IL-1 receptor blockade restores autophagy and reduces inflammation in chronic granulomatous disease in mice and in humans

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Patients with chronic granulomatous disease (CGD) have a mutated NADPH complex resulting in defective production of reactive oxygen species; these patients can develop severe colitis and are highly susceptible to invasive fungal infection. In CGD, defective mice, autophagy is defective but inflammasome activation is present despite lack of reactive oxygen species production. However, whether these processes are mutually regulated in CGD and whether defective autophagy is clinically relevant in patients with CGD is unknown. Here, we demonstrate that macrophages from CGD mice and blood monocytes from CGD patients display minimal recruitment of microtubule-associated protein 1 light chain 3 (LC3) to phagosomes. This defect in autophagy results in increased IL-1β release. Blocking IL-1 with the receptor antagonist (anakinra) decreases neutrophil recruitment and T helper 17 responses and protects CGD mice from colitis and also from invasive aspergillosis. In addition to decreased inflammasome activation, anakinra restored autophagy in CGD mice in vivo, with increased Aspergillus-induced LC3 recruitment and increased expression of autophagy genes. Anakinra also increased Aspergillus-induced LC3 recruitment from 23% to 51% (P < 0.01) in vitro in monocytes from CGD patients. The clinical relevance of these findings was assessed by treating CGD patients who had severe colitis with IL-1 receptor blockade using anakinra. Anakinra treatment resulted in a rapid and sustained improvement in colitis. Thus, inflammation in CGD is due to IL-1–dependent mechanisms, such as decreased autophagy and increased inflammasome activation, which are linked pathological conditions in CGD that can be restored by IL-1 receptor blockade.

Chronic granulomatous disease (CGD) is an immunodeficiency characterized by defective production of reactive oxygen species (ROS) (1) due to mutations in the proteins forming the NADPH complex (2, 3). The most frequent form of CGD is hereditary and X-linked, and is caused by a mutation in the gene CYBB, which encodes the protein p40phox, the catalytic subunit of the NADPH oxidase complex. In autosomal and recessive forms of CGD, the mutations affect the genes encoding p22phox, p40phox, p47phox, or p67phox, which are all part of the NADPH complex, resulting in a defective NADPH oxidase complex. As a result, patients who have CGD have defective microbial killing by phagocytic cells and an increased susceptibility to infections, especially Staphylococcus aureus and Aspergillus spp. (4, 5). Paradoxically, ROS deficiency in patients with CGD results in a hyper-inflammatory state (6), and one-third of the patients develop an inflammatory colitis indistinguishable from Crohn disease (7–10).

The hyperinflammatory state in CGD is linked to inflammation–some activation (11–13). Studies in mice and humans reveal that autophagy is crucial for IL-1β transcription (14) and processing of pro–IL-1β (15, 16); defects in autophagy result in increased secretion of IL-1β. ROS production is commonly believed to be necessary for autophagy (17), and mice deficient in autophagy (ATG16L1−/−) can develop severe colitis and exhibit increased production of IL-1β (18).

In the present study, we aimed to decipher the link between autophagy and inflammasome activation in CGD. ROS deficiency in CGD mice and patients with CGD resulted in defective autophagy, eventually leading to increased IL-1β secretion and IL-1–dependent inflammation. Blocking the IL-1 receptor (IL-1R) with anakinra (the recombinant form of the naturally occurring IL-1R antagonist) not only limited inflammasome activation but also restored protective autophagy in CGD.

Results

ROS Deficiency Results in Defective Autophagy. The autophago-some-associated protein light chain 3 (LC3) is recruited to macrophage phagosomes upon engulfment of bacteria (19). To test whether p40phox, encoding the p40phox subunit of NADPH oxidase, is required for LC3 recruitment to the autophagosomes,

Significance

Chronic granulomatous disease (CGD) has an immunodeficiency component and, in addition, an autoimmune-inflammatory component in which autophagy and inflammasome activation are linked and amenable to IL-1 blockade. This study provides a rationale to perform clinical trials to investigate the efficacy of blocking IL-1 in CGD colitis and expands the therapeutic potential of IL-1 antagonists to inflammatory diseases with defective autophagy.
peritoneal macrophages were isolated from LC3-GFP transgenic and p47phox−/− mice crossed with LC3-GFP mice and exposed to adherent and invasive Escherichia coli (AIEC) strain LF82. This Crohn disease-associated strain of E. coli has previously been shown to accumulate preferentially in epithelial cells deficient in autophagy (20). LC3-GFP is recruited to phagocytized AIEC within macrophages obtained from WT mice expressing LC3-GFP within 30 min of infection (Fig. 1A and Fig. S1A). However, in p47phox−/− mice, there is minimal recruitment of LC3 to internalized bacteria. Quantification of the fraction of internalized bacteria surrounded by LC3-GFP revealed significantly more recruitment in WT compared with p47phox−/− macrophages (P < 0.001) (Fig. 1A). Thus, p47phox−/−, which is necessary for NADPH-dependent ROS production, is required for LC3 recruitment to engulfed bacteria.

To investigate whether NADPH-dependent ROS in humans is important for LC3 recruitment to the phagosome, we assessed LC3 recruitment to the phagosome in cells isolated from patients with CGD and healthy controls (HCs). The cells were exposed to FITC-labeled S. aureus, a prominent pathogen in CGD (5), and were subsequently stained for LC3. The percentage of colocalization of S. aureus-FITC with labeled LC3 was determined (Fig. 1B and Fig. S1B). Monocytes from patients with CGD showed significantly less colocalization between LC3 and phagocytized S. aureus compared with monocytes from HCs (Fig. 1B). Therefore, the NADPH component p47phox in humans is required for LC3 recruitment upon engulfment of microorganisms.

**Defects in Autophagy in p47phox−/− Mice and Patients with CGD Result in Increased IL-1β Production.** Because autophagy inhibits IL-1β production (15, 16), we assessed whether defective autophagy in ROS-deficient mice is responsible for the increased IL-1β secretion. Bone marrow-derived macrophages (BMMs) in the presence (WT) or absence (p47phox−/−) of ROS were stimulated with LPS. As shown in Fig. 1C, LPS treatment induced IL-1β in both WT and p47phox−/− BMMs, but p47phox−/− BMMs produced nearly sixfold greater levels of the cytokine after stimulation (WT: 61 ± 20 pg/mL and p47phox−/−: 354 ± 50 pg/mL, P < 0.001). Blocking autophagy with 3-methyladenine (3MA) pretreatment enhanced LPS-induced IL-1β secretion in both WT and p47phox−/− BMMs (WT: 502 ± 100 pg/mL and p47phox−/−: 828 ± 86 pg/mL, P < 0.01; Fig. 1C). However, the IL-1β production due to inhibition of autophagy by 3MA was increased twofold in p47phox−/− BMMs compared with 8.5-fold increased in WT BMMs (Fig. 1C). Next, we investigated whether the same differences are present in patients with CGD. In cells of HCs, autophagy inhibition by 3MA significantly increases LPS-induced IL-1β production (P < 0.01) (Fig. 1D). In contrast, 3MA did not increase IL-1β secretion in LPS-stimulated peripheral blood mononuclear cells (PBMCs) from patients with CGD (Fig. 1D). Collectively, these data provide evidence that a defect in autophagy is responsible for the increased IL-1β production in patients with CGD.

**Blocking IL-1 Is Beneficial in p47phox−/− Mice with Colitis.** Colitis is a severe manifestation of CGD (21). To investigate whether the deficiency of NADPH-dependent ROS leading to defective autophagy and subsequently increased IL-1β production is relevant in vivo, we evaluated the effects of blocking IL-1 with anakinra in WT and p47phox−/− mice with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. We observed that TNBS-treated p47phox−/− mice have significantly more weight loss compared with WT mice (more than 20% loss of their initial body weight on day 5) upon TNBS treatment (Fig. 2A) and display severe inflammatory histopathology (Fig. 2B and Fig. S1A) and inflammatory cytokine responses (Fig. S1B). By contrast, TNBS-treated mice p47phox−/− mice regained weight after anakinra treatment, histological examination of colonic tissues and blinded histological scoring of colitis improved, and inflammatory cytokines were significantly lower (Fig. 2A and B and Fig. S1A and B). Thus, anakinra has significant beneficial effects on the outcome of colitis in CGD mice.

**Blocking IL-1 Protects p47phox−/− Mice from Invasive Aspergillosis.** A prominent complication of CGD is the increased susceptibility to pulmonary aspergillosis that is associated with a marked inflammatory response (22). To investigate whether blocking IL-1R would decrease detrimental inflammatory responses in CGD mice with aspergillosis, WT and p47phox−/− mice were infected with Aspergillus fumigatus and treated with anakinra daily. Mice were monitored for survival, local fungal growth, inflammatory cell recruitment in the bronchoalveolar lavage (BAL), and lung histopathology. In contrast to WT mice, the majority of p47phox−/− mice died of the infection (Fig. 2C), were unable to restrict fungal growth in the lung (Fig. 2D), and showed mycotic pneumonia at necropsy with more than half of the pulmonary parenchyma being involved (Fig. S2A), as well as BAL neutrophilia (Fig. S2A, Inset). Anakinra at 10 mg/kg, a dose that is known to be pharmacologically active in mice and mimics human therapeutic dosages (23), significantly increased survival (Fig. 2C), reduced fungal growth (Fig. 2D), decreased IL-1β (Fig. 2E), decreased BAL neutrophilia, decreased levels of MPO (Fig. 2F and Fig. S2A, Inset), and ameliorated lung pathology (Fig. S2A) in p47phox−/− mice. The extent of granuloma formation was also significantly reduced by anakinra in CGD mice, as observed by gross pathology. These data demonstrate that anakinra restores immunocompetence in CGD by inhibiting excess neutrophil influx in the lung during infection and restraining fungal growth in p47phox−/− mice.

The increased susceptibility of p47phox−/− mice to Aspergillus infection is associated with failure to activate protective T helper (Th) 1 and regulatory T-cell responses and the occurrence of inflammatory Th17 cells (22). Therefore, we also evaluated...
parameters of adaptive Th immunity in WT and p47phox−/− mice. Blocking the IL-1R with anakinra greatly reduced IL-17A (Fig. 3D) and increased IL-10 production in p47phox−/− mice (Fig. 3E). Anakinra treatment of p47phox−/− mice demonstrated that blocking IL-1 restores defective autophagy to Aspergillus SC in CGD (Fig. 3F).

Inhibiting IL-1 Restores Autophagy in Human CGD Cells. We investigated whether inhibition of IL-1 could also restore defective autophagy in human CGD cells. Monocytes isolated from patients with CGD show significantly less colocalization between LC3 and phagocytosed Aspergillus spores or SC compared with monocytes isolated from HCs (Fig. 4A). However, when the IL-1R was blocked, the capacity of monocytes from patients with CGD to recruit LC3 upon engulfment of Aspergillus increased to levels comparable to those with LC3 recruitment in monocytes from healthy subjects (Fig. 4B). Thus, blocking the IL-1 activity not only decreases inflammasome activation but restores defective autophagy in murine and human CGD cells.

Improved Clinical Outcome in Patients With CGD Who Had Colitis Treated With Anakinra. Two patients with CGD who had refractory colitis were treated s.c. with anakinra at a dosage of 100 mg daily for 3 mo. The first patient (p47phox−/− deficient) suffered from colitis, with 15–20 loose stools per day; over the 3-mo period with anakinra, there was progressive improvement, with a reduction in frequency to eight to 10 stools per day (Fig. 4C). The second patient (gp91phox−/− deficient), described by van de Veerdonk et al. (26), suffered from perirectal granulomas and abscesses, which were refractory to corticosteroid therapy. During the 3-mo treatment period, the inflammatory parameters improved and the patient showed a good clinical response (Fig. 4C), with resolution of the perirectal abscesses (Fig. 4D). After stopping anakinra, he was disease-free for several months; however, his colitis flared, and anakinra was restarted for a period of 4 wk after which he remained free of symptoms. Notably, although the first patient previously had a history of invasive pulmonary fungal disease with Esophila dermattidias and the second patient had multiple severe S. aureus infections (pneumonia and liver abscesses), no infections were observed during treatment with anakinra in these patients with CGD.

Discussion

Using murine and human cells, we demonstrate here that CGD is characterized by defective autophagy resulting in increased release of IL-1β. Although CGD is characterized by increased inflammasome activation (11–13), the present study expands on those findings by demonstrating that blocking IL-1 itself decreased IL-1β secretion and restored defective autophagy in CGD in vivo and in vitro settings. Clinically, we report here the beneficial effects observed in two patients suffering from CGD colitis treated with the IL-1R antagonist anakinra at the approved daily dose of 100 mg.
Autophagy defects resulting in inflammation appear to be a key feature in the pathogenesis of Crohn colitis, a disease that is indistinguishable from CGD colitis (27, 28). Given the suggested role for ROS in autophagy (29, 30), a defective autophagic process responsible for hyperproduction of IL-1β is consistent with NADPH deficiency. Indeed, defective autophagy (25) was observed in macrophages isolated from p40phox−/− mice, mimicking the intestinal inflammation of CGD (31). The defect in autophagy demonstrated here was accompanied by significantly higher secretion of IL-1β in the p40phox−/− macrophages compared with cells from WT mice and by increased secretion of IL-1β in PBMCs isolated from patients with CGD. The human data that defective autophagy contributes to the uncontrolled production of IL-1β in CGD cells is consistent with NADPH deficiency. Indeed, defective autophagy (25) was observed in macrophages isolated from p40phox−/− mice, mimicking the intestinal inflammation of CGD (31). The defect in autophagy demonstrated here was accompanied by significantly higher secretion of IL-1β in the p40phox−/− macrophages compared with cells from WT mice and by increased secretion of IL-1β in PBMCs isolated from patients with CGD. The human data that defective autophagy contributes to the uncontrolled production of IL-1β are also observed in macrophages from Atg16L1-deficient mice, which produce greater levels of IL-1β compared with WT mice (18). Moreover, loss-of-function polymorphisms in the autophagy genes ATG16L1 and IRGM increase susceptibility to Crohn disease (32, 33), and the risk allele is associated with increased production of IL-1β (34).

One unexpected finding in this report is that blocking the IL-1R with anakinra reduced IL-1β production and restored autophagy in vivo (24). Indeed, anakinra restored LC3 recruitment to near-normal levels in both mice and human NADPH oxidase-deficient cells in vitro, as well as in vivo in mice with invasive pulmonary aspergillosis, with both suggesting that inhibiting IL-1β is beneficial in CGD not only by dampening IL-1–mediated inflammation but by restoring defective LC3 recruitment. Although ROS deficiency leads to impaired pathogen killing (35, 36), the defect in autophagy observed in this study suggests a significant role for autophagy in the host defense mechanism against Aspergillus infections in CGD. Whereas promotion of autophagy restores CD8+ T-cell memory in CGD mice with aspergillosis (25), blocking autophagy in WT mice infected with Aspergillus resulted in increased inflammation similar to that of p47phox−/− mice (Fig. S4). Not surprisingly, impaired autophagy results in impaired killing of A. fumigatus (37). Thus, the beneficial effects of anakinra observed in CGD mice with invasive aspergillosis is not only due to the reduction of neutrophil influx and IL-17 production but to the restoration of autophagy, which increases the killing of A. fumigatus by host phagocytes.

With the autophagy defect and the IL-1β hyperproduction being fundamental to the inflammatory features of CGD, the management of the disease appears to have advanced, as shown in the two patients with CGD colitis treated with anakinra. Anakinra dampened the inflammatory reaction and improved the clinical condition in patients with CGD colitis, providing a proof of principle for this therapeutic approach in the management of CGD. Although anti-TNF antibodies improved CGD
can demonstrate that CGD is a disease associated with abnormal
experiments performed in PBMCs from two patients with CGD. *presence of anakinra (10 μg/mL). Data are representative of two separate
experiments performed in PBMCs from two patients with CGD in the absence or
CGD and two HCs. (B) Percentage of colocalization of LC3 with RC or SC in
the same PBMCs isolated from two patients with CGD harboring
homozygous mutations in the NCF1 gene (p47phox). To induce autophagy,
cells were incubated for 4 h in Earle's Balanced Salt Solution starvation
medium. After 4 h, IL-1β mRNA was assessed by quantitative RT-PCR. To
inhibit autophagy, cells were incubated with 10 mM 3MA for 24 h.

Mice. C57BL6 WT mice were purchased from Jackson Laboratories. Homo-
yzygous p47phox−/− mice on C57BL6 background were purchased (Harlan) and
bred under specific pathogen-free conditions. The p40phox−/− mice were
generated previously (31). The p40phox−/− × LC3-GFP mice were generated by
crossing p40phox−/− mice with LC3-GFP transgenic mice for two generations
(44) (SI Materials and Methods).

In Vitro Production of IL-1β in Mice. Bone marrow was harvested, and cells
were cultured for 5 d in M-CSF to drive macrophage differentiation (SI
Materials and Methods). On day 6, cells were harvested, counted, replated
for 3 h, and incubated with 3MA for 1 h before LPS stimulation.

Mouse Macrophage Infection and Induction of Autophagy. Peritoneal macro-
phages were harvested and allowed to adhere to the coverslips (SI Materials
and Methods).

Human Monocyte Infection and Induction of Autophagy. Monocytes were
isolated from PBMCs on anti-CD14-coated beads (MACS Miltenyi) and
allowed to adhere to glass coverslips for 1 h, after which they were exposed
to pathogens. The coverslips were then washed, fixed in cold methanol, and
examined by immunofluorescence (SI Materials and Methods).

TNBS-Induced Colitis. As reported previously, mice received 2.5 mg of TNBS
and were concomitantly treated i.p. with anakinra (10 mg/kg) daily. Weight
changes were recorded daily, and on day 5, mice were killed and tissues were
collected for histology, RNA analysis, and cytokine analysis. Colonic sections
were stained with H&E, and histology was scored as described elsewhere (45).

Experimental Invasive Pulmonary Aspergillosis in Mice. Details on this model
using viable conidia (5–15%) from the A. fumigatus Af293 strain are de-
scribed in SI Materials and Methods. Different doses of anakinra were ad-
ministered i.p. daily until the end of the experiment.

Cell Line Cultures, Transfection, and Autophagy. RAW 264.7 cells were tran-
siently transfected with the EGFP-LC3 plasmid (Addgene) for 48 h and ex-
posed to A. fumigatus SC at a cell/fungus ratio of 1:1 in the absence or
presence of different dosages of anakinra or rapamycin as a positive control.
LC3 staining, LC3b and p62 blotting, and gene transcription of autophagy
genomes were used to investigate autophagy (SI Materials and Methods).

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Materials and Methods

Ethics Statement. Patients and healthy volunteers gave written consent to
participate as approved by the Radboud University Institutional Review
Board. Experiments were performed according to the Italian Approved
Animal Welfare Assurance A-3143-01. The Subcommittee on Research Animal
Care approved the studies in Boston.

Patients and HCs. For cytokine production, PBMCs were isolated and stimu-
lated as previously described (43) from HCs and patients with CGD harboring
homozygous mutations in the NCF1 gene (p47phox). To induce autophagy,
cells were incubated for 4 h in Earle’s Balanced Salt Solution starvation
medium. After 4 h, IL-1β mRNA was assessed by quantitative RT-PCR. To
inhibit autophagy, cells were incubated with 10 mM 3MA for 24 h.

colitis, the treatment was accompanied by life-threatening in-
fecious complications (38). Anakinra is relatively safe (39), its
short t1/2 provides an exit in the event of an infectious process,
and blocking IL-1 deserves a trial in a larger cohort of patients
with CGD.

Not only can we explain the increased IL-1β production but we
can demonstrate that CGD is a disease associated with abnormal
states of IL-1-dependent mechanisms, such as decreased autopho-
yagy and increased inflammasome activation, that cannot be viewed as
separate pathological conditions in CGD. However, we cannot
conclude that IL-1 alone explains the pathology of CGD because,
Supporting Information

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SI Materials and Methods

Animals. Eight- to 10-wk-old C57BL6 WT mice were purchased from Jackson Laboratories, and breeding pairs of homozygous p47phox−/− mice raised on C57BL6 background were purchased from Harlan and bred under specific pathogen-free conditions at the breeding facilities of the University of Perugia. Mice were age- and gender-matched for each experiment. The p47phox−/− mice were generated as previously described (1). The p47phox−/− x light chain 3 (LC3)-GFP mice used for microscopy studies were generated crossing p47phox−/− and LC3-GFP transgenic mice for two generations (2). Mice were maintained on food and water ad libitum in specific pathogen-free facilities at the Massachusetts General Hospital.

In Vitro Production of IL-1β in Mice. Bone marrow was harvested into sterile complete RPMI Plus Glutamax (Gibco; supplemented with 10% FCS and 50 μg/mL gentamicin). Cells were treated with RBC lysing buffer (Sigma) for 7 min at room temperature and passed through a 70-μm filter to obtain a single-cell suspension. Cells were cultured for 5 d (37 °C in 5% CO2) in complete RPMI supplemented with 20 ng/mL recombinant murine M-CSF (Peprotech) to drive macrophage differentiation. Cultures were supplemented with fresh RPMI and M-CSF on day 3 to replenish the cytokine. On day 6, cells were harvested, counted, and replated on 96-well flat-bottom plates. A total of 1 × 106 cells were plated per condition (50 μL per well) and incubated for 3 h at 37 °C in 5% CO2, at which point the macrophages had reached the plate. Cells were incubated with 3-methyladenine (3MA) for 1 h before LPS stimulation. Cells not treated with 3MA were incubated in a similar volume of RPMI and rested for the pretreatment hour. LPS-treated samples were then stimulated with 100 ng/mL LPS (Invivogen) for 24 h at 37 °C in 5% CO2. Supernatants were harvested and frozen at −20 °C before ELISA analysis.

Mouse Macrophage Infection and Induction of Autophagy. Peritoneal macrophages were harvested by performing peritoneal lavage with 6 mL of GIBCO RPMI media (Invitrogen) containing 50 μg/mL gentamicin and applying 1 mL of lavage fluid to coverslips in 12-well plates. After 1 h, peritoneal macrophages had adhered to the coverslips and were washed with media and incubated at 37 °C in 5% CO2. Adherent and invasive Escherichia coli strain LF82 (a gift from A. Darfeuille-Michaud, Inserm/University of Auvergne, Clermont-Ferrand, France; INRA USC 2018) was grown overnight in Luria–Bertani 4919 broth containing 100 μg/mL ampicillin at 37 °C with aeration and were subcultured at a dilution of 1/33 for a further 3 h in Luria–Bertani broth. This culture was then labeled with Alexa Fluor 568 carboxylic acid, succinimidyl ester (Invitrogen) as follows. One milliliter of culture was washed in PBS and resuspended in 200 μL in PBS, Alexa Fluor 568 carboxylic acid, succinimidyl ester was added to a final concentration of 0.3 mg/mL and incubated at 37 °C for 10 min, washed twice in PBS, further diluted in RPMI medium without antibiotics to yield a multiplicity of infection of 100, and added to the cultured peritoneal macrophages. Infections were allowed to proceed for 15 min, and the cells were washed once in medium containing 100 g/mL gentamicin sulfate and then incubated in fresh high gentamicin medium for a further 30 min. Cells were washed in PBS and then fixed in 4% formalin for 15 min. Cells were permeabilized by incubation in PBS with 0.1% Triton X-100 and 1% BSA for 2 min. To enhance the endogenous GFP signal, cells were then stained with rabbit anti-GFP (Cell Signaling Technologies) and washed repeatedly in PBS. A secondary Alexa Fluor 488-conjugated goat-anti-rabbit antibody was then used, and cells were stained for DNA. Slides were viewed for counting under wide-field fluorescence illumination with a 100× objective lens (Zeiss Axioplan; Carl Zeiss MicroImaging). The total number of bacteria per cell and the number of LC3-GFP+ bacteria were assessed in randomly chosen fields with at least 50 cells counted in triplicate. The numbers of LC3-GFP+ bacteria were then calculated as a percentage of total bacteria. Significance was assessed using the two-tailed, unequal variance Student t test. Images were obtained using laser-scanning confocal microscopy with a 63× objective lens (Leica SP5; Leica Microsystems).

Human Monocyte Infection and Induction of Autophagy. Staphylococcus aureus (clinical isolate) was heat-killed at 100 °C for 90 min. To FITC-label the bacteria, they were incubated for 30 min at 4 °C on a tube roller in 0.1 M carbonate/bicarbonate buffer and 0.01 mg/mL FITC (Sigma). After labeling, the bacteria were washed three times with PBS to remove unbound FITC. Monocytes from healthy controls and patients with CGD were isolated from peripheral blood mononuclear cells using magnetic bead separation with anti-CD14–coated beads (MACS Miltenyi) according to the protocol provided by the manufacturer. The monocytes were resuspended in RPMI culture medium supplemented with 1% gentamicin, 1% l-glutamine, and 1% pyruvate (Life Technologies). The cells were counted in a Bürker counting chamber, and their number was adjusted to 1 × 107/mL. A total of 2 × 105 monocytes per condition in a final volume of 100 μL were allowed to adhere to glass coverslips (Ø12 mm) for 1 h, after which they were exposed to S. aureus-FITC (2 × 106) at 37 °C for 1 h. After stimulation, the coverslips were washed twice with PBS to remove medium and nonphagocytized bacteria, and cells were fixed on the coverslips for 15 min in 4% paraformaldehyde. Subsequently, the coverslips were washed with PBS, followed by a fixation in ice-cold methanol for 10 min, after which coverslips were stored in PBS at 4 °C until immunofluorescence staining was performed. For immunofluorescence imaging, cells were washed twice with PBS, permeabilized by using 0.1% saponin (Sigma–Aldrich), blocked for 30 min in PBS plus 2% BSA, incubated for 1 h with a mouse monoclonal antibody to LC3 (Nanotools), washed twice in PBS plus 2% BSA, and stained by a secondary Alexa Fluor 555 goat–anti-mouse antibody (Molecular Probes) and TOPRO 3 (Molecular Probes). After the washing steps, slides were mounted in Prolong Gold antifade media (Molecular Probes) and images were acquired using a Leica SP2 RS laser-scanning confocal microscope with an oil-immersion objective (40/1.4 N.A.; Leica) using identical gain settings. The same procedures were performed with monocytes isolated from patients with CGD who had been exposed to Aspergillus spores and swollen conidia (SC) in the presence or absence of 10 μg/mL anakinra.

Experimental Invasive Pulmonary Aspergillosis in Mice. Viable conidia (>95%) from the Aspergillus fumigatus AT293 strain were obtained by growth on Sabouraud dextrose agar (Difco Laboratories) at room temperature. SC were obtained as described by De Luca et al. (3). For infection, mice were anesthetized by i.p. injection of 2.5% avertin (Sigma–Aldrich) before the intranasal instillation of a suspension of 2 × 107 conidia per 20 μL of saline. Mice were monitored for fungal growth (cfu per organ, mean ± SE) and histopathology (periodic acid–Schiff and Gomori staining of lung tissue sections). Bronchoalveolar lavage fluid collection and morphometry were done as described (3). Histology sections
and cytoxin preparations were observed using a BX51 microscope (Olympus), and images were captured using a high-resolution DP71 camera (Olympus). Different doses of anakinra were administered i.p. daily until the end of experiment, and controls receive sterile saline.

**Cell Line Cultures, Transfection, and Autophagy.** RAW 264.7 cells (American Type Culture Collection) were seeded in a 100-mm Petri dish (3.5 × 10⁶) and transfected with the EGFP-LC3 plasmid (Addgene) using ExGen 500 in vitro Transfection Reagent (Fermentas) for 48 h, following the manufacturer’s instructions. Transiently transfected RAW 264.7 cells were exposed to *A. fumigatus* SC at a cell/fungi ratio of 1:1, 50 μg/mL Poly(I:C) (Sigma–Aldrich) to inhibit autophagosomal degradation by lysosomal enzymes. Cells were incubated for 4 h at 37 °C in 5% CO₂, as described by De Luca et al. (3). Cultures growing on coverslips were observed at a magnification of 100× with the Olympus BX51 fluorescence microscope using an FITC filter. Results are expressed as the number of cells with EGFP-LC3 puncta. An equal amount of cell lysate in 2× Laemmli buffer (Sigma–Aldrich) was probed with rabbit anti-GFP antibody (Abcam) and goat–anti-rabbit–IgG–HRP-conjugated secondary antibody (Sigma–Aldrich) after separation in 12% Tris/glycine SDS gel and transfer to a nitrocellulose membrane. Normalization was performed probing the membrane with mouse-anti-β-tubulin antibody (Sigma–Aldrich) and goat–anti-rabbit–IgG–HRP-conjugated secondary antibody. Chemiluminescence detection was performed with LiteAblotPlus chemiluminescence substrate (Euroclone S.p.A.), using the ChemiDocTM XRS+ Imaging system (Bio-Rad Laboratories Srl), and quantification was obtained by densitometry image analysis using Image Lab 3.1.1 software (Bio-Rad). For autophagy on lung cells, purified 1 × 10⁶ alveolar macrophages from naive mice were stimulated on glass slides in 24-multiwell plates with Poly(I:C), *Aspergillus* SC, and anakinra, as above, for 2 h at 37 °C in 5% CO₂. Cells were incubated with 1:200 diluted anti-LC3 antibody (Cell Signaling Technology) overnight at 4 °C in PBS containing 3% normal BSA, incubated with anti-rabbit–phycoerythrin secondary antibody (Sigma–Aldrich), and fixed for 20 min in PBS containing 4% paraformaldehyde. Images were acquired using the Olympus BX51 fluorescence microscope with a 40× objective and analySIS image processing software (Olympus). DAPI was used to detect nuclei.

**ELISA and Real-Time PCR.** Human and murine cytokines were measured using commercial ELISA kits (R&D Systems and BD Biosciences). Real-time RT-PCR was performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green chemistry (Finnzymes Oy). Cells were lysed, and total RNA was extracted using an RNeasy Mini Kit (QIAGEN) and reverse-transcribed as described by De Luca et al. (3). Amplification efficiencies were validated and normalized against *Gapdh*. The thermal profile for SYBR Green real-time PCR was at 95 °C for 3 min, followed by 40 cycles of denaturation for 30 s at 95 °C and an annealing/extension step of 30 s at 60 °C. Each data point was examined for integrity by analysis of the amplification plot. The mRNA-normalized data were expressed as relative cytokine mRNA in treated cells compared with that of unstimulated cells.

**Statistical Analyses.** The differences between groups were analyzed using the Mann–Whitney *U* test and the Wilcoxon signed rank test for unpaired and paired data, respectively. Differences were considered statistically significant when *P* ≤ 0.05. Data are presented as cumulative results of all experiments performed or as representative images (histology and Western blotting), and are given as mean ± SEM.


C57BL/6 or p47phox−/− mice (four to six per group) received 2.5 mg of 2,4,6-trinitrobenzene sulfonic acid and were concomitantly treated i.p. with anakinra (10 mg/kg) daily until the end of the experiment [5 d postinfection (dpi)]. Controls consisted of mice treated with 50% ethanol. (A) Representative H&E-stained colonic sections at 5 dpi. (Scale bars: 100 μm.) (Magnification: Inset, 100×.) (B) Cytokine production (ELISA) in colon homogenates of mice at 5 dpi. *P < 0.05; **P < 0.01.
C57BL/6 or p47phox−/− mice were infected intranasally with live A. fumigatus conidia (six to eight mice per group) and treated i.p. with anakinra (10 mg/kg) daily until the end of the experiment (21 dpi). (A) Lung histopathology [periodic acid–Schiff (PAS) staining and either May–Grunwald Giemsa or Gomori staining (Insets)] at different dpi in Aspergillus-infected mice treated with 10 mg/kg of anakinra. (Scale bars: 200 μm.) (Magnification: Insets, 100×.) (Insets) Red arrows in the May–Grunwald Giemsa-stained images indicate intracellular conidia. Cytokine production (ELISA) in lung homogenates (B) and pattern of cytokine gene expression (C) by RT-PCR on total lung cells at 20 dpi. (D) Mpo mRNA expression (by RT-PCR) in the lungs at 7 dpi. In A, numbers refer to the percentage of mononuclear (MNC) or polymorphonuclear (PMN) cells in the bronchoalveolar lavage (BAL). Data are representative of three experiments. *P < 0.05 (anakinra-treated vs. untreated mice).

Fig. 52. C57BL/6 or p47phox−/− mice were infected intranasally with live A. fumigatus conidia (six to eight mice per group) and treated i.p. with anakinra (10 mg/kg) daily until the end of the experiment (21 dpi). (A) Lung histopathology [periodic acid–Schiff (PAS) staining and either May–Grunwald Giemsa or Gomori staining (Insets)] at different dpi in Aspergillus-infected mice treated with 10 mg/kg of anakinra. (Scale bars: 200 μm.) (Magnification: Insets, 100×.) (Insets) Red arrows in the May–Grunwald Giemsa-stained images indicate intracellular conidia. Cytokine production (ELISA) in lung homogenates (B) and pattern of cytokine gene expression (C) by RT-PCR on total lung cells at 20 dpi. (D) Mpo mRNA expression (by RT-PCR) in the lungs at 7 dpi. In A, numbers refer to the percentage of mononuclear (MNC) or polymorphonuclear (PMN) cells in the bronchoalveolar lavage (BAL). Data are representative of three experiments. *P < 0.05 (anakinra-treated vs. untreated mice).
Fig. S3.  (A) Fluorescence images of EGFP-LC3–transfected RAW 264.7 cells exposed to A. fumigatus SC alone (None) or in the presence of anakinra (1 or 10 μg/mL) or rapamycin (50 μM) for 4 h. (Magnification: 100×.) Control cells, unexposed/untreated cells. (B) Quantification of LC3b and p62 in cell lysates by immunoblotting. Normalization was performed using β-tubulin (β-tub). Quantification was obtained by densitometry image analysis using Image Lab 3.1.1 software. (C) Cell lysates from EGFP-LC3–transfected RAW 264.7 cells stimulated as in A were subjected to immunoprecipitation with antibody to Casp-1 (normalized to β-tubulin). (D) Autophagy gene expression by RT-PCR in lungs of naive, infected untreated (None), or anakinra (10 mg/kg-treated mice at 7 dpi). Results are representative of three experiments. *P < 0.05; **P < 0.01.
Fig. S4. Blocking autophagy increases inflammasome-dependent inflammation in C57BL/6 mice. (A) Lung histopathology (PAS staining) of mice intranasally infected with live *A. fumigatus* conidia (six to eight mice per group) and treated i.p. with saline (none), chloroquine (15 mg/kg), or 3MA (24 mg/kg) from the day of the infection until the end of the experiment (1 wk later). (Scale bars: 200 μm.) (B) Cleaved forms of Casp-1 in the lungs at 7 dpi were analyzed using antibodies that identify both the full-length proform and cleaved form, and were normalized to β-tub. (C) Cytokine production (ELISA) in lung homogenates at 7 dpi. Results are representative of two experiments. *P* < 0.05.