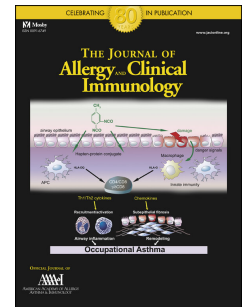


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Proinflammatory cytokine response towards fungi but not bacteria in Chronic Granulomatous Disease

Running title: proinflammatory cytokine response in CGD

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Capsule summary: Monocytes and neutrophils from CGD patients show an enhanced proinflammatory cytokine response towards fungal but not towards bacterial pathogens. The proinflammatory response may contribute to the inflammation reported in CGD.

To the editor,

Chronic Granulomatous Disease (CGD) is characterized by recurrent bacterial and fungal infections, granulomas and sometimes inflammatory bowel disease⁽¹⁾. Mutations in genes encoding the NADPH oxidase components have been identified as disease causing and result in defective Reactive Oxygen Species (ROS) production⁽¹⁾. Granulocytes and monocytes from CGD patients show a clear defect in the killing of bacterial and fungal microbes, leading to susceptibility to infections. Although purified ligands have been tested in the pro- and anti-inflammatory cytokine response by CGD leukocytes, the response towards intact viable bacterial and fungal pathogens have thus far not been reported.

To determine a role for the NADPH oxidase activity in monocyte and neutrophil cytokine responses towards bacterial and fungal pathogens, we analyzed ten CGD patients with previously described mutations in genes encoding for either the cytosolic NADPH oxidase proteins, *i.e.* p47^{phox} or p67^{phox} or the membrane-expressed cytochrome-*b*₅₅₈ subunit gp91^{phox}, all resulting in a failure to produce ROS (Repository Table 1)⁽¹⁾. The role of two known signaling pathways involved in bacterial and fungal host defense was determined in CARD9- and IRAK4-deficient patients. Cytokine release was measured upon stimulation with the TLR1/2 agonist Pam3Cys (P3C) and the TLR4 agonist LPS, as representative and most commonly used ligands from Gram-positive and Gram-negative pathogens, respectively. These were compared to the stimulation by *Staphylococcus aureus*, *Escherichia coli*, as well as *Candida albicans* and *Aspergillus fumigatus* of phagocytic cells isolated from CGD patients and healthy controls.

Pathogen-associated molecular patterns (PAMPs) including LPS can result in the assembly of the inflammasome and caspase-1 activation for processing pro-IL-1 β into the active pro-inflammatory cytokine IL-1 β ⁽²⁾. Monocytes from CGD patients released slightly more IL-1 β in response to P3C and LPS, as compared to control monocytes (Figure 1A). As reported before, inflammasome activity is increased in monocytes from CGD patients⁽²⁾. On the other hand, the CGD monocytes were impaired in the release of IL-1 β in response to Gram-positive *S. aureus* and Gram-negative *E. coli* bacteria (Figure 1A). In contrast to intact bacteria, we observed enhanced IL-1 β release by the patient monocytes in response to the fungi *C. albicans* and *A. fumigatus* (Figure 1A). Together these data indicate that ROS are not quenching the cytokine measurement but show that the response to different pathogens is variable. Recent work demonstrated that blocking IL-1R with anakinra reduced the IL-1 β levels in p47^{phox}-deficient mice and improved the colitis in two CGD patients⁽²⁾. These data suggest that fungal opportunists may trigger such *in-vivo* (auto)inflammatory responses in CGD rather than bacterial determinants.

We also assessed the pro-inflammatory IL-6 and anti-inflammatory IL-10 cytokines which are produced upon activation of the NF- κ B pathway⁽³⁾. Although CGD monocytes already released some background IL-6, stimulation with P3C, LPS, or *S. aureus* resulted in normal levels, whereas the IL-6 response to *E. coli* was reduced compared with control cells (Figure 1B). In contrast, the monocytes from CGD patients again released strongly elevated levels of IL-6 upon stimulation with *C. albicans* or *A. fumigatus* (Figure 1B), indicating that the IL-1 β release was part of a broader activation response. Whereas IL-10 is not produced in response to fungi, the CGD monocytes produced low IL-10 levels in response to P3C, LPS, *S. aureus* and *E. coli* as compared to controls (Figure 1C). In line with this latter observation,

a p47^{phox}-deficient mouse model showed reduced NF- κ B activation following *Pseudomonas* infection⁽⁴⁾. Others have reported upregulated pro-inflammatory genes in response to TLR agonists and an enhanced IL-6 production by CGD monocytes in response to formaldehyde-killed *S. aureus*^(3;5). These findings indicate that the role of NADPH oxidase activity in NF- κ B activation and cytokine production clearly depends on the stimulus.

Together, our data demonstrate for the first time that the pro-inflammatory cytokine response by CGD monocytes is enhanced in response to intact viable fungi but clearly impaired in response to intact bacterial pathogens.

Human neutrophils do not produce IL-6 and IL-10⁽⁶⁾ but can be induced to generate large amounts of pro-inflammatory IL-8 as well as the anti-inflammatory IL-1RA. Whereas the IL-8 response was not different from control neutrophils (Figure 2), CGD neutrophils released high IL-1RA levels under non-stimulated conditions, which was further enhanced upon stimulation by TLR agonists or bacterial and fungal microbes (Figure 2). Contrary to these findings, RNA transcripts for IL-8 were reported to be upregulated in unstimulated neutrophils from CGD patients⁽⁷⁾. However, most neutrophil-derived IL-8 production is post-transcriptionally regulated, and protein levels may therefore be very different from transcriptional activity⁽⁶⁾.

These data demonstrate a distinct role for the NADPH oxidase activity in the regulation of the cytokine response of CGD phagocytes to TLR agonists or intact bacterial and fungal pathogens. TLRs signal through the kinase IRAK4 for the activation of NF- κ B. Leukocytes from IRAK4-deficient patients demonstrated a complete defect in the cytokine response to TLR-agonists, intact bacterial and fungal pathogens under our test conditions, and these patients are selectively predisposed to bacterial infections (Repository-Figure 1)⁽⁸⁾. Fungi are recognized by C-type lectin receptors (CLRs), including dectin-1 for the activation of CARD9 and NF- κ B. CARD9 deficiency is clinically characterized by *Candida* infections and associated with an impaired cytokine response to yeast in particular (Repository-Figure 2)⁽⁹⁾. Serum opsonization of the microbes and conidia had surprisingly little additional effects under the conditions tested (data not shown), suggesting that the PAMP-sensing molecules expressed by the phagocytes determined the effects observed, instead of complement or IgG binding to their respective surface receptors.

In conclusion, we have demonstrated that monocytes and neutrophils from CGD patients show an exacerbated pro-inflammatory response to intact fungal but not to bacterial pathogens. These findings indicate that ROS production by the NADPH oxidase system may under normal conditions limit the NF- κ B- and inflammasome-mediated cytokine production by monocytes and neutrophils in response to fungal pathogens, whereas the intracellular mitochondrial ROS is supposed to enhance the initiation and activation of inflammasomes⁽²⁾. The cytokine response to intact bacterial and fungal pathogens was completely dependent on TLR-mediated signaling through IRAK4, while CARD9 was involved in the yeast-induced cytokine production, being regulated by dectin-1. The observed deregulated inflammatory response to intact pathogens instead of isolated PAMPs may contribute to the often persistent and overactive innate immune response in CGD following certain infections.

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Key words: CGD, NADPH oxidase, monocytes, neutrophils, bacterial and fungal pathogens, cytokine production, inflammasome activity

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Authorship contribution: T.W.K. is the principal investigator who conceived and designed the study. R.P.G., J.L.v.H., A.v.d.G., and L.H. performed the experiments. J.M.v.d.B., M.C., C.P. and J.R. assisted in acquisition of the patient samples. T.K.v.d.B. and D.R. contributed to the design of the study and helped with the interpretation and the presentation of the results. R.P.G. devised and performed the analyses and wrote the manuscript together with T.W.K.

Figure Legends**Figure 1. CGD monocytes show an enhanced pro-inflammatory cytokine response towards fungal but not towards bacterial pathogens**

PBMCs from the CGD patients and control subjects, were incubated overnight with P3C (20 µg/ml), LPS/LBP (20 ng/ml/50 ng/ml), *E. coli*, *S. aureus*, *C. albicans* or *A. fumigatus*. Afterwards, supernatants were collected and the concentrations of IL-1β (panel A), IL-6 (panel B) and IL-10 (panel C) were assessed by ELISA. Results are means ± SEM of measurements from at least three independent experiments.* p<0.05. Co-incubation of multiple PAMPs to activate simultaneously the different TLRs on monocytes did not result in enhanced cytokine responses as compared to single stimuli (data not shown).

Figure 2. Neutrophils from CGD patients have an enhanced pro- and anti-inflammatory cytokine response

PMNs from the CGD patients and control subjects, were incubated overnight with P3C (20 µg/ml), LPS/LBP (20 ng/ml/50 ng/ml), *E. coli*, *S. aureus*, *C. albicans* or *A. fumigatus*. Afterwards, supernatants were collected and the concentrations of IL-8 (panel A) and IL-1RA (panel B) were assessed by ELISA. Results are means ± SEM of measurement from at least three independent experiments.* p<0.05.

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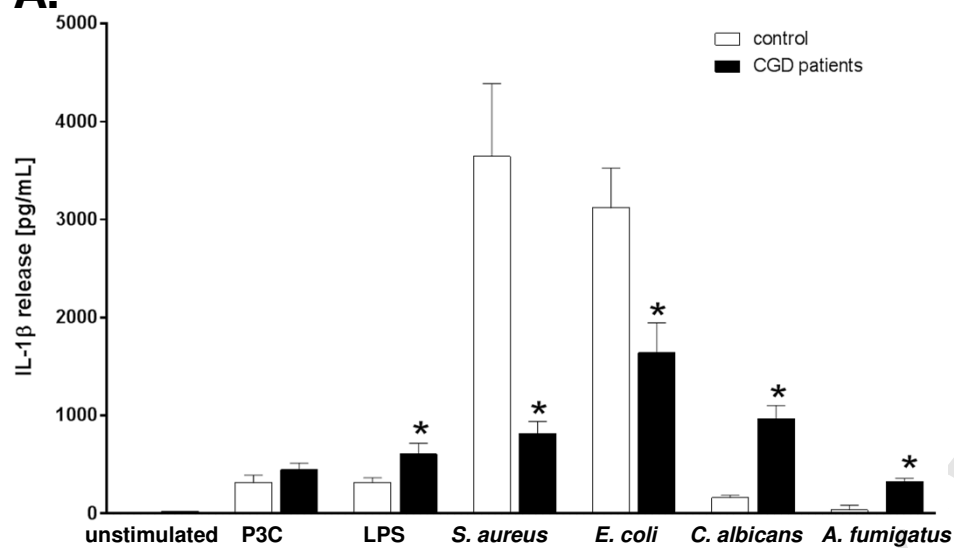
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Repository Table 1

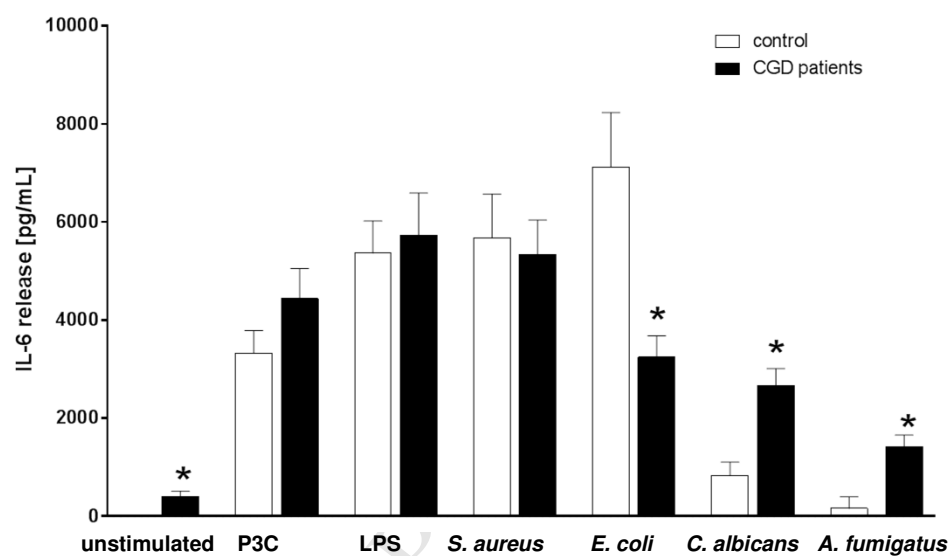
	Mutation	Gene	Clinical Symptoms
Patient A	c.781_782delCA, p.Gln261Valfs*22	<i>CYBB</i>	Recurrent bacterial and fungal infections, IBD
Patient B	c.1166G>C, p.Gly389Ala	<i>CYBB</i>	Recurrent bacterial and fungal infections, IBD
Patient C	del exons 6_13	<i>CYBB</i>	Recurrent bacterial and fungal infections, IBD
Patient D	c.318G>A, p.Trp106*	<i>CYBB</i>	Recurrent bacterial and fungal infections, IBD
Patient E	c.1499A>G, p.Asp500Gly	<i>CYBB</i>	Recurrent bacterial and fungal infections, IBD
Patient F	c.75_76delGT, p.Tyr26Hisfs*26	<i>NCF1</i>	Recurrent bacterial and fungal infections, IBD
Patient G	c.75_76delGT, p.Tyr26Hisfs*26	<i>NCF1</i>	Recurrent bacterial and fungal infections, IBD
Patient H	c.75_76delGT, p.Tyr26Hisfs*26	<i>NCF1</i>	Recurrent bacterial and fungal infections, IBD
Patient I	c.[75_76delGT];[.417_451+650del685] , p.[Tyr26Hisfs*26];[Thr133HisfsX66]	<i>NCF1</i>	Recurrent bacterial and fungal infections, IBD
Patient J	c.229C>T, p. Arg77*	<i>NCF2</i>	Recurrent bacterial and fungal infections, IBD
Patient K	c.[214G>A];[1118G>C], p.[Gly72Ser];[Arg373Pro]	<i>CARD9</i>	<i>Candida</i> meningitis
Patient L	c.883C>T, p.Gln295*	<i>CARD9</i>	<i>Candida</i> meningitis
Patient M	c.877C>T, p.Gln293*	<i>IRAK4</i>	bacterial infections
Patient N	c.877C>T, p.Gln293*	<i>IRAK4</i>	bacterial infections

Figure 1.

A.



B.



C.

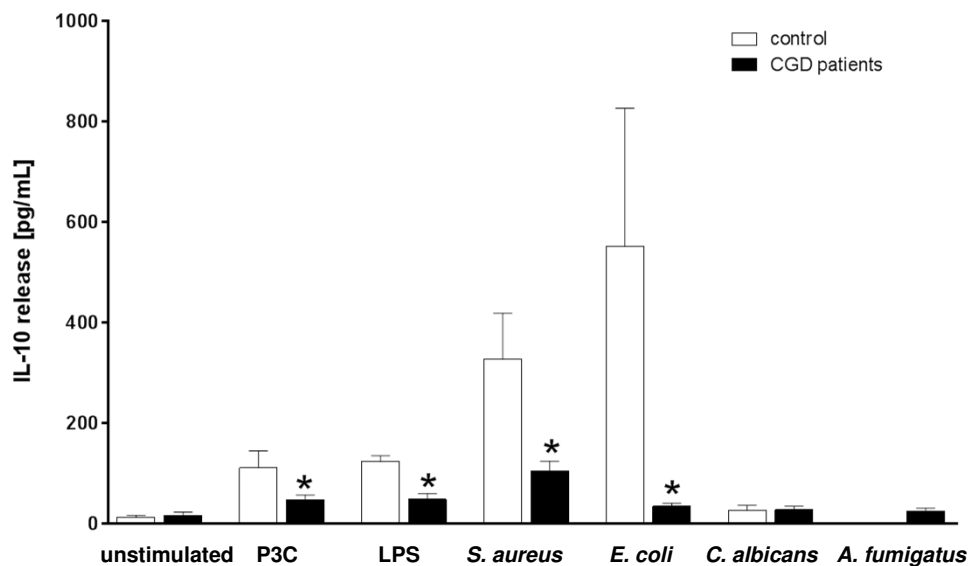
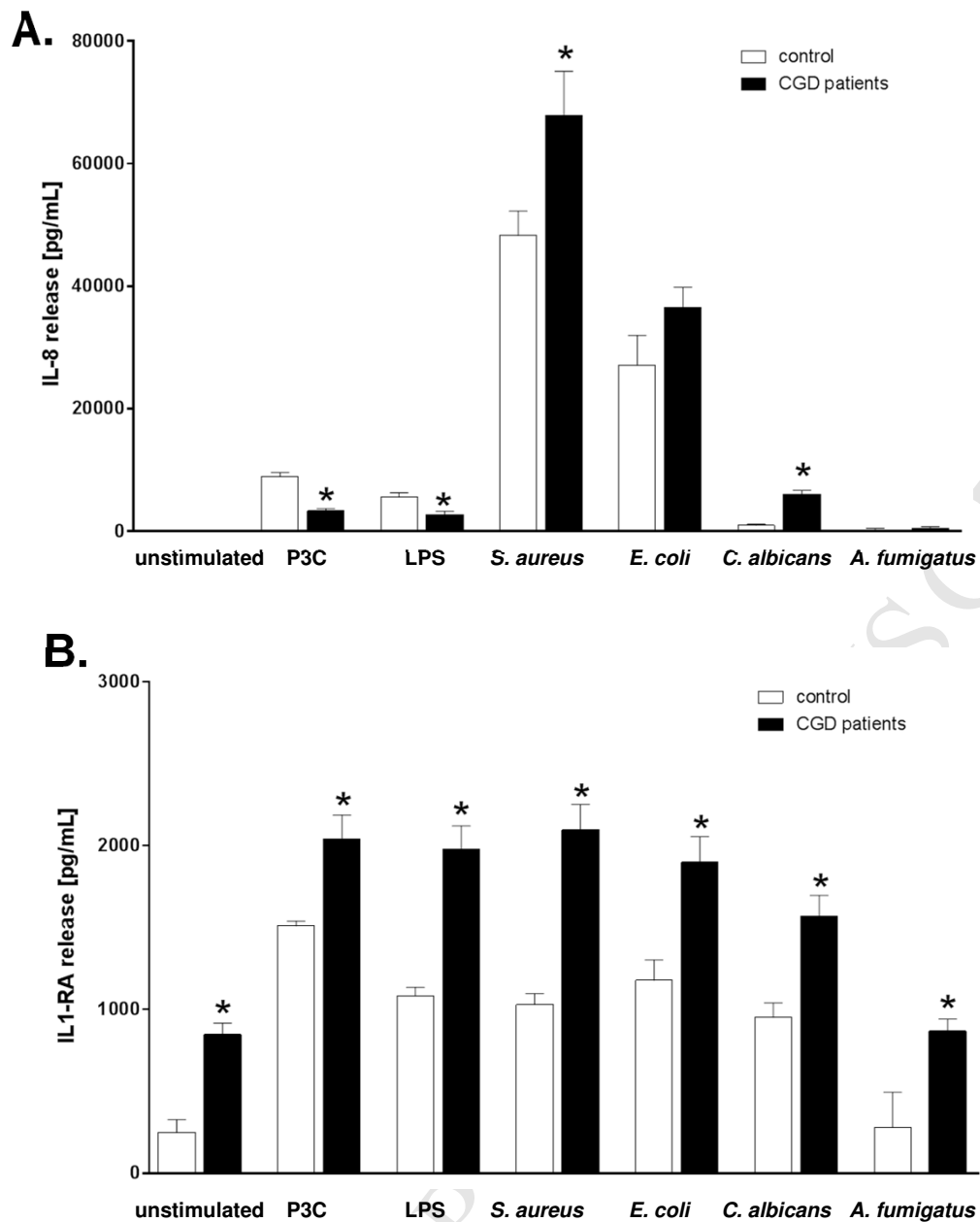


Figure 2.

Repository Table and Figure legends

Repository Table 1. Gene mutations and clinical features of patients. Hemizygous mutations in the X-linked CGD patients (cases A-E). All non-X-linked cases carry homozygous mutations for the indicated gene mutation in the Table, except for the compound heterozygous mutations from patient I and K. Note that all cases come from separate, unrelated families.

Repository Figure 1. PBMCs and PMNs from IRAK4-deficient patients show an impaired cytokine response to TLR agonist, bacterial and fungal pathogens

PBMCs or PMNs from two IRAK4-deficient patients and control subjects were incubated overnight with P3C (20 µg/ml), LPS/LBP (20 ng/ml/50 ng/ml), *S. aureus*, *E. coli*, *C. albicans* or *A. fumigatus*. Cultured supernatants were collected and the concentrations of IL-1β, IL-6 by PBMCs, IL-8 by PMNs were assessed by ELISA. Results are means ± SEM of measurements from three independent experiments. * p<0.05.

Repository Figure 2. PBMCs and PMNs from CARD9-deficient patients show an impaired cytokine response to *Candida*

PBMCs or PMNs from two CARD9-deficient patients and control subjects were incubated overnight with P3C (20 µg/ml), LPS/LBP (20 ng/ml/50 ng/ml), *S. aureus*, *C. albicans* or *A. fumigatus*. Cultured supernatants were collected and the concentrations of IL-1β and IL-6 by PBMCs were assessed by ELISA. Results are means ± SEM of measurements from three independent experiments. * p<0.05.

Repository materials and methods

CGD, IRAK4- and CARD9-deficient patients, control subjects and cell isolation

Heparinized blood was obtained, after informed consent, approval by the Sanquin Research Ethical Medical Committee (Amsterdam, The Netherlands) and in accordance with the Declaration of Helsinki (version Seoul 2008) from patients (Repository Table 1) and from healthy controls. Control subjects originated from the same hospital as the patients. These mutations and patients were reported before^{1,2}. The granulocytes and erythrocytes were separated from the mononuclear leukocytes and platelets by centrifugation over isotonic Percoll with a specific density of 1.076 g/ml and resuspended in Hepes-buffered saline solution³. PBMCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% (v/v) FCS (Life, Bleiswijk, The Netherlands).

Cell stimulation and cytokine measurement

For the cytokine response, PBMCs (0.25×10^6 monocytes/mL) or PMNs (5.0×10^6 cells/mL) were stimulated overnight with the indicated stimuli including Pam3Cys-SK4 (EMC Microcollections, Tübingen, Germany), LPS/LBP (isolated from *E. coli* strain 055:B5, Sigma, St. Louis, MO, USA; LBP from R&D Systems, Abingdon, UK). *Escherichia coli* (strain ML-35), *Staphylococcus aureus* (strain 502A), *Aspergillus fumigatus* (clinical isolate), *Candida albicans* (strain SC5314) were grown under aerobic conditions at 37°C (bacteria) or 30°C (yeast) in Lysogeny Broth (LB). Bacteria and yeasts were then collected by centrifugation, washed twice in PBS and resuspended in HEPES medium. After opsonization (10% (v/v) pooled serum, 15 min, at 37°C), bacteria and *Candida* were added at a ratio of 1:1 monocytes, 1:5 neutrophils and *Aspergillus* at ratio of 1:10 for monocytes, and 1:50 for neutrophils. Production of IL-6, IL-1 β , IL-10 and IL-8 was measured in the supernatants with a Pelikine ELISA (Sanquin Reagents, Amsterdam, The Netherlands) according to the manufacturer's protocol. IL-1RA was measured with an IL-1RA ELISA kit (Life).

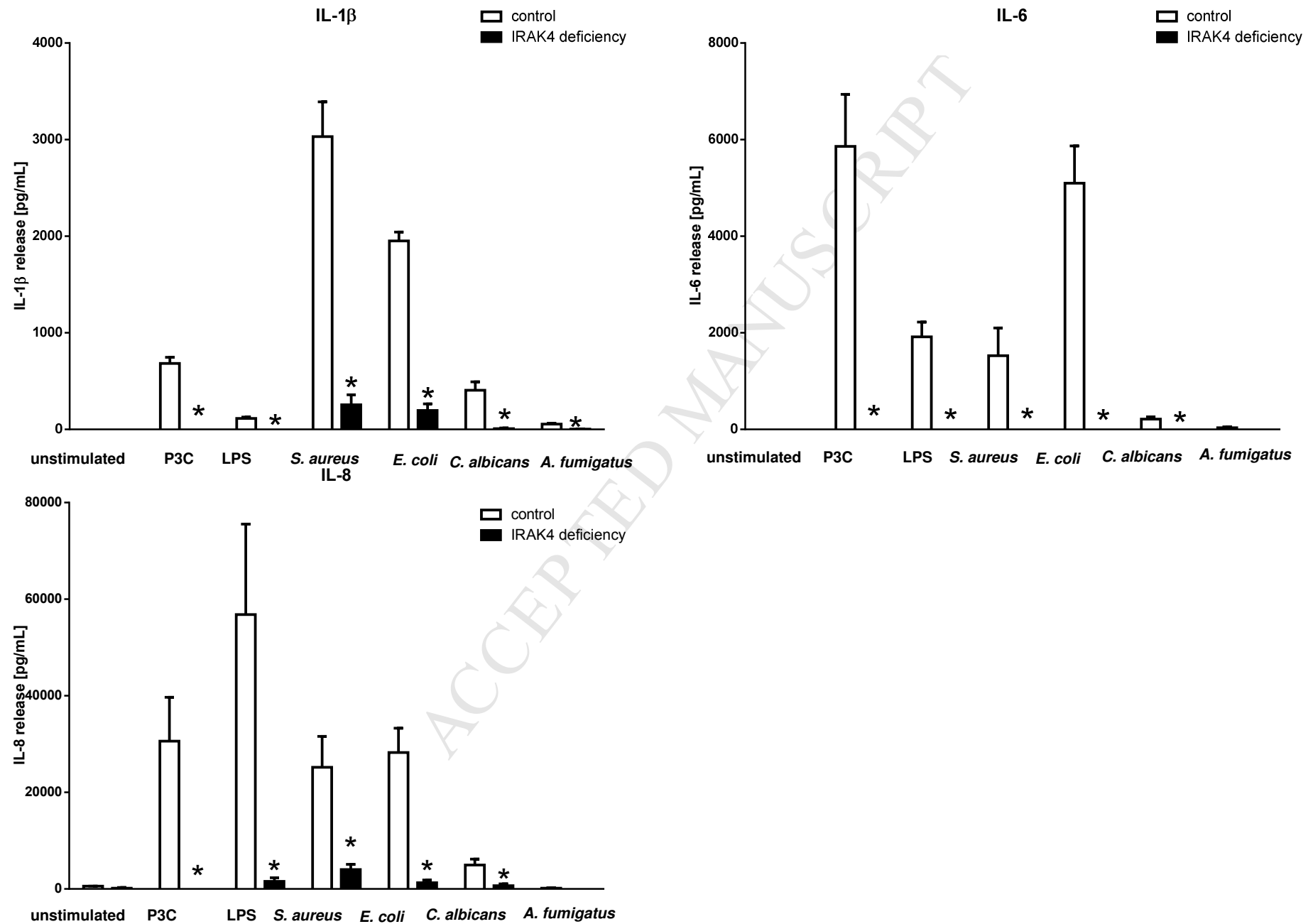
Statistics

Statistical analysis was performed with GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). Data were evaluated by two-tailed student's *t*-test and by the Mann-Whitney U test. The results are presented as the mean $\pm$ SEM, as indicated. Data were considered significant when $p < 0.05$.

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Repository Figure 1.



Repository Figure 2.

