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Supplemental Information

Noncanonical Fungal Autophagy Inhibits Inflammation in Response to IFN-γ via DAPK1

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SUPPLEMENTAL FIGURES

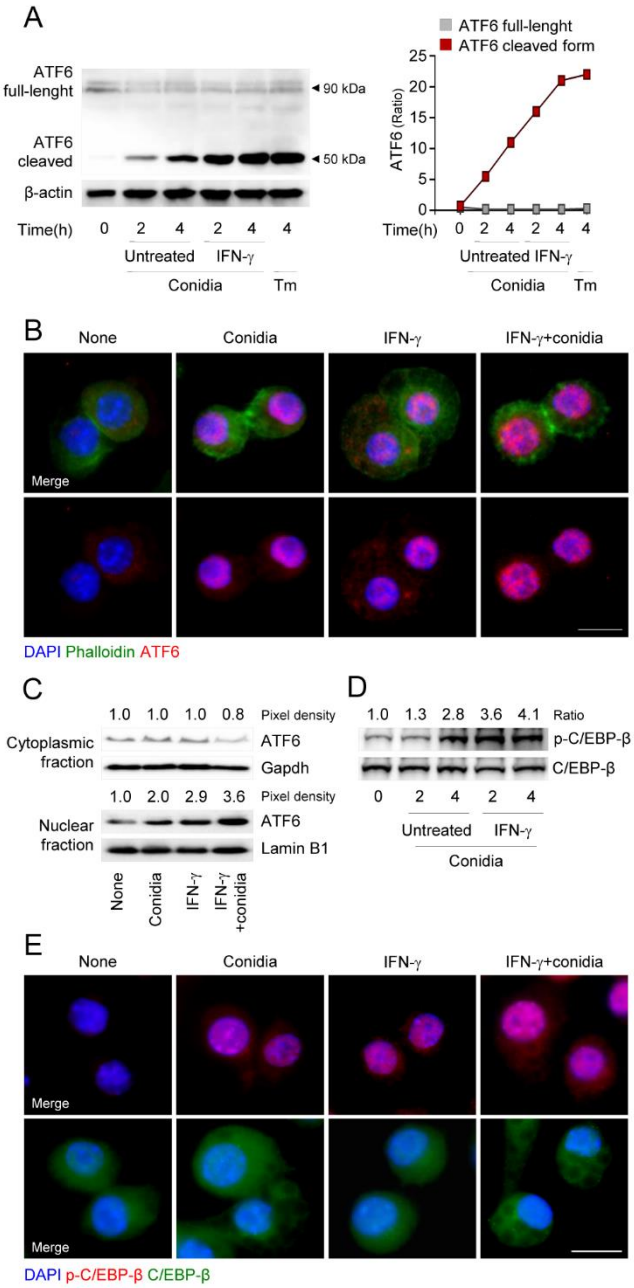

Figure S1, related to Figure 1. (A) ATF6 cleavage (immunoblotting) in RAW264.7 cells stimulated with live *A. fumigatus* conidia in the presence of rIFN-γ. (B and C) ATF6 nuclear translocation (immunofluorescence and immunoblotting of cytoplasmic or nuclear fraction) in RAW264.7 cells stimulated as in A. (D) C/EBP-β phosphorylation (immunoblotting) and (E) nuclear translocation (immunofluorescence) in RAW264.7 cells stimulated with live *A. fumigatus* conidia in the presence of rIFN-γ. For immunoblotting, normalization was performed on mouse β-actin, Laminin B1 or Gapdh (the corresponding pixel density or ratio is indicated). Photographs were taken with a high-resolution microscope (Olympus BX51) equipped with a ×40 objective, scale bars 100 μm. For immunofluorescence, nuclei were counterstained with DAPI. Tm, Tunicamycin. Representative images from three experiments.

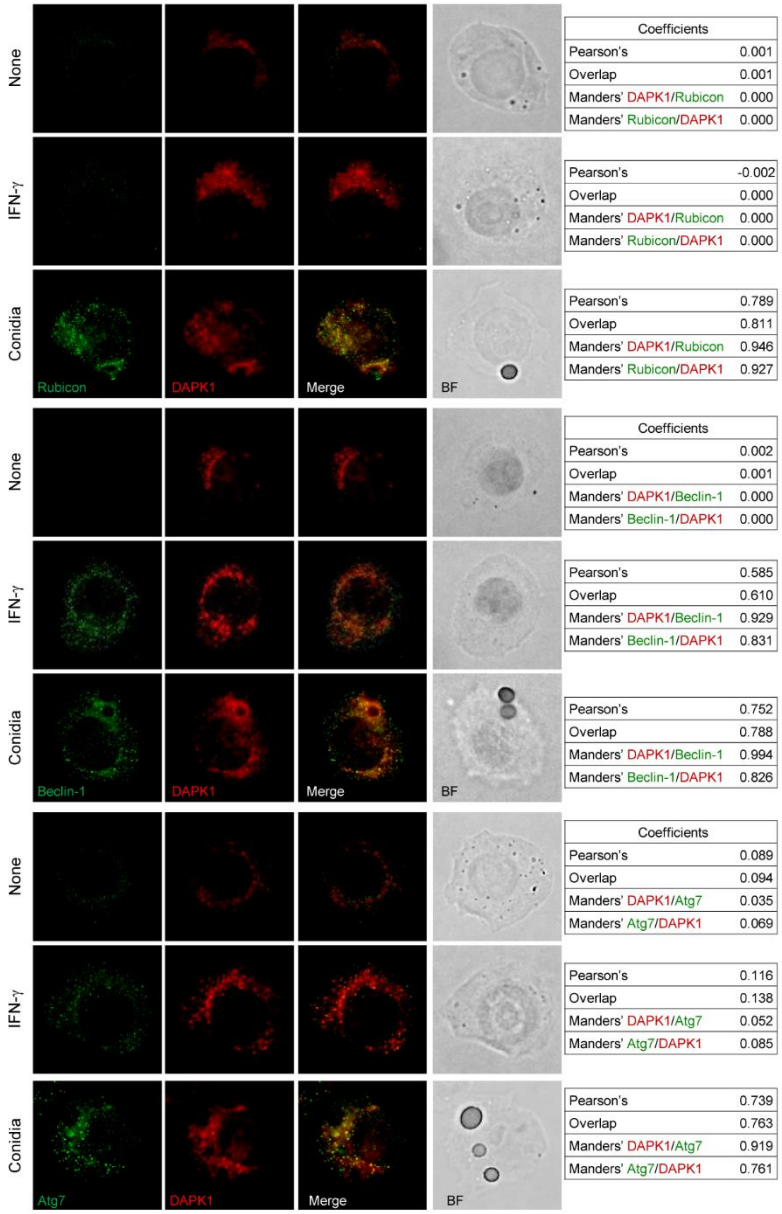

Figure S2, related to Figure 3. Immunofluorescence imaging of RAW264.7 cells untreated (None) or pulsed with *A. fumigatus* conidia or rIFN-γ. Photographs were taken with a high-resolution microscope (Olympus BX51) equipped with a ×100 objectives, scale bar 25 μm. BF, brightfield. Numbers refer to co-localization coefficients to quantify the overlap degree of DAPK1 and Beclin-1, Rubicon and Atg7. Representative images from three experiments.

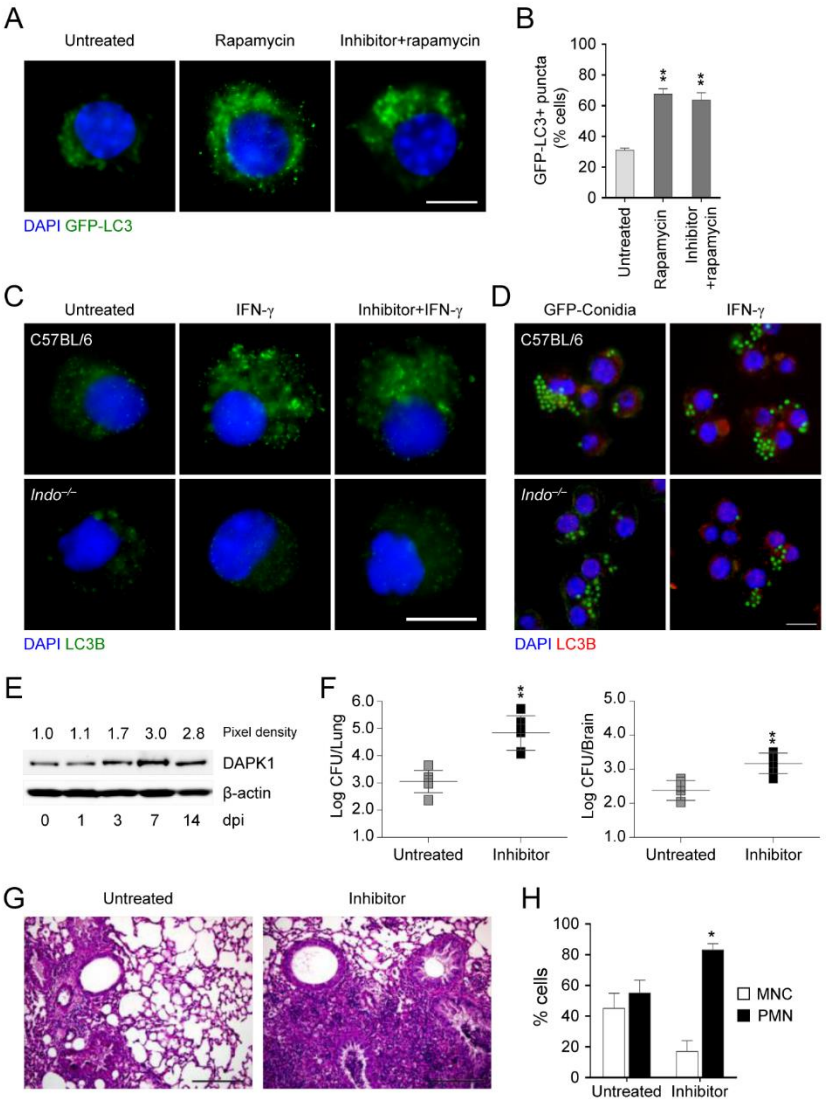

Figure S3, related to Figure 3. (A) GFP-LC3 punctae accumulation (immunofluorescence) and (B) percentage of GFP-LC3 positive cells in RAW-GFP-LC3 cells after stimulation with rapamycin in the presence of DAPK Inhibitor. (C) LC3B expression in C57BL/6 or *Indo*$^{-/-}$ lung macrophages exposed to rIFN-γ in the presence of Inhibitor or (D) pulsed for two hours with *A. fumigatus* conidia and rIFN-γ. (E) DAPK1 expression evaluated at indicated days after infection (dpi) in lung of *Indo*$^{-/-}$ mice either uninfected (0 dpi) or infected intranasally (i.n.) with *A. fumigatus* conidia. *Indo* $^{-/-}$ mice infected as in E and treated i.n. with Inhibitor daily starting three days before the infection, were evaluated at three dpi for (F) fungal load in the lung and in the brain, (G) lung histopathology and (H) bronchoalveolar lavage fluid morphometry. For immunofluorescence, nuclei were counterstained with DAPI. For immunoblotting, normalization was performed on mouse β-actin and corresponding pixel density is depicted. Photographs were taken with a high-resolution microscope (Olympus BX51) equipped with a ×40 and ×100 objectives, scale bars 100 and 25 μm. Data (mean values ± SD) represent pooled results or representative images (immunofluorescence and immunoblotting) from three experiments. *P< 0.05, **P< 0.01, DAPK Inhibitor treated *vs* untreated cells or mice. MNC, mononuclear cells. PMN, polymorphonuclear cells.

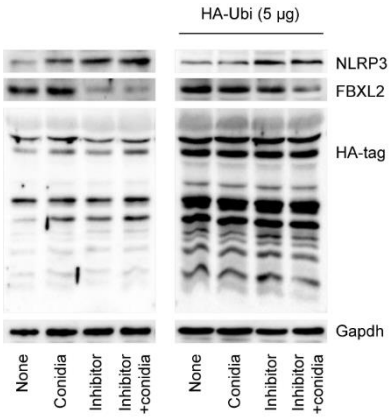

Figure S4, related to Figure 5. Expression of HA, NLRP3, FBXL2 and Gapdh by immunoblotting in RAW 264.7 cells transfected with HA-ubiquitin plasmid for 24h and stimulated with *A. fumigatus* conidia in the presence of DAPK Inhibitor. Normalization was performed on mouse Gapdh and corresponding pixel density is depicted. Representative images from three experiments. None, unpulsed and untreated cells.

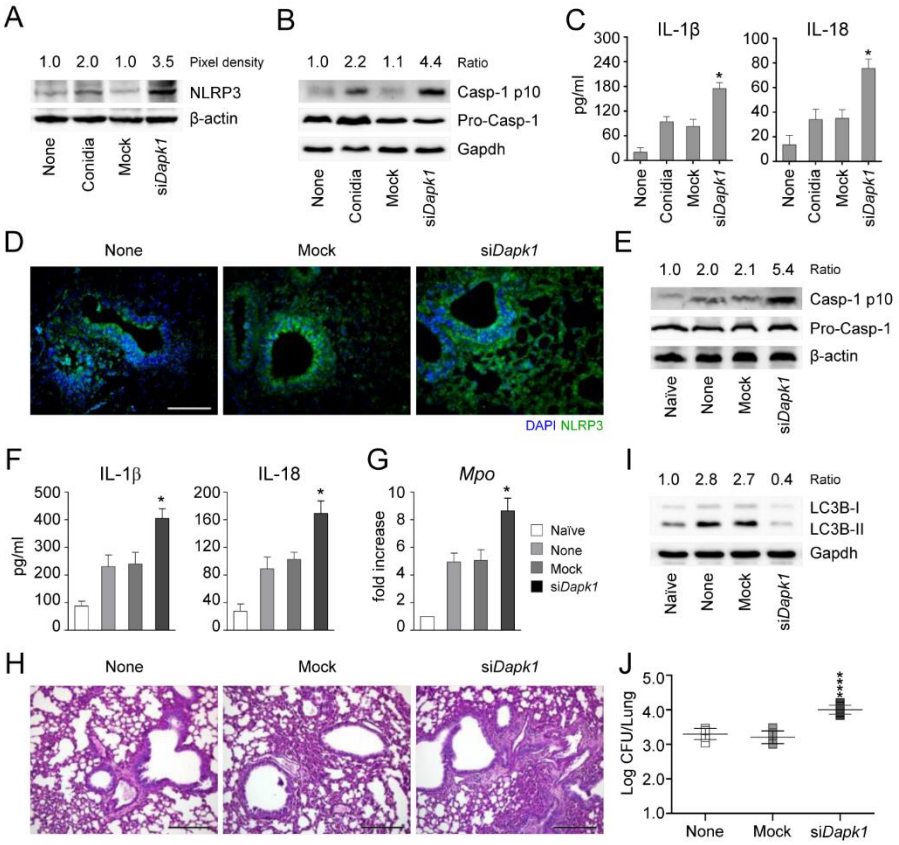

Figure S5, related to Figure 5. (A) NLRP3 (B) Caspase-1 cleavage and (C) cytokines production in RAW264.7 cells stimulated with *A. fumigatus* conidia in the presence of *Dapk1* specific siRNA (si*Dapk1*) or negative control (Mock). C57BL/6 mice were infected with *A. fumigatus* conidia and treated with si*Dapk1* or Mock daily until the sacrifice, starting three days before the infection. Mice were assessed three days post infection for: (D) NLRP3 expression, (E) Caspase-1 cleavage, (F) cytokines production on lung homogenates, (G) *Mpo* expression on total lung cells, (H) lung histology (period acid-Schiff staining), (I) lung LC3B-II/LC3B-I ratio and (J) fungal burden. For immunoblotting, normalization was performed on mouse β-actin or Gapdh and corresponding pixel density or ratio is depicted. For immunofluorescence, nuclei were counterstained with DAPI. Photographs were taken with a high-resolution microscope (Olympus BX51) equipped with a ×40 objective, scale bars 100 μm. Data (mean values ± SD) represent pooled results or representative images (immunofluorescence and immunoblotting) from three experiments. *P< 0.05, ****P< 0.0001, si*Dapk1* treated *vs* untreated RAW 264.7 cells or mice. None, unpulsed and untreated cells or untreated mice. Naive, uninfected mice.

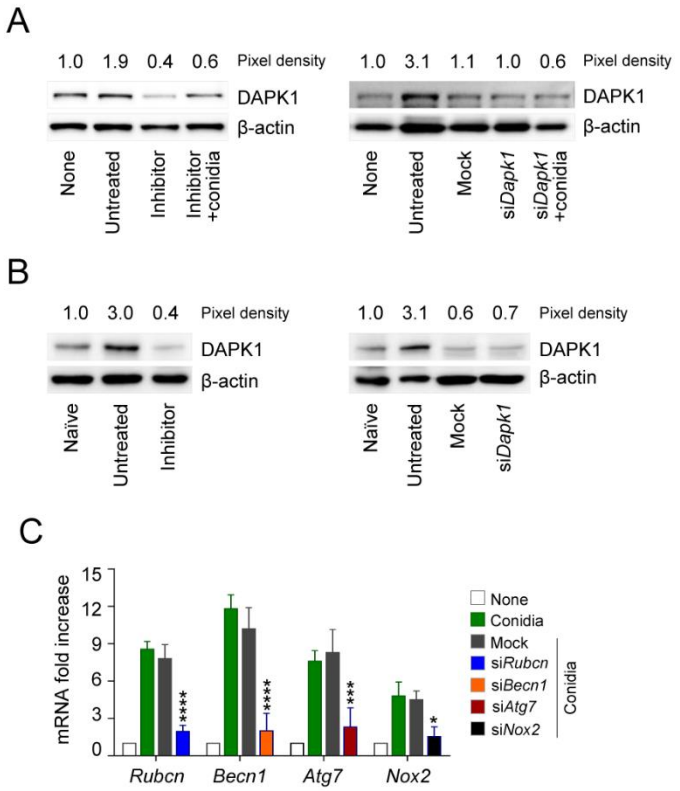

Figure S6, related to Figures 3, 5 and S5. (A) DAPK1 expression in RAW264.7 cells stimulated with *A. fumigatus* conidia in the presence of DAPK Inhibitor, *Dapk1* specific siRNA (si*Dapk1*) or negative control (Mock). (B) DAPK1 expression in total lung cells of C57BL/6 mice infected intranasally with *A. fumigatus* conidia and treated i.n. with Inhibitor, si*Dapk1* or Mock daily until the sacrifice, starting three days before the infection. Normalization was performed on mouse β-actin and corresponding pixel density is depicted. (C) Gene expression on RAW264.7 cells pulsed with *A. fumigatus* conidia and treated with the indicated siRNAs. None, untreated cells. Naive, uninfected mice.

SNP ID	Genotype	Recipient (N=246)	P-value	Donor (N=246)	P-value
rs1964911	A/A	8	0.066	8	0.429
	A/C	20		22	
	C/C	16		13	
rs4878104	C/C	11	0.832	11	0.998
	C/T	23		21	
	T/T	9		8	
rs4877365	G/G	16	0.827	14	0.856
	G/A	16		20	
	A/A	7		5	
rs7025760	A/A	27	0.944	27	0.918
	A/G	12		11	
	G/G	2		2	
rs1056719	A/A	18	0.833	15	0.872
	A/G	18		21	
	G/G	6		5	

Table S1 related to Figure 7. Univariate analysis of association of *DAPK1* SNPs in recipients and donors of HSCT with cumulative incidence of IA.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Ethic Statement

Murine experiments were performed according to the Italian Approved Animal Welfare Authorization 360/2015-PR and Legislative decree 26/2014 regarding the animal licence obtained by the Italian Ministry of Health lasting for five years (2015-2020). Infections were performed under avertin anesthesia, and all efforts were made to minimize suffering. Human studies approval was obtained from institutional review boards at each site and written informed consent was obtained from the participants, or, in case of minors, from parents or guardian.

Mice

Female C57BL/6 and BALB/c mice, 8-10 week old, were purchased from Charles River (Calco, Italy). Homozygous *Ifng*$^{-/-}$, *p47*$^{phox-/-}$, *Myd88*$^{-/-}$, *Dectin-1*$^{-/-}$, *Nlrp3*$^{-/-}$ mice on C57BL/6 background were bred under specific pathogen-free conditions at the Animal Facility of Perugia University, Perugia, Italy.

Fungal Strains, Infections, and Treatments

For infection, mice were anaesthetized in a plastic cage by inhalation of 3% isoflurane (Isofluran Forene Abbot) in oxygen before intranasal instillation (i.n.) of a suspension of 2 x 10^7/20 µl saline *A. fumigatus* 293 resting conidia. Recombinant mouse IFN-γ (rIFN-γ from R&D systems) at the dose of 20000 U/mouse was given subcutaneously (s.c) daily starting the day of the infection until the sacrifice of the animals. DAPK inhibitor (Calbiochem) was administered i.n. at the dose of 500µg/kg for 5 consecutive days starting 3 days before the infection. Control mice received the diluent alone. For *Dapk1* silencing, each mouse received intranasal administration of 10 mg/kg siRNA sequences specific for mouse *Dapk1* (Duplex name MMC.RNAI.N029653.12.1 (IDT); sense, 5'-CGAGUUUGGAUAUGACAAGGAUACA-3'; antisense, 5'-UGUAUCCUUGUCAUAUCCAAACUCGCC-3') or equivalent dose of nonspecific control siRNA duplex in a volume of 20 µl of duplex buffer (IDT). siRNA was given for two consecutive days before the infection. It is known that lung-specific siRNA delivery can be achieved by intranasal administration without the need for viral vectors or transfection agents in vivo.

Cell Preparation, Culture and Transfection

RAW264.7 (ATCC) and RAW-GFP-LC3 cells were grown in RPMI 1640 medium (Lonza) supplemented as described (De Luca et al., 2012). Alveolar macrophages from C57BL/6 and *p47*$^{phox-/-}$, *Myd88*$^{-/-}$, *Dectin-1*$^{-/-}$ and *Ifng*$^{-/-}$ lung cells were isolated after 2-hour plastic adherence at 37°C. Cells were pretreated overnight with 200U/ml rIFN-γ (as from preliminary experiments showing activity in the range of 100 – 400U/ml) before pulsing at different hours with *A. fumigatus* resting (RC) or swollen (SC) conidia (1:1 cell/fungus ratio). For DAPK1 inhibition, cells were pretreated for 90 minutes with 3 µM DAPK Inhibitor as described (Usui et al., 2012) before *A. fumigatus* stimulation. Rapamycin (Sigma Aldrich) was used at the concentration of 50 µM. The detection of ATF6, a nuclear–cytoplasmic fractionation, was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For NF-kB inhibition, SN50 (Calbiochem) was used at the concentration of 18 µM for 1hour before *A. fumigatus* pulsing. For in vitro silencing, cells were transfected with 40 nM of the following siRNAs: *Dapk1* (described above), *Rubcn* (Duplex name mm.Ri.1700021K19Rik.13.1; sense, 5'-GUACUUGACCGCUAGUAAAAUCATT-3'; antisense, 5'-GACAUGAACUGGCGAUCAUUUUAGUAA -3'), *Atg7* (Duplex name mm.Ri.Atg7.13.1; sense, 5'CUUGAUCAGUACGAGCGAGAAGGAT-3'; antisense, 5'-AAGAACUAGUCAUGCUCGCUCUUCCUA -3'), *Becn1* (Duplex name mm.Ri.Becn1.13.1; sense, 5'-GUAAUAUUAAACCACAUGUUUACAA-3'; antisense, 5'-CCCAUUAUAAUUUGGUGUACAAAUGUU -3'), *Nox2* (Duplex name mm.Ri.Cybb.13.1; sense, 5'-GUUCAAGGUCAGUUUAUUGAAUGAA-3'; antisense, 5'-CACAAGUUCCAGUCAAAUAACUUACUU -3') (all from IDT). Silencing was performed using TransIT-TKO® Transfection Reagent (Mirus) and incubated for 24 hours (as indicated by preliminary experiments performed at 12, 24, or 48 hours) at 37°C in 5% CO_2. The efficiency of gene silencing was assessed by RT-PCR and western blotting. Transfected cells were exposed to *A. fumigatus* or inert beads.

Immunoblotting and Immunoprecipitation

For immunoblotting, cells were lysed in Laemmli buffer. For immunoprecipitation, cells were lysed in RIPA buffer and the lysates were incubated and rotated with 1 µg of anti-ubiquitin, anti-FBXL2 antibody and then incubated with 30 µl of Protein G Sepharose beads. The lysate was separated in SDS-PAGE and transferred to a nitrocellulose membrane. Blot of cell lysates were incubated with the following antibodies: anti-mouse monoclonal polyclonal DAP Kinase 1 (antibodies-online.com), ATF6 full length and cleaved form (70B1413.1 Imgenex); polyclonal

C/EBPβ and p-C/EBPβ (Santa Cruz Biotechnology), polyclonal LC3B (Cell Signaling), polyclonal SQSTM1/p62 (Santa Cruz Biotechnology), polyclonal FBXL2 (Aviva), polyclonal HA (Bethyl), polyclonal Ubiquitin (Abcam), polyclonal Caspase-1 p10 (Santa Cruz Biotechnology), monoclonal Anti-Interferon gamma (EPR1108, Abcam), polyclonal Anti-Lamin B1-Nuclear Envelope Marker (Abcam), anti-human polyclonal DAPK1 (Sigma-Aldrich), rabbit monoclonal Caspase-1 (D7F10, Cell Signaling) and polyclonal NLRP3 (Abcam). Normalization was performed by probing the membrane with mouse/human-monoclonal anti-β-actin, anti-β-tubulin and anti-Gapdh antibody (Sigma-Aldrich). Phagosomes from RAW264.7 cells were obtained as previously described (Martinez et al., 2015). Briefly, after culture of cells with *A. fumigatus* swollen conidia or inert beads (LB30, Sigma Aldrich), the cells were washed in cold PBS, pelleted, resuspended in 1 ml of homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4), and homogenized on ice. Phagosomes were then isolated by flotation on a sucrose step gradient during centrifugation for 1 h at 100,000g at 4 °C. The phagosomal fraction was then collected from the interface of the 10% and 25% sucrose solutions and resuspended in RIPA buffer for protein immunoblot analysis. The entire phagosome purification was run on 7.5–12% SDS–PAGE gels. Membranes were sectioned according to the molecular weight marker, and proteins residing within that range of molecular weights were probed with anti-mouse polyclonal DAP Kinase 1, polyclonal LC3B, rabbit polyclonal Beclin 1 (Abcam), rabbit polyclonal Rubicon (Abcam) and rabbit polyclonal Atg7 (Abgent). Chemiluminescence detection was performed with LiteAblot Plus chemiluminescence substrate (EuroClone S.p.A), using the ChemiDocTM XRS+ Imaging System (Bio-Rad), and quantification was obtained by densitometry image analysis using Image Lab 5.1 software (Bio-Rad).

ELISA and Real-Time PCR

The level of cytokines in lung homogenates or cell culture supernatants was determined by mouse specific ELISAs (R&D Systems). Data were normalized to total protein levels for each sample as determined using the Bio-Rad Protein assay (Bio-Rad Laboratories). Real-time RT-PCR was performed using CFX96 Touch Real-Time PCR Detection System and SYBR Green chemistry (Biorad). Cells were lysed and total RNA was reverse transcribed with cDNA Synthesis Kit (BioRad), according to the manufacturer's instructions. The PCR primers were as follow: *Dapk1* Forward, 5'- CCT GGG TCT TGA GGC AGA TA -3'and Reverse, 5'- TCG CTA ATG TTT CTT GCT TGG -3'; *Ifng* Forward, 5'- ACT GGC AAA AGG ATG GTG AC -3' and Reverse, 5'- TGA GCT CAT TGA ATG CTT GG -3'; *IP10* Forward, 5'- AAG TGC TGC CGT CAT TTT CT -3' and Reverse, 5'- CCT ATG GCC CTC ATT CTC AC -3'; *Mpo* Forward, 5'- TTA CAC CCC AGG CAT AAA AA -3' and Reverse, 5'- TTC CAT ACA GCT CAG CAC AA -3'; *Rubcn* Forward, 5'- ATC CAT GTT TGC CAG AAA GC -3'and Reverse, 5'- GGA GGA GGA CCC AAA GTA GG -3'; *Becn1* Forward, 5'- CTG AAA CTG GAC ACG AGC TTC AAG -3'and Reverse, 5'- CCA GAA CAG TAT AAC GGC AAC TCC -3'; *Atg7* Forward, 5'- GCA CAA CAC CAA CAC ACT T -3'and Reverse, 5'- GAG AGC AGC ACC TGA CTT -3'; *Nox2* Forward, 5'- TGG TGT GTG AAT GCC AGA GT -3'and Reverse, 5'- CCC CTT CAG GTT CTT GAT T -3'. Amplification efficiencies were validated and normalized against Gapdh. Each data point was examined for integrity by analysis of the amplification plot.

Terminal Deoxynucleotidyl Transferase-mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) of Lung Sections

Lung sections were fixed in 4% buffered paraformaldehyde, pH 7.3, for 36 h and embedded in paraffin. Section were de-paraffinized, re-hydrated and treated with 0.1 M citrate buffer, pH 6.0, for 20 min in a water bath, washed and blocked in 0.1 M Tris/HCl buffer, pH 7.5, supplemented with 3% bovine serum albumin and 20% FCS. The slides were then incubated with fluorescein-coupled dUTP and TUNEL enzyme (Roche Diagnostics) in the presence of terminal deoxynucleotidyl transferase. The samples were then washed with PBS and incubated for 10 min at 70°C to remove unspecific binding. The sections were mounted and analyzed by fluorescent microscopy using a ×40 objective.

Immunofluorescence and Acridine Orange Staining

Cells were grown in supplemented RPMI and placed on microscope glass slides at 37°C for adhesion. Slides were then washed with PBS and fixed with 4% of paraformaldehyde. Cells were incubated in blocking solution (PBS/3% bovine serum albumin (BSA)/0.1% Triton X-100) with the following antibodies: anti DAPK1, ATF6, C/EBPβ, p-C/EBPβ, IFN-γ, LC3B (5F10, Nanotools), SQSTM1/p62, LAMP-1 (Sigma-Aldrich), Ubiquitin (Abcam), Beclin 1 (Abcam), Rubicon (Abcam) and Atg7 (Abgent). Alexa Fluor® 488 phalloidin was used for selective labeling of F-actin. Nuclei were counterstained with DAPI. After overnight staining with primary antibodies, slides were washed and incubated with anti-rabbit or anti-mouse IgG-TRITC and IgG-FITC (Sigma Aldrich). NLRP3 staining of lung sections were done as described (Moretti et al., 2014). 2.5 ng/ml Acridine Orange (Sigma-Aldrich) was used to detect the cellular acidic compartment by measuring the red fluorescence emission. Images were acquired using a fluorescence microscope (BX51 Olympus) and the analySIS image processing software (Olympus).

Human studies

Isolation and Culture of monocytes from human PBMC

Monocytes were isolated from PBMC of healthy donors or two CGD patients, harboring the mutations c.736C>T, p.Q246X and whole CYBB gene deletion (69,84 kb), followed informed consent, as described (de Luca et al., 2014). Plastic adherent cells were cultured for 4 hours with live *A. fumigatus* conidia in the presence of 200 U/ml rIFN-γ before assays.

Patients

The genetic study included 277 patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) at the University of Perugia (Perugia, Italy) between 2003 and 2011 and their respective donors (who were related in 98% of cases). Patient characteristics are summarized in Table 2. Grafts consisted of immunoselected CD34+ peripheral blood cells in all patients, and transplantation procedures, antifungal prophylaxis, and surveillance for fungal infection were performed as described previously(Iannitti et al., 2016). Probable/proven fungal infection was defined according to the revised standard criteria from the European Organization for Research and Treatment of Cancer/Mycology Study Group. Study approval was provided by the local ethics committee and informed written consent was obtained from all participants in accordance with the Declaration of Helsinki.

SNP selection and genotyping

SNPs were selected from a literature review and public databases based on 3 selection criteria: (i) published evidence of association with human diseases; (ii) localization to the promoter, untranslated, or coding regions; (iii) minor allele frequency (MAF) higher than 5% in the European-Caucasian population. Five *DAPK1* SNPs complied with the selection criteria: rs1964911, rs4878104, rs4877365, rs7025760, rs1056719. Genotyping was performed using KASPar assays.

Statistical Analysis

Data are expressed as mean ± SD. Horizontal bars indicate the means. For multiple comparisons, p values were calculated by a one-way ANOVA (Bonferroni's post hoc test). For single comparison, p values were calculated by a two tailed Student's t test. The data reported are either from one representative experiment (histology, TUNEL and western blotting) or pooled otherwise. The in vivo groups consisted of 6 mice/group. Data were analyzed by GraphPad Prism 4.03 program (GraphPad Software). Cell fluorescence intensity was measured by using the ImageJ software. The co-localization program Fiji with the JACoP Plugin was used to quantify the degree of overlap by calculating the co-localization coefficients (Pearson's correlation coefficient, Overlap coefficient according to Manders and the Overlap coefficients). The probability of IA according to DAPK1 variants was determined with the use of the cumulative incidence method, and DAPK1 status among patients with and those without infection was compared with the use of Gray's test (Grey, 1988). Cumulative incidences were computed with R software, version 2.10.1 (cmprsk package) (Scrucca et al., 2007), with censoring of data at the date of the last follow-up visit, and with relapse and death as competing risks. A period of 24 months was chosen to include all cases. Multivariate analysis was performed using the subdistribution regression model of Fine and Gray implemented in the cmprsk package (Scrucca et al., 2010).

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