Ibrutinib induces multiple functional defects in the neutrophil response against *Aspergillus fumigatus*

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ONLINE SUPPLEMENT METHODS

Patients. Thirty-three patients from 3 centers (Institut Gustave Roussy, Institut Curie, and La Pitié-Salpêtrière Hospital) treated with ibrutinib for a lymphoid malignancy were enrolled in the study (see Table 1). The characteristics of the patients and blood samples are shown in table 1. Main indications for ibrutinib treatment were CLL (n=23; 72% of patients) and mantle-cell lymphoma (n= 4; 12.5% of patients). Patients receiving corticoid therapy or granulocyte colony stimulating factors were not considered. Neutrophils were collected from patients just before ibrutinib treatment was initiated (M0; n=23) then approximately 1 month (M1; n=22) and 3 months later (M3; n=18). No patient was neutropenic. Because a complete longitudinal (M0, M1 and M3) follow up could not be obtained in all patients, the sample size may vary between experiments and time points. No available data have been substracted. This work did not aim to investigate the emergence of aspergillosis amongst patients but it should be noted that no IA case was diagnosed throughout the study. Other experiments were performed using blood samples obtained from healthy donors.

Neutrophil isolation. Neutrophils were isolated using the dextran-Ficoll method. Briefly, whole fresh blood was mixed with an equivalent volume of 2.0% dextran solution (Sigma Aldricht) in normal saline and the red blood cells were allowed to settle for 40 minutes at 4°C. The leucocyte-rich supernatant was then submitted to Ficoll (Eurobio) centrifuge separation for 30 minutes at 750 g; 4°C. After elimination of the remaining red blood cells, neutrophils in the pellet were recovered in RPMI medium and tested within 2 hours.

***Aspergillus* fumigatus strain.** An *Aspergillus fumigatus sensu stricto* strain isolated from clinical samples in La Pitié-Salpêtrière Hospital was used for the experiments. The strain was maintained on Sabouraud with chloramphenicol and gentamicin agar tubes at 37° for 5-7 days. Conidia were harvested in phosphate-buffered-saline (PBS) containing 0.05% Tween 20, washed three times and resuspended in PBS and counted.

Stimulation conditions. Five-hundred-microliter whole-blood samples were stimulated with either 10^6 *Aspergillus* germinating conidia or 5 ng/mL bacterial lipopolysaccharide (LPS) (Sigma Aldrich) or PBS as control for 2 hours at 37°C. For some experiments, ibrutinib (Sigma Aldrich) was added to blood for ten minutes at a final concentration of either 1 or 5 μM prior to stimulation and compared with blood in which same concentration of DMSO was added. For LPS condition, formyl-methionine-leucyl-phenylalanine (fMLP; final concentration 1 μM) was added 5 minutes before the end of stimulation.

Surface molecule expression of neutrophils. For cytometry analysis anti-human antibodies directed against the following human antigen were used: CD11b, CD14, CD15, CD16, CD62L, Dectin 1, TLR2 and TLR4 (BD Biosciences) and CD66b (Biolegned). Cytometry was performed on a Fortessa X20 flow cytometer (BD Biosciences) and results were analyzed using FlowJo software. Neutrophils were gated as CD15+, CD66+, CD16+, CD14- cells.

Measurement of neutrophil oxidative burst. For determination of reactive oxygen species (ROS) production, neutrophils contained in 500 μL heparinized whole-blood samples were incubated with 123-dihydro-rhodamine (Life Technologies) (final concentration 1.5 μg/mL) for 5 minutes at 37°C, then stimulated with the aforementioned stimuli for 2 hours at 37°C. Samples stained with surface antibodies were then analyzed by flow cytometry.

Intracellular cytokine analysis. 500 μL of whole-blood samples were stimulated with the above-mentioned stimuli for 4 hours at 37°C under kind agitation. Brefeldin A (final concentration 5 μg/mL) (Sigma-Aldrich) was added after 30 minutes of stimulation. Cells were stained with membrane antibodies, permeabilized using the Intracellular Fixation & Permeabilisation Buffer Set (eBioscience) according to manufacturer's recommendations and stained with the following anti-human antibodies: IL8, IL-6, TNFα (BD Biosciences) and IL1β (R&D Systems). IL-8 concentration was determined using Duo-Set ELISA kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's protocol.

Video microscopy experiments. Three thousand *Aspergillus fumigatus* conidia were seeded in black, 96-well clear-bottom plates (Greiner) in RPMI medium supplemented with fetal calf serum (final concentration: 2%) and allowed to germinate at 37°C for 7 hours. After this growth period, *Aspergillus* measuring approximately 15-20 µm can be considered either as germinating conidia or as small hyphae. After two washes, 48,000 isolated neutrophils were added in RPMI medium and Sytox green (final concentration 2 µM) in duplicate. For some experiments, germinating conidia were opsonized by autologous serum during 15 minutes at 37°C then washed before addition of neutrophils. Interactions were visualized during 16 hours of co-cultures at 37°C under 5% CO_2 using a Zeiss Axio Z1 fluorescent microscope (Carl Zeiss, Germany) using Zen software. Signals were acquired using the following light cube filters: DIC for transmitted light, ExBP 475/40, EmBP 530/50 for Sytox, and ExG365, EmBP 445/50 for DAPI. Pictures were taken every 3 minutes. Images were processed and analyzed using Imaris® software. Cell-fungus interactions were observed and killing of *Aspergillus* hyphae by the neutrophils was quantified. For chemotaxis assay, we used IncuCyte® ClearView 96-Well Chemotaxis Plates coated beforehand with 5% fetal calf serum during 1 hour at 37°C. Purified neutrophils stained by Hoechst (final concentration 10 µM) were placed in the membrane insert and fMLP as chemo-attractant (final concentration 10 µM) was put in the reservoir plate. Neutrophils migration from the insert to the reservoir was observed during 60 minutes (one picture taken every 30 seconds) and quantified using the Imaris®. tracking software. The migration index was defined as the proportion of neutrophils migrating into the pore against all neutrophils crossing a square zone of 260x260 µm centered by the pore of interest.

Statistical analysis. GraphPad Prism 6 was used for statistical analyses (GraphPad software, La Jolla, Calif).