Tryptophan catabolism is unaffected in chronic granulomatous disease

ARISING FROM L. Romani *et al. Nature* **451,** 211–215 (2008); doi:10.1038/nature06471

Chronic granulomatous disease (CGD) is an inherited disorder of phagocyte function, caused by a genetic defect in NADPH oxidase (NOX2), leading to an impaired ability of leukocytes to produce superoxide ($O_2^{-\bullet}$)[1]; CGD subjects are susceptible to chronic infections and hyperinflammation, although the mechanisms remain unclear. Romani *et al.*[2] reported an aberrant inflammatory response to pulmonary aspergillosis as well as sterile *Aspergillus fumigatus* to be mediated by a defective tryptophan catabolism to kynurenine caused by lack of $O_2^{-\bullet}$ in CGD mice. Kynurenine is formed by indoleamine 2,3-dioxygenase-1 (IDO1) in a reaction originally reported to depend on $O_2^{-\bullet}$ (ref. 3). Here we show that NOX2 deficiency does not attenuate IDO1-mediated tryptophan catabolism in human phagocytes and CGD mice with granulomas arising from an inflammatory response to *Aspergillus*. There is a Reply to this Brief Communications Arising by Romani, L. & Puccetti, P. *Nature* **514,** http://dx.doi.org/10.1038/nature13845 (2014).

Romani *et al.*[2] concluded that IDO-mediated tryptophan catabolism is blocked in CGD based on studies performed in *p47*$^{phox-/-}$ mice. They reported increased kynurenine in granuloma-containing lungs of wild-type but not *p47*$^{phox-/-}$ mice, and that interferon-γ (IFN-γ) stimulates IDO activity in lung phagocytes from wild-type but not *p47*$^{phox-/-}$ mice, despite the presence of IDO protein in both mouse strains.

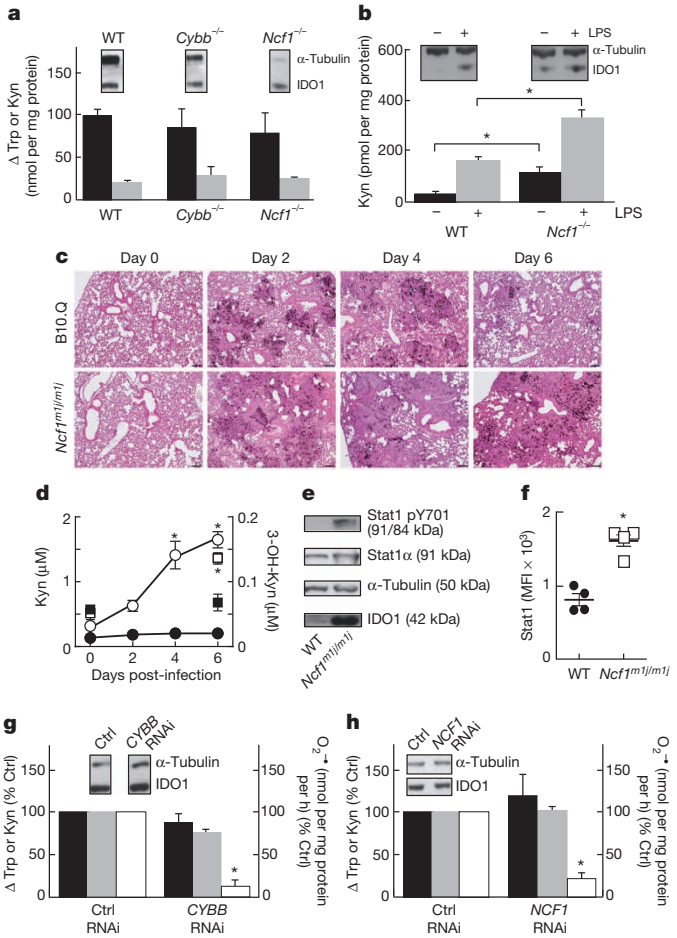

Figure 1 | Phagocyte NADPH oxidase activity is not required for IDO1 activity in inflammation. **a**, Lung PMN (purity > 85% by differential count) were isolated from wild-type (WT), *Ncf1*$^{-/-}$ or *Cybb*$^{-/-}$ mice 24 h after intraperitoneal injection of 7.5 mg per kg lipopolysaccharide 0111:B4 (LPS). PMN were then incubated for 48 h in RPMI medium containing 10% fetal bovine serum in the presence of 200 U ml^{-1} mouse recombinant IFN-γ and 400 μM L-N^G-monomethyl-arginine (nitric oxide synthase inhibitor). Consumption of tryptophan (ΔTrp, black) and accumulation of kynurenine (Kyn, grey) in the medium were then determined by high-performance liquid chromatography (HPLC) (mean ± s.e.m., $n = 3$); insets show representative α-tubulin (50 kDa) and IDO1 (42 kDa) proteins. PMN from CGD mice failed to generate $O_2^{-\bullet}$ upon stimulation with IFN-γ (72 ± 9 versus 0 ± 0 nmol $O_2^{-\bullet}$ per h per mg protein for WT versus *Ncf1*$^{-/-}$ or *Cybb*$^{-/-}$), mean ± s.e.m., $n = 3$ (data not shown). **b**, Lungs from WT and *Ncf1*$^{-/-}$ mice before (–) and 24 h after intra-peritoneal administration of 7.5 mg per kg LPS (+) were homogenized in 1 ml of 20 mM phosphate buffer pH 7.2 containing 140 mM KCl and 2× complete protease inhibitor cocktail (Roche), and Kyn determined by HPLC; insets show representative α-tubulin (50 kDa) and IDO1 (42 kDa) proteins. Data are mean ± s.e.m. of 3 mice in each group; $^*P < 0.05$ (Mann–Whitney rank sum test). **c**, Representative lung sections (5-μm thick) prepared from B10.Q and *Ncf1*$^{m1j/m1j}$ mice before (day 0) and 2, 4 and 6 days after intra-nasal instillation of 5 μg of sterile hyphal cell wall from *A. fumigatus*, and stained with haematoxylin and eosin. Scale bar, 100 μm. **d**, Lung Kyn (circles) and 3-hydroxykynurenine (3-OH-Kyn, squares) in B10.Q (WT, black symbols) and *Ncf1*$^{m1j/m1j}$ mice (white symbols) before (day 0) and 2, 4 and 6 days after instillation of sterile hyphal cell wall of *A. fumigatus*. Lungs were homogenized, proteins precipitated with 4% trichloroacetic acid, the mixture centrifuged and the resulting supernatant neutralised (1 M sodium phosphate, pH 7.4) and subjected to liquid chromatography with tandem mass spectrometry (LC/MS/MS) using *m/z* 209→146 and 225→208 transitions for Kyn and 3-OH-Kyn, respectively. Kyn and 3-OH-Kyn were separated on a 150 × 4.6 mm Luna C18 (2), 5 μm column (Phenomenex) using a gradient generated by mobile phase A (0.1% formic acid) and B (0.1% formic acid in 100% acetonitrile). The results show mean ± s.e.m., with $n = 7$–8 for Kyn and $n = 4$ for 3-OH-Kyn for each time point and genotype. $^*P < 0.05$ indicates significant difference between *Ncf1*$^{m1j/m1j}$ and B10.Q using two-way ANOVA followed by Sidak's multiple comparison test. **e**, Representative western blots of lung homogenates from B10.Q (WT) and *Ncf1*$^{m1j/m1j}$ mice 6 days after instillation of sterile hyphal cell wall from *A. fumigatus,* showing phosphorylated Stat1 (Stat1 pY701; 91 and 84 kDa), Stat1α (91 kDa), α-tubulin (50 kDa) and IDO1 (42 kDa) proteins. **f**, Cells from bronchoalveolar lavage fluid of B10.Q (WT) and *Ncf1*$^{m1j/m1j}$ mice 4 days after instillation of *A. fumigatus* were stained by cell surface markers, fixed and incubated with anti-Stat1 antibody (BD Biosciences). Expression of Stat1 in CD11b$^+$Ly6g$^+$ PMN was assessed by flow cytometry and is shown as median fluorescence intensity (MFI). Data are shown for individual animals as well as mean ± s.e.m. $^*P < 0.05$ (Mann–Whitney rank sum test). **g**, **h**, THP-1 cells (10^6 per well) were treated for 24 h with the respective control, *CYBB*-specific siRNA (5′-CGGAGGUCUUACUUUGAAGUCUUU-3′, Invitrogen) (**g**) or *NCF1*-specific siRNA (sc-29422; Santa Cruz Biotechnologies) (**h**), followed by 48 h incubation in the presence of 400 U ml^{-1} recombinant human IFN-γ. Trp lost from (ΔTrp, black) and Kyn accumulated in the medium (grey) was then determined by HPLC. Generation of $O_2^{-\bullet}$ (white) was determined by cytochrome *c* reduction after treatment of the cells with 200 ng ml^{-1} phorbol-12-myristate-13-acetate for 1 h. The 100% control-values for **g** and **h** were 15 ± 3.4 and 51 ± 9 nmol $O_2^{-\bullet}$ per h per mg protein, respectively (mean ± s.e.m., $n = 3$); insets show representative α-tubulin (50 kDa) and IDO1 (42 kDa) proteins. $^*P < 0.05$ (Mann–Whitney rank sum test).

However, recent studies have shown that cytochrome b_5 rather than $O_2^{-\bullet}$ activates cellular IDO[4]. Therefore, we re-examined tryptophan catabolism to kynurenine in several models of CGD, using *Ncf1*$^{-/-}$ (lacking

the Ncf1 protein, also known as $p47^{phox}$), $Cybb^{-/-}$ (lacking the catalytic subunit of NOX2, also known as $gp91^{phox}$)[5] and $Ncf1^{m1j/m1j}$ mice (with a single mutation in the *Ncf1* gene, resulting in a defective NCF1 protein leading to a lack of NOX2 activity)[6,7].

Polymorphonuclear leukocytes (PMN) from lungs of endotoxin-treated $Ncf1^{-/-}$ and $Cybb^{-/-}$ mice failed to generate $O_2^{-\bullet}$ upon activation, yet these cells, like wild-type cells, converted tryptophan to kynurenine (Fig. 1a). Moreover, endotoxin treatment increased pulmonary kynurenine similarly in wild-type and $Ncf1^{-/-}$ mice (Fig. 1b). To examine tryptophan catabolism in situations of hyper-inflammation, we administered sterile *Aspergillus fumigatus* to $Ncf1^{m1j/m1j}$ and their wild-type control mice (B10.Q), differing at only a single *Ncf1* mutation. As expected, this caused pulmonary granulomas in B10.Q and $Ncf1^{m1j/m1j}$ mice, and these granulomas resolved only in control animals (Fig. 1c). Strikingly, lung IDO1 protein, kynurenine as well as 3-hydroxykynurenine were higher with defective NCF1 (Fig. 1d, e). This was associated with an increase in pulmonary and bronchoalveolar lavage fluid PMN Stat1 protein (Fig. 1e, f), and lung phosphorylated Stat1 (Fig. 1e). Phosphorylation of Stat1 is a major NOX2 downstream pathway[8] that mediates IFN-γ-dependent IDO1 expression[9].

The above studies imply that $O_2^{-\bullet}$ is not required for *in vivo* IDO activity. Consistent with this, blood PMN isolated from CGD patients with a mutation in the *NCF1* or *CYBB* gene were unable to generate $O_2^{-\bullet}$, yet effectively degraded tryptophan to kynurenine (data not shown), as also reported recently by others[10,11]. Similarly, knockdown of NCF1 or CYBB protein in human monocytic THP-1 cells did not decrease tryptophan catabolism to kynurenine, although it blunted $O_2^{-\bullet}$ formation by ~80% (Fig. 1g, h).

Our observation of increased, as opposed to decreased[2], IDO activity and 3-hydroxykynurenine (an indicator of kynurenine metabolism) in infected CGD mice is consistent with studies reporting elevated plasma and urinary kynurenine and 3-hydroxykynurenine in CGD patients[10,12]. Also, gene therapy with *CYBB* in a CGD patient resulted in clearance of *Aspergillus* infection that was associated with restoration of 30% of normal NOX activity without increase of IDO activity, as assessed by plasma kynurenine[13]. The discrepancy between our cellular studies (Fig. 1a) and those of Romani *et al.*[2] may be explained in part by nitric oxide inhibiting IDO activity[14], as we observed IDO activity only when nitric oxide synthases were blocked. Moreover, the $Ncf1^{m1j/m1j}$ mice used here[7] better reflect human CGD in which NOX proteins are expressed, albeit as non-functional mutants, rather than being absent and with linked chromosomal fragment/s from the original 129 derived embryonic stem cell, which could also vary in different backcrossed strains, as in the case of the $Ncf1^{-/-}$ mice used[15].

We conclude that IDO1-mediated tryptophan catabolism to kynurenine does not require phagocyte NADPH oxidase derived $O_2^{-\bullet}$, nor is it defective in human or mouse CGD, even under conditions where hyper-inflammation exists. Therefore, blockade of this pathway is unlikely to explain the acute pulmonary inflammatory response observed by Romani *et al.*[2].

Methods

Lungs and lung PMN were isolated from mice and cells cultured as described previously[2,14]. Sterile *Aspergillus fumigatus* hyphal cell wall was administered intranasally to B10.Q and $Ncf1^{m1j/m1j}$ mice[5]. Human blood PMN were isolated from control or CGD patients by a Percoll gradient[16]. Knockdown of NCF1 or CYBB in THP-1 cells was achieved by short interfering RNA (siRNA) transfection. Tryptophan, kynurenine and 3-hydroxykynurenine in the medium of IFN-γ-treated cells and in lung homogenates were quantified by high-performance liquid chromatography[4] and liquid chromatography–tandem mass spectrometry (LC/MS/MS), respectively. Cellular NOX activity was determined by cytochrome *c* reduction following stimulation with phorbol-12-myristate-13-acetate.

All experiments involving animal and human subjects were performed with approval from the institutional ethics committees. We thank the CGD donors for their co-operation and donation of blood, and J. Ziegler for his help. We also thank E. Lönnblom, E. Chryssanthou and K. Selva Nandakumar for their help with the *Aspergillus* experiments. This work was supported by grants from the National Health and Medical Research Council of Australia (1003484 and 1037879 to RS and 455395 to R.S. and B.H.C.) and the European Commission Directorate-General for Research & Innovation (HEALTH-F2-2012-278611).

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Received 26 June; accepted 4 August 2014.

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Author Contributions G.J.M. designed and carried out most experiments. S.W. and R.H. designed and carried out studies involving *A. fumigatus*. B.W. carried out initial cellular studies and B.H.C. was responsible for studies involving CGD patients. R.S. conceived the study and wrote the manuscript with G.J.M. All authors read and contributed to the final version of the manuscript.

Competing Financial Interests Declared none.

doi:10.1038/nature13844

Romani & Puccetti reply

REPLYING TO G. J. Maghzal *et al*. *Nature* **514,** http://dx.doi.org/10.1038/nature13844 (2014)

After our initial observation of defective tryptophan catabolism in experimental chronic granulomatous disease (CGD)[1], several laboratories have been testing the indoleamine 2,3-dioxygenase (IDO1) competence of cells from CGD patients. In most instances, they found no impairment in IDO1 competence in terms of tryptophan catabolic activity *in vitro* by polymorphonuclear leukocytes and monocyte-derived dendritic cells[2,3], leading to the conclusion that there is no obvious defect in the production of kynurenine (the first by-product of tryptophan degradation)—hence in the IDO1-dependent mechanism of tolerogenesis as a whole in human CGD. In the accompanying Comment[4], Maghzal *et al.* report that tryptophan catabolism is unaffected in chronic granulomatous disease, again by measurements of kynurenine production.

However, a number of studies have now been providing evidence that the assay is not sufficiently informative as to the local versus systemic functioning of the IDO1 mechanism, particularly on considering the pleiotropic effects of IDO1 *in vivo*[5,6]. While we stand by our original observations in CGD mice with lethal pulmonary aspergillosis[1], it should be noted that, in all of the experimental models tested so far, lack of IDO1 competence does not result, *per se*, in spontaneous inflammatory pathology. Yet, the functional defect becomes obvious when mice not competent for IDO1 function are challenged with an inflammatory *noxa* recognized more usually by Toll-like receptors[7]. Additionally, a number of potential factors have now been identified that further substantiate the concept that a global defect in IDO1 functioning underlies the severe chronic inflammation in CGD, among which are local accumulation of peroxynitrites[8] (which compromise IFN-γ signalling necessary for IDO1 induction[9]) and IL-6 (ref. 8) (which promotes IDO1 proteasomal degradation[10]), lack of IDO1-dependent neutrophil apoptosis[11], loss of IDO1-driven non-canonical NF-κB activation (otherwise resulting in downregulation of proinflammatory cytokines, and upregulation of tolerogenic TGF-β), and probably defective IDO1 signalling activity[12].

Thus the problem is not whether cells from CGD patients and mice equally display defective tryptophan catabolism *in vitro*. Rather, when contextualized to the current knowledge, that is a matter of appreciation that the IDO1 mechanism, and the multiple downstream regulatory responses over which it presides—including control of the over-reactive responses to TLR signalling—are globally compromised. The situation—that is, elevated rather than suppressed circulating kynurenine and/or *in vitro* production in CGD patients[3]—may be similar to that of septic patients who display high levels of circulating kynurenine in the face of defective overall IDO1 functioning[13].

Substantial differences might occur between human and experimental CGD, and $p47^{phox}$-deficient mice with infection-related acute inflammatory lung injury may well be an extreme condition. Such as they are, those mice provide a sound proof-of-principle in their being a prototypic model of a specific condition, exemplifying how in experimental CGD the IDO1 mechanism of disease tolerance[13] is severely compromised at sites where it would mostly be beneficial to control local inflammatory reactions. The very nature of granulation formation could be but one of the multiple phenotypic manifestations of defective IDO1 functioning in infection-related pathology[14]. This Reply has been written on behalf of the entire original author list[1], most of the original authors are no longer working with us or on the specific subject.

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doi:10.1038/nature13845