European Journal of Immunology Cellular immune response

NADPH oxidase of human dendritic cells: Role in *Candida albicans* killing and regulation by interferons, dectin-1 and CD206

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Human monocyte-derived DC express the enzyme NADPH oxidase, responsible for ROS production. We show that *Candida albicans* did not activate NADPH oxidase in DC, and was poorly killed by these cells. However, *Candida*-killing activity increased upon DC stimulation with the NADPH oxidase activator PMA and was further enhanced by DC treatment with IFN-α or IFN-γ. This fungicidal activity took place at high DC-to-*Candida* ratio, but decreased at low DC-to-yeast ratio, when *Candida* inhibited the NADPH oxidase by contrasting the assembly of the enzyme on DC plasma membrane. The NADPH oxidase inhibitor diphenyliodonium chloride abrogated the PMA-dependent DC candidacidal capacity. Engagement of β-glucan receptor dectin-1 induced NADPH oxidase activation in DC that was depressed by mannose-binding receptor CD206 co-stimulation. *Candida* was internalized by DC through mannose-binding receptors, but not through dectin-1, thus explaining why *Candida* did not elicit NADPH oxidase activity. Our results indicate that NADPH oxidase is involved in DC *Candida*-killing activity, which is increased by IFN. However, *Candida* escapes the oxidative damage by inhibiting NADPH oxidase and by entering DC through receptors not involved in NADPH oxidase activation.

Received 26/7/06
Revised 5/12/06
Accepted 16/2/07

[DOI 10.1002/eji.200636532]

Key words: *Candida albicans* · Dectin-1 · Dendritic cells · Interferons · NADPH oxidase

Introduction

Dendritic cells (DC) play an important role in the initiation of immune responses [1–4]. In peripheral tissues, immature DC capture antigens by specialized receptors, undergo maturation and migrate to lymphoid organs where they present antigens to naive T cells [1–4]. Moreover, DC produce cytotoxic molecules limiting pathogen replication [5–8].

Recently, we reported that human monocyte-derived DC express NADPH oxidase [9], the enzyme of leukocytes responsible for ROS production, whose activation requires the association between cytosolic (p47phox, p67phox, p40phox, p21rac) and membrane (gp91phox, p22phox) components [10, 11]. ROS produced by NADPH oxidase of leukocytes are involved in pathogen killing, as demonstrated by the recurrent infections affecting individuals with chronic granulomatous disease, an inherited disorder in which the enzyme is not functional [10, 11], but are also recognized as signaling molecules [12]. We previously showed that NADPH oxidase is not involved in DC differentiation, LPS-induced maturation, cytokine production and induction of T cell proliferation, but is required for DC killing of intracellular bacteria [9].

The present study was undertaken to elucidate in more detail the regulation of DC NADPH oxidase activity and the role of this enzyme in pathogen-killing ability of

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Abbreviations: **DC-SIGN:** DC-specific intercellular adhesion molecule-grabbing nonintegrin · **DPI:** diphenyliodonium chloride

DC. For this purpose, we investigated the effect of cytokines able to modulate DC functions such as IFN-α [13–15] and IFN-γ [16, 17] on NADPH oxidase activity and ability of DC to kill *Candida albicans*. Moreover, we analyzed the DC receptors involved in *Candida* uptake and their role in NADPH oxidase activation.

Results

Regulation of NADPH oxidase activity by IFN-α and IFN-γ

We previously reported that PMA-stimulated human monocyte-derived DC release superoxide anion, which was nearly completely produced *via* NADPH oxidase activation [9]. Here we show that immature DC treatment with IFN-α or IFN-γ enhanced the PMA-induced NADPH oxidase activity (Fig. 1A, B). A surface phenotype analysis by flow cytometry demonstrated that this effect was not consequent to changes of DC maturation stage, as IFN-γ and IFN-α did not induce the expression of the maturation markers CD83, CD86 and CD80 (data not shown), as previously reported [14, 16, 18].

It is known that in leukocytes, changes of expression of NADPH oxidase components are involved in regulation of ROS production [19–21]. To investigate the molecular mechanisms of NADPH oxidase modulation by IFN, we analyzed gp91phox, p67phox, p47phox and

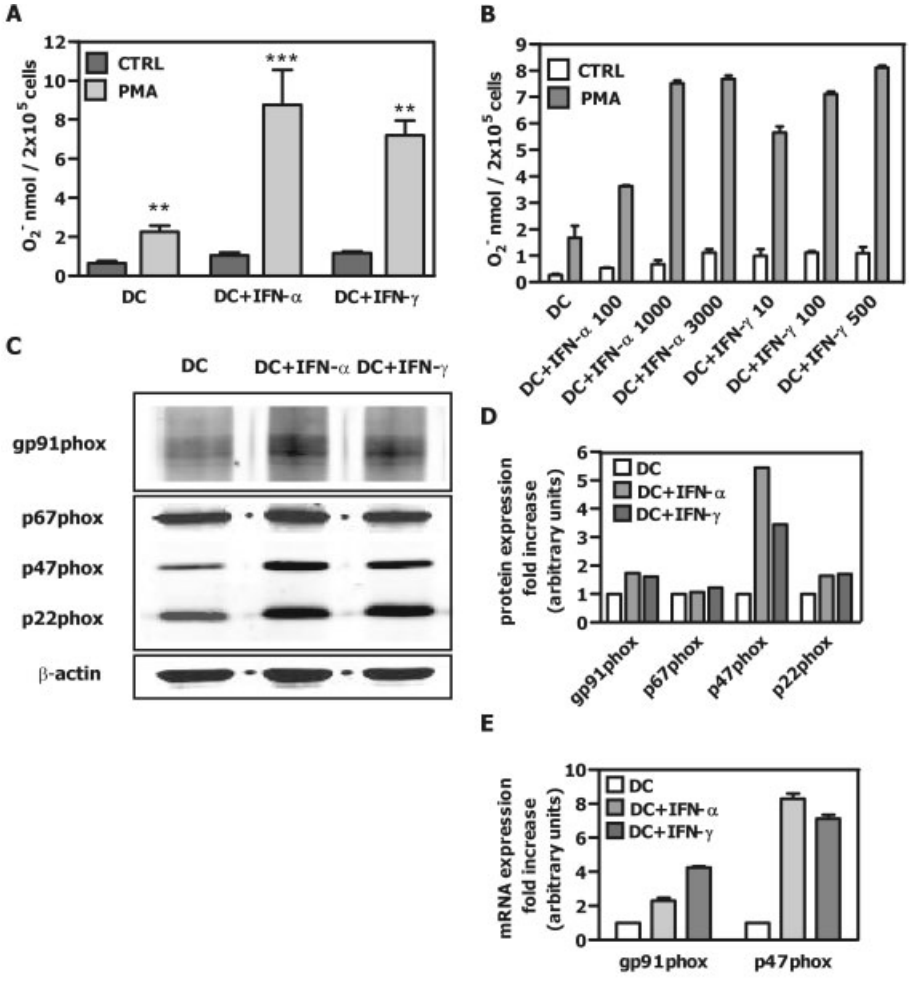

Figure 1. Effect of IFN-α and IFN-γ on NADPH oxidase activity and expression. (A) DC were cultured (24 h) in the absence or presence of 1000 IU/mL IFN-α, or 100 IU/mL IFN-γ. Superoxide anion production was measured after stimulation (3 h) with 20 ng/mL PMA; $^{**}p<0.01$, $^{***}p<0.001$, PMA-stimulated DC compared to untreated cells (CTRL). (B) DC were incubated (24 h) with the indicated doses (IU/mL) of IFN and stimulated (3 h) with PMA to assess O_2^- production. Results shown in (A) and (B) are expressed as the mean + SD of five and three experiments, respectively. (C) DC were cultured as in (A), lysed and subjected to Western blot analysis of NADPH oxidase components. The gp91phox appears as a broad smear because it is highly glycosylated. β-Actin was used to normalize protein levels. One experiment representative of five ones is shown. (D) Quantification of the bands shown in (C) using the LI-COR Odyssey system. Protein expression changes are calculated by comparing the values of IFN-treated DC with the ones of untreated cells. (E) gp91phox and p47phox mRNA expression was evaluated by RT-PCR. Values have been normalized to GAPDH mRNA levels. Results are shown as mean values + SD of three experiments (IFN-treated compared to untreated DC).

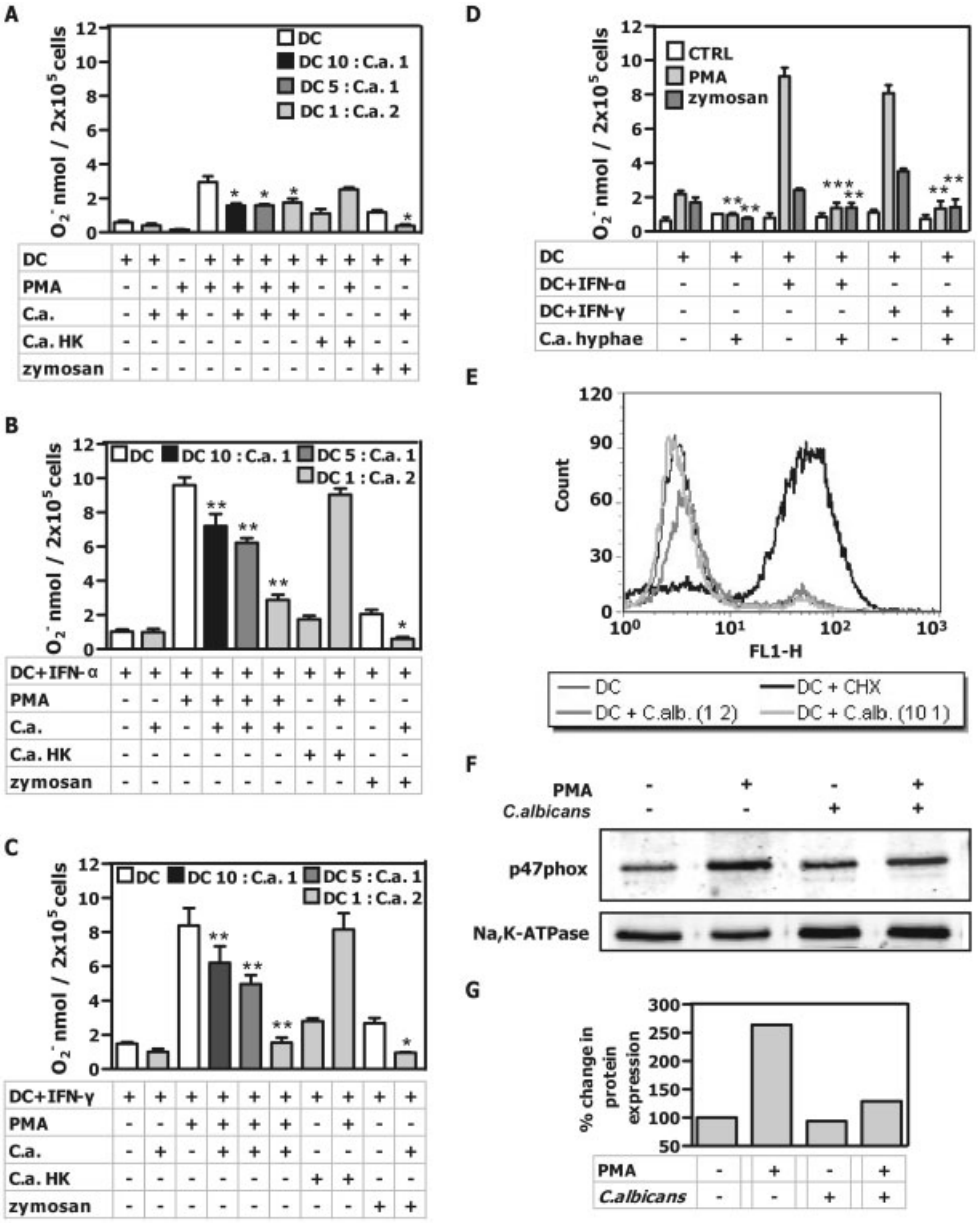

Figure 2. Effects of *C. albicans* on NADPH oxidase activity. O_2^- production was evaluated in DC cultured (24 h) in the absence (A) or presence of IFN-α (B) or IFN-γ (C) and incubated (3 h) with *C. albicans* yeasts (C.a.) or heat-killed *Candida* (C.a.HK) at the indicated DC-to-*Candida* ratios with or without PMA (20 ng/mL) or zymosan (200 μg/mL). Different amounts of *Candida* were added to a fixed DC number (2×10^5). *C. albicans* was stimulated with PMA in the absence of DC as a control (A). (D) DC were cultured (24 h) in absence or presence of IFN, incubated (3 h) with *Candida* hyphae (DC-to-*Candida* ratio 1:2) with or without PMA or zymosan to assess O_2^- production. Results shown in (A–D) are expressed as mean value + SD of five to six experiments; $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, stimulated DC with *Candida* compared to stimulated DC without *Candida*. (E) DC were cultured (3 h) with *Candida* at the indicated DC-to-yeast ratios and DC apoptosis has been evaluated by FACS analysis. DC treated (24 h) with cycloheximide (CHX) were used as positive control. One experiment representative of three is shown. (F) DC were cultured (3 h) with or without *C. albicans* (DC-to-*Candida* ratio 1:2) and/or PMA, lysed by sonication and loaded on a sucrose gradient. Membrane fractions were subjected to electrophoresis and immunoblotting. Proteins were visualized and processed with LI-COR Odyssey system. Plasma membrane marker Na,K-ATPase was used to normalize protein levels. One representative experiment out of four is shown. (G) Quantification of the bands shown in (F) using LI-COR Odyssey system. PMA- and/or *Candida*-treated DC were compared to untreated DC assumed as 100%.

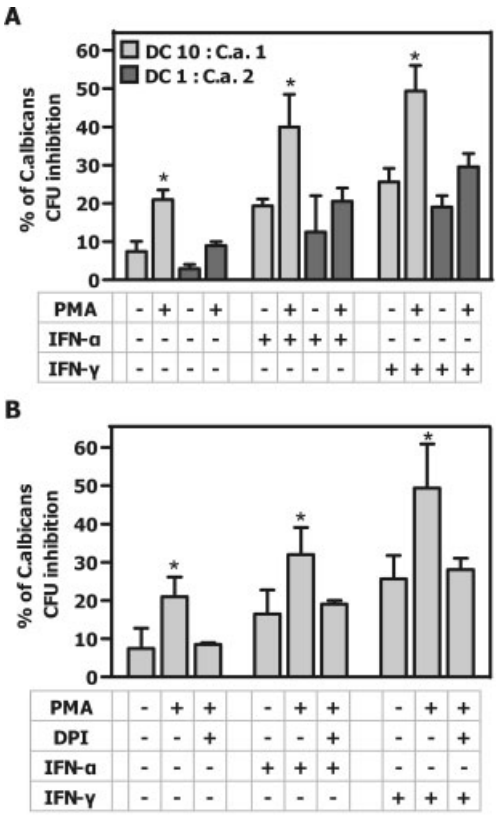

Figure 3. Role of NADPH oxidase on DC candidacidal capacity. (A) DC were cultured (24 h) with or without IFN and then incubated (3 h) with *C. albicans* yeasts (2×10^4) at the indicated DC-to-*Candida* ratios in absence or presence of 20 ng/mL PMA. DC were then lysed, and *Candida* CFU inhibition was determined. *$p<0.05$, PMA-stimulated DC *versus* unstimulated ones. (B) *Candida* yeasts and DC were cultured as in (A), but stimulation with PMA was performed in absence or presence of 10 μM DPI. *$p<0.05$, PMA-stimulated DC *versus* unstimulated ones. Results shown in (A, B) are expressed as the mean value + SD of three experiments.

p22phox expression by Western blot (Fig. 1C, D) and gp91phox and p47phox expression by quantitative PCR (Fig. 1E). We found that IFN-α or IFN-γ increased gp91phox, p47phox and p22phox, but not p67phox expression. Therefore, intracellular changes of some NADPH oxidase components could have a role in IFN-induced modulation of NADPH oxidase activity.

C. albicans inhibits NADPH oxidase activity

C. albicans influences some functions of human DC [22–26]. Therefore, we investigated whether *Candida* affects the NADPH oxidase activity of DC. For this purpose DC were cultured in the absence or presence of IFN-α or IFN-γ before incubation with unopsonized *Candida* cells, and O_2^- production was analyzed in absence or presence of PMA by cytochrome C reduction assay. We found that *C. albicans* alone was unable to reduce cytochrome C either in absence (data not shown) or presence of PMA (Fig. 2A). Moreover, addition of *Candida* to DC pretreated or not with IFN-α or IFN-γ did not lead to O_2^- production (Fig. 2A–C). However, *Candida* inhibited the PMA-induced NADPH oxidase activity in untreated (Fig. 2A), IFN-α- (Fig. 2B) or IFN-γ- (Fig. 2C) treated DC. In IFN-stimulated DC, the *Candida*-mediated inhibitory effect was slight at a DC-to-yeast ratio of 10:1, but raised by increasing the amount of *Candida* (Fig. 2B, C). At a DC-to-yeast ratio of 1:2, we found 41%, 71% and 82% inhibition of PMA-induced NADPH oxidase activity in untreated, IFN-α- and IFN-γ-treated DC, respectively (Fig. 2A–C). Interestingly, *Candida* inhibited the zymosan-induced NADPH oxidase activity (67%, 70% and 65% inhibition in untreated, IFN-α- and IFN-γ-treated DC, respectively; Fig. 2A–C), suggesting that the yeast affects a mechanism of NADPH oxidase activation shared by PMA and zymosan.

Similarly to yeasts, *Candida* hyphae did not activate the NADPH oxidase, but inhibited the PMA- and zymosan-induced activity of the enzyme both in untreated and IFN-treated DC (Fig. 2D). Heat-killed *Candida* used as a control did not affect PMA-dependent NADPH oxidase activity, but induced a slight but significant ($p<0.05$) O_2^- production in unstimulated DC (Fig. 2A–C). This effect could be due to heat-induced changes of yeast cell wall [27]. We also investigated the effect of yeast germination on NADPH oxidase activity. We found that *Candida* phagocytosis by DC followed by intracellular yeast germination did not lead to NADPH oxidase activation. Moreover, intracellular *Candida* germination induced before DC stimulation inhibited both PMA- and zymosan-dependent NADPH oxidase activity (data not shown).

The NADPH oxidase inhibition by *Candida* cannot be ascribed to yeast-induced DC apoptosis, as annexin-5-FLUOS staining assay showed that cell viability at high DC-to-yeast ratio was equivalent to that observed at low DC-to-*Candida* ratio (Fig. 2E). Moreover, this inhibition was not due to oxidant-scavenging molecules released by *Candida* [28], as conditioned medium obtained from DC/*Candida* co-cultures (ratio 1:2) did not affect ROS production by PMA-stimulated DC (results not shown). Fig. 2F, G shows that *Candida* affects the PMA-induced p47phox association with DC plasma membrane, thus hampering the assembly of NADPH oxidase components leading to O_2^- production [10, 11].

NADPH oxidase is involved in *Candida*-killing activity of DC

Leukocytes kill *Candida* essentially via oxygen-dependent mechanisms [29, 30]. We then investigated whether NADPH oxidase is involved in *Candida*-killing activity of DC. For this purpose, DC were cultured with

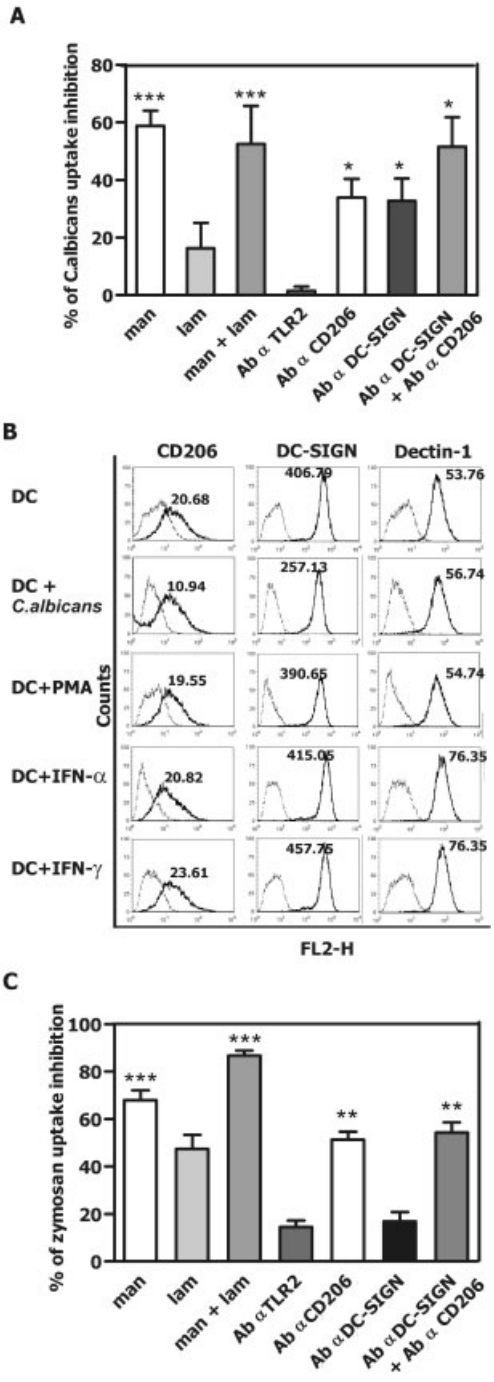

Figure 4. Receptors involved in *C. albicans* phagocytosis. (A) DC were cultured (20 min) in the presence of 3 mg/mL mannan (man), 1 mg/mL laminarin (lam) or 10 μg/mL TLR2-, CD206-, DC-SIGN-blocking antibodies. Phagocytosis was assayed after 40-min DC incubation with *Candida* yeasts. The percentage of uptake inhibition was calculated by comparing phagocytosis of inhibitor-treated DC with the one of untreated cells, assumed as 100%. Results are expressed as the mean value + SD of five experiments; $^{***}p<0.001$, compared to untreated control; $^{*}p<0.05$, $^{**}p<0.01$, blocking antibodies *versus* IgG control antibody. (B) DC were incubated with or without *C. albicans* (3 h, DC-to-yeast ratio 1:2), PMA (3 h) or IFN (24 h). CD206, DC-SIGN and dectin-1 surface expression was analyzed by flow cytometry. The value indicated on the histogram is the MFI of the cells stained with the specific antibody. Isotype-matched antibodies were used as controls (thin-line histograms). The data shown are representative of four experiments. (C) Zymosan phagocytosis by DC was evaluated as in (A). Results are expressed as the mean value + SD of three experiments.

or without IFN before incubation with unopsonized *Candida* cells in the presence or absence of PMA. *Candida* killing was analyzed by evaluation of CFU number. We found that the fungicidal activity of unstimulated DC was very low; however, DC treatment with PMA increased *Candida* killing (Fig. 3A). In the absence of DC, PMA was unable to kill *Candida* (results not shown). Of note, DC pretreatment with IFN-α or IFN-γ enhanced both the PMA-independent and the PMA-dependent killing of *Candida*. The effect of PMA was evident at a DC-to-yeast ratio of 10:1, but became irrelevant when an excess (ratio 1:2) of *Candida* was added to the culture (Fig. 3A).

The lack of a PMA-dependent enhancement of *Candida* killing at a DC-to-yeast ratio of 1:2 could be due to the above-reported NADPH oxidase inhibition by high *Candida* amounts (Fig. 2). Therefore, we performed killing experiments in the presence of diphenyliodonium chloride (DPI), an inhibitor of flavoproteins including the NADPH oxidase. We previously demonstrated that NADPH oxidase is the main source of O_2^- in DC, because DC from chronic granulomatous disease patients bearing a defective enzyme did not produce superoxide [9]. Moreover, DPI-treated DC lack O_2^- production in response to PMA [9]. Therefore, although it is not a specific inhibitor, DPI is a useful reagent to investigate NADPH oxidase activity in DC. Fig. 3B shows that DPI abrogated the PMA-dependent *Candida*-killing ability of DC observed at a DC-to-yeast ratio of 10:1, either in absence or in presence of IFN, thus reproducing the effect of high *Candida* amounts. Similar results have been obtained by treating the DC with the ROS scavenger superoxide dismutase (data not shown). These findings indicate that ROS produced by NADPH oxidase play an important role in DC fungicidal activity, and that *Candida* inhibits NADPH oxidase to escape oxidative damage.

DC receptors involved in *C. albicans* uptake

The reason why DC challenge with *C. albicans* did not lead to NADPH oxidase activation could rely on receptors involved in *Candida* recognition by DC. It is known that DC interact with yeasts essentially by the mannose-binding receptors CD206 [22, 31] and DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) [32, 33], the β-glucan receptor

dectin-1 [27, 32, 34–37], and TLR2 [32, 38, 39]. Therefore, we analyzed *Candida* uptake in the presence of mannan, which binds both CD206 and DC-SIGN [40, 41] and interferes with particle internalization by these receptors [42], or laminarin, a specific dectin-1 inhibitor [27, 35, 40, 41, 43]. Fig. 4A shows that *Candida* uptake was depressed by mannan (58.7% inhibition) but not by laminarin (16.2% inhibition). The simultaneous addition of mannan and laminarin caused a decrease of *Candida* phagocytosis comparable to that obtained with mannan alone (52.4%) (Fig. 4A). Anti-DC-SIGN or anti-CD206 antibodies depressed *Candida* uptake (32.7% and 33.8%, respectively) and their simultaneous addition caused a decrease of *Candida* uptake similar to that observed with mannan, suggesting that DC-SIGN and CD206 cooperate to *Candida* internalization by DC (Fig. 4A). Anti-TLR2 antibodies did not affect *Candida* uptake by DC (Fig. 4A).

These findings are reinforced by a FACS analysis performed with specific anti-CD206, anti-DC-SIGN and

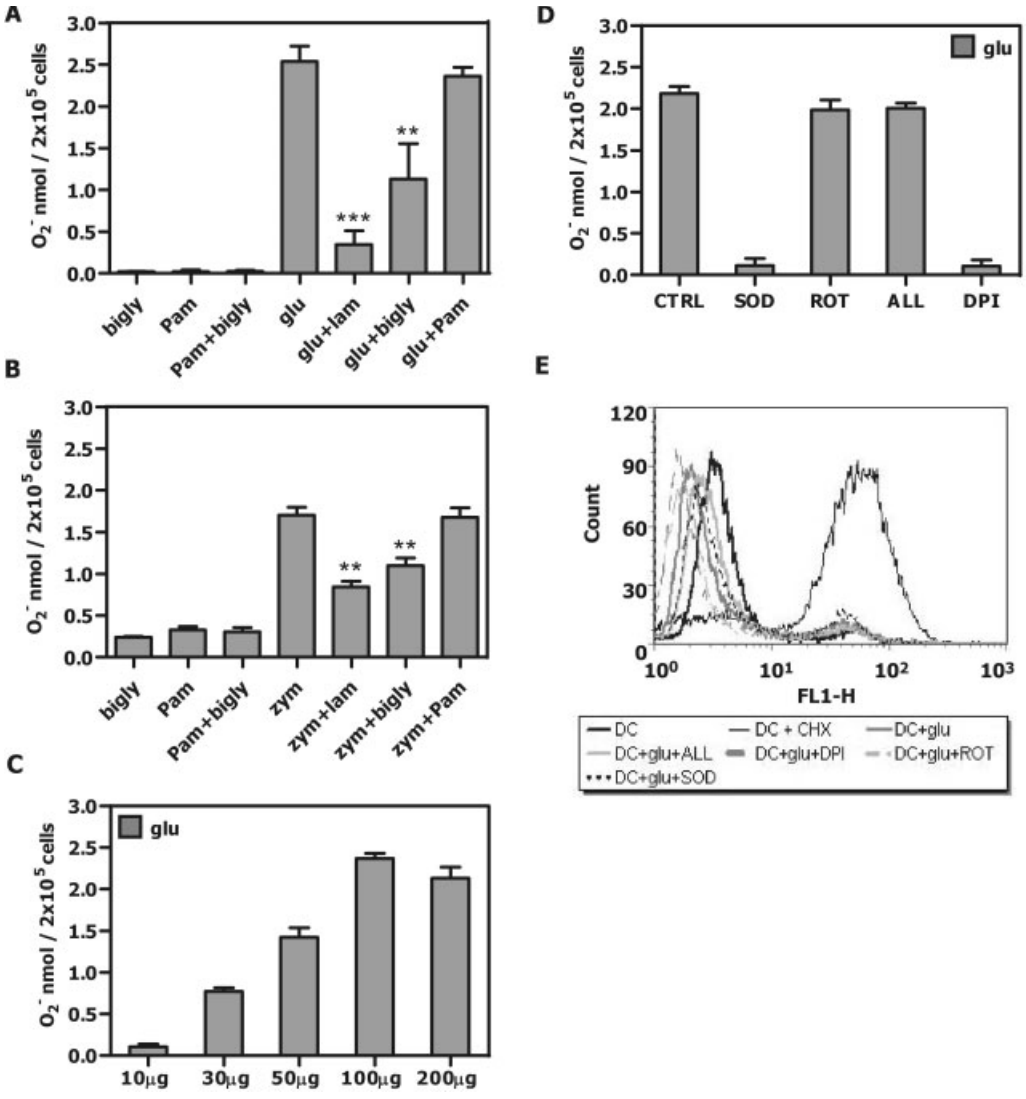

Figure 5. Effect of soluble receptor agonists on NADPH oxidase activity. (A) O_2^- production was measured in DC stimulated (1 h) with 100 μg/mL biglycan (bigly), 200 ng/mL Pam_3CSK4 (Pam), 100 μg/mL glucan (glu) alone or in combination. When required, DC were treated (20 min) with 1 mg/mL laminarin (lam) before stimulation with glucan. (B) O_2^- production was measured in IFN-α-treated DC stimulated (3 h) with 200 μg/mL zymosan (zym), biglycan (bigly) or Pam_3CSK4 (Pam) alone or in combination. When required, DC were treated (20 min) with laminarin (lam) before stimulation with zymosan. Results are shown as the mean value + SD of five (A) and six (B) experiments; $^{***}p<0.001$, $^{**}p<0.01$, simultaneous addition of glucan (A) or zymosan (B) and other agonists compared to glucan or zymosan alone. (C) O_2^- release was assayed after 1-h DC stimulation with the indicated doses of glucan. (D) DC were incubated (15 min) in the absence (CTRL) or presence of 50 μg/mL superoxide dismutase (SOD), 10 μM rotenone (ROT), 200 μM allopurinol (ALL), and 10 μM DPI. O_2^- generation was measured after 1-h treatment with glucan. Results shown in (C) and (D) are the mean values + SD of three experiments. (E) DC were treated as in (D) and apoptosis was evaluated by FACS analysis. DC treated (24 h) with cycloheximide (CHX) were used as positive control. The data shown are representative of three experiments.

anti-dectin-1 antibodies showing that upon *Candida* phagocytosis, dectin-1 receptors remained expressed on DC surface, whereas CD206 and DC-SIGN decreased (Fig. 4B), indicating that these mannose-binding receptors, but not dectin-1, are internalized during *Candida* uptake. Figure 4B also shows that PMA and IFN did not affect CD206, DC-SIGN and dectin-1 expression. Accordingly, we found that *Candida* phagocytosis was not influenced by DC treatment with IFN (results not shown).

Taken together, our findings indicate that *Candida* enters DC mainly *via* CD206 and DC-SIGN. As *Candida* is unable to activate NADPH oxidase of DC, it is conceivable that these receptors are not involved in the activation of this enzyme. In contrast to *Candida*, the NADPH oxidase-activating zymosan particles are internalized by both dectin-1 and mannose-binding receptors (Fig. 4C), confirming early studies [39, 42]. Consistently with reports showing that dectin-1 induces ROS production in murine macrophages [27, 39], this finding suggests that dectin-1 could mediate NADPH oxidase activation in human DC.

Dectin-1 mediates NADPH oxidase activation

To investigate the receptors involved in NADPH oxidase activation, DC were stimulated with purified biglycan, glucan and Pam_3CSK4, specific agonists of CD206, dectin-1 and TLR2, respectively [44–46]. We found that biglycan and Pam_3CSK4, alone or in combination, did not activate ROS production (Fig. 5A). In contrast, DC treatment with glucan induced an high O_2^- production that was abrogated (91% inhibition) by laminarin, demonstrating that it is specifically due to dectin-1 receptor activation (Fig. 5A, C). Simultaneous addition of biglycan and glucan decreased O_2^- production (53.2% inhibition), whereas contemporaneous DC stimulation with glucan plus Pam_3CSK4 induced an O_2^- production comparable to that observed with glucan alone (Fig. 5A). Of note, ROS release elicited by zymosan was also inhibited by laminarin and biglycan, but not by Pam_3CSK4 (Fig. 5B). Since zymosan alone induces a scarce ROS production, we performed the experiments shown in Fig. 5B with interferon-treated DC to enhance their responsiveness to zymosan.

Taken together, these findings suggest that dectin-1, but not TLR2, is involved in NADPH oxidase activation, whereas CD206 stimulation activates anti-inflammatory signaling pathways inhibiting dectin-1-dependent ROS production. This result is in agreement with previous investigations showing that CD206 generates inhibitory signals on the release of pro-inflammatory cytokines [40, 44, 47] and can explain why *Candida*, which enters DC via mannose-binding receptors and avoids dectin-1, did not activate the NADPH oxidase. A possible role of DC-SIGN on NADPH oxidase activity remains to be elucidated. Ostensibly, no specific DC-SIGN agonists are commercially available. Fig. 5D shows that superoxide dismutase and DPI abrogated glucan-induced ROS release, whereas neither rotenone (an inhibitor of mitochondria respiratory chain) nor allopurinol (a xanthine oxidase inhibitor) affected glucan-elicited O_2^- production, indicating that NADPH oxidase is the source of oxidants produced upon DC stimulation with glucan. Fig. 5E shows that, at the doses used in our experiments, none of these inhibitors induced DC apoptosis.

Discussion

Here we show that IFN-α and IFN-γ enhance NADPH oxidase activity in DC, and this effect could be dependent on increased gp91phox, p22phox and p47phox expression. In fact, in spite of the absence of an absolute relationship between the NADPH oxidase component protein levels and ROS production, p47phox, gp91phox and p22phox expression and NADPH oxidase activity are regulated in a coordinated manner [19–21, 48].

To understand the biological relevance of IFN-induced enhancement of NADPH oxidase activity, we investigated the oxidative response and the fungicidal capacity of DC challenged with *C. albicans* in the absence or presence of IFN-α and IFN-γ. We found that *Candida* interacts with DC without activating the NADPH oxidase and inducing a poor fungicidal activity in these cells. These results are consistent with reports showing that *Candida* is poorly killed by DC [25], but not with other studies indicating that this yeast is efficiently killed by DC [49]. These discrepancies could be due to differences in the methodology as discussed by Netea *et al.* [25].

We also found that stimulation with PMA enhances DC fungicidal activity by inducing ROS production. It is worth to point out that, although it is not a physiological agonist, PMA directly activates the protein kinase C-dependent phosphorylation of NADPH oxidase components, thus reproducing the final step of NADPH oxidase activation by a large number of physiological stimulators of this enzyme [11]. DC pretreatment with IFN-α or IFN-γ increases both the PMA-dependent and the poor PMA-independent fungicidal activity.

Interestingly, at low DC-to-yeast ratio, *C. albicans* strongly inhibits only the predominant PMA-dependent DC candidacidal capacity by contrasting the assembly of NADPH oxidase on DC plasma membranes, but is unable to decrease the oxygen-independent fungicidal activity of DC induced by IFN. Although *in vitro* studies may not reflect the *in vivo* DC reactivity, these findings suggest that in some circumstances *Candida* could escape the oxidative damage by DC in spite of the IFN-dependent

enhancement of NADPH oxidase activity, but IFN-induced oxygen-independent mechanisms could still affect this dangerous yeast. Newman and Holly [49] report that human DC kill *Candida* by oxygen-independent mechanisms, probably *via* lysosomal hydrolases activation. These mechanisms could correspond to those observed by us in the absence of DC stimulation with PMA. Savina *et al.* [50] suggest that in murine DC, NADPH oxidase is mainly involved in antigen processing. Our results indicate that NADPH oxidase also plays a role in *Candida*-killing activity of DC, in particular when ROS production is enhanced by IFN.

Here we also show that dectin-1 mediates the activation of DC NADPH oxidase, whereas CD206 stimulation inhibits the dectin-1-dependent NADPH oxidase activity. These results agree with the reports that in murine macrophages dectin-1 plays a role in ROS production [27, 39], and with previous findings demonstrating an inhibitory effect of CD206 on pro-inflammatory cytokine release [40, 44, 47]. Moreover, these results may explain why *Candida* does not elicit the NADPH oxidase activity in DC. In fact, here we confirm the previous reports [22, 31], indicating that *Candida* is phagocytosed by DC *via* mannose-binding receptors rather than by dectin-1. Obviously, we cannot exclude that receptors other than those investigated by us could play a role in *Candida* uptake. Of note, *Candida* hyphae, expressing little amounts of β-glucan on their surface [27], failed to activate NADPH oxidase, whereas heat-killed *Candida*, showing an increased exposure of β-glucan on the surface [27], elicited a slight NADPH oxidase activity in DC.

Materials and methods

Reagents and antibodies

The following reagents were used: RPMI 1640 and low-endotoxin FCS (BioWhittaker, Verviers, Belgium); recombinant human GM-CSF and IL-4 (PeproTech, Rocky Hill, NJ); IFN-γ (R&D Systems, Minneapolis, MN); IFN-α (Roferon; Roche Laboratories, Nutley, NJ); Pam_3CSK4 (Invivogen, San Diego, CA); biglycan from bovine cartilage, glucan and mannan from baker's yeast (*Saccharomyces cerevisiae*), zymosan from *S. cerevisiae*, laminarin from *Laminaria digitata*, PMA, superoxide dismutase, allopurinol, rotenone, DPI and diisopropyl fluorophosphate (Sigma, St. Louis, MO). All the reagents contained less than 0.125 endotoxin units/mL, as checked by the Limulus Amebocyte assay (Microbiological Associates, Walkersville, MD).

Rabbit antibodies were anti-gp91phox, anti-p67phox, anti-p47phox and anti-p22phox (kindly provided by Dr. F. B. Wientjes, Department of Medicine, University College, London, UK); anti-β-actin (Sigma). Mouse anti-human antibodies were anti-CD206 (human mannose receptor, IgG1, HM2056; Hycult Biotechnology, Uden, The Netherlands); anti-dectin-1 (IgG2, MAB1859; R&D Systems); anti-TLR2 (mAb 2392; Genentech, San Francisco, CA); anti-DC-SIGN (IgG2b, ab13487; Abcam, Cambridge, UK), anti-Na,K-ATPase (IgG2a, 464.8; Abcam).

C. albicans strains and culture condition

C. albicans strain ATCC24433 was routinely maintained on Sabouraud agar. For experimental purposes, *Candida* was cultured as yeast in liquid Sabouraud medium at 30°C, washed twice in ice-cold HBSS, counted in a hemocytometer, resuspended in HBSS and kept on ice. Heat-killed cells were prepared by boiling for 30 min *C. albicans* resuspended in water [51]. *Candida* was grown in the hyphal form in HBSS, 0.5 mM $CaCl_2$, 1 mg/mL glucose, 10% FBS, pH 7.4 at 37°C, 5% CO_2 for 3 h (short hyphal filaments emerging from yeast form and three to five times as long as the yeast of origin) [52]. These culture conditions were also used to obtain yeast germination after *Candida* phagocytosis by DC.

DC preparation

DC were generated from monocytes isolated from the blood of healthy donors as previously described [53] and incubated at 37°C, 5% CO_2 for 5–6 days at 1×10^6/mL in RPMI 1640 supplemented with heat-inactivated 10% FCS, 2 mM L-glutamine, 50 ng/mL GM-CSF and 20 ng/mL IL-4.

Superoxide anion production

O_2^- release was estimated by cytochrome C reduction as previously described [9]. Briefly, after cell culture the medium was replaced with HBSS pH 7.4 containing 80 μM ferricytochrome C (Sigma) and the required stimulus. Cytochrome C reduction was evaluated at 550 nm.

Electrophoresis and immunoblotting

Cells were lysed and subjected to electrophoresis and immunoblotting as previously described [9]. Membrane preparation for translocation experiments was done as in [54]. The blots were incubated with anti-gp91phox, anti-p67phox, anti-p47phox, anti-p22phox, anti-actin and anti-Na,K-ATPase antibodies and then probed with goat anti-mouse or anti-rabbit antibodies conjugated to Alexa Fluor 680 (Molecular Probes, Leiden, The Netherlands) or IRdye 800 (Rockland Immunochemicals). Blotted proteins were detected and quantified using the Odyssey infrared imaging system LI-COR.

Quantitative real-time PCR

Total RNA was extracted from 10^6 DC using the RNeasy mini kit (Qiagen, Crawley, UK). All samples were reverse-transcribed under the same conditions to minimize differences in RT efficiency. Oligonucleotide primers (purchased from Invitrogen) were: p47phox forward: TTGAGAAGCGCTTCGTACCC, p47phox reverse: CGTCAAACCACTTGGGAGCT; gp91phox forward: GCCAGTGAAGATGTGTTCAGCT, gp91phox reverse: GCACAGCCAGTAGAAGTAGATCT. The

cDNA was amplified by using DNA Engine Opticon 2 System (MJ Research, Waltham, MA) in the presence of SYBR Green Ex Taq (Takara, Tokio, Japan). Amplification plots were analyzed using Opticon Monitor software Version 2.02 (MJ Research), and data were calculated with Q-Gene software (www.BioTechniques.com). mRNA expression levels are reported as the number of gene copies per copies of the control mRNA. GAPDH was selected as a normalizing gene according to its stable expression levels.

Flow cytometric analysis

Cells were washed twice with PBS and incubated (30 min) with 10% human serum to prevent non-specific binding. For surface receptors analysis, mouse anti-human CD206, dectin-1 and DC-SIGN were used. Bound antibodies were detected with goat anti-mouse IgG-RPE (1031-09; Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed with FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software. The Vybrant FAM Caspase-3 and -7 Assay Kit (V35118; Molecular Probes) was used to study apoptosis.

Phagocytosis assay

For zymosan phagocytosis, DC were incubated (40 min at 37°C) in 5% CO_2 with ten particles per cell in complete medium. For *C. albicans* phagocytosis, DC (5×10^5/300 μl) were incubated (37°C, 40 min) with $1.5X10^6$ yeasts in HBSS/0.5 mM $CaCl_2$ pH 7.4, washed twice with PBS, transferred onto glass slides and stained with May–Grünwald Giemsa before examination under light microscopy.

Candidacidal assay

C. albicans yeasts (2×10^4) were plated in 96-well plates and incubated (3 h, 37°C) with different amounts of DC pretreated or not with IFN-α or IFN-γ in 200 μL HBSS/0.5 mM $CaCl_2$ pH 7.4, in the presence or absence of PMA. DC-free yeast incubations were included as a control for *Candida* viability. Cell lysis was obtained by adding 20 μL Triton X-100 to the wells. Microscopic examination of culture plates showed a complete removal of DC. Serial tenfold dilutions in distilled water were prepared, and aliquots (100 μL) of three replicate samples were spread on Sabouraud dextrose agar plates. *C. albicans* CFU were counted after 24 h incubation at 37°C. The percentage of yeasts killed by DC was determined as follows: (1 – (CFU after incubation with DC/CFU control culture)) × 100.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). Student's *t*-test was used to analyze the data for statistical significance, and results were considered significant at a p level of <0.05.

Acknowledgements: This work was supported by Ministero dell'Istruzione, Università e Ricerca, and Fondazione Cassa di Risparmio di Verona (Bando 2003 e Bando 2004 – Integrazione tra tecnologia e sviluppo di settore).

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