Macrophage Depletion Inhibits Experimental Choroidal Neovascularization

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OBJECTIVE. To investigate the role of macrophages in the development of laser-induced choroidal neovascularization (CNV) by selective depletion with liposomal clodronate (Cl2MDP-LIP).

METHODS. Laser photoagulation was used to induce CNV in wild-type C57BL/6j mice. Animals were treated with intravenous (IV) and/or subconjunctival (SC) Cl2MDP-LIP or PBS-LIP at the following time points: 2 days before, immediately after, 2 days before and immediately after, or 2 days after laser injury. CNV responses were compared on the basis of en masse volumetric measurements and fluorescein angiography after laser photoagulation. Macrophages were identified by immunostaining for F4/80, and vascular endothelial growth factor (VEGF) expression was quantified by ELISA.

RESULTS. Macrophages invaded the site of laser injury within 1 day of photoagulation and peaked at 3 days. IV Cl2MDP-LIP significantly decreased the volume of CNV and angiographic leakage when administered 2 days before and/or immediately after laser injury, but not when administered 2 days after injury. SC Cl2MDP-LIP significantly decreased lesion volume when coadministered with IV PBS-LIP but not IV Cl2MDP-LIP. IV Cl2MDP-LIP was significantly more beneficial when administered 2 days before laser injury than immediately after, but combining SC Cl2MDP-LIP with IV treatment eliminated this difference. Reduction in CNV volume correlated with VEGF protein levels and number of infiltrating macrophages.

CONCLUSIONS. Generalized macrophage depletion reduced the size and leakage of laser-induced CNV and was associated with decreased macrophage infiltration and VEGF protein. These findings define the role of the macrophage as a critical component in initiating the laser-induced CNV response. (Invest Ophthal Vis Sc. 2003;44:3578–3585) DOI:10.1167/iovs.03-0097

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Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among the elderly in most industrialized nations,1 yet little is known about the molecular mechanisms of choroidal neovascularization (CNV), the angioenic process responsible for most severe visual loss in patients with AMD.2

The presence of macrophages in histologic studies of CNV has elicited interest in their role in the development of neovascular AMD. The spatiotemporal distribution of macrophages correlates with aborizing CNV in humans3 and in animal models.4 In patients with AMD, macrophages are in proximity to thinned and perforated areas of Bruch’s membrane5,6 and participate in digesting the outer collagenous zone of Bruch’s membrane,6 both of which facilitate the subretinal entry of CNV.

To test directly the hypothesis that macrophages play a causal rather than coincidental role in the development of CNV, we used the technique of pharmacological macrophage depletion with liposomal clodronate (Cl2MDP-LIP) in the laser-induced model of CNV, which captures many salient pathologic and molecular features of neovascular AMD. Although free Cl2MDP does not penetrate cell membranes and has a short circulating half-life, Cl2MDP-LIP is phagocytosed by macrophages and rapidly induces apoptosis7,8 without secretion of proinflammatory cytokines by the dying macrophages.9 Moreover, Cl2MDP-LIP appears to have a very selective effect on macrophages and phagocytic dendritic cells. Neutrophils and lymphocytes are not directly affected by this drug.10–12

We sought to assess the differential contribution of macrophages in the local (submandibular) lymph nodes versus that of the circulating, splenic, and hepatic macrophages to the development of CNV in this model. We administered Cl2MDP-LIP by intravenous (IV) injection, which leads to near complete depletion of splenic and hepatic macrophages and marginal zone dendritic cells within 24 hours, persisting for 1 to 2 weeks in mice,10 and/or subconjunctival (SC) injection, which leads to the depletion of macrophages from the draining submandibular lymph nodes.13

We attempted to differentiate the contribution of circulating versus resident retinal macrophages to CNV. Cl2MDP-LIP does not cross the blood–brain barrier (and presumably the blood–retinal barrier) until it is damaged by an inflammatory response.14 Therefore, we administered IV Cl2MDP-LIP before laser injury and/or after laser injury, because the latter but not the former would permit the drug access to resident macrophages in the retina. We also correlated the macrophage response to laser injury with the level of vascular endothelial growth factor (VEGF), which is operative in the development of CNV in mice15–21 to deduce a mechanism for the effect of macrophage depletion in this process.

METHODS

Animals

All animal experiments were in accordance with the guidelines of the University of Kentucky IACUC and ARVO Statement for the Use of
Animals in Ophthalmic and Vision Research. Male wild-type C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) between 6 and 8 weeks of age were used to minimize variability, because age22 and sex (Tanemura M, et al. IOVS 2001;42:ARVO Abstract 5300) can influence susceptibility to CNV. For all procedures, anesthesia was achieved by intramuscular injection of 50 mg/kg ketamine HCl (Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg xylazine (Phoenix Scientific, St. Joseph, MO), and pupils were dilated with topical 1% tropicamide (Alcon, Fort Worth, TX).

**Induction of CNV**

Laser photocoagulation (532 nm, 200 mW, 100 ms, 75 μm; Oculight GL, Iridex, Mountain View, CA) was performed on both eyes of each animal on day 0 by a single individual masked to drug group assignment. Laser spots were applied in a standardized fashion around the optic nerve, using a slit lamp delivery system and a coverslip as a contact lens. The morphologic end point of the laser injury was the appearance of a cavitation bubble, a sign thought to correlate with the disruption of Bruch’s membrane.

**Liposomes**

Clodronate (dichloromethylene diphosphonate; Cl2MDP) was a gift of Roche Diagnostics GmbH, Mannheim, Germany. Clodronate-liposomes (Cl2MDP-LIP) were prepared as follows. In short, 86 mg phosphatidylcholine (Lipoid EPC; Lipoid, Ludwigshafen, Germany) and 8 mg cholesterol (Sigma-Aldrich, St. Louis, MO) were combined with 10 mL of a clodronate (0.7 M) solution and sonicated gently. The resultant liposomes were then washed to eliminate free drug. Empty liposomes were prepared under the same conditions with phosphate-buffered saline (PBS; Invitrogen/Gibco, Grand Island, NY) instead of the clodronate solution. Animals received 200 μL Cl2MDP-LIP or PBS-LIP through the tail vein with a 30-gauge needle on day –2 (group 1), day 0 (immediately after laser injury; group 2), day –2 and day 0 (group 3), or day +2 (group 4). At these same time points, animals received 10 μL Cl2MDP-LIP in one eye and 10 μL PBS-LIP in the other injected into the subconjunctival space with a syringe (Hamilton, Reno, NV). Injections were performed in a masked fashion.

**Fluorescein Angiography**

Fluorescein angiography was performed with a camera and imaging system (TRC 50 IA camera; ImageNet 2.01 system; Topcon, Paramus, NJ) at 1 week after laser photocoagulation. The photographs were captured with a 20-D lens in contact with the fundus camera lens after intraperitoneal injection of 0.1 mL of 2.5% fluorescein sodium (Akorn, Decatur, IL). A retina specialist not involved in the laser photocoagulation or angiography evaluated the fluorescein angiograms at a single sitting in masked fashion.

**Volume of CNV**

One week after laser injury, eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4°C. Eye cups obtained by removing anterior segments were washed three times in PBS, followed by dehydration and rehydration through a methanol series. After blocking twice with buffer (PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich)) for 30 minutes at room temperature, eye cups were incubated overnight at 4°C with 0.5% FITC-isolectin B4 (Vector Laboratories, Burlingame, CA), which binds to terminal β-D-galactose residues on the surface of endothelial cells and selectively labels the murine vasculature, diluted with PBS containing 0.2% BSA and 0.1% Triton X-100. After two washings with PBS containing 0.1% Triton X-100, the neurosensory retina was gently detached and severed from the optic nerve. Four relaxing radial incisions were made, and the remaining RPE-choroid-sclera complex was flatmounted in antifade medium (Immuno-Mount Vectashield Mounting Medium; Vector Laboratories) and coverslipped.

Flamentous were examined with a scanning laser confocal microscope (TCS SP; Leica, Heidelberg, Germany). Vessels were visualized by exciting with blue argon laser wavelength (488 nm) and capturing emission between 515 to 545 nm. A 40× oil-immersion objective was used for all imaging studies. Horizontal optical sections (1 μm step) were obtained from the surface of the RPE-choroid-sclera complex. The deepest focal plane in which the surrounding choroidal vascular network connecting to the lesion could be identified was judged to be the floor of the lesion. Any vessel in the laser treated area and superficial to this reference plane was judged as CNV. Images of each section were digitally stored. The area of CNV-related fluorescence was measured by computerized image analysis with the microscope software (TCS SP; Leica). The summation of whole fluorescent area in each horizontal section was used as an index for the volume of CNV. Imaging was performed by an operator masked to treatment group assignment.

**Immunostaining**

At various times during the first week after laser injury, animals were injected with 1 mL FITC-isolectin B4 through the tail vein, and retinal-RPE-choroid-scleral or RPE-choroid-scleral flatmounts were prepared 30 minutes later. These were stained with antibodies against F4/80 (5 μg/mL; Serotec, Oxford, UK), expressed by retinal microglia and all macrophages save those in lymphoid organs,53 or leukocytes common antigen CD45.2 (5 μg/mL; eBioscience, San Diego, CA), which also identifies retinal microglia.24 Flatmounts were examined by scanning laser confocal microscopy. An optical density plot of the selected area was generated by a histogram graphing tool in the image-analysis software (Photoshop, ver. 6.0; Adobe Systems, Mountain View, CA) to obtain a quantitative index of macrophage numbers, as described previously.25 Image analysis was performed by an operator masked to treatment group assignment.

**VEGF ELISA**

At 3 days after injury by 12 laser spots, the RPE-choroid complex was sonicated in lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl2, 10 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM NaN3, and 1 mM EDTA with protease inhibitor; Sigma-Aldrich) on ice for 15 minutes. VEGF protein levels in the supernatant were determined by an ELISA kit (threshold of detection 3 pg/mL; R&D Systems, Minneapolis, MN) that recognizes all splice variants, at 450 to 570 nm (Emax Molecular Devices, Sunnyvale, CA). Duplicate measurements were performed in a masked fashion by an operator not involved in photocogulation, imaging, or fluorescence angiography.

**Statistics**

**Volume of CNV.** The dependent variable in the analysis was the average lesion volume per eye per mouse. A linear mixed model for a split plot design was constructed with two whole plot (between mice) factors. These are treatments (i.e., timing of the treatments relative to the laser injury) and intravenous (IV) administration (Cl2MDP-LIP or PBS-LIP). The split plot (within mice) factor was subcutaneous (SC) administration (Cl2MDP-LIP or PBS-LIP). Post hoc comparison of means was performed with a contrast among the means constructed with error terms, depending on whether the contrast was between or within mice, respectively. Because the variability among mice treated with IV Cl2MDP-LIP differed substantially from the variability among mice treated with IV PBS-LIP, the linear mixed model contained different variance components for these groups. Statistical significance was determined at the 0.05 level.

**F4/80 and VEGF.** Quantitative immunostaining and VEGF protein data were analyzed by ANOVA with the Dunnett multiple comparison test. Results were considered significant at P < 0.05.
RESULTS

Macrophages invaded the site of laser injury within 1 day, with a peak response at 3 days, followed by rapid disappearance by 5 to 7 days (Fig. 1A). IV C12MDP-LIP administration nearly abolished macrophage recruitment, whereas SC C12MDP-LIP blunted the macrophage response when administered with IV PBS-LIP, but did not confer added benefit to IV C12MDP-LIP (Fig. 1B). The peak macrophage number paralleled the maximal amount of VEGF protein detected in the laser lesion at 3 days ($r^2 = 0.988$; Fig. 1C) and the volume of CNV at 7 days ($r^2 = 0.986$).

Confocal planar analysis revealed marked spatial colocalization of macrophages and endothelial cells at the site of laser injury (Fig. 2). In addition, IV C12MDP-LIP, and SC C12MDP-LIP to a lesser extent, decreased both the peak number of macrophages and endothelial cell coverage in parallel. We also observed that, whereas macrophages were found in areas without endothelial cells, the converse was rarely the case, supporting the notion that temporarily macrophages precede and promote endothelial cell proliferation. During the first 3 days after laser injury, macrophages were concentrated in the choroidal base and central substance of the CNV lesion, but were sparse near its retinal apex (Fig. 3). Also, there was no difference in the density of macrophages and retinal microglia in the retina adjacent to the laser scar compared with the remainder of the retina, using either F4/80 (Fig. 3C) or CD45.2 (similar data not shown).

The stereotypical CNV response to laser injury was markedly inhibited by IV C12MDP-LIP and to a lesser extent by SC C12MDP-LIP (Fig. 4). Combined IV and SC C12MDP-LIP treatment 2 days before laser injury decreased CNV volume by 90.8% $\pm$ 3.1% ($P < 0.0001$), immediately after laser injury by 79.2% $\pm$ 11.1% ($P < 0.0001$), and before and immediately after laser injury by 91.7% $\pm$ 4.7% ($P < 0.0001$), compared with combined IV and SC PBS-LIP treatment (Fig. 5). However, when IV and SC C12MDP-LIP were administered 2 days after laser injury CNV volume was not significantly reduced ($P = 0.11$). IV C12MDP-LIP treatment reduced CNV volumes in eyes treated with SC C12MDP-LIP or SC PBS-LIP. When coadministered with IV PBS-LIP, SC C12MDP-LIP treatment 2 days before laser injury decreased CNV volumes by 24.5% $\pm$ 7.2% ($P = 0.05$), immediately after laser injury by 27.8% $\pm$ 8.2% ($P = 0.05$), and before and immediately after laser injury by 35.5% $\pm$ 7.9% ($P = 0.007$), compared with SC PBS-LIP; however, SC C12MDP-LIP did not augment the antiangiogenic effect of IV C12MDP-LIP.

Pair-wise comparison of the different groups (timing of administration) revealed that IV C12MDP-LIP was more effective when administered 2 days before laser injury than immediately after ($P = 0.05$) or 2 days after ($P < 0.0001$; Fig. 5E). Administering IV C12MDP-LIP immediately after laser injury did not provide added benefit when it was administered 2 days before as well ($P = 0.83$). When SC C12MDP-LIP was combined with IV C12MDP-LIP, drug treatment 2 days before was no more effective than immediately after ($P = 0.20$), but both were more effective than 2 days after ($P < 0.0001$).

At 1 week after laser photocoagulation, fewer lesions in C12MDP-treated animals exhibited fluorescein leakage (Fig. 6). Greater suppression of angiographic leakage was found when C12MDP-LIP was administered both before and immediately after laser injury.

DISCUSSION

To our knowledge, this study is the first to demonstrate that macrophage depletion by C12MDP-LIP inhibits the development of laser-induced CNV, validating our hypothesis that macrophages play a pivotal role in this process. C12MDP-LIP decreased the peak macrophage response in parallel with VEGF protein levels and total CNV volume. C12MDP-LIP administered before and/or immediately after laser injury inhibited CNV, whereas it did not exhibit any effect when administered 2 days after laser injury. This is presumably because macrophage depletion occurs roughly 24 hours after C12MDP-LIP exposure, by which time the peak macrophage response at the site of laser injury has occurred. These data show that macrophages, which previous histopathological studies of experimental and clinical CNV have shown to be closely associated with new vessels, play a causal not a coincidental role in the development of laser-induced CNV.

IV C12MDP-LIP–induced inhibition of CNV was not augmented by SC C12MDP-LIP, whereas the latter was observed to inhibit CNV partially when coadministered with IV PBS-LIP. The total volume of SC C12MDP-LIP administered to any single animal did not exceed 20 µl, which is insufficient to deplete splenic or hepatic macrophages. In a model of experimental autoimmune pigment-epithelial uveitis (EAPU), IV C12MDP-LIP, but not SC C12MDP-LIP, inhibited EAPU, suggesting that SC
FIGURE 2. Macrophages recruited after laser injury colocalized with endothelial cells, and both responses were inhibited by Cl2MDP-LIP. (A) Three days after laser injury in an animal treated with IV PBS-LIP, macrophages (arrows) stained by Cy5-F4/80 (blue) colocalized (arrowheads) with endothelial cells stained by FITC-Isolectin B4 (green). Colocalization by merging yielded a cyan color. (B) SC Cl2MDP-LIP partially inhibited the number of macrophages (arrows) and CNV volume. (C) IV Cl2MDP-LIP nearly abolished macrophage (arrows) and CNV response. The 1-μm sections with the greatest density of F4/80+ staining within laser scars are shown. Scale bar, 50 μm.

FIGURE 3. Macrophages in CNV were not recruited from the resident retinal population. (A) Three days after laser injury, numerous macrophages (arrow) stained by Cy5-F4/80 (blue) were present near the choroidal base of the CNV lesion (endothelial cells stained by FITC-Isolectin B4 appear green). (B) The highest density of macrophages (arrow), many of which colocalized with endothelial cells (merge yields cyan color; arrowheads) was present in the middle of the CNV lesion. (C) A paucity of macrophages were found at the retinal surface of the CNV lesion (perimeter outlined in white) and in the adjacent retina (R). One-micrometer-thick sections are shown. Scale bar, 50 μm.

FIGURE 4. Cl2MDP-LIP inhibited CNV 1 week after laser injury. IV Cl2MDP-LIP administered 2 days before and immediately after laser injury suppressed CNV volume (A) to a greater degree than when administered 2 days after laser injury (B). SC Cl2MDP-LIP administered 2 days before and immediately after laser injury in the presence of IV PBS-LIP partially inhibited CNV volume (C) compared with IV and SC PBS-LIP treatments at the same times (D). Stacked confocal images (1 μm sections) of FITC-Isolectin B4 labeled tissue within laser scars are shown. Scale bar, 100 μm.
The delivery of liposomes does not exert a systemic effect. Therefore, the observed beneficial effect of SC Cl2MDP-LIP on CNV may be attributed to depletion of regional lymph node macrophages. In aggregate, these observations suggest that the predominant pool of macrophages that infiltrate areas of laser-induced CNV is derived from the systemic circulation, although submandibular nodes make a minor contribution.

The origin of macrophages observed after laser injury has been the subject of much inquiry. We found that administering IV Cl2MDP-LIP immediately after laser injury, which provides access to resident macrophages, did not augment the inhibition of CNV induced by IV Cl2MDP-LIP 2 days before injury. We also found no infiltration of macrophages and microglia in the retina adjacent to the laser scar. Our data provide

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**Figure 5.** CNV volume was markedly diminished in Cl2MDP-LIP–treated mice 1 week after laser injury. Cl2MDP-LIP administered 2 days before laser injury (A), 2 days before and immediately after laser injury (B), and immediately after laser injury (C) demonstrated potent inhibition of CNV volume when Cl2MDP-LIP was administered IV and mild inhibition when it was administered SC. SC Cl2MDP-LIP did not provide added inhibition when administered with IV Cl2MDP-LIP, but provided moderate inhibition when coadministered with IV PBS-LIP. Neither route of administration provided significant inhibition when administered 2 days after laser injury (D). (E) Pair-wise comparison of CNV volumes between group 1 (treatment 2 days before), group 2 (2 days before and immediately after), group 3 (immediately after), and group 4 (2 days after) are presented. IV Cl2MDP-LIP was more effective when administered 2 days before laser injury (group 1 or 2) than immediately after (group 3) or 2 days after (group 4). When SC Cl2MDP-LIP was combined with IV Cl2MDP-LIP, drug treatments 2 days before (group 1 or 2) or immediately after (group 3) were more effective than at 2 days after (group 4). Administering IV Cl2MDP-LIP immediately after laser injury did not provide added benefit when it was administered 2 days before, as well (group 1 versus group 2). *P < 0.05 versus group 3, #P < 0.0001 versus group 4. All other differences NS. n = 5 for all groups.
direct anatomic and functional evidence that circulating rather than resident macrophages are the primary culprit in laser-induced CNV. This is consistent with the in vitro finding of polarized secretion of macrophage chemoattractant protein (MCP)-1 from the RPE into the choroid rather than the retina \(^{31}\) and the in vivo finding of MCP-1 in RPE and choroid, but not in the retina, of eyes with AMD.\(^{32}\)

A consensus has yet to emerge in quantifying experimental CNV: Both anatomic and functional metrics have been used. The former include measuring thickness and area on serial sections or volumes by confocal microscopy on RPE-choroidal flatmounts, aided by an endothelial cell marker. En masse volumetric measurements are less susceptible to nonorthogonality and loss or poor quality of sections than serial sectioning. Fluorescein angiography, which correlates with visual acuity in patients with AMD\(^{33–35}\) and also permits longitudinal evaluation of the evolution of the laser lesions unlike histopathological examination, reflects on the leakage of these lesions, which presumably correlates with their activity. We used both anatomic and functional metrics of measuring CNV to corroborate our findings: Cl\(_2\)MDP-LIP inhibited both the anatomic volume and the angiographic leakage of laser-induced CNV.

We have shown that the leukocyte adhesion molecules CD18 and intercellular adhesion molecule (ICAM)-1 play a key role in laser-induced CNV.\(^{36}\) Because liposomes do not interfere with leukocyte adhesion\(^{37}\) or rolling\(^{38}\) and PBS-LIP did not inhibit CNV, the antiangiogenic effects of Cl\(_2\)MDP-LIP can be attributed to depletion of macrophages alone. We infer therefore that the paracrine signals produced by macrophages promote the development of CNV. A likely signaling candidate is VEGF, as its levels were suppressed by Cl\(_2\)MDP-LIP in tandem with the number of macrophages, particularly because VEGF has been shown to be operative in CNV.\(^{35–41}\)

We postulate that Cl\(_2\)MDP-LIP aborted the early-phase response to laser injury, mediated by macrophage migration, perhaps in response to overexpression of MCP-1, a stereotyped wounding response\(^{39}\) that also occurs in RPE cells of AMD eyes (Spandau \(U, \text{et al.} IOVS 2000;41:ARVO Abstract 4440)\(^{37}\). In support of this hypothesis, we have demonstrated that genetic ablation of MCP-1 or its cognate receptor CCR2 markedly inhibits laser-induced CNV (data not shown). However, the inhibition of CNV volume in MCP-1- or CCR2-deficient mice (~75%) did not match the near abolition induced by maximal Cl\(_2\)MDP-LIP treatment, probably due to depletion by Cl\(_2\)MDP-LIP of macrophages responsive not only to MCP-1 but to other chemokines, such as macrophage inflammatory proteins-1\(\alpha\) and \(\beta\), that may play minor roles in recruiting macrophages.

Administering Cl\(_2\)MDP-LIP before or immediately after injury sharply reduced the number of macrophages in the site of laser injury, preventing the paracrine effects of these cells on endothelial cell migration and proliferation. VEGF, a major product of activated macrophages was reduced in parallel with the decrease in the number of macrophages. Laser photocoagulation leads to VEGF production by RPE cells,\(^{40}\) predominantly on the choroidal side,\(^{41}\) which itself can act as a macrophage chemoattractant.\(^{42–44}\) However, because VEGF was reduced by Cl\(_2\)MDP-LIP, it seems that macrophages contribute perhaps more toward upregulation of VEGF than RPE or that RPE secretion of VEGF may be induced, in part, by macrophage–RPE interaction. Through their own VEGF release\(^{44}\) macrophages can amplify the local VEGF response. Also macrophage-derived cytokines can stimulate VEGF production in RPE cells\(^{45}\) and choroidal fibroblasts.\(^{46}\) Macrophages can perpetuate their ingress by stimulating RPE cells to secrete MCP-1 into the choroid in a polarized gradient.\(^{31}\) In addition to VEGF, macrophages also may produce matrix metalloproteinases (MMPs) directly\(^{47}\) or through VEGF, which induces MMP expression in endothelial cells.\(^{48}\) These MMPs, which have been found in CNV in AMD,\(^{49}\) can facilitate endothelial cell migration during angiogenesis.

These findings may have some relevance to CNV in AMD, for although the laser injury model may involve processes not relevant to AMD, it captures many of the important features of the human condition. Laser photocoagulation that disrupts Bruch’s membrane can induce CNV in humans.\(^{50}\) Both in experimental models and in AMD, newly formed vessels that are functionally incompetent\(^{51,52}\) project into the subretinal space through defects in Bruch’s membrane. Aggregation of leukocytes near arborizing neovascular tufts\(^{53–55}\) is another shared feature of experimental and clinical CNV. Immunostaining has demonstrated the presence of VEGF and its receptors,\(^{40,55}\) basic fibroblast growth factor,\(^{54,55}\) transforming growth factor-\(\beta\),\(^{34,56}\) tumor necrosis factor-\(\alpha\),\(^{48}\) Fas, and Fas-ligand\(^{57,58}\) in cells of the CNV membranes in both conditions.

Because angiogenesis is a complex process with multiple redundant and intertwined cascades, it is remarkable that macrophage depletion alone nearly abolished CNV. This suggests, at least in this model of CNV, that macrophages and cytokines derived from them are requisite in this process and buttresses the growing body of evidence implicating leukocytes in the initiation of angiogenesis. Although macrophage inactivation could lead to immunosuppression, no overt infection was observed in our study involving transient macrophage depletion or by other investigators.\(^{59–61}\) Although the clinical implications of transient, partial depletion of macrophages with Cl\(_2\)MDP-LIP will be apparent only in human trials, MCP-1 or CCR2 may be attractive molecular targets, particularly with local drug delivery.\(^{59}\)

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