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Functional Manipulation of Dendritic Cells by Photoswitchable Generation of Intracellular Reactive Oxygen Species

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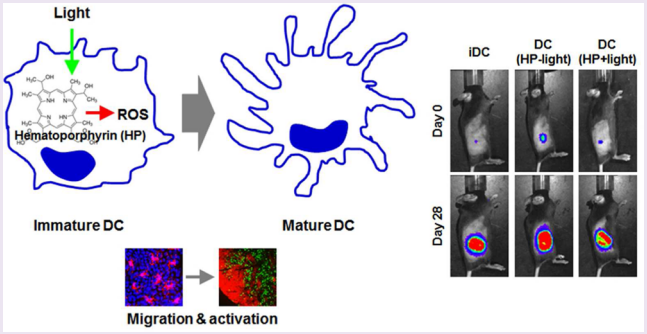
Supporting Information

ABSTRACT: Reactive oxygen species (ROS) play an important role in cellular signaling as second messengers. However, studying the role of ROS in physiological redox signaling has been hampered by technical difficulties in controlling their generation within cells. Here, we utilize two inert components, a photosensitizer and light, to finely manipulate the generation of intracellular ROS and examine their specific role in activating dendritic cells (DCs). Photoswitchable generation of intracellular ROS rapidly induced cytosolic mobilization of Ca^{2+}, differential activation of mitogen-activated protein kinases, and nuclear translocation of NF-κB. Moreover, a transient intracellular ROS surge could activate immature DCs to mature and potently enhance migration *in vitro* and *in vivo*. Finally, we observed that intracellular ROS-stimulated DCs enhanced antigen specific T-cell responses *in vitro* and *in vivo*, which led to delayed tumor growth and prolonged survival of tumor-bearing mice when immunized with a specific tumor antigen. Therefore, a transient intracellular ROS surge alone, if properly manipulated, can cause immature DCs to differentiate into a motile state and mature forms that are sufficient to initiate adaptive T cell responses *in vivo*.



Reactive oxygen species (ROS), which include highly reactive free oxygen radicals (e.g., $O_2^{\bullet}$ and OH^-) and nonradical oxidants (e.g., H_2O_2), are generated during mitochondrial respiration and cellular responses to diverse stimulation such as growth factors and pathogen infection.[1,2] Although excess ROS causes oxidative stress resulting in macromolecular damage and various disease states including cancer and aging, increasing evidence indicates that ROS also serve as critical signaling molecules in cell proliferation, differentiation, and survival.[1−3] In particular, ROS is directly involved in the activation of various cellular signaling pathways,[2] such as MAP kinase[4] and tyrosine kinase[5] signaling cascades via oxidation of redox-sensitive cysteine residues of target proteins. Transcription factors, including AP-1 and NF-κB, are also subject to redox regulation and lead to many biological changes, ranging from responding to growth factors to inflammatory responses.[3] Thus, it is now widely accepted that ROS function as important second messengers of intracellular signaling pathways.

Signaling ROS are generated at the cell surface or within intracellular compartments by multiple NADPH oxidases in response to diverse stimuli and then enter the cytoplasm.[1,6] Recent evidence suggests that ROS might preferentially enter the cell through specific plasma membrane aquaporin channels.[7] Additionally, generation of mitochondrial ROS has been shown to be tightly regulated and participates in physiological cell signaling associated with various stresses.[8] Within the cytoplasm, intracellular ROS potentially modifies cysteine residues of over 500 proteins, as revealed by large scale

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proteomic approaches,[9] thereby affecting a wide range of biological processes. Recent studies investigating the biological roles of intracellular ROS, however, have generally employed negative technical approaches. Technical drawbacks of using chemical antioxidants to scavenge ROS include nonspecific actions as they have multiple potential targets beyond ROS.[10] Single gene knockouts of ROS-generating enzymes also have limitations since intracellular ROS can be generated by multiple enzymes that respond to specific stimuli.[1,6,11] Exogenous addition of oxidants, such as H_2O_2, to a biological system could be used to monitor ROS-specific activation. Since the timing and location of ROS chemistry is tightly regulated in living systems, however, it is hard to mimic the biological system via simple addition of exogenous ROS or ROS-generating agents. Given that ROS quantity can determine specificity and function, tight regulation of ROS in timing and location is critical for their participation in physiological cell signaling.[8,12] Previously, several interesting techniques have been developed to produce intracellular ROS on demand and in a controlled fashion using light and a photosensitizer,[13] organelle-specific peptide conjugates with photosensitizers,[14] or a photocaged hydrogen peroxide generator.[15] They demonstrated that precisely controlled photogeneration of ROS induces differential activation of MAP kinases, cellular gene expression, and migration.[12] Here, we applied this technology to investigate the specific role of intracellular ROS in the functional regulation of dendritic cells (DCs), crucial sentinels orchestrating the innate and adaptive immune systems.[16]

ROS have been implicated in various physiological activities of DCs: their development from hematopoietic progenitor cells,[17] differentiation from monocytes,[18] maturation and antigen presentation after exogenous stimuli,[19,20] and cellular migration.[21] However, most of these studies, as mentioned above, utilized indirect technical approaches using ROS scavengers or single gene knockout to modulate the specific roles of ROS in DC functions. In order to investigate whether intracellular ROS alone can modulate DC activity, we examined cellular responses and the ability of DCs to induce antigen-specific T cell responses after regulated photogeneration of intracellular ROS.

RESULTS AND DISCUSSION

ROS Generation upon Photosensitization of Hematoporphyrin within DCs. We first measured the loading efficacy of a photosensitizer, hematoporphyrin (HP), in DCs after incubation in culture media. Since photosensitizers can be incorporated into diverse intracellular compartments[22] and have unique fluorescence properties,[23] we measured the relative uptake of HP in DCs by flow cytometry and its localization within cells by confocal microscopy (Figure 1A). HP was rapidly taken up by DCs, localized throughout the cytoplasm and nucleus, and saturated after 1 h of incubation. Immunofluorescence analysis showed that HP was barely colocalized with specific cellular organelles such as endosomes, lysosomes, or mitochondria (Supporting Information Figure S1). A total of 1 μg/mL of photosensitizer was sufficient to label more than 60% of DCs, and 4 μg/mL was sufficient to label almost 100% of DCs (Figure 1B). After 1 h of incubation of the cells with 1 μg/mL of HP, intracellular concentration of the photosensitizer was $0.66 \pm 0.04 \times 10^{-11}$ mol/10^6 cells. Next, the photogeneration of intracellular ROS was examined *in vitro* by phosphorescence decay signal[24] in DCs loaded with HP and a ROS-sensitive fluorescent dye, 2′,7′-dichlorodihydro-

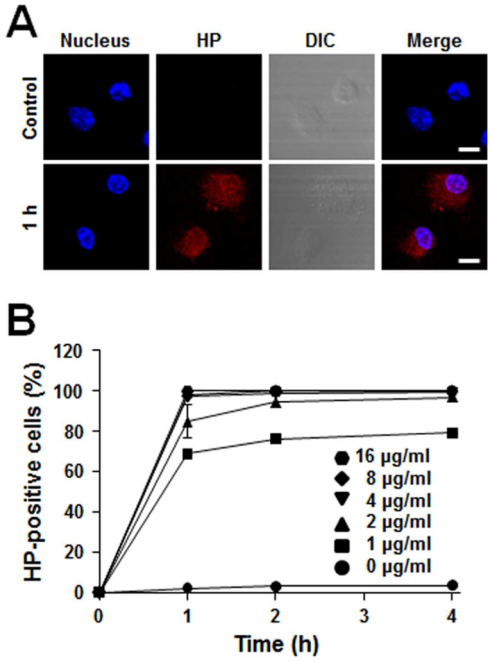

Figure 1. Intracellular delivery of HP into DCs. (A) DCs were incubated with 1 μg/mL of HP for 1 h, washed three times, fixed with 4% paraformaldehyde for 20 min, and then stained with DAPI for 10 min to detect nuclei of the cells. Intracellular distribution of HP was detected by confocal microscopy. Scale bar, 10 μm. (B) DCs were incubated with the indicated amounts of HP for the indicated time periods and then analyzed by flow cytometry. Data from three separate experiments are presented. Error bars, mean ± SD.

fluorescein diacetate (DCFDA) reactive to H_2O_2 and hydroxyl radical ($HO^{\bullet}$),[25] or Singlet Oxygen Sensor Green Reagent responsive to singlet oxygen ($^1O_2$). When labeled DCs were exposed to light from a light emitting diode (LED, peak wavelength 517 nm, power output 3.1 mW/cm^2), both of the ROS-responsive fluorescence gradually increased, demonstrating photogeneration of ROS throughout the illumination period (Figure 2A). In contrast, cells without photosensitizer did not show any changes in fluorescence levels during illumination. DCs loaded with increasing amounts of HP produced more intense fluorescence upon light exposure, indicating a correlation of photosensitizer concentration with increasing ROS generation. As a control experiment, we also measured intracellular ROS levels in DCs treated with different concentrations of exogenous H_2O_2 after labeling the cells with the ROS-sensitive dyes (Supporting Information Figure S2). Consistent with a previous report,[26] the intracellular H_2O_2 and hydroxyl radical signal was rapidly increased and saturated within a minute after the addition of exogenous H_2O_2 (Supporting Information Figure S2A and B). The relative fluorescent intensities representing the intracellular H_2O_2 and hydroxyl radical level were increased 1.6−2.4-fold depending on the concentration of H_2O_2 added in the media and gradually decreased at 5 min after incubation. A similar level of fluorescent intensities could be attained in HP-loaded DCs when the cells were exposed to light for 3 to 6 min depending of the amount of HP used for labeling (Supporting Information Figure S2C). It is notable that there is no detectable induction of singlet oxygen signal during the incubation with exogenous H_2O_2. In order to examine whether increased intracellular ROS generated by photosensitizer in DCs can induce cellular

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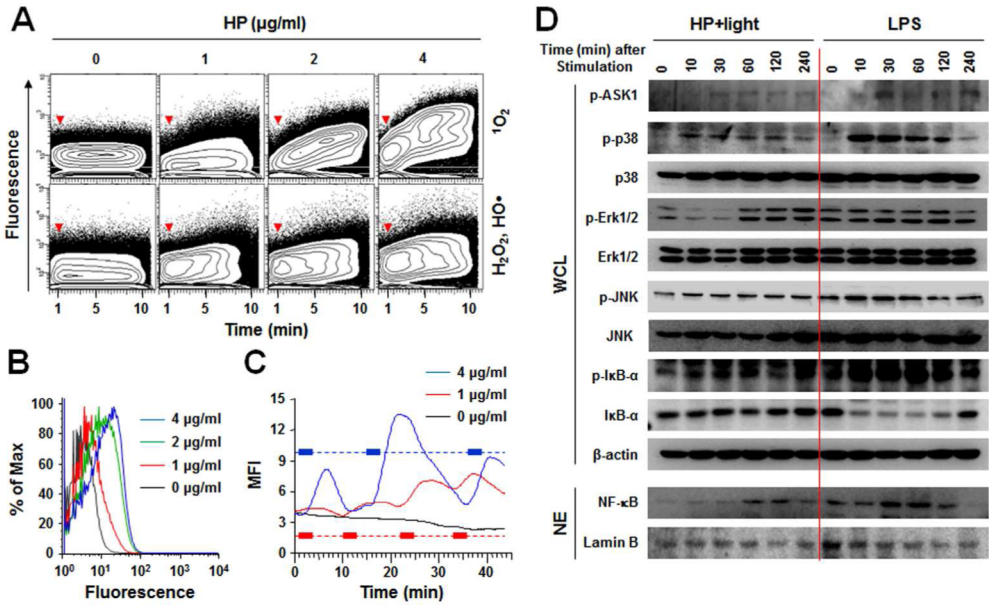

Figure 2. Activation of cellular signaling by photoswitchable generation of intracellular ROS within DCs. (A) Generation of intracellular ROS within HP-pulsed DCs by LED illumination. ROS level was monitored by loading the cells with ROS-sensitive fluorescent dyes, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) or *trans*-1-(2′-methoxyvinyl)pyrene (MVP). Red arrow indicates the start of LED illumination. (B) Intracellular calcium mobilization was monitored in HP-pulsed DCs after 3 min of illumination. Cytosolic calcium level was measured by loading DCs with a calcium-sensitive fluorescent dye, Fluo-4 AM. (C) Photoswitchable control of intracellular calcium level in HP-loaded DCs. DCs loaded with the indicated amount of HP and Fluo-4 AM were monitored by flow cytometry while the cells were exposed to LED light (3 min, thick lines) or in dark condition (dashed lines). (D) DCs were loaded with 1 μg/mL of HP, illuminated with LED for 3 min, and then incubated in dark for the indicated time periods. Cells were lysed and analyzed by immunoblot assays using the indicated antibodies. These data are representative of three independent experiments. LPS (1 μg/mL)-stimulated DCs were used as positive control. WCL, whole cell lysate; NE, nuclear extract.

signaling, we examined intracellular calcium levels, an important second messenger, by illumination of DCs loaded with photosensitizer and a calcium-sensitive fluorescent dye, Fluo-4 AM. Upon 3 min of light exposure, intracellular calcium levels increased in a HP concentration dependent manner (Figure 2B), indicating that photogeneration of ROS induces cellular signaling. Interestingly, intracellular calcium rapidly declined upon turning off the LED (Figure 2C). A reversible increase of intracellular calcium level was observed with re-exposure to the LED, suggesting that repeated photoswitchable generation of intracellular ROS can be used to manipulate calcium levels in DCs. In contrast to DCs loaded with 1 μg/mL of photosensitizer, which showed a gradual increase of intracellular calcium upon repeated light exposure, robust calcium mobilization was followed by reduced calcium response in DCs loaded with 4 μg/mL of HP. This might be due to cell death triggered by cellular calcium discharge and robust ROS generation.[27] Even though changes in intracellular calcium concentration are key triggers for diverse cellular functions, the molecular target for reversible calcium regulation by intracellular redox potential is largely unknown.[28] Some transient receptor potential (TRP) channels[29] or direct oxidation of cytoplasmic cyteins in the calcium channels might be responsible for the reversible changes of intracellular calcium by oxidation of regulatory proteins.[30] TRPM2 channels, located in endolysosomal vesicles within DCs and required for DC maturation and migration, might be potential regulators of ROS-mediated calcium release in DCs.[31,32]

To establish the optimal range of ROS photogeneration for cellular signaling, we monitored photocytotoxicity of DCs loaded with HP. The photosensitizer (up to 4 μg/mL) in the absence of light did not show any cytotoxicity for up to 7 days (Supporting Information Figure S3A). However, cell viability gradually declined with increasing concentrations (1−8 μg/mL) of photosensitizer or with increasing length (1−10 min) of illumination at 18 h after illumination (Supporting Information Figure S3B). In cells labeled with more than 2 μg/mL of HP, apoptotic cell death was significantly increased between 1 and 2 h after 3 min of illumination, as measured by annexin V staining (Supporting Information Figure S3C). In contrast, we did not observe any changes in apoptotic cell death after 3 min of illumination of DCs labeled with less than 2 μg/mL of photosensitizer.

Induction of DC Activation and Enhanced Migration by Photogeneration of Intracellular ROS. To examine the effects of photogenerated intracellular ROS on cellular activation, DCs incubated with 1 μg/mL of HP were exposed to light for 3 min, incubated in the dark for the indicated time points, and then lysed for the analysis of signaling activation (Figure 2D). Bacterial lipopolysaccharide (LPS), a potent DC activator, was used as a positive control. Substantial increases of phosphorylation of p38 and JNK MAP kinases were observed as early as 10 min after stimulation and were sustained for up to 4 h. Since p38 activation in DCs requires the activation of apoptosis signal-regulating kinase 1 (ASK1) by ROS,[33] which triggers the dissociation of thioredoxin from inactive ASK1 complex,[34] phosphorylation of ASK1 was also examined. Although the activation of ASK1 by intracellular ROS surge was weaker than that of cells stimulated with LPS, phosphorylation of ASK1 did increase, peaking at 30 min after stimulation. In contrast, ERK underwent rapid dephosphorylation 30 min after light exposure and gradually rephosphorylated until 4 h. A similar pattern of differential activation of MAP kinases was previously reported in mouse

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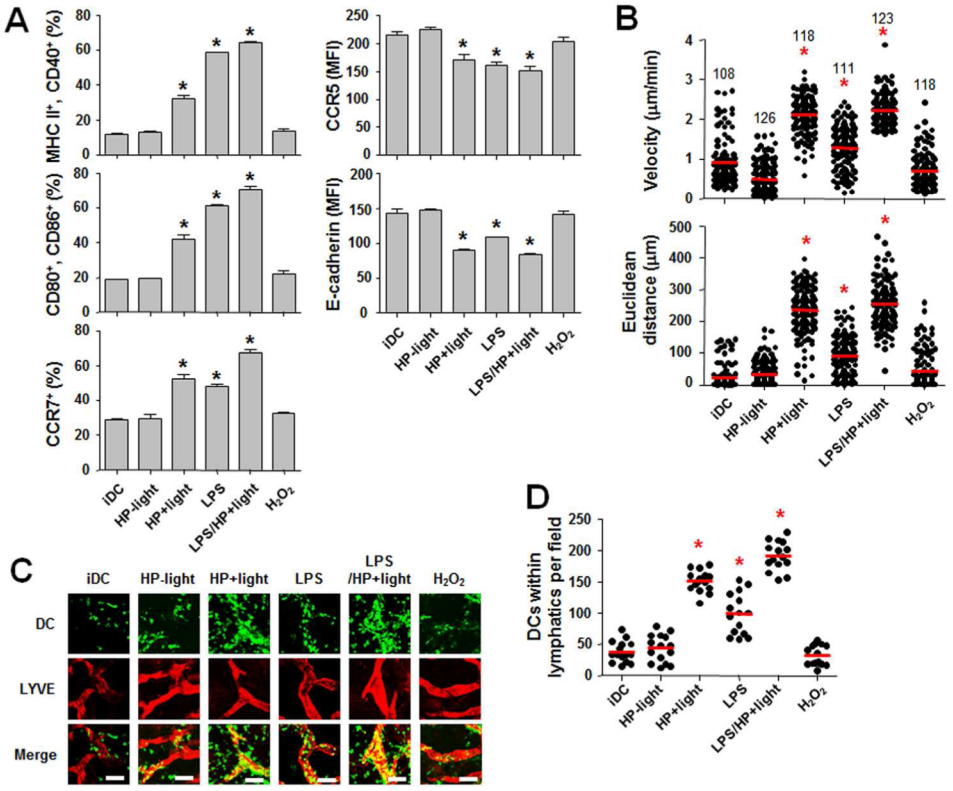

Figure 3. Activation and enhanced *in vitro* migration of DCs by photogeneration of intracellular ROS. (A) Activation of DCs was examined by flow cytometry measuring surface expression of the indicated activation markers at 18 h after various stimulation. Data are from three independent experiments. Error bars, mean ± SD. (B) *In vitro* chemotactic migration of DCs toward CCL19 gradient in 3D collagen matrix was measured at 18 h after the indicated stimulation. Velocity and Euclidean distance parameters were calculated and compared between the experimental groups. Numbers indicate the cell numbers analyzed. Red line, mean. (C and D) *Ex vivo* migration of DCs into lymphatic vessels was determined using crawl-in assay. DCs were incubated with the indicated stimuli for 18 h, stained with CFSE (green), placed onto the dermis of ear explants for 2 h. Lymphatic vessels were stained with anti-LYVE-1 antibody (red) after fixation (C). The number of DCs within lymphatic vessels was counted in 15 different fields of images. Red line, mean (D). iDC, unstimulated DCs; HP-light, HP-loaded DCs incubated under dark conditions; HP+light, HP-loaded DCs illuminated 3 min and then incubated in dark; LPS, LPS (1 μg/mL)-stimulated DCs; LPS/HP+light, HP-loaded DCs stimulated with LPS and 3 min of illumination; H_2O_2, DCs stimulated with H_2O_2 (300 μM). Scale bar, 100 μm. p values were calculated using one-way ANOVA followed by Tukey comparisons test. *, $p < 0.05$ when compared to iDC.

melanoma cells by photogeneration of intracellular ROS.[13] Additionally, we observed a slight increase of IκB phosphorylation and nuclear translocation NF-κB in DCs after transient illumination, suggesting a potential activation of DCs by photogeneration of intracellular ROS (Figure 2D). There were no detectable changes in these signaling molecules in the absence of illumination (data not shown).

Since we observed significant activation of cellular signaling in DCs by transient photogeneration of ROS, maturation of DCs was examined by measuring surface markers (MHC II, CD40, CD80, CD86, CCR7, CCR5, and E-cadherin) at 18 h after 3 min of illumination (Figure 3A and Supporting Information Figure S4). Substantial increases (more than 2-fold) of activation markers (MHC II, CD40, CD80, and CD86) were detected in DCs after photogeneration of intracellular ROS (HP+light), when compared to untreated control (iDC) or HP-treated DCs without illumination (HP-light), though the proportion of activated cells was around 20% lower than LPS-stimulated DCs (LPS). The activation level of DC(HP+light) was unaffected by increased concentration of HP (~8 μg/mL) or by extended illumination time (~10 min; Supporting Information Figure S5A and B). Increased activation of DCs by intracellular ROS surge was abrogated by pretreatment of cells with an antioxidant, N-actyl cysteine (NAC, 25 mM), suggesting that cellular activation might be mediated by oxidative stress (Supporting Information Figure S5C). In addition to the activation markers, expression of chemokine receptors and adhesion molecules also changed during DC maturation. Of note, CCR7, responsible for chemotactic migration of DCs from local inflamed tissue to draining lymph nodes,[35] was induced by transient intracellular ROS similar to LPS (Figure 3A). In contrast, surface expression of CCR5 and E-cadherin, which are down-regulated to mobilize the sentinel cells from the inflamed tissue upon activation,[36−38] were decreased in DCs stimulated with intracellular ROS or LPS (Figure 3A).

To examine whether exogenous ROS can induce DC activation, we added different concentrations of H_2O_2 in the culture media and measured the surface expression of activation markers at 18 h after ROS stimulation (Supporting Information Figure S5D and E). Although cellular viability rapidly declined in the presence of more than 2 mM H_2O_2, surface expression of activation markers was not significantly changed at any concentration of H_2O_2, in agreement with a previous study.[19] These results suggest that DCs are responsive to and activated by photosensitization of intracellular HP but poorly by exogenous H_2O_2. When cells were stimulated by transient photogeneration of intracellular ROS with LPS (LPS/HP

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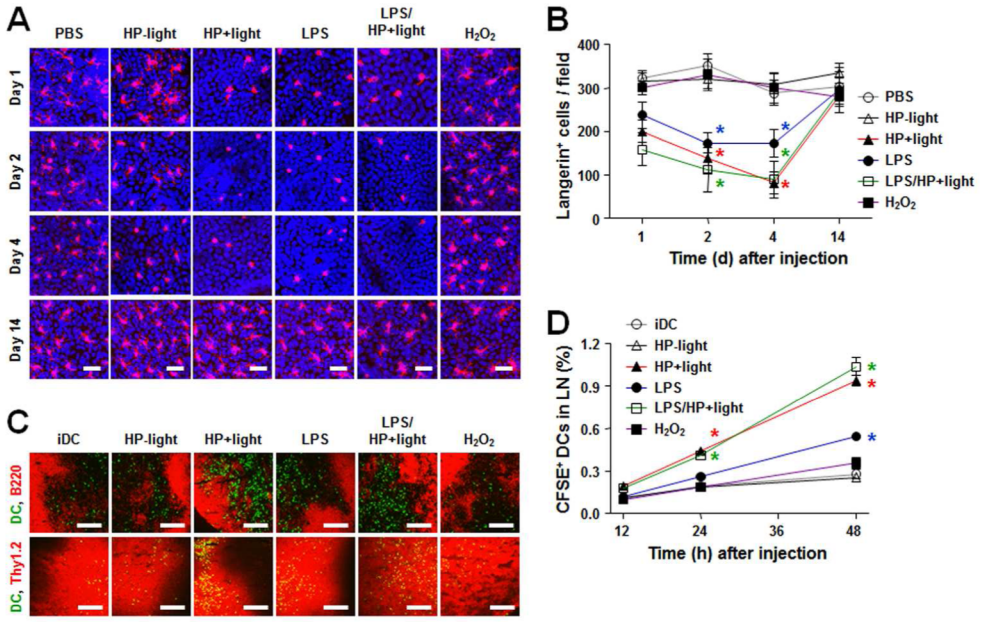

Figure 4. Enhanced *in vivo* migration of DCs by photogeneration of intracellular ROS. (A and B) Exit of Langerhans cells from ear epidermis in which the indicated agents were directly injected with or without illumination. C57BL/6 mice were then maintained in a dark cage for the indicated periods. Epidermal sheets from the mice were stained with antilangerin antibody (red) to detect Langerhans cells and ToPro-3 dye (blue) to identify cell nuclei (A), and the number of Langerhans cells in the ear epidermis was randomly calculated in 15 different fields of images. Scale bar, 20 μm (B). (C and D) DCs were treated with the indicated stimuli, stained with CFSE (green), and then injected subcutaneously into hind footpads of C57BL/6 mice. Popliteal lymph nodes were dissected at 2 days after injection and then stained with anti-B220 antibody (top) or anti-Thy1.2 antibody (bottom). Scale bar, 200 μm (C). After the indicated times after DC injection, percentile of CFSE-labeled $CD11c^+$ cells in popliteal lymph nodes was examined by flow cytometry (D). Data are from four independent experiments. Experimental groups are the same as in Figure 3. Statistical analysis was performed using two-way ANOVA followed by a Tukey comparison test. Error bars, mean ± SD. *, $p < 0.05$ when compared to iDC.

+light), surface expression of activation markers were further upregulated (Figure 3A). Pretreatment of cells with NAC abrogated the enhanced surface expression of activation markers under any stimulation conditions, suggesting an active role of oxidative stress in DC activation (Supporting Information Figure S5F).

The ability of activated DCs to migrate to secondary lymphoid organs where naïve T cells reside is a crucial step in the generation of primary T cell responses. DC migration to regional lymph nodes is a complex process composed of multiple steps, including movement to the tissue interstitium, entry into lymphatic vessels, and extravasation from the lymphatic system into lymph nodes.[39] In order to investigate the effect of intracellular ROS on DC migration, we used 3D collagen gels to mimic the interstitial microenvironment, and cells were exposed to a diffusion gradient of CCL19, a ligand for CCR7.[40] As shown in Figure 3B, the velocity and migration distance of DC(HP+light) were significantly enhanced when compared to those of iDC or DC(HP-light) ($p < 0.0001$). This enhanced migration by ROS photogeneration was even better than that of DC(LPS) ($p < 0.0001$) and comparable to that of DC(LPS/HP+light) ($p = 0.7209$). However, the migration of cells stimulated by exogenous H_2O_2 was similar to that of iDC. In order to examine the entry of DCs into lymphatic vessels, we used crawl-in assays in which fluorescently labeled DCs were placed on the dermis of ear explants. After incubation for 2 h, the numbers of DCs localized within the LYVE-1^+ lymphatic vessels were counted and compared (Figure 3C and D). As observed in the 3D collagen matrix, the *ex vivo* migration of DCs into lymphatic vessels was remarkably enhanced by intracellular ROS stimulation ($p < 0.0001$), whereas photosensitizer treatment without illumination or exogenous H_2O_2 stimulation did not. To confirm the enhanced migration of DCs *in vivo*, we employed two different methods. First, we directly injected HP (1 μg in 20 μL of phosphate-buffered saline, PBS) into ear dermises, left them in a dark cage for 2 h, and then illuminated the injection site for 3 min. Groups included mice injected with PBS (20 μL) plus illumination (PBS), photosensitizer without illumination (HP-light), LPS (1 μg in 20 μL of PBS), LPS plus HP with illumination (LPS/HP+light), and H_2O_2 (300 μM in PBS). At the indicated time point, ear epidermis at the injection site was examined for the distribution of residential Langerhans cells, an epidermal DC subtype (Figure 4A and B). Groups stimulated with HP+light, LPS, or LPS/HP+light showed a gradual reduction in the number of Langerhans cells up to 4 days after injection, and the number of cells completely recovered at 14 days after injection. The degree of DC reduction was most dramatic in mice stimulated with HP+light, whereas no significant change was observed in the control groups (PBS, HP-light, or H_2O_2). Second, fluorescently labeled DCs stimulated with the indicated agents were injected in the footpads of mice, and their popliteal lymph nodes were analyzed up to 2 days after injection to examine *in vivo* migration of DCs (Figure 4C and D). Again, we observed a more rapid increase of fluorescently labeled DCs in popliteal lymph nodes when cells were stimulated with intracellular ROS, compared to control groups (Figure 4D). We confirmed the increased DC migration into the T cell zone of lymph nodes by immunohistochemistry (Figure 4C). The consistent and enhanced chemotactic migration of DCs stimulated by transient intracellular ROS surge clearly indicates that intracellular ROS itself is sufficient to induce DCs to develop into a

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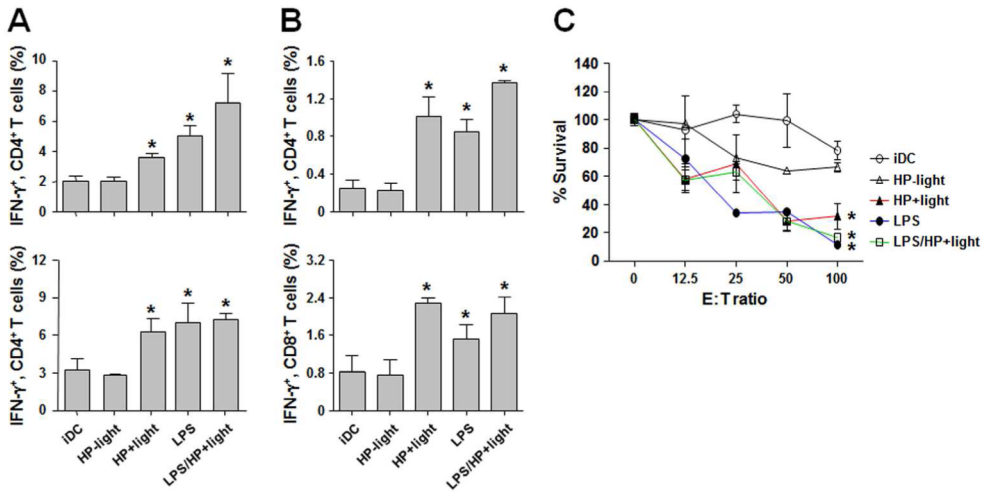

Figure 5. Induction of antigen-specific cellular immunity by ROS-stimulated DCs. (A) $CD4^+$ T cells were purified from the spleens of OT-II mice and cultured with stimulated DCs for 3 days with 20 μg/mL of endotoxin-free OVA. Then, cells were stimulated with 50 ng/mL of PMA and 1 μg/mL of ionomycin for 2 h and further incubated with 1 μg/mL of GolgiPlug for 2 h. The percentiles of OVA-specific IFN-γ^+CD4^+ T cells were determined by flow cytometry (upper panel). For *in vivo* stimulation assay, OT-II mice were immunized at the tail base with stimulated DCs loaded with $OVA_{323-339}$ peptides two times at weekly intervals. At 7 days after the second immunization, percentiles of OVA-specific IFN-γ^+CD4^+ T cells in spleens were determined by flow cytometry (lower panel). (B) C57BL/6 mice were immunized with DCs stimulated with the indicated agents twice at weekly intervals. At 7 days after the second immunization, percentiles of CEA-specific IFN-γ^+CD4^+ (upper panel) or IFN-γ^+CD8^+ (lower panel) T cells in spleen were determined by flow cytometry. Data are from three independent experiments. Experimental groups are the same as in Figure 3. (C) CTL assay was performed based upon crystal violet absorbance. CTLs were generated by stimulating the splenocytes with synthetic CEA peptide (EAQNTTYL) and IL-2 (20 U/ml) for 3 days followed by incubation with MC38/CEA cells as a target for 24 h. After washing, the target cells were stained with crystal violet (4 mg mL^{-1} in PBS) for 30 min at RT. The plate was then washed with PBS and the cells lysed using methanol. Target cell survival was analyzed by measuring the absorbance at 570 nm. Data are from three independent experiments. Statistical analysis was performed using two-way ANOVA followed by a Tukey comparisons\ test. Error bars, mean ± SD. *, $p < 0.05$ when compared to iDC.

mature and motile state, thereby increasing chemotactic efficiency. Previously, intracellular ROS surge[13] and ionizing radiation (potentially by the generation of ROS)[41] were shown to enhance cancer cell motility and cutaneous DC migration, respectively, but far less is known about the changes in the genetic programs that control DC migration during activation.[42,43] More detailed mechanisms of enhanced migration of DCs by intracellular ROS-stimulation need to be followed.

Induction of Adaptive Immunity and Antitumor Responses by Intracellular ROS-Stimulated DCs. Since mature DCs capable of priming naïve T cells not only express costimulatory molecules on their surfaces but also produce inflammatory cytokines such as IL-12,[16] we also examined proinflammatory cytokines produced by DC cultures stimulated with intracellular ROS surge and observed that substantial amounts of inflammatory cytokines such as IL-6, IL-10, IL-12, as well as TNF-α were secreted into the culture media (Supporting Information Table S1). Considering that DCs stimulated with exogenous H_2O_2 were shown to fail to express these cytokines except TNF-α,[44] our current data confirmed that intracellular ROS generated by photosensitization of HP is more potent than exogenous H_2O_2 in activating DCs. To investigate whether DCs stimulated with transient intracellular ROS surge can induce adaptive immunity, stimulated DCs were cocultured *in vitro* with $CD4^+$ T cells from OT-II mice in the presence of $OVA_{323-339}$. Flow cytometry analysis revealed a significantly increased number of IFN-γ-secreting $CD4^+$ T cells after incubation with DC(HP+light), DC(LPS), or DC(LPS/HP+light) compared with iDC or DC(HP-light) (Figure 5A, upper panel and Supporting Information Figure S6A). DC(LPS) significantly induced IFN-γ-secreting $CD4^+$ T cells to a greater extent than DC(HP+light). We also examined the Th1/Th2 responses in the DC-$CD4^+$ T cell cultures by measuring cytokines in the culture supernatant (Supporting Information Figure S7). IFN-γ and IL-4 levels were significantly increased and decreased, respectively, at 72 h after incubation with DC(HP+light), DC(LPS), or DC(LPS/HP+light) compared with iDC or DC(HP-light), suggesting that the stimulated DCs induce potent Th1 responses *in vitro*. When OT-II mice were immunized with DCs loaded with $OVA_{323-339}$ *in vivo*, the frequency of IFN-γ-secreting $CD4^+$ T cells in spleens was also significantly increased at 7 days after second immunization in DC(HP+light), DC(LPS), or DC(LPS/HP+light)-vaccinated groups compared with iDC or DC(HP-light)-immunized groups (Figure 5A, lower panel and Supporting Information Figure S6B). It is notable that the frequency of IFN-γ-secreting $CD4^+$ T cells in DC(HP+light)-immunized mice was comparable to those of DC(LPS) group after *in vivo* immunization.

To further prove the functional significance of adaptive immunity generated by DCs stimulated with an intracellular ROS surge, we immunized mice with DCs loaded with a tumor antigen, carcinoembryonic antigen (CEA)-derived T cell epitopes.[45,46] At 7 days after DC immunization twice at a weekly interval, mice immunized with DC(HP+light) showed comparable levels of CEA-specific $CD4^+$ and $CD8^+$ T cell responses to that of groups immunized with DC(LPS) and DC(LPS/HP+light) (Figure 5B). The frequencies of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells in spleens of the immunized groups were approximately 2- to 3-fold higher than that of mice immunized with iDC and DC(HP-light). In addition, DC(HP+light), DC(LPS), or DC(LPS/HP+light) vaccination induced efficient tumor cell-specific CTL responses compared with iDC or DC(HP-light) (Figure 5C). These results indicate that DCs

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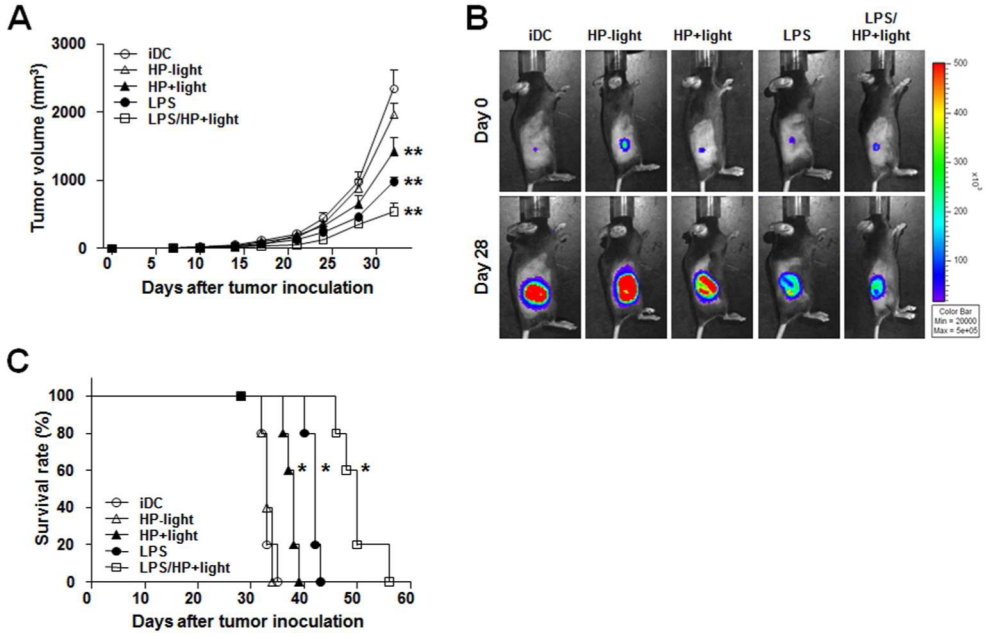

Figure 6. Tumor growth and survival of mice immunized with DCs. Mice (five mice per group) were inoculated with MC38 cells expressing CEA and firefly luciferase (1×10^5 cells/mouse) in the right flank. Mice were immunized with DCs stimulated with the indicated agents and loaded with CEA-derived peptides four times at weekly intervals starting from 1 week after injection. (A) Tumor growth was calculated by digital caliper. (B) Representative tumor bioluminescence imaging. (C) Survival rate of immunized mice was monitored until all the mice were sacrificed. Statistical analysis was performed using one-way ANOVA followed by a Tukey comparison test for tumor volume and the Kaplan−Meier method (log-rank test) for survival rate. Error bars, mean ± SD. *, $p < 0.01$; **, $p < 0.001$ when compared to iDC.

stimulated only with transient intracellular ROS can induce antigen-specific T cell responses as efficiently as LPS-stimulated DCs after repeated immunization. Consistent with the induction of systemic T cell immunity by DCs stimulated with intracellular ROS, CEA-expressing tumor growth was significantly delayed in mice immunized with DC(LPS/HP +light), when compared to mice immunized with iDC (p = 0.0001) (Figure 6A and B). Even though DC(LPS) immunization showed slightly enhanced suppression of tumor growth than DC(HP+light) (p = 0.1035), DC(LPS/HP+light) was superior to DC(LPS) (p = 0.0683) and DC(HP+light) (p < 0.0001). In correlation with these results, DC(HP+light) immunization significantly extended the mean survival period to 38 days from 33 days of tumor-bearing mice, compared to control groups, iDC and DC(HP-light) (p = 0.002 and 0.0026 respectively; Figure 6C). The mean survival period was further extended to 50 days in mice immunized with DC(LPS/HP +light). Therefore, transient photogeneration of intracellular ROS can induce the functional activation of DCs to prime antigen-specific and protective T cell responses *in vivo* and enhance the efficacy of DC vaccines when combined with a conventional immune adjuvant.

Photogeneration of ROS has long been employed in the field of photodynamic therapy for cancer treatment,[47] but most research has focused on the direct effects on cell death pathways[22] and the indirect inflammatory effects on antitumor immunity.[23] Here, we applied the technology in a new direction: controlled and functional manipulation of DCs, a potent antigen-presenting cell that orchestrates both innate and adaptive immunity.[16] Although several studies showed that controlled photogeneration of ROS in a mammalian cells can be used to manipulate cellular signaling and functions,[12,13] no other reports to our knowledge demonstrate the functional modulation of DCs by photoswitchable intracellular ROS generation despite their crucial role in the immune system. By using two inert components, a photosensitizer and light, transient photogeneration of intracellular ROS can efficiently induce DC activation and maturation without significant cellular death. It is interesting to note that incubation of DCs with exogenous H_2O_2 also rapidly increased intracellular H_2O_2 or oxygen radicals with different kinetics and amplitude compared to those generated by photosensitization of HP (Supporting Information Figure 2) but failed to induce DC activation. In addition, exogenous H_2O_2 did not generate intracellular singlet oxygen, which is efficiently formed by photosensitization. Even though it remained impossible to discriminate between the oxidative signaling effects of different ROS, it would be possible that free radicals and singlet oxygen may mediate a differential effect on cellular signaling *in vivo*.[48] Nevertheless, our current approach may not only provide a valuable tool for studying the role of intracellular ROS in cellular redox signaling of immune cells, but also contributes to the development of effective vaccine adjuvants that manipulate the immune system.

METHODS

Mice and Cells. C57BL/10NAGCSnAi-(KO) Rag2 (H-2^b) mice (Taconic Farms), OT-II TCR transgenic (H-2b) mice (Jackson Laboratory), and C57BL/6 (H-2^b) mice (Orient Bio) were housed and maintained in the specific pathogen-free facility at Seoul National University (SNU) College of Medicine. Animal experiments were performed after approval by the SNU IACUC (permission ID: SNU-090805-5). Tumor-bearing mice were humanly sacrificed when tumors reached 2 cm. The MC38/CEA/Luc cell line expressing human CEA and firefly luciferase was cultured and maintained in Dubecco's Modified Eagle Medium (DMEM; Welgene) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. DCs were generated from the bone marrow of 6- to 12-week-old Rag2 knockout mice and cultured as previously described.[49] In brief, the bone marrow

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cells were flushed out of the femurs and tibias with serum-free Iscove's modified Eagle medium (IMDM; Gibco Invitrogen). The single cell suspension was then filtered through a nylon cell strainer (70-μm Nylon mesh; BD Biosciences); washed twice with complete IMDM supplemented with recombinant mouse GM-CSF (1.5 ng/mL), mouse IL-4 (1.5 ng/mL; PeproTech), penicillin (100 units/ml), streptomycin (100 μg/mL), gentamicin (50 μg/mL), L-glutamine glutamine (2 mM), and β-mercaptoethanol (50 nM; Gibco Invitrogen); and seeded at a concentration of 1×10^6 cells per well in a 24-well plate in a final volume of 2 mL of complete IMDM medium. Half of the medium was replaced every other day with an equal volume of complete IMDM medium for 6 days. These *in vitro* DC cultures derived from bone marrow cells were analyzed by flow cytometry, which showed a typical yield of 90–93% of $CD11c^+$ DCs.

■ ASSOCIATED CONTENT

Supporting Information

The details of the other experimental methods are described in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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