer S, Michel CS, Habermeier A, Luckner-Minden C, Giese T, et al. Granulocyte functions are independent of arginine availability. Journal of Leukocyte Biology. 2014 Dec 1;96(6):1047–53.

2. Prüfer S, Weber M, Sasca D, Teschner D, Wölfel C, Stein P, et al. Distinct signaling cascades of TREM-1, TLR and NLR in neutrophils and monocytic cells. J Innate Immun. 2014;6(3):339–52.

3. Prüfer S, Weber M, Stein P, Bosmann M, Stassen M, Kreft A, et al. Oxidative burst and neutrophil elastase contribute to clearance of Aspergillus fumigatus pneumonia in mice. Immunobiology. 2014 Feb;219(2):87–96.