Photoreceptor Protection after Photodynamic Therapy Using Dexamethasone in a Rat Model of Choroidal Neovascularization

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PURPOSE. To study whether corticosteroids protect photoreceptors when combined with photodynamic therapy (PDT) in a laser-induced model of choroidal neovascularization (CNV).

METHODS. PDT was performed in 36 Brown-Norway rats 2 weeks after laser induction of CNV. The expression of several cytokines and chemokines in the CNV lesions after PDT was measured by real-time PCR in combination with laser-capture microdissection. Immunostaining for monocyte chemoattractant protein (MCP)-1, C-C chemokine receptor 2 (CCR2), interleukin (IL)-1β, and myeloperoxidase (MPO) were performed. To study the effect of corticosteroids in combination with PDT, either dexamethasone (100 mg/kg) or control was injected intraperitoneally 1 hour before PDT. Animals were killed 24 hours or 1 week after PDT. CNV was examined by fluorescein angiography and choroidal flatmount. Photoreceptor degeneration was evaluated by TUNEL assay.

RESULTS. MCP-1 and IL-1β was increased in CNV lesions 24 hours after PDT. CCR2 was also expressed in laser-induced CNV but did not increase after PDT. Twenty-four hours after PDT, MPO-positive cells were noted in the CNV lesions. Dexamethasone-treated animals had significantly fewer TUNEL-positive photoreceptors in the photoreceptor layer than did the control animals (P < 0.05) after PDT. Fluorescein angiographic grading of CNV closure 6 days after PDT showed a closure rate of the dexamethasone-treated group of 31% (15/48 lesions) compared to 10% (4/42 lesions) in the control group (P < 0.05). CNV size was significantly smaller in the dexamethasone-treated group 1 week after PDT compared with the control (P < 0.05).

CONCLUSIONS. Systemic administration of dexamethasone combined with PDT reduces photoreceptor apoptosis, increases angiographic closure, and reduces CNV size compared with PDT alone in a rat model. (Invest Ophthalmol Vis Sci. 2008;49: 5008–5014) DOI:10.1167/iovs.07-1154

Photodynamic therapy (PDT) with verteporfin is an established treatment for subfoveal choroidal neovascularization (CNV) secondary to age-related macular degeneration (AMD).1–4 PDT offers greater selectivity than does traditional laser photocoagulation5 or transpupillary thermotherapy6 and now is part of the treatment regimen for CNV worldwide. The treatment is well tolerated, and severe visual loss can often be prevented.4 However, patients still lose vision over time with treatment, perhaps because of cumulative damage to the RPE and photoreceptors and also because of recurrent CNV.2,7 Thus, it is reasonable to make efforts to improve the efficiency and minimize the side effects of PDT, such as photoreceptor damage.

Preclinical studies of PDT in animal models have shown that there is limited damage to the retina in the rat8,9 and the monkey.10–14 Recently, we found that PDT causes TUNEL-positive photoreceptor degeneration in the rat and monkey, and one of the mechanisms of PDT-induced photoreceptor death is the increased expression of inducible nitric oxide synthase (iNOS) in macrophages after PDT.15 In an experimental cancer model, PDT was shown to induce a rapid inflammatory response, including infiltration of leukocytes and increased expression of cytokines, which cause further damage to the tissues.16 Up to now, the cellular response to PDT in the retina has not been investigated. It has been reported recently that PDT itself may cause an increase of VEGF expression in retina pigment epithelium (RPE).17,18 PDT triggers inflammation and angiogenic factor expression may cause damage to the normal tissues and even recurrence of CNV.

Corticosteroids have anti-inflammatory and antiangiogenic effects. Intravitreal triamcinolone acetonide (IVTA)19 and systemic administration of dexamethasone19 have been shown to reduce experimental CNV in animal models. However, clinical studies with IVTA alone used to treat CNV secondary to AMD has shown some anatomic effects, but has fail to demonstrate any visual benefits,20,21 and corticosteroids alone appear insufficient to treat CNV. In contrast, combining corticosteroids with PDT seem to yield better visual results and decrease retreatment rates.22–25 It has been hypothesized that PDT-induced inflammation may compromise the visual outcome of PDT despite its otherwise good selectivity for the target tissue. The factors regulating this inflammation and how inflammation compromises the visual outcome of PDT remain to be elucidated.

The purpose of this study was to investigate PDT-induced inflammation in experimental CNV and the molecular basis for dexamethasone modulation of PDT’s effects, including photoreceptor apoptosis and CNV regression.

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METHODS

Induction of Choroidal Neovascularization

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and using protocols approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Thirty-six male Brown-Norway rats (BN rats) 5 to 5 months of age were used for the studies. Rats were anesthetized with an intraperitoneal injection of 0.2 to 0.3 mL of a 50:50 mixture of 100 mg/mL ketamine and 20 mg/mL xylazine. Pups were dilated with 5.0% phenylephrine and 0.8% tropicamide.

CNV was induced in the eyes of rats with a 532-nm laser (OcuLight GLx; Iridex, Mountain View, CA), as previously described.8,15,26 Six laser spots (150 mW, 100 μm, 100 ms) were placed in each eye of the rat with a slit lamp delivery system and a cover slip serving as a contact lens. Production of a bubble at the time of laser confirmed the rupture of Bruch’s membrane. If significant hemorrhage occurred, the eye was excluded.

Photodynamic Therapy

PDT was performed as previously described for rats.8,15,26 Briefly, 2 weeks after laser injury, 6 mg/m2 verteporfin (Visudyne; Novartis, Basel, Switzerland) was injected in the tail vein as a bolus. Laser light of 690 nm was administered using a diode laser (Visulas 690s; Carl Zeiss Meditec, Jena, Germany) delivered through a slit lamp adaptor 12 to 20 minutes after verteporfin injection. The laser spot size was set at 800 μm and was confirmed with a micrometer. The irradiance used was 600 mW/cm2, which was delivered for 42 seconds to yield a fluence of 25 J/cm2.

Our rationale for systemic administration versus intraocular injection of this corticosteroid, pertaining to this animal model is as follows. Although intravitreous injections can be performed in rat eyes, the large lens, and small vitreous make it more likely to cause local injury than it does in human eyes. In this study, our purpose was to clarify the roles of PDT-induced inflammation, and we felt that systemic administration of dexamethasone was a reasonable approach to this question. One hour before PDT, dexamethasone (1 mg/kg; Sigma-Aldrich, St. Louis, MO) or vehicle was injected intraperitoneally. Dexamethasone was first dissolved in 100% ethanol alcohol to 1 mg/mL and then diluted in Dulbecco’s phosphated-buffered saline (DPBS) to 0.5 mg/mL. Fifty percent ethanol alcohol in DPBS was used as vehicle control. Rats were killed at 24 hours or 7 days after PDT.

Evaluation of CNV Size and Leakage in Rats

Six rats treated with dexamethasone and PDT and five control rats treated with vehicle and PDT were used for evaluation of CNV size and leakage. After 0.2 mL of 2% fluorescein sodium was injected intraperitoneally, fluorescein angiography was performed 1 and 6 days after PDT with a digital fundus camera (TICR501i; Topcon, Paramus, NJ). A choroidal neovascular membrane was defined as being closed after treatment if there was early hypofluorescence in the area of treated membrane.8,15 The angiograms were graded by two masked readers. Seven days after PDT, the size of the CNV lesions was measured in choroidal flatmount tissue with a small modification to methods reported previously.15,27 Briefly, the rats were anesthetized and perfused through the left ventricle with 20 mL PBS followed by 20 mL of 5 mg/mL fluorescein labeled dextran (FITC-dextran, MW = 2 × 106; Sigma-Aldrich) in 1% gelatin. The eyes were enucleated and fixed in 4% paraformaldehyde for 3 hours. The anterior segment and retina were removed from the eye cup. Four to six relaxing radial incisions were made, and the remaining RPE-choroid-sclera complex was flatmounted (Vectashield; Vector Laboratories, Burlingame, CA) and coverslipped. Pictures of the choroidal flatmounts were taken by microscope (Leica Microsystems, Wetzler, Germany). A computer program (OpenLab; Improvision, Boston, MA) was used by two masked investigators to measure the magnitude of the hyperfluorescent areas corresponding to the CNV lesions. The average size of CNV lesions in each animal was determined.

Laser-Capture Microdissection

To investigate the gene expression in CNV lesions, laser-capture microdissection (LCM) was performed in five rats, as previously described.28 Cells of interest were selectively removed from a tissue section and analyzed for RNA levels or other quantification. Briefly, 24 hours after PDT, the eyes were enucleated and embedded in OCT compound (Tissue Tek; Sakura Finetec, Tokyo, Japan). Sections (12 μm) were cut with a cryostat (Micron, Munich, Germany) and mounted on glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Sections were rehydrated in 75% ethanol and diethylyprocarbonate RNase-free (DEPC)-treated water twice, followed by dehydration with 75%, 95%, and 100% ethanol for 1 minute each and xylene for 5 minutes. LCM was performed at 70 mW with a spot size of 7.5 μm for 0.75 seconds to capture the cells in CNV lesions.

In the right eye of each animal, three CNV lesions in the superior retina were examined after PDT and CNV without PDT (three lesions in the inferior retina) served as the control. Two sections from each lesion that cut through the center of the CNV lesions were used.

RNA Extraction and Real-Time PCR

RNA was extracted (PicoPre RNA Isolation Kit; Arcturus, Mountain View, CA) followed by DNase treatment (Qiagen, Valencia, CA). Total RNA was eluted in 30 μL of elution buffer and 24 μL of the total RNA was subjected to reverse transcription (RT; SuperScript III First-Strand Synthesis System; Invitrogen, Carlsbad, CA). First-strand cDNAs were amplified with a real-time PCR thermal cycle (model 7700; Applied Biosystems, Inc. [ABI], Foster City, CA). Quantitative real-time polymerase chain reaction (qPCR) was performed (Sybergreen PCR core kit; ABI), as previously reported.29 The primers used are listed in Table 1. PCR products were confirmed by agarose gel electrophoresis. For relative comparison, we analyzed the Ct of the real-time PCR data with the comparative CT (ΔΔCt) method according to the manufacturer’s instructions (ABI). To normalize the amount of sample cDNA added to each reaction, the Ct value of the endogenous control (18sRNA) was subtracted from the Cts of each target gene.

Immunohistochemistry

Immunohistochemistry was performed as previously reported.15,20–21 Ten rats with CNV—five treated by PDT and five as control animals—were killed for immunohistochemistry. The eyes were fixed in 4% paraformaldehyde at 4°C overnight. The anterior segment and the lens were then removed, and the remaining eye cup was cryoprotected with 20% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4, 0.15 M NaCl). The eye cups were then embedded in OCT compound. Sections were cut at 10 μm with a cryostat (Leica Microsystems, Wetzler, Germany). After incubation with blocking buffer (PBS containing 10% goat serum, 0.5% gelatin, 3% bovine serum albumin, and 0.2% Tween 20) for 1 hour, the sections were incubated with one of the following primary antibodies: polyclonal rabbit anti-monocyte chemotactant protein (MCP)-1 (1:500; Peprotech, Rocky Hill, NJ), anti-myeloperoxidase (1:100; MPO, Laboratory Vision, Fremont CA), inter-leukin (IL)-β (1:200; Pierce Biotechnology, Rockford, IL), or goat anti-CCR2 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Slides incubated without any primary antibody were used as the control. The sections were then incubated with fluorescence-conjugated secondary antibody: goat anti-rabbit or rabbit anti-goat IgG Alexa Fluor 488 (Invitrogen-Molecular Probes, Carlsbad, CA). Sections were mounted with mounting medium with DAPI (Vectashield Vector Laboratories, Burlingame, CA). Pictures were taken by microscope (Leica). MPO-positive cells in the CNV lesions of rat eyes before and 24 hours after PDT were counted by two masked investigators and normalized by CNV area. The area of CNV was measured on computer (OpenLab; Improvision). The average of three slides from one CNV
lesion was calculated as well as the average of all the lesions from one animal.

TUNEL Assay
TUNEL staining was performed according to the manufacturer’s protocol (ApopTag Fluorescein In Situ Apoptosis Detection Kit; Chemicon, Temecula, CA) as previously described.15,28–30 Only sections that were cut through the middle of the CNV lesions were included. Briefly, cryosections of the eye cups were washed with PBS twice and then incubated with TdT enzyme at 37°C for 1 hour. The sections were washed three times in PBS for 1 minute and incubated with antidigoxigenin conjugate (FITC) in a humidified chamber for 30 minutes at room temperature, followed by three rinses with 0.1 M PBS. Sections were mounted with mounting medium with DAPI (Vectashield; Vector Laboratories).

TUNEL-positive cells in the CNV lesions of rat eyes at 24 hours after PDT with or without dexamethasone injection were counted by two masked investigators and normalized by CNV area. The area of CNV was measured using Openlab software. The average of three slides from one CNV lesion was calculated, as were the average of all the lesions from one animal.

Statistics
Unpaired Student’s t-test was used to compare the CNV size between different groups and paired Student’s t-test was used to analyze the change of cytokines after PDT. The χ² test was used to compare the CNV closure rate after PDT. Statistical significance was set at P < 0.05.

RESULTS

PDT-Induced Expressional Changes of Cytokines and Growth Factors in CNV
To study whether PDT causes an inflammatory response in CNV, we investigated the expressional change of inflammatory cytokines and the recruitment of immune cells after PDT. First, to investigate which inflammatory factors were upregulated in CNV after PDT, we performed LCM in combination with qPCR. The change of the mRNA levels of cytokines and growth factors in the CNV 24 hours after PDT quantified by real-time PCR

<table>
<thead>
<tr>
<th>Gene Names</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>GATGAAACGTGGCATACTGCTGATT</td>
<td>CGTGCGCGATTTTTGAGTCA</td>
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<td>MCP-1</td>
<td>ATGGGACGCTGGTGATCTCCTG</td>
<td>GACACCTCGCAGGTTGATCTCCT</td>
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<td>IL-1β</td>
<td>TGGCGGAGGCGGACGTGCTGATTG</td>
<td>ACACTAGTAGGCTGCTGATCAGT</td>
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<td>CCR2</td>
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<td>HGF</td>
<td>AGATGAGCATGACCAAGATGCTAT</td>
<td>AGGTGACAACTTCTGGACAC</td>
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<td>IGF-1</td>
<td>TTCAAGTCCTCGGTGTGGCACAAGG</td>
<td>GCTTCGCGGAGACGAGTCACATCT</td>
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As shown in Figure 1, MCP-1 was significantly increased in CNV 24 hours after PDT, MCP-1 (arrows) and IL-1β (asterisks) signals were detected in the CNV. In contrast, CCR2 signal was detected both in the cells in the CNV lesion (arrowheads) and the inner nuclear layer (INL) before PDT and did not seem to change afterward. Both the semiquantitative data at the mRNA level and the qualitative data at the protein level suggest that MCP-1 and IL-1β are increased in CNV after PDT.

Neutrophil Accumulation in CNV Lesions after PDT
As shown in Figure 1, MCP-1 was significantly increased in CNV after PDT. One of the well-known functions of MCP-1 is to recruit immune cells, such as neutrophils, monocytes, and lymphocytes. We have previously found that macrophage numbers did not change after PDT in the rat model. Here, to study whether PDT causes an inflammatory response in CNV, we counted the number of MPO-positive neutrophils before and after PDT by immunohistochemistry (Fig. 3). Before PDT, there were no MPO-positive cells in the CNV lesion, but 24 hours after PDT, numerous MPO-positive cells accumulated in the CNV lesion and the difference was significant (P < 0.001). This signal in the CNV lesions before PDT. Twenty-four hours after PDT, MCP-1 (arrows) and IL-1β (asterisks) signals were detected in the CNV. In contrast, CCR2 signal was detected both in the cells in the CNV lesion (arrowheads) and the inner nuclear layer (INL) before PDT and did not seem to change afterward. Both the semiquantitative data at the mRNA level and the qualitative data at the protein level suggest that MCP-1 and IL-1β are increased in CNV after PDT.

FIGURE 1. The change of the mRNA levels of cytokines and growth factors in the CNV 24 hours after PDT quantified by real-time PCR combined with LCM. The y-axis represents the ratio of the mRNA level in PDT-treated CNV lesions to those of control CNV lesions in the same eye. The right eyes of five rats were included. *P < 0.05 compared with control. MCP-1, macrophage chemoattractant protein 1; IL-1β, interleukin 1β; CCR2, C-C chemokine receptor 2; TNF-α, tumor necrosis factor; MIP-1α, macrophage inflammatory protein 1α; TGF-β2, transforming growth factor β2; Angio-1, angiotensin-1; Angio-2, angiotensin-2; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1.
suggests that PDT, acting perhaps through MCP-1, causes neutrophil recruitment to CNV lesion after PDT.

Reduction in Photoreceptor Apoptosis after PDT

To study whether corticosteroids can affect photoreceptor apoptosis after PDT, dexamethasone (1 mg/kg) was injected intraperitoneally 1 hour before PDT, and the number of TUNEL-positive cells in the photoreceptor layer 24 hours after PDT was quantified. The data showed that dexamethasone significantly reduced the number of TUNEL-positive cells in the photoreceptor layer 24 hours after PDT (Fig. 4) with 594 ± 157/mm² in the dexamethasone/PDT combination group compared to 1286 ± 182/mm² in the PDT only group (P < 0.05). Dexamethasone did not affect apoptosis in CNV after PDT. The number of TUNEL-positive cells in CNV lesions was 2688 ± 174/mm² in the combination group and 2037 ± 183/mm² in the PDT-only group (P > 0.05), respectively, at this time point. These data suggest that anti-inflammation treatment combined with PDT reduces photoreceptor degeneration after PDT without decreasing apoptosis in CNV.
Decreased CNV Leakage and Size after PDT

To investigate whether dexamethasone treatment has any effect on CNV closure after PDT, fluorescein angiography was performed 1 and 6 days after PDT. As shown in Table 2, CNV closure rate at 1 day after PDT was similar in the dexamethasone/PDT and the PDT only groups (P > 0.05). At 6 days after PDT, the combination group had a higher CNV closure rate (31%) than the control group (10%, P < 0.05). Measurement of CNV size after FITC-dextran perfusion of flatmount showed that dexamethasone/PDT combination (0.011 ± 0.001 mm²) significantly reduced CNV size 7 days after PDT compared to control (0.019 ± 0.002 mm², P < 0.05; Fig. 6).

DISCUSSION

To investigate whether an inflammatory response occurred after PDT in an animal model of CNV, we examined the expression of inflammatory cytokines and chemokines, and the recruitment of inflammatory cells (neutrophils). A variety of inflammatory cytokines have been reported to be increased after PDT of tumors. In the present study, we showed upregulation of MCP-1 and IL-1β in cells in CNV after PDT. MCP-1 is a prototypic inflammatory chemokine, which targets monocytes, T lymphocytes, and other cells expressing the C-C chemokine receptor (CCR2). Remarkably, MCP-1 not only provides chemotactic cues for the recruitment of immune cells from the bloodstream to the tissue but is also responsible for activation of residual immune cells. IL-1β mainly affects inflammatory processes, and recent observations have shown that IL-1β may act as a potent proangiogenic cytokine. In an in vitro system, IL-1β stimulates RPE and leads to more tube formation of the cocultured choroidal endothelial cells. Blocking of IL-1β inhibits tumor growth and neovascularization and CNV in animal models. IL-1β upregulates the expression of MCP-1 in activated RPE and MCP-1 plays an important role in IL-1β-induced angiogenesis in corneal and tumor angiogenesis via recruitment of immune cells. Thus, collaboration of IL-1β and MCP-1 is critical for the recruitment of immune cells in various tissues during the inflammatory response. Although others have shown the contribution of neutrophils and macrophages in the formation of laser-induced CNV in animal models, and our unpublished data showed a transient increase of neutrophils during CNV formation, the present study now demonstrates that neutrophils are also recruited to CNV after PDT. How these immune cells contribute to PDT treatment for CNV requires further study. However, the fact that the anti-inflammatory drug dexamethasone reduces PDT-induced photoreceptor death suggests that MCP-1, IL-1β, and the recruited immune cells play an important role in the PDT-induced inflammatory response and may promote both inflammation and angiogenesis, which could lead to further injury of photoreceptors and recurrence of CNV.

In experimental studies of PDT-induced tumor destruction, it is known that the cellular targets of PDT include tumor cells and microvasculature with a subsequent inflammatory and immune reaction. The inflammatory factors released and the inflammatory cells accumulated after PDT are very important for tumor destruction. PDT causes mild photoreceptor degeneration in experimental models. Recently, we showed that while the number of macrophages do not increase after PDT of CNV, activated macrophages cause photoreceptor degeneration via increased iNOS expression after PDT.

Table 2. CNV Closure Rate after PDT Graded on Fluorescein Angiography

<table>
<thead>
<tr>
<th>Lesions Closed at 1 Day</th>
<th>Lesions Closed at 6 Days</th>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>PDT + vehicle</td>
<td>42/48 (88%)</td>
</tr>
<tr>
<td>PDT + dexamethasone</td>
<td>32/36 (89%)</td>
</tr>
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</table>

* The number of CNV lesions graded as closed.
† The number of all the CNV lesions in that group which have gradable angiograms. There were six rats with 72 CNV lesions in the dexamethasone/PDT group and five rats with 60 CNV lesions in the PDT-only group.
In the present study, we found a significant increase of IL-1β and MCP-1 in CNV after PDT, and the presence of the MCP-1 receptor CCR2. In addition, intraperitoneal injection of dexamethasone reduced PDT-induced photoreceptor degeneration. This suggests that PDT upregulates inflammatory cytokines, which act in part by increasing iNOS activation in macrophages, resulting in photoreceptor degeneration.

The primary purpose of PDT is to destroy the endothelial cells in CNV. Our results show that dexamethasone/PDT combination therapy did not affect angiographic closure of CNV at 1 day after PDT, suggesting that inflammation is not critical for the initial closure of CNV after PDT. However, combined therapy did increase the CNV closure rate at 6 days after PDT, suggesting that inflammation may increase the reopening of CNV. It has been reported that there is a significant increase of VEGF in human RPE after PDT, and it is known that proinflammatory cytokines upregulate VEGF expression in various tissues. These suggest that PDT-induced inflammation may stimulate angiogenesis and cause CNV recurrence.

Although there is a potential possibility of unwanted influence of one lesion on the other, to collect enough tissue for PCR and to make sure the control and treated lesions are under same condition, we placed six CNV lesions in each eye: three lesions treated with PDT and three as control lesions. We have tried to place the lesions significantly distant from each other and PDT-treated and -untreated lesions in different quadrants. Using LCM combined with quantitative real-time PCR and immunohistochemistry, we found that MCP-1 and IL-1β were upregulated in the CNV after PDT and that numerous neutrophils accumulated in CNV after PDT, suggesting that PDT indeed causes a specific inflammatory response. Dexamethasone, combined with PDT reduced photoreceptor apoptosis, increased angiographic closure, and reduced the size of CNV at 1 week after PDT. Overall, anti-inflammatory treatment combined with PDT was beneficial compared with PDT alone in an experimental model. These findings may provide some insight into the mechanism of PDT of CNV and also provide the basis to develop new combination treatments, including anti-MCP-1, anti-IL-1β, and antineutrophil treatment to improve the efficacy of PDT and prevent unwanted side effects, such as photoreceptor damage.

**References**


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