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Toll Receptor-Mediated Regulation of NADPH Oxidase in Human Dendritic Cells[1]

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Activation of NADPH oxidase represents an essential mechanism of defense against pathogens. Dendritic cells (DC) are phagocytic cells specialized in Ag presentation rather than in bacteria killing. Human monocyte-derived DC were found to express the NADPH oxidase components and to release superoxide anions in response to phorbol esters and phagocytic agonists. The NADPH oxidase components p47^{phox} and gp91^{phox} were down-regulated during monocyte differentiation to DC, and maturation of DC with pathogen-derived molecules, known to activate TLRs, increased p47^{phox} and gp91^{phox} expression and enhanced superoxide anions release. Similar results were obtained with plasmacytoid DC following maturation with influenza virus. In contrast, activation of DC by immune stimuli (CD40 ligand) did not regulate NADPH oxidase components or respiratory burst. NADPH oxidase-derived oxygen radicals did not play any role in DC differentiation, maturation, cytokine production, and induction of T cell proliferation, as based on the normal function of DC generated from chronic granulomatous disease patients and the use of an oxygen radical scavenger. However, NADPH oxidase activation was required for DC killing of intracellular *Escherichia coli*. It is likely that the selective regulation of oxygen radicals production by pathogen-activated DC may function to limit pathogen dissemination during DC trafficking to secondary lymphoid tissues. *The Journal of Immunology,* **2004, 173: 5749–5756.**

Dendritic cells (DC)[4] are bone marrow-derived APCs that play a unique role in the initiation of primary specific immune responses (1, 2). DC are located at the interface between innate and adaptive immunity (3–5). At the immature stage, DC reside in peripheral tissues to detect and capture Ags by specialized endocytic and pattern recognition receptors (1, 2). Following activation, DC leave peripheral tissues and migrate to regional lymph nodes to present the processed Ags to naive T cells (6, 7). In addition to myeloid DC, blood DC comprise also plasmacytoid DC (P-DC). P-DC have a morphology resembling plasma cells, are devoid of myeloid markers, and can be distinguished from myeloid DC on the basis of membrane markers like CD123, BDCA-2, and BDCA-4. Following virus infection, P-DC migrate from the blood to lymph nodes where they produce high concentrations of type I IFN (8). Although the primary biological function of DC is the initiation of specific immune responses, DC, similarly to other phagocytic cells, have the potential to secrete cytotoxic molecules that may function to limit pathogen replication (9–11).

Phagocytic cells (e.g., neutrophils and macrophages) possess the NADPH oxidase, a specialized multicomponent enzyme system responsible for the release of reactive oxygen radicals in a process known as respiratory burst (12, 13). The functional significance of this process is extensively demonstrated by the recurrent infections observed in individuals with chronic granulomatous disease (CGD), an inherited disorder in which the oxidase is nonfunctional due to a deficiency in one or more of its components (14, 15). In resting cells, the NADPH oxidase is disassembled and inactive. Upon stimulation, a protein complex consisting of both cytosolic components (p47^{phox}, p67^{phox}, p40^{phox}, and the small GTP-binding protein p21^{rac}) and membrane proteins (gp91^{phox}, gp22^{phox}) is assembled and becomes functional (12, 16, 17).

In addition to the cytotoxic potential, oxygen radicals are recognized as signaling molecules for cells that localize at the site of inflammation (18, 19). In this respect, oxygen radicals act as mediators of cell apoptosis (20, 21) and as regulators of gene expression by their action on redox-regulated transcription factors, like NF-κB and AP-1 (22, 23). It was reported that oxidative stress induced by the in vitro cell exposure to H_2O_2 could influence DC maturation and function through the up-regulation of MHC class II molecule expression and the induction of CXCL8 and TNF production by immature DC (24–27).

This study was undertaken with the aim to elucidate whether human DC express the NADPH oxidase components and to investigate the expression and the activity of this enzyme complex during DC differentiation and maturation. The role of endogenous oxygen radicals on DC biology was investigated by the use of inhibitors and using DC obtained from CGD patients. The results reported here show that DC express the NADPH oxidase components and present a peculiar regulation of this enzyme during DC

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[4] Abbreviations used in this paper: DC. dendritic cell; P-DC, plasmacytoid DC; CGD, chronic granulomatous disease; PGN, peptidoglycan; SOD, superoxide dismutase; DPI, diphenyliodonium chloride; CD40L, CD40 ligand; mDC, mature DC, iDC, immature DC.

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differentiation. In addition, this study shows that Toll receptor-, but not CD40 ligand (CD40L)-, induced maturation increases the production of cytotoxic oxygen radicals by DC. It is tempting to speculate that this regulation may be important in controlling infection dissemination.

Materials and Methods

Cell culture media and reagents

The following reagents were used: pyrogen-free saline (S.A.L.F., Bergamo, Italy), RPMI 1640 (Biochrom, Berlin, Germany), and aseptically collected FCS (HyClone, Logan, UT). Peptidoglycan (PGN) from *Staphylococcus aureus* was purchased from Fluka (Buchs, Switzerland). Poly(I:C) was obtained from Amersham Biosciences (Amersham, U.K.). Flagellin, from *Salmonella typhimurium*, and trypsin-inactivated flagellin (flagellin-control) were a kind gift from Dr. J.-C. Sirard (Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland) (28). CpG oligodeoxynucleotide motif GTCGTT (2006) was provided by Invitrogen Life Technologies (Rockville, MD). LPS from *Escherichia coli* strain 055:B5 (LPS) was obtained from Difco (Detroit, MI). fMLP, dihydrocytochalasin B, PMA, C5a, superoxide dismutase (SOD), allopurinol, rotenone and diphenyleneiodonium chloride (DPI) were purchased from Sigma-Aldrich (St. Louis, MO). Human recombinant GM-CSF was a gift from Novartis (Milan, Italy). Human IL-13 was a gift from Dr. A. Minty (Sanofi Elf BioRecherches, Labège, France). CXCL8, CCL2, CCL3, CCL19, and CXCL12 were obtained from PeproTech (Rocky Hill, NJ). All reagents contained <0.125 endotoxin units/ml, as checked by the *Limulus* amebocyte assay (Microbiological Associates, Walkersville, MD). Cytokines and chemokines were detected by ELISA using specific Duo-Set kits (R&D Systems, Minneapolis, MN).

DC preparation

DC were generated in vitro as previously described (29). Highly enriched blood monocytes were obtained from buffy coats (through the courtesy of the Centro Trasfusionale, Ospedale Sacco, Milan, Italy) by Ficoll (Biochrom, Berlin, Germany) and Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Monocytes were cultured for 6 days at 1×10^6/ml in six-well tissue culture plates (Falcon; BD Biosciences, Lincoln Park, NJ) in RPMI 1640 complemented with 10% FCS, 50 ng/ml GM-CSF, and 20 ng/ml IL-13. Where indicated, DC were further cultured in the presence of 100 ng/ml LPS for 24 h, or as otherwise specified. CD40L-transfected J558L cells or mock-transfected control cells were cultured with DC at a 1:4 ratio. Incubation of DC with the J558L mock-transfected cells did not induce cell maturation or chemokine production (data not shown). Monocyte-derived macrophages were generated by incubating monocytes in Petriperm dishes in RPMI 1640 medium in the presence of autologous serum for 7 days (30). Heparinized blood from CGD patients (age range, 4–27 years) was derived from six patients with X-linked CGD ($X91^0$-CGD) and one female (CGD#2) with the autosomal recessive form (AR^0-CGD). Diagnosis was based on clinical history of recurrent infections and absence of granulocyte respiratory burst as measured by at least two concordant assays: nitroblue tetrazolium reduction test, dihydrorhodamine 123 flow cytometric assay, or cytochrome *c* reduction. CGD genotyped was defined on the basis of $gp91^{phox}$ molecular genetics, of the carrier state of female relatives of the patient, and/or immunoblotting for NADPH oxidase subunits (31). Approval for these studies was obtained from S. Civili of the Brescia Institutional Review Board. PBMC were obtained by Ficoll gradients. Adherent cells obtained after a 2-h incubation at 37°C were cultured in the presence of GM-CSF and IL-13 as detailed above. P-DC were magnetically sorted from Ficoll-purified mononuclear cells by the BDCA-4 cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) (32). P-DC were cultured in medium containing 20 ng/ml IL-3 (BD Pharmingen, San Diego, CA) and stimulated with 20 ng of hemagglutinin/ml inactivated influenza virus strain A/Moscow/10/99 (a kind gift from Dr. T. De Magistris, Istituto Superiore di Sanità, Rome, Italy) for 24 h (32).

Superoxide anion production

O^-_2 release was estimated by cytochrome *c* reduction as previously described (33). DC (2×10^6/ml) were resuspended in HBSS (pH 7.4) containing 80 μM ferricytochrome *c* type III (Sigma-Aldrich) and the stimulus indicated in each experiment. Cytochrome *c* reduction was evaluated at 550 nm at different time points.

Electrophoresis and immunoblotting

Cells were suspended in HBSS containing 1 mM diisopropyl fluorophosphate (Sigma-Aldrich). After 5 min, the cells were recovered, lysed with electrophoresis sample buffer (60 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, and 2% (v/v) 2-ME, pH 6.8), and boiled for 5 min. Cell lysates were subjected to SDS-PAGE on 12% gels and then transferred to nitrocellulose membranes (Amersham Biosciences). The blots were then rinsed in TBS-T (50 mM Tris, 170 mM NaCl, and 0.2% (v/v) Tween 20, pH 7.5) and incubated for 90 min in TBS-T containing 5% BSA (pH 7.5, blocking buffer) before incubation overnight (4°C) with rabbit anti-$gp91^{phox}$, anti-$p47^{phox}$, anti-$p67^{phox}$, or anti-$p40^{phox}$ Abs (kindly provided by Dr. F. B. Wientjes, Department of Medicine, University College, London, U.K.), diluted 1/500 in TBS-T containing 1 mg/ml BSA. The blots were incubated with HRP-conjugated anti-rabbit IgG (Amersham Biosciences). Bound Abs were detected by ECL Western blotting detection reagents (Amersham Biosciences) (34).

Mixed Leukocyte Reaction

Irradiated LPS-stimulated DC (LPS-mature DC (mDC)) were added in graded doses to 2×10^5 purified allogeneic T cells in 96-well round-bottom microtest plates. Each group was performed in triplicate. [^{3}H]Thymidine incorporation was measured on day 5 after a 16-h pulse (5 Ci/μm; Amersham Biosciences).

Flow cytometric analysis

Surface phenotype analysis was performed using the following Abs: anti-CD83 mAb (IgG1, HB/5e; BD Pharmingen), L243 (IgG2a, anti-MHC class II; American Type Culture Collection, Manassas, VA), NA1/34 (IgG2a, anti-CD1a; DakoCytomation, Glostrup, Denmark), and UCHM1 (IgG2a, anti-CD14; American Type Culture Collection). Apoptosis was detected by staining with Annexin V^{FITC} (BD Pharmingen) according to the manufacturer's protocol. Cells were analyzed with a FACScan flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software.

Bacteria killing assay

E. coli ATCC11129 were grown overnight on tryptic soy agar. Colonies from agar were grown in tryptic soy broth (Merck, Darmstadt, Germany) for 6 h at 37°C to 2×10^8/ml and washed twice in 0.9% saline. Mature DC were resuspended to 1×10^6/ml in medium without antibiotics, dispensed in triplicate (100 μl/well) in 96-well polypropylene plates, and incubated with *E. coli* at a ratio of 25:1 for 1 h at 37°C under shaking conditions. Extracellular bacteria were washed off and, where specified, cells were incubated in gentamicin containing medium to kill any remaining extracellular bacteria for an additional 2 h (11). Plates were washed twice with PBS to remove gentamicin and cells were lysed in 0.5% Nonidet P-40 for 10 min at room temperature. CFU were counted after serial dilutions of cellular lysates on Luria-Bertani agar to quantify the number of live intracellular bacteria. Where indicated the killing assay was performed in the presence of 100 ng/ml PMA or with the preincubation with 10 μM DPI (35, 36) for 10 min followed by cell stimulation.

Results

Activation of the oxidative burst in DC

In a first set of experiments, DC were tested for their ability to release O^-_2 in response to PMA, a nonreceptor activator of NADPH oxidase in phagocytic cells (13). As shown in Fig. 1*A*, immature DC (iDC) released low but detectable levels of O^-_2, starting 90 min after stimulation. mDC obtained by 24-h LPS (LPS-mDC) stimulation were stronger producers than iDC with O^-_2 levels already detectable after 60 min of stimulation. After 2 h of PMA stimulation, LPS-mDC released 8.2 ± 0.8 nmol $O^-_2/10^6$ cells, whereas iDC produced 1.6 ± 0.3 nmol $O^-_2/10^6$ cells ($n = 7$; $p < 0.01$ by Student's *t* test). On the contrary, DC maturation induced by CD40L (up to 48 h) resulted in a complete loss of the ability of DC to produce O^-_2 following PMA stimulation (Fig. 1*A*). Similar results were obtained using opsonized zymosan instead of PMA. In LPS-mDC, opsonized zymosan induced a concentration-dependent O^-_2 release that, at the concentration of 30 μg/ml, was 22 ± 5 nmol $O^-_2/10^6$ by LPS-mDC after 2 h of stimulation ($n = 4$). Of note, both LPS- and CD40L-mDC expressed a similar mature membrane phenotype, as evaluated by CD83 expression (>85% $CD83^+$; data not shown). None of the chemotactic agonists tested (i.e., C5a, fMLP, CCL2, CXCL8, CXCL12, CCL19) was able to induce oxygen radical production by iDC or

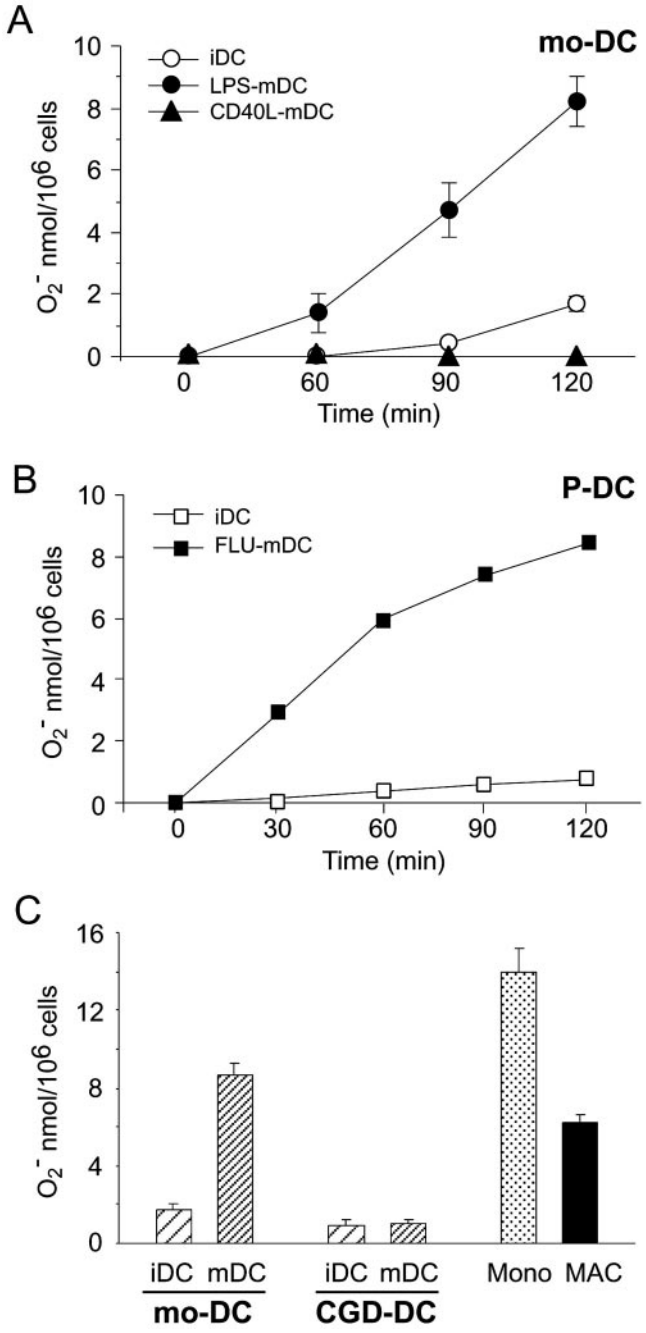

FIGURE 1. Activation of the respiratory burst in DC. *A*, DC were cultured in the absence or presence of LPS or CD40L for 24 h and then assayed for their ability to release O^-_2 after stimulation with PMA (10 ng/ml) at different time points. Results expressed as the mean ± SD of seven independent experiments are shown. *B*, Immature or influenza virus (FLU)-stimulated P-DC were tested for their ability to release O^-_2 after stimulation with PMA (10 ng/ml) at different time points. *C*, iDC and mDC (LPS-mDC) from healthy subjects (mo-DC) and CGD patients (CGD-DC), monocytes (Mono) and macrophages (MAC) were prepared as described in *Materials and Methods*, and the release of O^-_2 was measured after a 120-min stimulation with PMA (10 ng/ml). Results expressed as the mean ± SD of three to seven independent experiments.

mDC irrespective of the presence of dihydrocytochalasin B, a known coactivator of NADPH oxidase in phagocytic cells (data not shown). Immature P-DC were also poor producers of oxygen radicals. However, when these cells were induced to mature in the presence of influenza virus, they released O^-_2 levels that where comparable to those produced by myeloid DC (Fig. 1*B*).

Fig. 1*C* shows that the levels of O^-_2 produced by iDC were lower than those released by other mononuclear phagocytes, like monocytes and monocyte-derived macrophages (7.3- and 3.3-fold lower, respectively). However, the O^-_2 release by LPS-mDC was 1.4-fold higher than that of macrophages, but still 1.6-fold lower than that of monocytes.

Multiple enzymatic pathways may contribute to the release of oxygen radicals (19). Therefore, inhibitors of different enzymatic pathways were used to investigate the cellular source of O^-_2 production in LPS-mDC. Fig. 2*A* shows that SOD (an O^-_2 scavenger) and DPI (an inhibitor of flavoproteins, including the NADPH oxidase) completely suppressed oxygen radical release by mDC, whereas, neither rotenone (an inhibitor of mitochondria respiratory chain) nor allopurinol (a xanthine oxidase inhibitor) affected O^-_2 release. To prove in a more direct manner the role of NADPH oxidase in O^-_2 production by DC, experiments were performed using DC obtained from CGD patients. CGD is an inherited disease in which multiple genetic alterations at the level of NADPH oxidase components affect the enzyme activity (14). As shown in Fig. 1*C*, both immature and mature CGD DC did not produce oxygen radicals when challenged with phorbol esters. These results demonstrate that NADPH oxidase is the source of superoxide anions in LPS-mDC.

NADPH oxidase is a multicomponent enzyme that comprises $gp22^{phox}$, $gp91^{phox}$, $p40^{phox}$ $p47^{phox}$, $p67^{phox}$, and the small GTP-binding protein Rac (12, 16, 17). Fig. 2*B* shows that $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ proteins are expressed by iDC although at lower levels than those present in monocytes. DC maturation induced by LPS was associated with a dramatic increase in the expression of $p47^{phox}$ and $gp91^{phox}$ to levels comparable to those observed in freshly isolated monocytes, and no apparent change in $p67^{phox}$ and $p40^{phox}$ protein levels. On the contrary, maturation of DC by CD40L did not modify in a detectable manner the levels of any of the four proteins investigated. A quantitative analysis of $p47^{phox}$ protein expression shows a 3.4-fold increase of $p47^{phox}$ in LPS-mDC over control values (Fig. 2*C*). These results suggest that the NADPH oxidase components are down-regulated during the differentiation of monocytes to iDC and that the subsequent exposure of DC to LPS induces a selective up-regulation of $p47^{phox}$ and $gp91^{phox}$.

Up-regulation of DC respiratory burst by TLR ligands

LPS exerts its biological function through the interaction with TLR4 (37, 38). DC express a wide spectrum of TLRs (37, 38). TLRs activate similar signaling pathways (39, 40), but different members of the TLR family differ in their capacity to activate collateral signaling molecules (e.g., MyD88 adaptor-like/TIR domain-containing adaptor protein) and to induce production of downstream effectors (38–42). Therefore, it was important to assess whether different TLR agonists could up-regulate O_2^- production in DC. Fig. 3, *A* and B, shows that when DC were cultured in the presence of PGN (TLR2), poly(I:C) (TLR3), flagellin (TLR5), and CpG (TLR9) for 24 h, and subsequently challenged with an optimal concentration of PMA, they produced superoxide levels that were higher than those produced by iDC. No marked difference was detected in the levels of O^-_2 production in cells stimulated with TLR2, TLR3, TLR5, TLR9 vs TLR4 ligands. As expected on the basis of the previous results (Fig. 2*B*), all of these agonists also induced an increase in the expression of $p47^{phox}$ and $p91^{phox}$ as assessed by Western blot experiments (Fig. 3*C*).

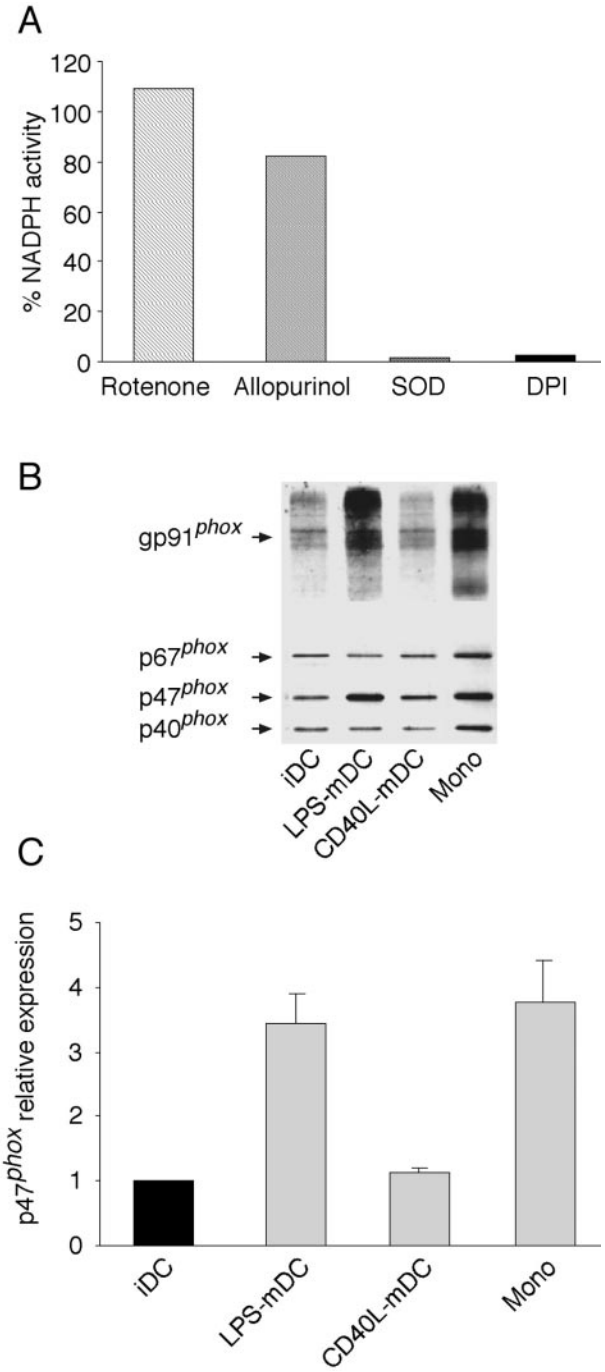

FIGURE 2. NADPH oxidase involvement in DC respiratory burst. *A,* DC were preincubated for 15 min with rotenone (10 μM), allopurinol (200 μM), SOD (50 μg/ml), and DPI (10 μM). Release of O^-_2 was measured after a 90-min stimulation with PMA (10 ng/ml). Results are the mean values of three independent experiments. Variability between the experiments was <15%. *B,* DC were cultured in the absence or presence of LPS or CD40L for 24 h, then lysed and immunoblotted with Abs raised against the indicated NADPH oxidase components. Lysates of freshly isolated human blood monocytes (Mono) were included for comparison. One blot representative of three independent experiments is shown. *C,* Densitometric analysis of relative $p47^{phox}$ expression. Results expressed as fold of increase of control values and are the mean ± SD of three independent experiments.

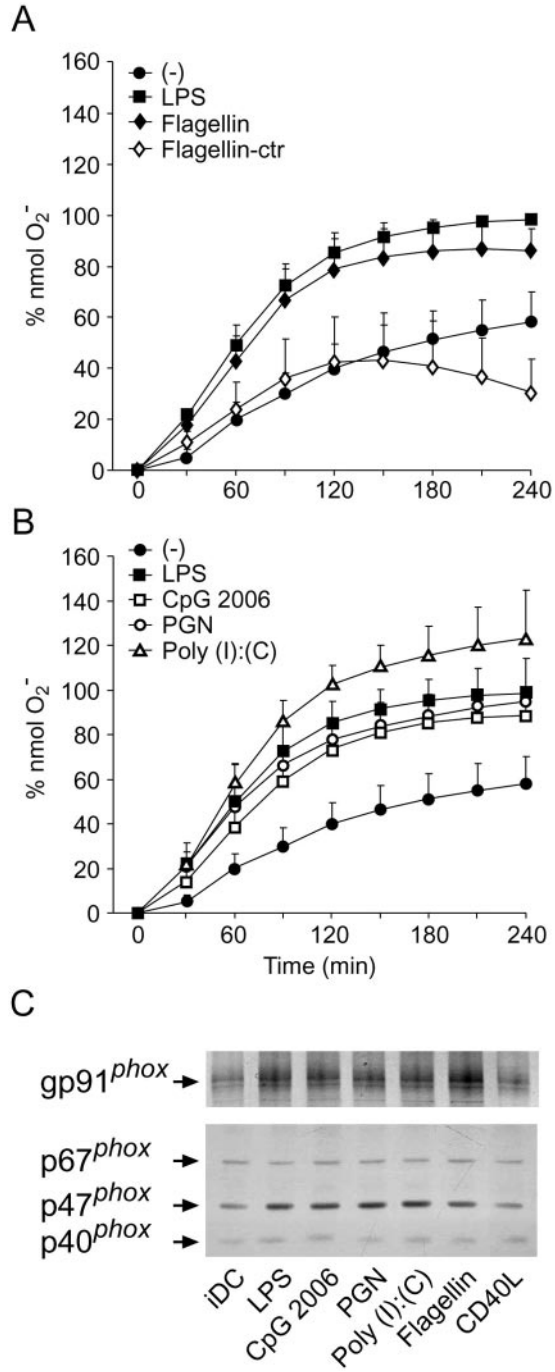

FIGURE 3. Induction of DC respiratory burst by TLR ligands. *A* and *B,* Monocyte-derived DC were cultured in the presence of different TLR ligands for 24 h and then assayed for their ability to release O^-_2 after stimulation with PMA (10 ng/ml) at different time points. Optimal stimuli concentrations used were: 100 ng/ml LPS, 1 μg/ml flagellin, 200 μg/ml inactivated flagellin (Flagellin-ctr), 2 μg/ml CpG 2006, 10 μg/ml PGN, and 10 μg/ml poly(I:C). Results are expressed as the mean ± SD of three independent experiments. For each experiment, LPS maximal value was assumed as 100%. *C,* DC were cultured in the presence of TLR ligands for 24 h, then lysed and immunoblotted with Abs raised against the indicated NADPH oxidase components. One blot representative of two independent experiments is shown.

Generation and function of DC from CGD patients

Oxygen radicals were reported to modulate APC differentiation and function (24–27, 43). To investigate the role of NADPH-derived oxygen radicals in DC biology, DC generated from monocytes obtained from CGD patients were tested. For these experiments, patients carrying different genetic alterations were evaluated (Table I). At the end of the 6-day culture protocol, cells

Table I. *Membrane phenotype of DC generated from CGD patients with different genetic abnormalities*[a]

Patient	Genetic Defect	iDC			LPS-mDC
		CD14	CD1a	MHC-II	CD83
Control	-	10.4 ± 4.2 (37 ± 6)	97.6 ± 5.1 (600 ± 120)	99.8 ± 1.1 (1523 ± 810)	97.5 ± 3.6 (50 ± 12)
CGD#1	$X91^0$-CGD	16.3 (53)	92.6 (317)	99.2 (1571)	97.9 (44)
CGD#2	AR^0-CGD	26.0 (61)	91.5 (800)	ND[b]	90.1 (31)
CGD#3	$X91^0$-CGD	38.8 (101)	80.6 (296)	99.5 (2828)	82.4 (30)
CGD#4	$X91^0$-CGD	5.4 (28)	89.0 (566)	ND	65.8 (33)
CGD#5	$X91^0$-CGD	4.4 (23)	74.1 (1072)	ND	31.4 (40)
CGD#6	$X91^0$-CGD	5.4 (26)	70.4 (781)	ND	36.9 (21)
CGD#7	$X91^0$-CGD	8.6 (30)	97.0 (2471)	99.7 (2452)	92.0 (45)

[a] Results are expressed as percentage of positive cells, mean fluorence intensity values are shown in parentheses. Results of single experiments are provided. For healthy donors (Control) average values ± SD are shown ($n = 15$).
[b] ND, Not done.

generated from CGD monocytes presented the characteristic morphology of iDC with abundant cytoplasm and membrane ruffling (data not shown). As shown in Table I, iDC were $CD14^{dim}$ and $CD1a^+$. CD83 was expressed by LPS-mDC. Normal expression of β_2 integrins (i.e., CD18) was also observed in all of the patients investigated (data not shown).

DC maturation is associated with the increased production of many cytokines and chemokines (7, 29). Fig. 4 reports the levels of IL-8/CXCL8, macrophage-derived chemokine/CCL22, IL-10, TNF, and IL-12 in the supernatants of LPS-mDC from CGD patients. Results obtained with three CGD patients showed no defect in cytokine and chemokine production by CGD DC. Indeed, the tendency of CGD DC was to release higher concentrations of cytokines. These results exclude a positive role for NADPH oxidase-dependent oxygen radicals in DC cytokine production.

To further investigate the function of CGD DC, LPS-mDC were tested for their ability to induce T lymphocyte proliferation. Fig. 5*A* shows that CGD DC were as efficient as control DC in inducing the proliferation of allogeneic T lymphocytes. Interestingly, at low DC:T ratio (i.e., 1%) five of six patients exhibited a stronger ability in inducing T cell proliferation compared with DC from healthy donors. To further gain insight on the role of oxygen radicals in MLR response, MLR experiments were performed using DC from normal subjects in the presence of nontoxic concentrations of SOD. As reported in Fig. 5*B*, the presence of the O^-_2 scavenger during MLR did not affect the induction of T cell proliferation.

Since oxidative stress was implicated in the induction of apoptosis (20, 21), we investigated the percentage of annexin V-positive cells in CGD DC by flow cytometry analysis. Results obtained with six CGD patients showed no difference in the degree of spontaneous apoptosis in CGD DC when compared with control DC (data not shown). Taken together, these results indicate that monocytes from CGD patients can differentiate into normal DC and that the lack of production of NADPH oxidase-dependent oxygen radicals does not affect DC functions.

NADPH oxidase-dependent killing of E. coli *by LPS-treated DC*

To evaluate the biological significance of O^-_2 production by DC, mDC were tested for their ability to kill intracellular *E. coli*, one of the pathogens responsible for recurrent infections in CGD patients (15). For these experiments DC were activated in the presence of LPS or CD40L. The different treatments did not alter the ability of DC to capture bacteria at the end of the 1-h incubation period (2.0×10^6 and 2.3×10^6 CFU, $n = 2$, for LPS and CD40L, respectively). Fig. 6*A* shows that stimulation of LPS-mDC by PMA activated the microbicidal activity of DC as documented by the dramatic reduction (>70%) of the number of CFU along the entire kinetics investigated (1–4 h). In the same experimental conditions, CD40L-DC were not efficient in bacteria killing at 1- and 2-h assays, although a certain degree of PMA-independent cytotoxicity was observed at longer time points (e.g., 4 h). Bacteria killing was abolished by the presence of DPI and was minimal when CGD DC were used (33 and 25% for CGD#1 and CGD#2, respectively; Fig. 6*B*). Similarly, PMA did not activate bacteria killing in iDC from normal subjects (data not shown).

Discussion

This study reports that DC matured in the presence of TLR ligands release oxygen radicals and kill intracellular pathogens through the activation of the NADPH oxidase. DC at the immature state are weak producers of oxygen radicals. However, maturation induced by LPS, or other TLR ligands, strongly increased their ability to release oxygen radicals when stimulated with PMA or with phagocytic stimuli like opsonized zymosan. Oxygen radicals produced under these experimental conditions derive from the activation of the NADPH oxidase, as established by the use of DC generated from CGD patients, an inherited disease in which phagocytes are unable to produce O^-_2 due to the defective expression of the

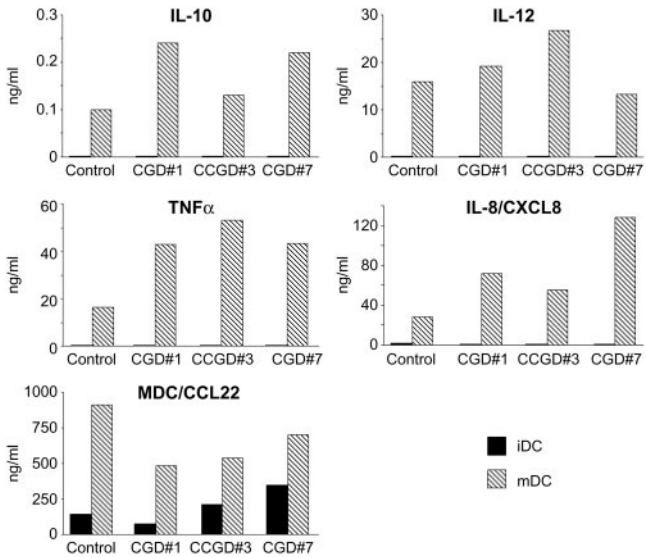

FIGURE 4. Cytokine and chemokine production by DC generated from CGD patients. Monocytes from three CGD patients were used to generate DC in vitro. DC were cultured in the absence (iDC) or the presence of 100 ng/ml LPS (mDC) for 24 h and supernatants were tested for cytokine and chemokine production by specific ELISA.

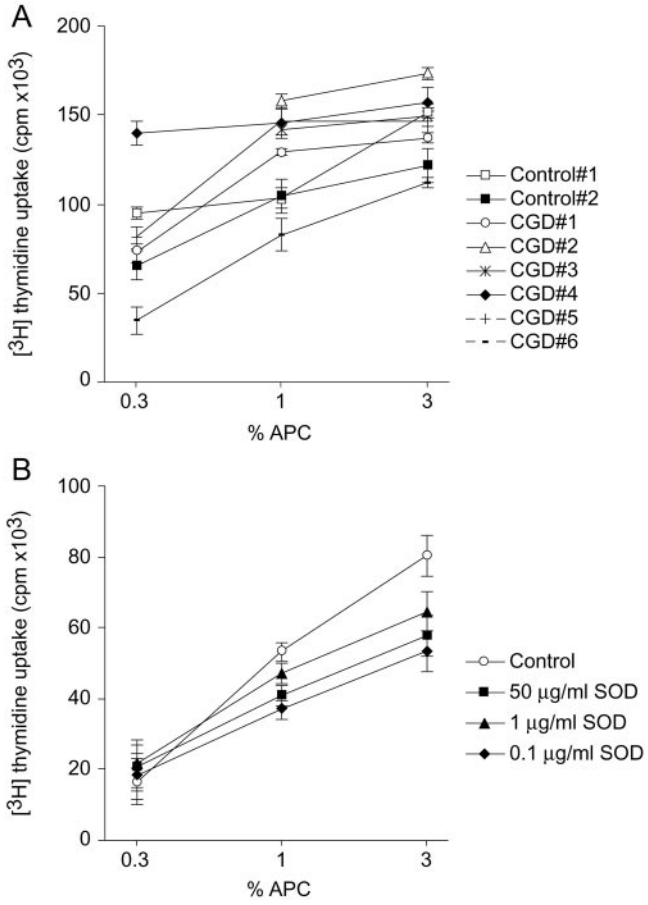

FIGURE 5. MLR using DC from CGD patients. *A,* Different concentrations of irradiated LPS-mDC were cocultured with 2×10^5 allogeneic purified T cells. Proliferation was assayed as uptake of [^{3}H]thymidine added in the last 16 h of a 6-day culture assay. *B,* iDC from healthy donors were matured with LPS for 24 h in the absence or presence of different concentrations of SOD and used as APC in MLR assays.

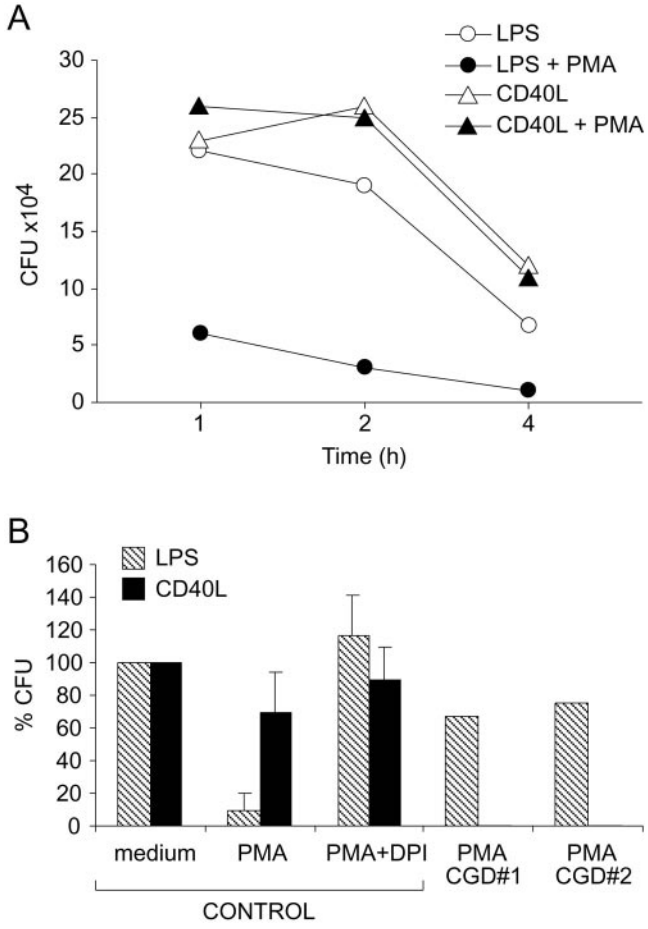

FIGURE 6. NADPH oxidase-dependent killing of *E. coli* by LPS-treated DC. *A,* LPS- and CD40L- mDC (1×10^6/ml) obtained from healthy donors were infected with *E. coli* at the ratio of 25:1 for 1 h. Gentamicin was added to kill extracellular bacteria. Where indicated, cells were stimulated with 100 ng/ml PMA. Then at different times postinfection, the number of intracellular *E. coli* was evaluated by viable plate count of CFU as described in *Materials and Methods*. Results are the mean of three independent experiments. *B,* LPS- and CD40L-mDC obtained from healthy donors and from two CGD patients were infected as detailed above. Where indicated, 100 ng/ml PMA or 10 μM DPI (10-min preincubation) were used. Two hours postinfection, the number of intracellular bacteria was evaluated by viable plate count of CFU. Results are expressed as percentage of CFU. The percentage of CFU obtained with LPS-mDC (from healthy subjects) stimulated with PMA was assumed as 100%. Results are the mean values of three independent experiments. For CGD DC, single experiments are shown.

NADPH oxidase protein complex (14, 15), and by the use of inhibitors. Accordingly, allopurinol and rotenone, two inhibitors of the xanthine oxidase system and the mitochondria respiratory chain, respectively, did not alter O^-_2 production by LPS-mDC. Otherwise, O^-_2 release by LPS-mDC was abolished by SOD (an O^-_2 scavenger) and DPI, a NADPH oxidase nonspecific inhibitor. This study also shows that influenza virus-activated P-DC release oxygen radicals when stimulated by PMA.

In comparison with monocytes, iDC show both reduced O^-_2 production and reduced NADPH oxidase protein expression. LPS-mDC released higher levels of O^-_2 than iDC and CD40L-mDC, and parallel experiments showed that the expression of p47^{phox} and gp91^{phox}, but not of p67^{phox} and p40^{phox}, was increased following TLR ligand-, but not CD40L-induced maturation. Therefore, the expression of p47^{phox} and gp91^{phox} apparently correlated with the ability of DC to release oxygen radicals at different stages of differentiation/maturation. Many studies, including ours, have shown the absence of an absolute relationship between the NADPH oxidase component protein levels and the respiratory burst (34, 44, 45). Whereas in other studies it was reported that p47^{phox} and/or gp91^{phox} levels and NADPH oxidase activity are regulated in a coordinate manner (34, 46–49). Although it is conceivable that additional factor(s) are involved in the regulation of O^-_2 production by LPS-mDC, the results reported here strongly suggest that the up-regulation of p47^{phox} and gp91^{phox} represents one of the factors involved in the increased production of oxygen radicals observed in TLR ligand-activated DC.

Reactive oxygen species are known to participate in cellular signaling through the regulation of transcription factors, such as AP-1 and NF-κB (22, 23), and various enzyme systems (e.g., protein tyrosine phosphates, protein kinase C, and mitogen-activated protein kinases) (50, 51). In DC, oxygen radicals were implicated in both DC differentiation (43) and maturation (26, 27) and in IL-8/CXCL8 production (24, 25). Controversial data are present in the literature about the inhibitory action (52) or the positive role (26, 53) of oxygen radicals on the ability of DC to stimulate T cell response. To evaluate the contribution of NADPH oxidase-dependent oxygen radicals on DC functions, we generated DC from monocytes obtained from CGD patients. Monocytes from CGD patients differentiated normally into both immature and mature

DC. Mature CGD DC produced normal, if not increased, levels of cytokines (TNF, IL-10, and IL-12) and chemokines (IL-8/CXCL8 and macrophage-derived chemokine/CCL22) and were fully competent in inducing T cell proliferation. These results are in agreement with recent reports showing an increased production of inflammatory cytokines, such as IL-1, TNF, and KC, and IL-10 in CGD-mice and in whole blood samples from CGD patients challenged with *Aspergillus fumigatus* (15, 54, 55). The finding that CGD DC induce a normal proliferation of T lymphocytes was further confirmed by the lack of effect of SOD, a radical scavenger, in MLR assays performed with normal DC. Since reactive oxygen species were reported to be involved in apoptosis of hemopoietic cells (20, 21), the percentage of annexin V-positive cells in iDC and mDC obtained from normal subjects and CGD patients was evaluated. No difference in the number of apoptotic (annexin V-positive), necrotic (propidium iodide-positive), or double-positive cells was detected between the two experimental groups. Taken together, these results indicate that NADPH-derived oxygen radicals are not essential for DC differentiation, maturation, survival, or T cell priming. However, these results do not exclude a role for reactive oxygen species produced by other enzyme systems.

Chemotactic agonists, including chemokines, are known to induce the activation of NADPH oxidase in phagocytic cells (56–59). However, none of the agonists tested in this study (i.e., C5a, fMLP, CCL2, CXCL8, CXCL12, CCL19) was able to induce O^-_2 release by LPS-mDC. Thus, chemotactic signals do not promote the activation of the respiratory burst in DC.

It is interesting to note that maturation induced by TLR ligands, but not by an immune signal like CD40 ligation, makes DC competent for the production of oxygen radicals. These results indicate that alternative pathways of DC maturation have a different action on the regulation of NADPH oxidase activation. TLRs represent a particular family of pattern-recognition receptors that are capable of discriminating between the molecular signature of different pathogens. DC express many TLRs, including TLR2, TLR3, TLR4, and TLR5 (38–42). Activation of DC by pathogens that engage TLR results in the migration of DC to T cell areas of secondary lymphoid organs. Activation of TLR also promotes the production of cytokines that will properly orient the generation of adaptive immunity. Pathogens have a different ability to induce cytokine production (e.g., IL-10 and IL-12) by DC (38, 41, 42). In this study, DC maturation was induced using different TLR ligands, namely, PGN (TLR2), poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), and CpG (TLR9). All of these agonists generated mature DC able to release oxygen radicals. Following activation, TLRs associate with the cytosolic transducer protein MyD88. However, not all of the TLRs have the same requirement for MyD88. For instance, DC maturation is achieved in a MyD88-independent manner when induced through TLR4. In contrast, it is a MyD88-dependent process when elicited by TLR9 agonists (38). The results presented here show that all of the TLR agonists used induce a mature DC phenotype associated with the activation of the respiratory burst. Therefore, it seems likely that the regulation of the NADPH components follows either a MyD88-independent pathway or proceeds through both MyD88-dependent and -independent pathways.

The levels of O^-_2 released by mDC were lower than those produced by professional phagocytes and were delayed in their kinetics. However, oxygen radicals released by mDC are sufficient for the killing of intracellular *E. coli*, a catalase-positive bacteria known to cause recurrent infections in CGD patients (15). The relevance of NADPH oxidase activity in bacteria killing is suggested by several observations: 1) bacteria killing is suppressed by DPI, a NADPH oxidase inhibitor; 2) bacteria killing is minimal in CD40L-mDC; and 3) bacteria killing is weakly inducible by PMA in CGD DC. In agreement with this finding, Aline et al. (10) have demonstrated that the intracellular replication of *Toxoplasma gondii* in DC is inhibited by an oxygen-dependent mechanism. Thus, even if DC might have a reduced ability to kill pathogens when compared with professional phagocytes (60), these cells posses the ability to clear intracellular pathogens by an oxygen-dependent mechanism.

In summary, this study reports that NADPH oxidase is selectively induced in DC through the activation of TLRs. The levels of oxygen radicals produced by DC are limited but sufficient to kill intracellular bacteria like *E. coli*. Because of its peculiar regulation, it is tempting to speculate that this oxygen-dependent mechanism of bacterial killing of mDC is functional to limit the dissemination of pathogens during DC migration from periphery to secondary lymphoid organs.

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