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Reactive Oxygen Species Level Defines Two Functionally Distinctive Stages of Inflammatory Dendritic Cell Development from Mouse Bone Marrow

Kuo-Ching Sheng,* Geoffrey A. Pietersz,† Choon Kit Tang,† Paul A. Ramsland,‡ and Vasso Apostolopoulos*

Reactive oxygen species (ROS) have been implicated in various physiological activities. However, their role in dendritic cell (DC) activation and generation has not been investigated. Using the bone marrow-derived GM-CSF–induced ex vivo DC model, we characterize how induction of ROS correlates with inflammatory DC functionality and expansion. We describe that the functionality of GM-CSF–induced DCs is distinct in two developmental stages. Whereas division of DC-committed hematopoietic progenitor cells (HPCs) neared completion by day 6, the level of ROS soared after day 4. Day 3 ROSlo DCs were highly responsive to TLR stimuli such as LPS and zymosan by rapid upregulation of CD80, CD86, and MHC class II, in contrast to the low response of day 6 ROShi DCs. ROShi DCs could not initiate and sustain a significant level of NF-κB phosphorylation in response to LPS and zymosan, although demonstrating hyperactivation of p38 MAPK by LPS, in a fashion disparate to ROSlo DCs. ROSlo DCs stimulated a higher level of allogeneic and OVA-specific T cell proliferative responses, although ROShi DCs were much more proficient in processing OVA. In response to pathogenic stimuli, ROSlo DCs also demonstrated rapid cellular adhesion and H2O2 release, indicating their role in immediate microbial targeting. Moreover, HPC expansion and DC generation were dependent on the surge of ROS in an NADPH oxidase-independent manner. These findings point to the potential role of cellular ROS in mediating functionality and development of DCs from HPCs during inflammation. The Journal of Immunology, 2010, 184: 2863–2872.

Dendritic cells (DCs) as key immune regulators strategically localize at tissue niches and comprise a range of subtypes to induce distinct T cell immune responses (1, 2). Under inflammatory conditions, development of inflammatory DCs is continuous owing to high levels of proinflammatory cytokines produced at the site of inflammation (3). This DC subtype is highly proficient in production of cytokines such as TNF-α, IL-12, and IL-23, with strong propensity to induce Th1 cells (4), and has been described as a TNF-α and inducible NO synthase-producing (Tip) DC (5). Based on several in vivo studies with microbial infections, Tip DCs are believed to derive from monocytes under the influence of GM-CSF. These DCs demonstrate a high level of functional and phenotypical resemblance to DCs that are derived from hematopoietic progenitor cells (HPCs), with the potential to differentiate and converge into the DC lineage in bone marrow (BM) or blood monocytes in the presence of GM-CSF (5). Consequently, ex vivo GM-CSF–induced DCs (GMDCs) derived from culture conditions are considered equivalents of in vivo Tip DCs (2, 5). GMDCs fulfill several criteria set out in the classical view of DCs. They are larger and more granular than other DC subtypes, with distinct dendritic morphology (5–7). GMDCs express a wide range of pattern recognition receptors such as TLRs and C-type lectins, which recognize pathogen-associated molecular patterns (PAMPs) and in turn enhance/regulate phenotypic maturation and/or uptake of PAMP-associated Ags (1). They are abundantly produced in culture and easy to manipulate. In comparison with in vivo isolated DCs, GMDCs do not undergo spontaneous maturation. These characteristics, together with a strong capacity in induction of T cell responses, have led to the fidelity of their use in immunological studies and ex vivo DC immunotherapy.

Hematopoietic growth factors such as GM-CSF for DC induction have been shown to signal through formation of reactive oxygen species (ROS) (8, 9). ROS represent a group of molecular entities that are either free radicals containing unpaired electrons, such as superoxide, NO, and hydroxyl radical, or nonradical oxidizing agents, such as H2O2 and the NO derivative peroxynitrite (10). Cellular ROS contain a pool of by-products generated from biological reactions during oxygen metabolism in cellular elements such as mitochondria and peroxisomes. They are also generated by NADPH oxidase within different types of cells in response to various stimuli (11, 12). Owing to the highly reactive nature to, and avidity for, carbohydrates, lipids, nucleic acids, and proteins, ROS have been considered significant contributors to cellular damage or to impaired or abnormal cellular functions, leading to their implication in aging, cancer, diabetes, atherosclerosis, and chronic infections (11, 13). However, over the past decades, ROS

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Abbreviations used in this paper: BM, bone marrow; CBA, cytometric bead array; CM-H2DCFDA, 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DC, dendritic cell; GMDC, GM-CSF–induced DC; GSH, reduced glutathione; HPC, hematopoietic progenitor cell; MFI, mean fluorescence intensity; MR, mannose receptor; NAc-cys, N-acetylcysteine; NS, not stimulated; NT, no treatment; p, phosphorylated; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; Tip, TNF-α and inducible NO synthase producing.

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have also emerged as crucial regulators in cellular motility, differentiation, and apoptosis through a direct influence on signal-transducing proteins such as phosphatases and kinases, protein ubiquitination, or cytoskeleton (14–17). Moreover, in phagocytes, NADPH oxidase-induced ROS are indispensable for microbial killing, inactivation, and pH regulation in phagosomes (10, 11, 18). Given the range of cellular activities involved with ROS, how induction of ROS by GM-CSF correlates with DC functions and development from HPCs during inflammation is not known.

The inflammatory condition induced with GM-CSF ex vivo provides a scenario for studies on the role of ROS in DC functionality and generation during inflammation. GMDCs, although considered an integrated population of inflammatory DCs by day 6, can emerge as early as day 1. A DC functionality assay can be performed at any time point when sufficient numbers of DCs are generated, although high DC purity is not reached until day 6 or 7. Whether the developmental stage of inflammatory DCs can affect their functionality has yet to be determined. In the current study, we describe the possible connection of ROS induction with DC functionality and generation. The level of ROS surged between day 4 and day 6 in GM-CSF BM culture, when expansion of HPCs into DCs was most rapid. Day 3 ROShi DCs were highly responsive to LPS and zymosan stimulation by upregulating costimulatory molecules CD80, CD86, and MHC class II, in comparison with the low response of day 6 ROSlo DCs. ROShi DCs failed to initiate a significant level of NF-κB p65 activation, although exhibiting hyperactivation of p38 MAPK, in contrast to ROSlo DCs. ROShi DCs were much more proficient in OVA uptake and processing; however, their capability in OVA-specific T cell stimulation was weaker than that of ROSlo DCs. ROShi DCs were CD11b+ and, in response to LPS and zymosan stimulation, they rapidly exhibited strong adhesion and spreading morphology and released a significant level of H2O2, suggesting a role for immediate microbial targeting. However, given that day 3 and day 6 DCs demonstrate different functional properties, the developmental relationship between these two populations remains largely unknown. Finally, ROS escalation was indispensable for late-stage HPC expansion, DC generation, and survival that were independent of NADPH oxidase. These findings propose a potential role for ROS in DC functionality and generation under the GM-CSF–associated inflammatory condition.

Materials and Methods

Animals

C57BL/6, C3H/He, OTI, and OTII mice (aged 6–10 wk) used throughout this study were purchased from the animal facilities of the Walter and Eliza Hall Institute and Precinct Animal Center in Alfred Medical Research and Education Precinct (Melbourne, Australia).

DC generation and purification

BM cells from femurs and tibias of C57BL/6 mice were collected and treated with ACK lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Mn2+EDTA) to lyse erythrocytes. Cells were washed and cultured with complete RPMI 1640 media (2% HEPES buffer, 0.1 mM 2-ME, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% FCS) at 37°C and incubated with anti-CD11c MAC beads (10 min. DCs were purified by magnetic cell sorting. Briefly, cells were pelleted collected at specific days by gentle pipetting. In most experiments where total and 6. In other experiments, it was conducted every day from day 3. Cells were slowly replenished with media containing 20 ng/ml GM-CSF, with or without GMDCs, although considered 2% HEPES buffer, 0.1 mM 2-ME, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% FCS) at 5% and incubated with anti-CD11c MAC beads (10 min). The purity of T cells was at least 94%.

Detection of the intracellular redox state

Splenocytes from OTI, OTII, or C3H/He mice were collected, washed, and incubated in ACK lysis buffer at 37°C for 5 min. Cells were incubated with Ab mix, which contained in-house–produced rat anti-mouse Gr-1 (RB6-8C5), anti-CD11b (M1/70.15), anti-erythrocyte (TER-119), and anti-MHC class II (M5/114.15.2) at 4°C for 30 min. To purify CD4+ and CD8+ T cells, rat anti-mouse CD8a (YTS169.4) and anti-CD4 (GK1.5) were included in the Ab mix, respectively. Labeled cells were depleted with two rounds of bead separation. In each round, cells were incubated with BioMag goat anti-rat Ig magnetic beads (8 beads/cell) (Qiagen, Melbourne, Australia) at 4°C for 25 min. Cells were washed, and those that bound to the beads were removed by magnetic attraction. The purity of T cells was at least 94%.

Induction of DC maturation

DCs were stimulated with LPS (1 µg/ml) derived from Escherichia coli (0111:B4; Sigma-Aldrich, St. Louis, MO) or zymosan A (20 µg/ml) of Saccharomyces cerevisiae (Sigma-Aldrich) and collected at specific time points at 37°C. Cells (5 × 105) were washed and resuspended with in-house FITC-conjugated anti-CD80 (16.10.A1), anti-CD86 (GL1), and MHC class II (IAb) (M5/114.15.2), together with APC-conjugated anti-CD11c (BD Biosciences) at 4°C for 30 min. In flow cytometry analysis, dead cells were separated by positive PI staining, and live CD11c+ DCs were gated and the DC maturation state was determined by FITC fluorescence intensity.

Mannose receptor and TLR expression

To detect mannose receptor (MR) expression, cells prelabeled with anti-CD11c–APC were incubated with 50 µg/ml FITC-conjugated mannose-BSA (Sigma-Aldrich) at 4°C for 1 h. To determine the surface expression of TLR2, CD11c+ cells were incubated with 5 µg/ml mouse anti-human/mouse TLR2 (T2.5) (eBioscience, San Diego, CA) and labeled with FITC-conjugated anti-mouse Ig F(ab’2) (Chemicon, Melbourne, Australia) at 4°C for 1 h. To detect TLR4 expression, CD11c+ cells were incubated with 20 µg/ml rat anti-mouse TLR4/MD2 (MTS510) (BioLegend, San Diego, CA) and labeled with FITC-conjugated polyclonal anti-rat Ig (BD Biosciences) at 4°C for 1 h. The FITC fluorescence intensity of live CD11c+ DCs was determined by flow cytometry analysis.

T cell proliferation

To evaluate the capacity of DCs to stimulate peptide-specific T cells, OTI and OTII cells (2 × 105) were cultured with purified day 3 or day 6 DCs (1 × 104 cells, which were preloaded with H2Kb-restricted SIINFEKL (0.1 µg/ml), IAα-restricted ISQVHAHAAHINAEGR (OVA231-240) (10 µg/ml), and endotoxin-depleted OVA (40 µg/ml) for 3 h in quadruplicates in 96-well plates. Proliferation of T cells was monitored by addition of 1 µCi [3H] thymidine from day 1 to day 6. The radioactivity was measured in counts per minute. Peak proliferation of OTI (on day 2) and OTII (on day 3) T cells was compared. To evaluate the capacity of day 3 or day 6 DCs to stimulate allogeneic T cells in the MHC-mismatched MLR, purified DCs were seeded with C3H/He T cells at the same ratios as described. Proliferation was monitored from day 2 to day 7. Peak proliferation at day 6 was compared.

Carboxyfluorescein succinimidyl ester-labeled HPC expansion

Erythrocyte-depleted BM cells were labeled with 5 µM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) in PBS containing 2% FCS at 37°C for 10 min. Cells were then washed with ice-cold 2% FCS in PBS three times. Labeled cells were seeded for DC generation as described. The division of HPCs that were responsive to GM-CSF was demonstrated in flow cytometry with FITC fluorescence. The CD11c+ cell number was calculated based on its percentage in total cells by anti-CD11c–APC labeling. The kinetics of HPC expansion and DC generation were monitored along culture development.

System (Miltenyi Biotec) following the manufacturer’s instructions. The purity of the CD11c+ cells was >97%.

ROS surge defines DC development

ROS was estimated by subtracting the background mean fluorescence intensity (%) of the nonfluorescent sample (negative control) from the measured MFI values of fluorescent samples in flow cytometry analysis.

T cell stimulation

Proliferation of T cells was monitored by addition of 1 µCi [3H] thymidine from day 1 to day 6. The radioactivity was measured in counts per minute. Peak proliferation of OTI (on day 2) and OTII (on day 3) T cells was compared. To evaluate the capacity of day 3 or day 6 DCs to stimulate allogeneic T cells in the MHC-mismatched MLR, purified DCs were seeded with C3H/He T cells at the same ratios as described. Proliferation was monitored from day 2 to day 7. Peak proliferation at day 6 was compared.
Detection of NF-κB p65 and p38 MAPK

To evaluate the intracellular presence of total NF-κB p65 and p38 MAPK subunits, nonstimulated DCs labeled with anti-CD11c–APC were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min and permeabilized with 5% (w/v) saponin in PBS for 10 min. Cells were washed and incubated with the FITC-conjugated mouse anti-NF-κB p65 (F-6) (Santa Cruz Biotechnology, Santa Cruz, CA) or with p38 (H-147) rabbit polyclonal IgG (Santa Cruz Biotechnology), followed by FITC-conjugated sheep anti-rabbit Ig (Silenus Labs, Melbourne, Australia) labeling at 4°C for 30 min. To determine activation of NF-κB p65, DCs were prestimulated with LPS (1 μg/ml) plus zymosan (20 μg/ml) (19, 20) and harvested at specified time points. DCs were permeabilized and incubated with p-NF-κB p65 (Ser276) rabbit polyclonal IgG (Santa Cruz Biotechnology), followed by FITC-conjugated sheep anti-rabbit Ig Glue. Similarly, for activated p38 MAPK detection, DCs prestimulated with LPS (1 μg/ml) (21) were permeabilized and incubated with p-p38 MAPK (Thr180/Tyr182) (28B10) mouse mAb, followed by FITC-conjugated anti-mouse Ig F(ab')2 (Chemicon) labeling. The phospho-level of a kinase subunit was determined by subtracting the background MFI of the secondary (Ab only) from the reflected MFI of FITC. The phospho-increase of a kinase was estimated by subtracting the steady-state phospho-level from the measured MFI.

OVA binding, uptake, and processing

DCs were incubated with FITC-OVA (40 μg/ml) at 4°C for 30 min and at 37°C for 5 min to determine the Ag binding and uptake capacities of DCs, respectively. To assess Ag processing, DCs were incubated with DQ-OVA (Molecular Probes) (1 μg/ml) at 37°C and harvested after 30 min. Because DQ-OVA as a self-quenched, low background conjugate exhibited bright fluorescence only upon proteolytic degradation. In all Ag assays, live CD11c+ cells were gated and FITC fluorescence determined by flow cytometry. The estimated level of DQ-OVA proteolysis was determined by subtracting the MFI for background binding at 4°C (0 min time point).

DC adhesion

To avoid activation of the adhesion mechanism due to lengthened handling, DCs were not purified. In the 24-well DC culture, nonadherent or loosely adherent cells were removed from three wells of day 3 or 6 culture by gentle pipetting and slowly released into a well of a 6-well plate (BD Biosciences) for microscopic examination. Cells were stimulated with LPS (1 μg/ml) or zymosan (20 μg/ml) at 37°C for 2 h and imaged with a 50x water immersion lens by the Leitz DMIRE2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) in the bright field. Readily distinguished floating DCs were large, with or without dendrite appearances. Adherent DCs, which emerged after cell plating at 37°C, were characterized by spreading morphology.

Nitrite and H2O2 detection

Purified DCs (2 × 10^5) in 200 μl were seeded in the 48-well plate and stimulated with LPS (1 μg/ml) or zymosan (20 μg/ml) in triplicates at 37°C for various lengths of time. Nitrite release was measured by mixing 50 μl culture media with 10 μl of the mixture of Griess reagent (2-naphthylamine-5-sulfonic acid (Sigma-Aldrich) at room temperature for 10 min. Titrated sodium nitrite (0–50 μM) was used as a standard. The OD of purple/magenta color formation was measured at 540 nm. H2O2 release was detected by DCs that were incubated with luminol chemiluminescence with a modified protocol (22). Briefly, 50 μl culture media or titrated H2O2 (0–60 μM) as the standard was incubated with 40 μl enhanced luminol reagent (PerkinElmer Life Sciences, Boston, MA) and 10 μl streptavidin HRP (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.) (1:2500 final dilution) for 5 min. The luminescence was measured by the FluoroStar Optima plate reader (BMG Labtech, Chicago, IL).

Cytokine detection

To detect the capability of DCs to produce cytokines, purified DCs (1 × 10^5) in 100 μl were seeded in a 48-well plate and stimulated with LPS (1 μg/ml) or zymosan (20 μg/ml) in quadruplicates at 37°C for 20 h. The supernatant was collected and analyzed by cytometric bead array (CBA) for inflammation and Th1/Th2 cytokines (BD Biosciences). To detect Th cell cytokine production induced by DCs, purified OTII T cells (2 × 10^5) in 100 μl were incubated with 2 × 10^5 DCs in the presence of OVA323-339 (10 μg/ml) or OVA (40 μg/ml) at 37°C in quadruplicates. At day 5, the supernatant was collected and analyzed by CBA, following the manufacturer’s instructions. Briefly, 25 μl culture supernatant or standard cytokine solutions (40 μg/ml) were incubated with 25 μl mixture of cytokine capture beads, together with PE detection reagent for 2 h. Acquisition involving scatter and compensation was standardized with cytometer setup beads, prior to analysis of detection array beads. Estimated values of cytokines were obtained from the FACarray software (BD Biosciences) based on the fluorescence intensity.

ROS scavenging and NADPH oxidase inhibition

N-acetyl cysteine (NAC-cys) as a powerful antioxidant was used, along with the reducing agent reduced glutathione (GSH), to scavenge ROS. Before experimentation, NAC-cys and GSH stock solutions were adjusted to pH 7.0 with 1 M NaOH. At day 3, NAC-cys and GSH in specified concentrations (7.5–30 mM) were added into the DC culture in triplicates. At day 6, culture was harvested and total cell numbers were determined by cell count. CD11c+ cell percents, together with ROS levels, were determined by flow cytometry. Apocynin, as an NADPH oxidase inhibitor shown to suppress the phagocytic oxidative burst, was used to test the role of NADPH oxidase in ROS production of HPCs and DCs (11). Apocynin was added into the day 3 culture in the titrated manner. At day 6, the levels of CD11c expression and ROS fluorescence were analyzed as described.

Statistics

All data are shown as the mean ± SEM. The data generated in this study were analyzed by Student t test. Significance of difference was determined by the p value (∗p < 0.05; **p < 0.01).

Results

The level of ROS soars when DC expansion is rapid

To investigate how induction of ROS correlated with HPC expansion into DCs, we first established the numbers of total and CD11c+ cells generated in relation to the division state of CFSE-labeled, GM-CSF–responsive HPCs along the progress of culture. Although the number of total cells increased steadily (10^5 cells/day), DC generation accelerated toward the end of culture, at a rate from ~10^5 cells/day between day 1 and day 4 to ≥ 10^6 cells/day between day 4 and day 6 (Fig. 1A). At the beginning of culture, in parallel with version of immediate progenitors into DCs, the majority of HPCs underwent replication, whereas a relatively small portion of cells were unresponsive to GM-CSF. In each round of HPC replication, DCs were continuously generated, resulting in smearlike CFSE fluorescence display (Fig. 1B). Between day 4 and day 6, rather than further replicating, HPCs were mostly converted into DCs, explaining the rapid DC expansion toward the end of culture (Fig. 1A, 1B). GM-CSF was shown to induce a low level of ROS in HPCs (8, 9). We next measured ROS during DC generation by the probe CM-H2DCFDA. Whereas DCs indeed produced a low level of ROS at day 3, day 6 DCs contained a surprisingly high level of ROS, in the absence or presence of the permeable control H2O2 (Fig. 1C). In the kinetic study, before day 4, the level of ROS was relatively low, until day 5, when a rapid increase in ROS was observed (Fig. 1C). Thus, the surge of ROS coincides with rapid DC generation.

Early developed GMDCs show enhanced maturation in response to TLR stimuli

To distinguish whether the developmental stage had an impact on DC functionality in consideration of the ROS level, the maturation capacity of DCs in response to TLR stimuli was evaluated. As shown in Fig. 2A, day 6 DCs induced with GM-CSF or GM-CSF/IL-4 showed dramatically reduced upregulation of costimulatory molecules CD80 and CD86, and MHC class II, after 16 h LPS or zymosan stimulation. Similar results were obtained when DCs were stimulated with CpG1668 and polyinosinic-polycytidylic acid (not shown). Interestingly, GM/IL-4DCs constitutively demonstrated a more mature phenotype than did GDMDCs in the steady-state, showing enhanced CD80, CD86, and MHC class II expression. To investigate whether reduced maturation was due to medium exhaustion, DCs were grown under daily medium and 2× cytokine replenishment from day 3 on. The maturation capacity of day 3 DCs was robust, with high levels of CD80, CD86, and MHC class II upregulation after only 8 h LPS stimulation, however,
regardless of medium/cytokine replenishment, such capacity declined in the following days (Fig. 2B). This trend was also evident when day 7 and day 8 DCs were compared after 24 h LPS stimulation. Day 8 DCs that were stimulated for 48 h could not reach the maturation level demonstrated by the day 3 counterpart stimulated merely for 8 h (Fig. 2B). To clarify whether TLR expression accounted for reduced maturation, surface TLR2 and TLR4 as corresponding signaling receptors for zymosan and LPS in DC maturation were examined. No significant differences in levels of TLR2 and TLR4 expression were observed between day 3 and day 6 DCs (Fig. 2C).

Day 6 ROShi DCs demonstrate hypoactivation of NF-κB p65, but hyperactivation of p38 MAPK

To provide mechanistic insights into reduced maturation at the signaling level, both NF-κB and p38 MAPK activations shown to dominate expression of DC maturation markers were investigated (23, 24). Among NF-κB subunits, RelA or p65 has been shown to be significantly involved in DC activation and cytokine production (25, 26). Subsequently, the expression and activation of NF-κB p65 and p38 MAPK in the steady-state and activated state of DCs were examined. As shown in Fig. 3A, day 6 ROShi DCs expressed a higher level of the NF-κB p65 subunit and an equal level of p38, in comparison with day 3 ROSlo DCs, indicating a basal difference in signaling between two DC groups. To further evaluate NF-κB p65 activation, phosphorylation on Ser276 was adopted as a model, which required activation of Raf-1, the regulatory signal for IL-10 production (19, 20). Hence, zymosan as a ligand for dectin-1 was used together with LPS to stimulate DCs in this model. In contrast, LPS alone was sufficient to stimulate p38 MAPK phosphorylation on Thr180 and Tyr182 (21). Before TLR stimulation, interestingly, day 3 ROSlo DCs showed a higher level of background NF-κB p65 phosphorylation and a lower level of p38 MAPK activation than did day 6 ROShi DCs, suggesting the constitutive presence of a diverse

**FIGURE 1.** ROS surge coincides with rapid DC generation. A, BM cells (5 × 10^5 cells/ml) were cultured with GM-CSF in 24-well plates. Medium/cytokine replenishment was performed at day 3. Total and CD11c+ cell numbers from three replicates were determined by cell counting and FACS analysis from day 1 to day 6. B, BM cells were labeled with CFSE prior to culture with GM-CSF. Cell division was determined each day by CFSE fluorescence, along with which DC generation was characterized by CD11c-APC labeling. The percentages of CD11c+ and CD11c- populations were determined. C, CD11c+ DCs derived from GM-CSF culture with no treatment or with 15 min 0.025% H2O2 treatment (H2O2) were incubated for 15 min with CM-H2DCFDA. The presence of ROS was determined by FITC fluorescence intensity each day. The histograms of ROS fluorescence at day 3 (dotted line) and day 6 (solid line) are compared in the left panel. The shaded area represents pooled non-CM-H2DCFDA-treated cells. The true level of ROS was calculated each day by subtracting the background (shaded area) MFI from the measured MFI. The kinetics of the ROS level in the absence or presence of H2O2 is shown in the right panel. The experiments were performed at least twice with similar results. NT, no treatment.

**FIGURE 2.** GMDCs show reduced maturation in response to the TLR stimulus along development. A, BM cells were cultured with GM-CSF or GM-CSF/IL-4. Medium/cytokine replenishment was performed on day 3. DCs at day 3 or day 6 were stimulated with LPS or zymosan for 16 h. The maturation status of CD11c+ cells with the stimulant (solid line) or without (dotted line) was determined by CD80, CD86, and MHC class II expression in the histogram. B, BM cells cultured with GM-CSF received daily medium/cytokine replenishment from day 3 on. Cells were stimulated with LPS for 8 h at days 3, 4, 5, and 6, for 24 h at days 7 and 8, or for 48 h at day 8. After stimulation, the levels of CD86 expression of CD11c+ cells were determined and compared. In both A and B, the shaded area represents the isotype control Ab. C. Cells harvested at day 3 and day 6 were labeled with primary anti-mouse TLR2 and TLR4/MD2 and the corresponding FITC-conjugated secondary Ab, as described in Materials and Methods. The levels of TLR2 and TLR4 expression of CD11c+ cells were determined by FITC fluorescence intensity. The shaded area represents cells stained with the respective secondary Ab. Data shown are representative of four experiments.
signaling mechanism between two rest groups of inflammatory DCs (Fig. 3B, 3C). After stimulation with LPS and zymosan for 90 min, phosphorylation of NF-κB p65 on Ser276 was barely detected in day 6 DCs, in contrast to the day 3 counterpart (Fig. 3C, 3D). Conversely, day 6 DCs showed ~2-fold higher level of p38 MAPK phosphorylation on Thr180 and Tyr182 than did day 3 DCs, after 15 min LPS stimulation (Fig. 3C, 3D). The kinetic studies also demonstrated that day 6 DCs could initiate only a relatively low level of NF-κB p65 activation peaking at 60 min, in comparison with a robust and sustained level of activation of day 3 DCs at 90 min poststimulation, whereas day 6 DCs exhibited a high level of p38 activation at 15 min LPS stimulation, with kinetics similar to that of day 3 DCs (Fig. 3D).

Day 6 ROShi DCs are highly efficient in MR-mediated Ag uptake and processing

The OVA system was used to study the Ag capacity of DCs. As cellular binding and uptake of OVA was shown to be MR mediated (27), we first detected the level of MR expression on DCs. Day 6 DCs showed a higher level of MR expression than did day 3 DCs (Fig. 4A). Consequently, these DCs demonstrated enhanced immediate binding of OVA-FITC at 4°C (Fig. 4B). As OVA-FITC was reported to be relatively unstable in the intracellular acidic compartment, its uptake was monitored only for 5 min at 37°C. Day 6 DCs again showed a greater level of overall OVA loading (Fig. 4B). In contrast to OVA-FITC, the fluorescence of DQ-OVA was self-quenched until proteolytic degradation took place. The degraded DQ-OVA product was shown to be highly resistant to low pH. As shown in Fig. 4C, DQ-OVA demonstrated little background fluorescence upon binding at 4°C (0 min time point). The fluorescence began to rise when it was degraded in DCs at 37°C. In comparison with day 3 ROShi DCs, day 6 ROShi DCs were more highly efficient in OVA processing, demonstrating a much more rapid shift in fluorescence. When the level of DQ-OVA proteolysis was estimated, day 6 ROShi DCs again showed at least a 4-fold elevated activity in 1 h. (Fig. 4C).

Day 3 ROSlo DCs are more efficient in T cell stimulation than are day 6 ROShi DCs

Because day 3 and day 6 DCs demonstrated diversities in maturation and Ag uptake/processing capacities, we investigated whether their capacities in T cell stimulation were different. In the MLR, day 3 DCs were more potent in stimulating allogeneic C3H/He T cells than were day 6 DCs, by stimulating a 5-fold stronger T cell response (Fig. 5A). This observation was consistent with the phenotypic maturation data (Fig. 2A), as MLR is known to be highly dependent on B7-mediated costimulation (28). Moreover, when the capacity of DCs to stimulate T cells in the MHC context was evaluated, day 3 DCs loaded with SIINFEKL and OVA323–339 peptides were more stimulatory to OVA-specific CD8 (OTI) and CD4 (OTII) T cells than were day 6 DCs (Fig. 5B). Given that day 3 DCs were much less efficient in taking up and processing the OVA Ag, after OVA loading, they also stimulated higher levels of CD4 and CD8 T cell proliferation (Fig. 5B).

Day 3 ROSlo and day 6 ROShi DCs show diverse capacities in cytokine production and induction

To characterize the capacity of DCs to produce cytokines, they were stimulated with LPS or zymosan. Day 3 ROSlo DCs produced substantial amounts of MCP-1, IL-12p70, and IL-2, which were largely reduced or abolished in day 6 DCs (Fig. 6A). Given that day 6 ROShi DCs remained highly active in generating IL-6 and TNF-α, day 3 DCs were more efficient in producing proinflammatory cytokines in general. Interestingly, although day 6 DCs produced more IL-10 in response to LPS, they produced much less IL-10 in response to zymosan, in comparison with day 3 DCs. It was noted that zymosan appeared to be a strong inducer for all the cytokines examined, whereas LPS was particularly potent in induction of IL-6 and TNF-α. To further investigate the capacity of DCs to induce cytokine production from Th cells, OTI T cells were incubated with DCs in the presence of the helper peptide OVA323–339 or OVA. As shown in Fig. 6B, both day 3 and day 6 DCs induced strong IFN-γ production when loaded with the helper peptide, but did not elicit IL-4 production, indicating their Th1 polarizing capacity. No cytokine was produced when the peptide

**FIGURE 3.** ROSlo DCs demonstrate altered NF-κB p65 or p38 MAPK expression and phosphorylation profiles in the steady-state or activated state. A, Cells from day 3 or day 6 GM-CSF culture were labeled with anti-CD11c-APC, fixed, and permeabilized. Cells were washed and labeled with FITC-conjugated mAb NF-κB p65 or with p38 (H-147) rabbit polyclonal IgG, followed by secondary FITC-conjugated sheep anti-rabbit Ig. The histograms compare NF-κB p65 (left) and p38 (right) expression between day 3 (dotted line) and day 6 (solid line). The shaded area for the NF-κB p65 histogram represents nonstained cells, and for p38 detection it represents the secondary Ab alone. B–D, To detect activation of NF-κB p65 or p38 MAPK, cells prestimulated with LPS plus zymosan or LPS alone were permeabilized, labeled with anti-CD11c-APC, and incubated with the p-NF-κB p65 (Ser276) or p-p38 MAPK (Thr180/Tyr182) mouse Ab, followed by labeling with the corresponding FITC-conjugated secondary Ab. The phosphorylated subunit of the kinase was detected and analyzed in the histogram, by which the true level of phosphorylation was calculated, as described in Materials and Methods. The shaded area represents the cells stained with the secondary Ab alone. The steady-state level of p-NF-κB p65 (Ser276) or p-p38 MAPK (Thr180/Tyr182) (phospho-level) of day 3 and day 6 DCs was compared (B) and is shown as a dotted line in the histogram (C), which also shows the activated level after TLR stimulation (solid line) at 90 min for NF-κB p65 (Ser276) and at 15 min for p38 MAPK (Thr180/Tyr182). The increase in phosphorylation level (phospho-increase) was determined at specific time points along stimulation and its kinetics was demonstrated (D). Data shown are representative of at least three experiments. p, phosphorylated.
or Ag was not absent. Notably, only day 3 DCs were able to stimulate significant IFN-γ production when loaded with OVA. Day 3 DCs are also much more potent in IL-2 induction than are day 6 DCs when loaded with OVA323–339.

**Day 6 ROShi DCs exhibit rapid innate cellular responses to pathogenic stimuli**

In addition to adaptive T cell stimulation, two innate aspects of DC functionality were investigated. DC spreading, which was observed after nonspecific cellular adhesion onto the tissue culture polystyrene surface, was first evaluated to indicate the capacity of DCs in initiating CD11b-mediated innate mechanisms (29–31). As shown in Fig. 7A, day 3 DCs, which represented ~35% of culture cells, adhered less frequently to the 6-well polystyrene surface after 2 h transfer than did day 6 DCs. Day 6 DCs strongly adhered to the culture surface with distinctive spreading morphology after 2 h stimulation with LPS or zymosan. In contrast, only ~50–60% day 3 DCs were adherent, with less spreading morphology (Fig. 7A). Subsequently, it was demonstrated that day 6 DCs expressed a significantly higher level of CD11b than did day 3 DCs (Fig. 7B). It is also noted that in contrast to the day 3 culture, day 0 BM cells contained a small CD11cloint population (2.5%), which could have minimally contributed to the day 3 DC population (Fig. 7B). In addition to DC adhesion, we examined the ability of DCs to release microbicidal ROS, including nitrite and H2O2. In response to LPS and zymosan, day 3 and day 6 DCs produced minimal levels of nitrite in 5 h. At 24 h of stimulation, day 3 DCs produced a higher level of nitrite than did day 6 DCs, although LPS appeared to be a much stronger nitrite inducer than zymosan (Fig. 7C). Day 6 DCs failed to produce a significant level of nitrite in the presence of zymosan (Fig. 7C); however, they produced a large amount of H2O2 in comparison with day 3 DCs (Fig. 7D). Although LPS-treated day 6 DCs generated high levels of H2O2 in 3 h, DCs with no stimulation also produced similar amounts. We propose that DC purification procedures could have triggered the cellular mechanism for H2O2 release. Zymosan appeared to be a stronger stimulator for H2O2 production than did LPS in day 6 DCs, perhaps owing to active induction of phagocytosis (Fig. 7D).

Having demonstrated that day 3 and day 6 DCs differed in H2O2 production, we tested how this should correspond with intracellular ROS. As shown in Fig. 7E, in contrast to day 3 DCs which showed upregulation of ROS within 5 h LPS or zymosan stimulation, day 6 DCs demonstrated overall reduction of ROS in the unpurified DC culture. Taken together, these results suggest that day 3 and day 6 DCs possessed differential adhesion and ROS mechanisms to target pathogenic stimuli. The surge of ROS is required for late-stage DC generation in an NADPH oxidase-independent manner

In addition to DC functionality, we evaluated whether ROS played a role in regulating DC generation. NAc-cys and GSH were used to scavenge ROS. Reduction of ROS was apparent when a dose of 7.5 mM or above was used. NAc-cys appeared to be more effective in scavenging ROS than was GSH (Fig. 7F). NAc-cys (15 and 30 mM) and GSH (30 mM) effectively reduced ROS generation of DCs and precursors in prolonged inflammation. Notably, severe ROS scavenging by NAc-cys at 30 mM resulted in drastic reduction of day 6 total or DC numbers, which were lower than initial day 3 counterparts, indicating extensive deprivation of ROS-caused cell death (Fig. 7G). In phagocytes, ROS generation was shown to be primarily NADPH oxidase mediated (11, 12, 32).
Accordingly, we investigated how blockade of NADPH oxidase could affect HPC expansion into DCs. When the NADPH oxidase inhibitor apocynin was used in a high concentration (400 μM), ROS production and the DC percentage were diminishingly affected (Fig. 8D). Total cell numbers in culture were unaffected by titrated apocynin (Fig. 8E). These data rule out the possible role of the NADPH oxidase protein family in regulating late-stage inflammatory DC expansion.

**Discussion**

ROS have been shown to mediate a wide range of cellular activities, such as activation, adhesion, proliferation, and apoptosis, through direct and indirect effects on signaling (14–17). Their role in innate immunity has been largely attributed to the control of microbial invasion by phagocytes (10). In this study, we report that the surge of ROS aligns with several cellular events of inflammatory GMDCs, including rapid cell expansion, altered signaling strengths, damped maturation, reduced T cell stimulation, increased expression of CD86 upregulation of GM or GM/IL-4 DCs (37–41), whereas others have shown intermediate to maximal activation (27, 42, 43) after TLR stimulation. In accordance with the maturation data, the capacity of late-stage DCs to stimulate allogeneic T cells is considered to maximally upregulated (35). In comparison with monocyte precursors, DCs display greater numbers of mitochondria. Rotenone, the respiratory chain complex I inhibitor, suppresses DC generation through inhibiting mitochondrial production of ROS (most likely H$_2$O$_2$) (35).

In pursuing the release of ROS during DC activation, it is demonstrated that a significant portion of high intracellular ROS in day 6 DCs is H$_2$O$_2$. Even after H$_2$O$_2$ release induced by 5 h LPS or zymosan stimulation, the ROS level remains higher in day 6 DCs in comparison with day 3 DCs (Fig. 7E). As ROS release from phagocytes is a relatively rapid process (22), this suggests that an additional fraction of ROS is constitutively present in day 6 DCs. ROS influences various physiological activities within different cellular compartments. In the steady-state, the nitrogen ROS are minimally produced in GMDCs unless nNOS is upregulated (10); hence, it is most likely that other ROS, such as the superoxide derivative H$_2$O$_2$, but not NO derivatives, constitute this fraction. Because ROS can react with a wide range of proteins that are involved in the processes of a cell cycle, including transcription, nucleotide metabolism, phosphorylation, and ubiquitylation (36), an additional amount of ROS elicited after day 4 may have an influence on any of these processes, enhancing the kinetics of HPC differentiation into GMDCs at the late developmental stage. To support this idea, ROS like H$_2$O$_2$ enhance tyrosine phosphorylation of the GM-CSF receptor β-chain and STAT5, leading to enhanced G$_1$- to S-phase transition in the cell cycle. Removal of ROS during GM-CSF stimulation causes reduced growth and viability of cells (8). Consequently, ROS scavenging with NAc-cys and GSH indeed reduces late-stage HPC expansion and DC generation (Fig. 8).

In comparison with day 3 ROS$^{lo}$ DCs, day 6 ROS$^{hi}$ DCs are low-maturation responders to LPS and zymosan stimulation. This observation urges critical revision of inconsistency in DC maturation studies, of which some have demonstrated minimal CD80 and/or CD86 upregulation of GM or GM/IL-4 DCs (37–41), whereas others have shown intermediate to maximal activation (27, 42, 43) after TLR stimulation. In accordance with the maturation data, the capacity of late-stage DCs to stimulate allogeneic T cells is considerably reduced in MLR. Furthermore, the nitrite response to LPS or zymosan, as well as the capability of ROS$^{lo}$ DCs in production of proinflammatory cytokines such as IL-6, IL-12, and MCP-1, is also diminished in ROS$^{hi}$ DCs (Figs. 6A, 7C). These observations underscore the effect of DC developmental stages on immunological assays.

**FIGURE 6.** Day 3 ROS$^{lo}$ and Day 6 ROS$^{hi}$ DCs demonstrate diverse capabilities in cytokine production and induction. A, Purified DCs (10$^5$) of day 3 or day 6 DCs in 100 μl were not stimulated or stimulated with LPS or zymosan for 20 h. The supernatant was collected and analyzed for IL-6, TNF-α, MCP-1, IL-12p70, IL-10, and IL-2 production by the CBA array. B, Purified T cells (2 × 10$^6$) in 100 μl were incubated with 2 × 10$^3$ DCs in the presence of media alone (not shown), OVA_{323-339}, or OVA. At day 5, the supernatant was collected and analyzed for IL-2, IFN-γ, and IL-4 production. *p < 0.05; **p < 0.01. NS, not stimulated.
In signaling studies, although the abnormality of steady-state signaling in day 6 DCs may be the outcome of homeostasis in the high ROS environment, the abnormality in the activated state is likely to be the result of the change in signaling equilibrium between NF-κB and MAPK subunits in the presence of ROS, leading to the reduced maturation response in day 6 DCs. To this end, the ability of ROS to mediate NF-κB and MAPK signaling has been well documented. ROS like H₂O₂ enhance NF-κB and MAPK signaling pathways in various cell types (44, 45). Conversely, exposure to H₂O₂ has been shown to diminish NF-κB activation in the crucial innate phagocytes, neutrophils (46). The role of ROS in cell physiology appears to be dependent on parameters such as ROS compartmentalization and cell types. Hence, in late-stage inflammatory DCs, it is possible that the excessive ROS (most likely H₂O₂) suppress NF-κB and enhance p38 MAPK activation after LPS and/or zymosan stimulation. Consequently, whether the change of steady-state signaling pathways of NF-κB and MAPK is attributable to the alteration of MR and CD11b expression (Figs. 4A, 7B) and how the change of activated signaling pathways correlates with the dramatic modification of cytokine responses to TLR ligands in DCs (Fig. 6A) remain to be determined.

In contrast to reduced maturation, ROS hi DCs demonstrate a strong capacity to process OVA in contrast to ROS lo DCs. However, their ability to stimulate OVA-specific CD4 and CD8 T cells is lower than that of ROS lo DCs. The difference in OTII T cell stimulation is particularly notable between OVA-loaded ROS hi and ROS lo DCs, most likely owing to the low capacity of ROS hi DCs to upregulate surface expression of MHC class II and costimulatory molecules (Fig. 5A). The reduced capacity of ROS hi DCs to stimulate Ag-specific T cells is in line with reduced MLR, which is

**FIGURE 7.** Day 6 ROS hi DCs are more capable of inducing immediate innate responses to microbial stimuli than are day 3 ROS lo DCs. A, Nonadherent or loosely adherent cells were gently removed from day 3 or day 6 culture and released into the 6-well plate. Cells were stimulated with LPS or zymosan at 37°C for 2 h. The adhesion or spreading capacity of stimulated and NS cells was examined with a 50× water immersion lens in the bright field under the Leitz DMRBE fluorescence microscope. DCs that were either floating (black arrow) or adherent (white arrow) in day 3 culture cells were identified by the distinctive size and/or dendrite morphology, whereas day 6 DCs, which constitute >80% of confluent culture cells, were not indicated. B, Cells from day 0, 1, 3, and 6 cultures, stained with anti-CD11b-PE and anti-CD11c-APC, were displayed in dot plots. C, Day 3 or day 6 purified DCs (2 × 10⁵) in 200 μl were seeded in the 48-well plate and stimulated with LPS or zymosan at 37°C for 5 and 24 h. Nitrite release by NS or stimulated cells was determined by the reaction with Griess reagent and measured by 540-nm absorbance. The concentration of nitrite was determined by the titrated sodium nitrite as a standard. D, Similarly, purified DCs were stimulated with LPS or zymosan for 1–3 h. H₂O₂ release by stimulated or NS DCs was detected by HRP-induced luminol chemiluminescence. The concentration of H₂O₂ was determined by the titrated standard H₂O₂. E, The redox state of day 3 or day 6 DCs that were NS or stimulated with LPS or zymosan for 5 h was examined by CM-H₂DCFDA, as shown and compared in the histogram. The shaded area represents non–CM-H₂DCFDA-treated cells. Data shown are representative of at least two experiments. **p < 0.01. NS, not stimulated.
largely costimulation dependent (28). Consequently, ROS\textsuperscript{hi} DCs are less efficient in IL-2 induction from T cells, resulting in low T cell expression and ROS fluorescence is displayed in the dot plot (A). Cells collected from each well were counted as total (B). DC numbers were also calculated based on the CD11c\textsuperscript{+} percentage in total cells (C). D, Titrated apocynin (50–400 \textmu M) and control solvent DMSO were added into DC culture in triplicates at day 3. Similarly, cells were collected at day 6 and labeled with anti-CD11c–APC, and the redox state was detected as described. E, Total cell numbers also were determined. Data shown are representative of two experiments.

Given that ROS\textsuperscript{hi} DCs are not as efficient as ROS\textsuperscript{lo} DCs in T cell stimulation, they express higher levels of MR and CD11b, which are involved in various innate functions of DCs. As a pattern recognition receptor, MR plays a role in Ag processing/presentation, pathogen recognition, and clearance. It is also implicated in cell migration and induction of cytokines (47). CD11b coupled with CD18 is the most promiscuous member of the integrin family in ligand recognition. It interacts with cellular matrix proteins, coagulant proteins, and the complement unit iC3b. Although CD11b mediates several leukocyte activities, such as phagocytosis and microbial recognition, to achieve innate defense, it is also critical for cellular adhesion and transendothelial migration (29–31). In assessment of cellular adhesion, ROS\textsuperscript{hi} DCs indeed demonstrate a high capacity to rapidly adhere and spread across the polystyrene tissue culture vessel. In line with this observation, ROS have been shown to be essential mediators of cell adhesion. Integrin-induced ROS enhance FAK phosphorylation and downstream events such as MAPK phosphorylation, leading to focal adhesion and cell spreading (14). In addition, ROS\textsuperscript{hi} DCs are highly capable of immediate H\textsubscript{2}O\textsubscript{2} release upon activation (Fig. 7D), which is indispensable for phagocytes to destroy invading pathogens (11). These observations point to a role for ROS\textsuperscript{hi} DCs in immediate microbial targeting.

By tracking cell division and development with CFSE and CD11b/CD11c labeling, respectively, GMDC ontogeny is partially characterized. The change in the cell distribution from day 0 to day 6 indicates that a large portion of CD11b\textsuperscript{lo} cells at day 0 may have the potential to differentiate into CD11b\textsuperscript{hi} and CD11b\textsuperscript{lo} DCs or CD11b\textsuperscript{lo} cells, as demonstrated at day 1 and day 3 (Fig. 7B). Whereas purified day 3 DCs could survive until day 6 with minimal cell death in the presence of GM-CSF (data not shown), the developmental capacity of day 3 CD11b\textsuperscript{lo} to become day 6 CD11b\textsuperscript{hi} remains unclear (Fig. 7B). Nonetheless, the transition of CD11b expression from day 0 to day 6 suggests that GMDCs are, in fact, a pool of cells derived from various developmental routes. For different types of HPCs—such as common myeloid progenitors, DC-committed progenitors, and monocytes—to become DCs, the number of division varies. In early culture, the rise of the DC percentage at day 1 (~15%) from day 0 (2–3%), with no decrease of CFSE fluorescence, indicates that some precursor cells are transformed into DCs without division (Figs. 1B, 7B). After day 1, HPCs undergo synchronized replication, in which DCs are continuously generated (Fig. 1B). The divisional hierarchy among HPCs and the transition of CD11b expression during culture development implicate the complexity associated with GMDC generation, which can potentially contribute to a level of heterogeneity at various developmental stages, given that they have been considered monocyte-derived Tip DC equivalents in their entirety.

In vivo, the development of inflammatory DCs is relatively fast. In GM-CSF–mediated inflammation, DCs can be generated in as little as 48 h (2). Under the pathogenic threat, it would appear critical that newly developed DCs efficiently sense PAMPs, prompt a strong cellular response, and effectively stimulate T cells, when the signaling pathways that mediate maturation and activation are optimal. The physiological significance of functional divergence between early- and late-stage DCs is intriguing. Late-stage GMDCs are capable of rapid adhesion, most likely because of high CD11b expression; however, these DCs are inefficient in migrating into the draining lymph node, in comparison with Fli-3L–induced steady-state DCs (42). In the course of microbial infection, as steady-state (resident) DCs migrate to the lymph node after the pathogen encounter, inflammatory ROS\textsuperscript{lo} DCs may be the early DCs derived from immediate precursors such as monocytes in a low number and rapidly recruited to the site of inflammation. They are highly responsive to PAMPs and highly capable of upregulating B7 and MHC class II molecules, with moderate capabilities in Ag uptake/processing and pathogen killing due to the dependence on ROS upregulation and nitrite production. ROS\textsuperscript{lo} DCs are potent in stimulating T cells, particularly CD4\textsuperscript{+} T cells. At the later stage (between day 4 and day 6) of infection, ROS\textsuperscript{hi} DCs can derive from accumulated precursors in high numbers owing to prolonged systemic GM-CSF exposure. ROS\textsuperscript{hi} DCs are specialized in direct microbial targeting but are less potent in T cell stimulation, as perhaps pathogen-specific T cell responses have been enhanced earlier by ROS\textsuperscript{lo} DCs. Therefore, ROS regulation in DCs may have a role in mediating the balance of innate and adaptive immune responses against microbial infection. In chronic inflammation in which GM-CSF is constantly produced, the capacity of DCs in T cell stimulation may be compromised because of high ROS induction. Although the proposed theory requires further validation, this is the first report demonstrating the role of ROS surge in DC generation and functions. Manipulating ex vivo GMDCs through their
developmental stage in association with ROS may provide a potential avenue to selectively enhance their functions, to achieve specific therapeutic applications.

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References