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Macrophages Restrict *Pseudomonas aeruginosa* Growth, Regulate Polymorphonuclear Neutrophil Influx, and Balance Pro- and Anti-Inflammatory Cytokines in BALB/c Mice

Sharon A. McClellan, Xi Huang, Ronald P. Barrett, Nico van Rooijen, and Linda D. Hazlett

The role of macrophages in *Pseudomonas aeruginosa* corneal infection in susceptible (cornea perforates), C57BL/6 (B6) vs resistant (cornea heals), BALB/c mice was tested by depleting macrophages using subconjunctival injections of clodronate-containing liposomes before corneal infection. Both groups of inbred mice treated with clodronate-liposomes compared with PBS-liposomes (controls) exhibited more severe disease. In B6 mice, the cornea perforated and the eye became extremely shrunken, whereas in BALB/c mice, the cornea perforated rather than healed. The myeloperoxidase assay detected significantly more PMN in the cornea of both groups of mice treated with clodronate-liposomes vs PBS-liposomes. In independent experiments, ELISA analysis showed that protein levels for IL-1β, macrophage-inflammatory protein 2, and macrophage-inflammatory protein 1α, all regulators of PMN chemotaxis, also were elevated in both groups of mice treated with clodronate-liposomes. Bacterial plate counts in B6 mice treated with clodronate-liposomes were unchanged at 3 days and were higher in control-treated mice at 5 days postinfection (p.i.), whereas in BALB/c mice, bacterial load was significantly elevated in the cornea of mice treated with clodronate-liposomes at both 3 and 5 days p.i. mRNA expression levels for pro (IFN-γ and TNF-α)- and anti (IL-4 and IL-10)-inflammatory cytokines also were determined in BALB/c mice treated with clodronate-liposomes vs control-treated mice. Expression levels for IFN-γ were significantly elevated in mice treated with clodronate-liposomes at 3 and 5 days p.i., while IL-10 levels (mRNA and protein) were reduced. These data provide evidence that macrophages control resistance to *P. aeruginosa* corneal infection through regulation of PMN number, bacterial killing and balancing pro- and anti-inflammatory cytokine levels.

In *Pseudomonas aeruginosa* corneal infections that can cause sight-threatening disease (1), experimental studies have centered on the roles of cytokines and chemokines (2–17) as well as APC (18, 19), polymorphonuclear neutrophils (PMN) (4, 5, 7, 8, 20), and T cells (5, 8, 21, 22) in generation of the susceptibility (cornea perforates) vs resistance (cornea heals) phenotype. B6 mice are susceptible to the infection and mounting evidence suggests that up-regulation and persistence of cytokines including, but not limited to, IL-1β (7, 11, 14), macrophage-inflammatory protein (MIP) 2 (4, 7, 17), IL-12p40 (9), IFN-γ (9, 10), and MIP-1α (8) are critical factors regulating this outcome. In addition, the B6 model, APC such as Langerhans cells (LC) provide for Ag presentation to CD4+ Th1 T cells (18), and it is the activated T cell that contributes directly to persistence of the PMN in the cornea and the corneal stromal destruction that ensues in B6 mice (8). In contrast, BALB/c mice, after similar infection, fail to up-regulate IL-12p40 and activated CD4+ Th1 T cells are not observed in the infected cornea of these mice (10), even using sensitive immuno-staining procedures. In addition, BALB/c, when compared with B6 mice, have more efficient up- and down-regulation of cytokines such as IL-1β and IFN-γ, and it is IL-18 that drives IFN-γ production in the cornea. IFN-γ was shown as necessary for efficient bacterial killing in the BALB/c cornea, but the mechanism remains undefined (10).

Studies have shown that if APC such as LC are induced into the cornea of BALB/c mice before infection, the disease response is altered from resistance to a susceptible (perforation) phenotype (19). This is accompanied by an influx into the stroma of not only PMN, as expected, but also by an increased number of macrophages and activated CD4+ Th1 T cells.

To study the role of macrophages in the response of susceptible and resistant mice to *P. aeruginosa* infection, we took advantage of the fact that macrophages can be selectively depleted through the administration of liposomes containing dichloromethylene diphosphonate (L-Cl2 MDP) or clodronate. Treatment with this drug from the liposomes leads to macrophage apoptosis by an unknown mechanism (27, 28). After their elimination, repopulation of the cell is variable, ranging from 1 wk to ~5 mo, depending on the site of depletion (24). In this study, we present clear evidence that in vivo depletion of these cells by subconjunctival injections of clodronate-containing liposomes leads to increased PMN in the cornea, failure to control bacterial growth, imbalance in pro- vs anti-inflammatory cytokines, and corneal perforation in BALB/c mice.

**Materials and Methods**

**Infection of mice**

Eight-week-old female B6 and BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized with isofluorane (Aerrane; Anaquest, Madison, WI), were placed beneath a stereoscopic microscope at ×40 magnification, and the cornea of the left eye was wounded with three 1-mm
incisions using a sterile 25-gauge needle (7, 21). A bacterial suspension (5 μl) containing 1 × 10^{5} CFU of *P. aeruginosa* ATCC strain 19660, prepared as described before (7), was applied to the eye surface. Eyes were examined at 24 h postinfection (p.i.) and/or at times described below to ensure that mice were similarly infected and to monitor disease. Animals were treated humanely and in compliance with the Association for Research in Vision and Ophthalmology resolution on usage and treatment of animals in research.

**Ocular response after infection**

Corneal disease was graded as described before (29): 0 = clear or slight opacity, partially or fully covering the pupil; +1 = slight opacity, fully covering the anterior segment; +2 = dense opacity, partially or fully covering the pupil; +3 = dense opacity, covering the entire anterior segment; and +4 = corneal perforation or phthisis. Mice (n = 5/group/time) treated with liposomes containing clodronate, PBS-liposomes, or sterile PBS were examined and a mean clinical score was calculated for each group to express disease severity (30). Slit-lamp photography was used to illustrate the disease response.

**Macrophage depletion**

Liposomes, composed of phospholipid bilayers and containing dichloromethylene-diphosphonate (clodronate), or PBS (control liposomes), were prepared as described before (26). Clodronate was provided by Roche Diagnostics (Mannheim, Germany). A total of 8 μl of the clodronate-containing liposome suspension was injected subconjunctivally in B6 and BALB/c mice using a 50-μl Hamilton syringe with a 30-gauge needle as generally described (28). This route of injection has been shown previously to deplete macrophages in the bulbar conjunctiva (28). On day −4 (day 0 = day of infection), clodronate-containing liposomes (5 μl/mouse/group) were injected; on day −2, 3 μl more was injected similarly. Corneas were infected (day 0) as described above. Control mice for each mouse strain received similar injections of either PBS-liposomes or sterile PBS. The latter was done to rule out the possible effect of nonspecific activation of macrophages by the PBS-liposomes. Since no significant difference in disease response was observed between these two groups, representative control data are shown only for PBS-liposome treatment. That macrophages were depleted/reduced in the anterior segment for up to 1 wk after clodronate-liposome injection was confirmed by acid phosphatase staining, as described before (19).

**Histopathology**

Eyes from mice treated with clodronate-liposomes or PBS-liposomes were enucleated at 1 and 3 days p.i. (B6) or at 5 days p.i. (BALB/c) (n = 3/group/time), rinsed in PBS, and fixed in 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorensen’s phosphate buffer (pH 7.4; 1:1:1) at 4°C for 3 h. Specimens were dehydrated in graded ethanols and embedded in plastic, and 1.5-μm sections were cut, stained, and photographed as described before (3, 4, 21).

**Myeloperoxidase (MPO)**

A MPO assay (31) was used to quantify PMN number in the infected cornea of B6 and BALB/c mice after injection of clodronate-liposomes or PBS-liposomes. Corneas were excised (n = 5/group/time) at 1 and 3 days p.i. and homogenized with glass tissue grinders in 1.0 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed three times and after centrifugation, a 0.1-ml aliquot of the supernatant was added to 2.9 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml) and hydrogen peroxide (0.0005%). The change in absorbancy at 460 nm was determined for each sample and the slope of the line was determined for each sample and at 3 and 5 days p.i. Individual corneas were homogenized in sterile 0.9% saline containing 0.25% BSA. Serial 10-fold dilutions of the samples were plated on *Pseudomonas* isolation agar (Difco, Detroit, MI) in triplicate and plates were incubated overnight at 37°C. Results are reported as log_{10} number of CFU/cornea.

**Bacterial load**

Bacteria were quantitated in the infected cornea of B6 and BALB/c mice after clodronate-liposome or PBS-liposome injection. Corneas (n = 5/group/time) were collected at 3 and 5 days p.i. Individual corneas were homogenized in sterile 0.9% saline containing 0.25% BSA. Serial 10-fold dilutions of the samples were plated on *Pseudomonas* isolation agar (Difco, Detroit, MI) in triplicate and plates were incubated overnight at 37°C. Results are reported as log_{10} number of CFU/cornea.

**RT-PCR**

Total RNA was isolated from individual corneas of B6 mice following injection of clodronate-liposomes or PBS-liposomes (n = 5/group/time) at 1, 3, and 5 days p.i. RNA (200 ng) was reverse transcribed using random primers and reverse transcriptase in the presence of 10 U of RNase inhibitor (Invitrogen, Grand Island, NY). Amplification of cDNA was conducted with Taq polymerase and specific primers for INF-γ, TNF-α, IL-4, and IL-10, all synthesized by Invitrogen, in a GeneMate Thermal cycler (ISC BioExpress, Kaysville, UT). Optimum conditions for the RT-PCR were established using routine methods (32). Conditions for RT-PCR were 94°C for 40 s, 60°C for 50 s, and 72°C for 1 min for 42, 30, 30 cycles for IFN-γ, TNF-α, IL-4, and IL-10, respectively, with a final extension at 72°C for 10 min. The primers used were 5′-TGATCATTG GCCGTGAGGCTTCTTCTTCTATTG-3′ (sense) and 5′-TGGACCTGT GGGTTGTTGACCTCAAACTTGGC-3′ (antisense) for INF-γ; 5′-GACA AGCCTGACCTCTGCTGACACTAAGTGACCGG-3′ (sense) and 5′-GCTC TAGAATGAGATAACAACTCGGTAGCGG-3′ (antisense) for TNF-α; 5′-GGGGGATTTGTGATCATCTCTTG-3′ (sense) and 5′-CATTCT CGTGGGTGTTCTCTCTCTGT-3′ (antisense) for IL-4; 5′-ACTCATATAGA ATTCGGGTTGCAAGGGCTATCTG G-3′ (sense) and 5′-CGTAAAGACA TCTGATTTCCGAGAGGCTTAAAGGCG-3′ (antisense) for IL-10; and 5′-GTTGGCGCGCTCCTAGGCCACCA-3′ (sense) and 5′-CCTTCT GATGTACCCGACATTTCC-3′ (antisense) for β-actin. The same amount of RNA (without RT reaction) was subjected to PCR amplification as a negative control to verify the absence of DNA contamination in samples. Twenty microliters of final PCR product was analyzed by electrophoresis (1.2% agarose gel with ethidium bromide). Bands were visualized under UV transillumination and quantitated using an Alphalager 2000 Documenta-
tion and Analysis System (Alpha Inotech, San Leandro, CA). Integrated density values for PCR products were corrected for the amount of β-actin on each sample. Data are expressed as the mean integrated density value of at least three samples from separate mice.

**Statistical analysis**

An unpaired Student’s t-test was used to determine the significance of the mean clinical score, MPO, bacterial load, protein, and mRNA assays. Data were considered significant at p < 0.05. All experiments were repeated at least twice and representative data, typical of a single experiment, are shown.

**Results**

**Infection in B6 mice after macrophage depletion**

To verify that macrophages were depleted by clodronate-liposome treatment, infected corneas were stained with acid phosphatase. Macrophages were reduced/absent at 1 (data not shown), 3, and 5 days p.i. in the clodronate- versus PBS-liposome-treated animals (Fig. 1). Disease severity was graded in B6 mice after macrophage depletion and the data are shown in Fig. 2. At 1 day p.i., mean clinical scores were similar in mice treated with clodronate-liposomes or PBS-liposomes (control; p = 0.6264). However, by 3 days p.i., mice treated with clodronate-liposomes had significantly increased disease severity (grade = +4, or perforated) when compared with the response of control mice (grades +2 to +3; p = 0.0032). Nonetheless, disease severity continued to progress in control-treated B6 mice and, as expected, ocular disease scores of +4 were observed by 5–7 days p.i.

Representative eyes from mice treated either with clodronate-liposomes or PBS-liposomes were photographed at 5 days p.i. using slit-lamp microscopy and these data are shown in Fig. 3. The eyes of the B6 mice treated with clodronate-liposomes were severely shrunk (phthisical; Fig. 3A), while PBS-liposome-treated
mice exhibited corneal perforation, with little shrinkage and appeared, in general, to be more vascularized (Fig. 3B).

**Histopathology**

Based upon the slit-lamp data, histopathology was used to further evaluate eyes from B6 mice treated with either clodronate-liposomes or PBS-liposomes and the data are shown in Fig. 4. At 1 day p.i., the eyes of PBS-liposome-injected mice (Fig. 4A) had an intense infiltrate present in the cornea that was concentrated in the superficial stroma. Centrally, the epithelium was denuded and rarely were infiltrating cells detected in the anterior chamber. In contrast, mice treated with clodronate-liposomes (Fig. 4B) exhibited a dense cellular infiltrate peripherally in the cornea and inflammatory cells were present in the deep stroma and anterior chamber. Epithelial defect also was observed centrally. By 3 days p.i., the epithelium was completely denuded in both PBS-liposome (Fig. 4C)- and clodronate-liposome-injected (Fig. 4D) B6 mice. An intense inflammatory infiltrate was present in the cornea and anterior chamber, but corneal stromal destruction was more pronounced (near perforation) in mice treated with clodronate-liposomes.

**Quantitation of PMN in cornea**

Because of the pronounced morphological changes and advanced disease and stromal destruction observed in B6 mice treated with clodronate-liposomes, an MPO assay was used to quantitate the PMN number in the cornea at 1 and 3 days p.i.. These data, expressed as units of MPO/cornea, are shown in Fig. 5. No significant difference in MPO levels between B6 mice injected with clodronate-liposomes or PBS-liposomes was detected at 1 day p.i. ($p = 0.4704$), but by 3 days p.i., the corneas of mice treated with clodronate-liposomes had significantly elevated MPO levels when compared with PBS-liposome-treated mice ($p = 0.0485$).

**Macrophage regulation of proinflammatory cytokines/chemokines**

Next, we determined whether cytokines and chemokines known to regulate PMN influx into the cornea were dysregulated in mice treated with clodronate-liposomes. For this, ELISA for IL-1β, MIP-1α, and MIP-2 proteins were used at 1, 3, and 5 days p.i. and

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**FIGURE 1.** Acid phosphatase staining in the cornea of B6 mice injected with clodronate-liposomes (A) or PBS-liposomes (B) at 3 days p.i. Magnification, ×35.

**FIGURE 2.** Ocular disease response in infected B6 mice after injection of clodronate-liposomes or PBS-liposomes. Ocular disease grades were averaged at individual times and results are reported as mean clinical score ± SEM ($p = 0.6264, 0.0032, 0.0892$, and $1.000$ at 1, 3, 5, and 7 days p.i. respectively).

**FIGURE 3.** Slit-lamp photomicrographs of *P. aeruginosa*-infected B6 eyes after injection of clodronate-liposomes (A) or PBS-liposomes (B). Representative eyes from each experimental group were photographed at 5 days p.i. Magnification, ×7.

**FIGURE 4.** Histopathology. Corneal sections from B6 mice treated with clodronate-liposomes or PBS-liposomes at 1 (A and B, respectively) and 3 (C and D, respectively) days p.i. In PBS-liposome-treated mice, inflammatory cells were evenly distributed throughout the cornea (A) with a heavier infiltrate at 3 days p.i. (C). Clodronate-liposome-injected mice exhibited inflammatory cells localized to the peripheral cornea at 1 day p.i. (B); by 3 days p.i., the macrophage-depleted cornea was thinned and near perforation (D). Magnification, ×18.
these data are shown in Fig. 6. In mice treated with clodronate-liposomes vs PBS-liposomes, protein levels for IL-1β (Fig. 6A) were elevated at 3 and 5 days p.i., but were significant only at 3 days p.i. (p = 0.0925, 0.0091, and 0.2608 at 1, 3, and 5 days, respectively). Similarly, MIP-1α protein levels (Fig. 6B) were elevated at 3 and 5 days p.i. in mice treated with clodronate-liposomes vs PBS-liposomes, but differences again were significant only at 3 days p.i. (p = 0.5945, 0.0023, and 0.1155 at 1, 3, and 5 days, respectively). Protein levels of MIP-2 (C) were significantly elevated in mice treated with clodronate-liposomes at 3 and 5 days p.i. (p = 0.0604, 0.0001, and 0.0479 at 1, 3, and 5 days p.i., respectively). Protein levels in the uninfected normal cornea were not detected for any of the cytokines/chemokines.

Quantitation of viable bacteria in infected cornea

Since clodronate-liposome injection resulted in elevated PMN number in the cornea at 3 days p.i., we quantitated viable bacteria in the cornea at 3 and 5 days p.i. with the expectation that bacterial load would be reduced. These data are shown in Fig. 7. No difference in bacterial load in the cornea was detected between the two groups (clodronate-liposome vs PBS-liposome) of mice at 3 days p.i., and by 5 days p.i., bacterial counts were significantly elevated (1 log increase) in PBS-liposome-injected B6 mice (p = 0.5296 and 0.0023 at 3 and 5 days p.i., respectively).

Infection in BALB/c mice after clodronate treatment

In light of a recent report that macrophages from B6 (a prototypic Th1 responder) vs BALB/c (a prototypic Th2 responder) mice may be dissimilar (cells designated as M1 vs M2 in type) (33), we next performed similar studies in BALB/c mice. Before these studies, we confirmed that macrophages were depleted by clodronate-liposome treatment by staining with acid phosphatase as described above for B6 mice. Macrophages were reduced/absent at 1–5 days p.i. (data not shown) in the cornea of clodronate- vs PBS-liposome-treated animals. No difference in mean clinical scores (Fig. 8) was observed at 1 day p.i. in mice treated with clodronate-liposomes vs PBS-liposomes (+1, p = 1.000). However, by 3 days p.i., mice treated with clodronate-liposomes exhibited increasingly more severe disease grades (+3 and +4) when compared with grades of +1 and +2 in mice injected with PBS-liposomes (p = 0.0003). By 5 days p.i., the corneas of all BALB/c mice treated...
with clodronate-liposomes had perforated when compared with disease grades of +1 to +2 in PBS-liposome-treated animals \( (p = 0.0001) \).

To document mean clinical score data, slit-lamp microscopy was used to visualize representative disease responses in mice treated with clodronate-liposomes or PBS-liposomes at 5 days p.i. and these data are shown in Fig. 9. Eyes of BALB/c mice treated with clodronate-liposomes had a dense inflammatory infiltrate and the cornea was thinned and/or perforated (Fig. 9A), whereas the eyes of PBS-liposome-treated mice exhibited a dense opacity covering the pupil \((+2)\) (Fig. 9B). Both of the corneas were vascularized. The severity of disease also was examined histopathologically at 5 days p.i. (Fig. 10). The corneas of mice treated with clodronate-liposomes were swollen, denuded of epithelium, and had a dense cellular infiltrate in the corneal stroma and anterior chamber (Fig. 10A). In contrast, the corneas of PBS-liposome-treated mice were less swollen, exhibited fewer inflammatory cells in the cornea and anterior chamber, and the epithelium was only partially denuded (Fig. 10B).

### Quantitation of PMN in cornea

Since slit lamp and histopathology provided evidence that macrophage depletion in BALB/c mice resulted in increased corneal disease and a greater inflammatory cell response, the MPO assay was used to quantitate PMN number in the cornea at 1 and 3 days p.i.. The data (Fig. 11) showed that MPO levels in the cornea of mice treated with clodronate-liposomes were significantly increased when compared with PBS-liposome-treated mice at 1 and 3 days p.i. \( (p = 0.0067 \text{ and } 0.0004, \text{ respectively}) \).

### ELISA analysis of IL-1β, MIP-1α, and MIP-2 protein levels

Significantly elevated levels of IL-1β \( (A, p = 0.0001 \text{ and } 0.0002) \), MIP-1α \( (B, p = 0.0001 \text{ and } 0.0003) \), and MIP-2 \( (C, p = 0.003 \text{ and } 0.00002) \) were detected in clodronate-liposome- vs control-injected mice at 1 and 3 days p.i.. Protein levels in the uninfected normal cornea were not detected for any of the cytokines/chemokines.
Macrophage regulation of PMN attracting cytokines

To determine whether increased PMN number in cornea following macrophage depletion in BALB/c mice resulted from dysregulation of cytokines/chemokines that are chemoattractants for PMN, protein levels for IL-1β, MIP-1α, and MIP-2 were quantified by ELISA at 1 and 3 days p.i. and these data are shown in Fig. 12. In macrophage-depleted vs PBS-liposome-treated mice, protein levels were significantly elevated at 1 and 3 days p.i. for IL-1β (Fig. 12A; p = 0.0001 and 0.0002, respectively), MIP-1α (Fig. 12B; p = 0.0001 and 0.003, respectively), and MIP-2 (Fig. 12C; p = 0.003 and 0.00002, respectively). Basal levels of proteins for these cytokines were not detected in the normal uninfected cornea.

Quantitation of viable bacteria

Since PMN were elevated after macrophage depletion, direct plate count was used to quantitate bacterial load in the cornea of BALB/c mice treated with clodronate-liposomes vs PBS-liposomes and these data are shown in Fig. 13. Approximately 2 logs more viable bacteria were recovered from the cornea of macrophage-depleted vs control-treated mice at 3 days (11.6-fold greater CFU, p = 0.0261) and 5 days (80.4-fold greater CFU, p = 0.0149) p.i.

Expression of Th1 vs Th2 cytokine mRNA and IL-10 protein levels

To determine whether injection of clodronate-liposomes shifted the production and/or balance of prototypic Th1 (IFN-γ, TNF-α)- and Th2 (IL-4, IL-10)-type cytokines in cornea, RT-PCR was used and the data are shown in Fig. 14. Significantly elevated mRNA expression levels for IFN-γ (Fig. 14A) were detected in cornea from mice treated with clodronate-liposomes at 3 days (p = 0.001) and 5 days (p = 0.006) p.i., whereas no significant difference in TNF-α (Fig. 14B) or IL-4 (Fig. 14C) mRNA expression levels were detected. In contrast, mice treated with clodronate-liposomes had significantly lower mRNA expression levels of IL-10 (Fig. 14D) in the cornea at 1, 3, and 5 days p.i. (p = 0.003, 0.024, and 0.012, respectively) when compared with mice treated with PBS-liposomes. Basal mRNA expression levels for each of the cytokines were not detected in the normal uninfected cornea. Protein levels of IL-10 in cornea also were determined in clodronate-liposome- and PBS-liposome-treated mice (Fig. 15). Clodronate-liposome-treated mice exhibited reduced IL-10 protein levels at both 3 and 5 days p.i. (p = 0.24 and 0.001, respectively), but only the latter time was significant.

Discussion

Macrophages are essential for host defense (33–36). They participate in both innate and specific immunity and have numerous functions including phagocytosis, Ag processing/presentation, secretion of pro- and anti-inflammatory cytokines, and production of...
reactive oxygen and nitrogen intermediates. After stimulation with microbial products, macrophages secrete several proinflammatory products such as TNF-α, IL-12, IL-1, IL-6, and NO, followed later by secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (37). Despite their general beneficial role in host defense, sustained production of cytokines and NO by these cells can lead to serious pathological conditions such as septic shock, inflammatory bowel disease, and respiratory distress syndrome (38).

Compelling evidence suggests that macrophages from Th1 (e.g., B6) T cell responder mouse strains are more easily activated than those from Th2 (e.g., BALB/c) strains (39, 40) and that the cells express distinct metabolic programs (33, 41). That macrophages themselves may determine immunologic outcomes is also suggested by results showing that Leishmania infection of macrophages can increase their ability to stimulate a Th2- instead of a Th1-type immune response (42). With these considerations in mind, the role of the macrophage in the host response to P. aeruginosa ocular challenge was tested in B6-susceptible (cornea perforates) and -resistant (cornea heals) BALB/c mice by depletion of macrophages by subconjunctival injections of liposomes containing clodronate before infection. This treatment dramatically increased the onset and/or severity of disease in both mouse strains. B6 corneas perforated earlier (3 vs 5–7 days p.i.) and eye shrinkage in the macrophage-depleted group, confirmed by slit lamp and histopathology, was exacerbated. In BALB/c mice, the corneas of macrophage-depleted mice perforated by 5–7 days p.i., changing their usual resistance response to susceptibility. These data are similar to the effects of macrophage depletion in a Chinese hamster model of Acanthamoeba-induced disease which resulted in enhanced incidence, severity, and chronicity of keratitis (43). As in the P. aeruginosa model described herein, macrophage-depleted animals had greater clinical disease (similar to B6 and BALB/c), with an earlier onset (similar to B6) and a prolonged chronic course (similar to BALB/c), suggesting that macrophages play an important role in both of these models. In contrast, in experimental pulmonary tuberculosis in mice, macrophage depletion by intratracheal administration of clodronate-containing liposomes resulted in improved clearance of M. tuberculosis bacilli, reduced outgrowth of mycobacteria in the lungs and liver, and a polarized production of type 1 cytokines in the lung that was protective (44).

Further support for the role of the macrophage in controlling microbial replication in the eye is provided in a model of viral infection of the eye. Cheng et al. (28) investigated the role that macrophages play in HSV-1 replication after infection of the mouse cornea. They found that BALB/c mice given subconjunctival injections of liposomes containing clodronate before viral infection had ocular virus titers 105-fold higher than seen in PBS-liposome-treated mice. They concluded that macrophages are important in restricting HSV-1 growth after infection and that macrophages appeared to be required for development of an acquired immune response, probably by functioning in Ag processing and presentation. Other studies examined the effects of HSV-1 corneal infection in the trigeminal ganglion, a site which the virus invades after corneal infection where it establishes latency in the neurons. Macrophage depletion showed that these cells are the main source of TNF-α and inducible NO synthase and their depletion led to increased viral titers in the ganglion (45).

In B6 vs BALB/c cornea, PMN persistence has been associated with increased tissue destruction and perforation (4, 5, 7, 8, 20). Because macrophage depletion induced profound differences in the disease response in both groups of mice, we next asked whether depletion of these cells affected other inflammatory cell populations, such as the PMN, a cell of major importance in P. aeruginosa bacterial keratitis (4, 5, 7, 8, 20). PMN were quantitated by the MPO assay in the cornea of both mouse strains after injection of clodronate-liposomes or PBS-liposomes. In B6 mice, no difference in PMN number was observed at 1 day p.i., but by 3 days p.i., the cornea of mice injected with clodronate-liposomes had significantly elevated MPO levels. In BALB/c mice, MPO assays showed that after macrophage depletion, the PMN number was significantly elevated at both 1 and 3 days p.i., suggesting that macrophages regulate the number of PMN in the cornea in both of these mouse strains, but that regulation is reduced/delayed in B6 mice. These data, together with our slit-lamp and histopathology data, suggest that in both groups of mice, PMN corneal damage is escalated after macrophage depletion and that the macrophage regulates the influx and persistence of these cells in the cornea.

In acute P. aeruginosa-induced pneumonia in mice, clodronate-liposome depletion of alveolar macrophages was evaluated at 48 h after infection. Unlike the work reported herein, this study was limited to early time periods after infection. Nonetheless, it also showed that depletion of macrophages decreased bacterial clearance, delayed movement of PMN from the site of inflammation, and aggravated lung injury (46).

We next tested whether depletion of macrophages also resulted in dysregulation of cytokines that chemoattract PMN into the cornea. In both B6 and BALB/c mice, ELISA analysis for IL-1β (7, 11, 24), MIP-2 (4, 7, 17), and MIP-1α (8) proteins was performed. In B6 mice, IL-1β and MIP-1α proteins were significantly up-regulated at 3 days p.i. in mice treated with clodronate-liposomes vs PBS-liposomes, while MIP-2 protein levels were significantly elevated at both 3 and 5 days p.i. Similarly analysis in BALB/c mice showed that protein levels for all of the cytokines/chemokines tested were significantly up-regulated as early as 1 and remained up-regulated at 3 days p.i. in mice treated with clodronate-liposomes. These data suggest that secretion of cytokines is at least one mechanism by which the macrophage regulates PMN influx into the bacterially infected cornea. Alternatively, it is also possible that in the absence of the macrophage, PMN, whose number is increased in the cornea, contribute to the higher levels of cytokines and chemokines, as in other nonocular systems (47).

Since in both groups of mice treated with clodronate-liposomes, the PMN number was increased, we hypothesized that corneal bacterial load would be lower than in PBS-liposome-treated mice. Unexpectedly, B6 mice treated with clodronate-liposomes had no significant difference in bacterial plate counts at 3 days p.i., indicating that despite the increased number of cells, PMN failed to control bacterial load in these mice. Furthermore, by 5 days p.i., when the eyes of B6 mice treated with clodronate-liposomes had severely shrunken (Fig. 3), bacterial plate counts were higher in the cornea of mice treated with PBS-liposomes. We hypothesize that this occurred because more corneal tissue remained intact in the PBS-liposome-treated mouse cornea to support bacterial growth, whereas in the shrunken, macrophage-depleted eye, little corneal tissue remained to allow bacterial growth. In contrast, in the BALB/c mouse, in which there was no apparent difference in eye size between the two treatment groups at any time p.i., ~2 logs more bacteria were recovered from the cornea of mice treated with clodronate-liposomes vs PBS-liposomes at 3 and 5 days p.i. When expressed as fold differences, about an 11.6-fold (3 day) and an 80.4-fold (5 day) greater number of bacteria were cultured from the cornea of these mice, suggesting that in the BALB/c mouse, the macrophage is important in elimination of bacteria from the cornea, a feature that would be critical to the resistance response.

In a P. aeruginosa-induced chronic lung infection model, others have shown that the PMN is the predominant cell type to respond in B6 mice, whereas macrophages constituted the majority of cells in BALB/c mice at 7 days after infection. They concluded that an
exaggerated inflammatory response dominated by the PMN correlated with susceptibility while a modest inflammatory response dominated by macrophages correlated with resistance (48). Although these data are somewhat similar to our ocular model, a major difference exists in that both B6 and BALB/c mice respond initially after ocular bacterial infection with a predominantly PMN response that persists in B6, but is down-regulated in BALB/c mice. Furthermore, when LC are present in the central cornea before ocular bacterial infection in BALB/c mice, the macrophage response is enhanced and normally resistant mice become susceptible (cornea perforates) (19).

Since macrophages also control the balance of pro- vs anti-inflammatory cytokines, we next used semiquantitative RT-PCR an approach with some limitation, to test for mRNA expression levels for prototypic cytokines of the Th1 vs Th2 pathway. Significant increased expression levels for IFN-γ were detected in the cornea of BALB/c mice injected with clodronate-liposomes at both 3 and 5 days p.i.. However, no difference was seen in mRNA expression levels for TNF-α or of a prototypic Th2 cytokine, IL-4, in the macrophage-depleted vs PBS-liposome-injected group. In contrast, mRNA expression levels of IL-10 were significantly lower in the cornea of BALB/c mice treated with clodronate-liposomes at 1, 3, and 5 days p.i.. Protein levels for IL-10 also were reduced in clodronate-liposome-treated mice at 3 and 5 days p.i., but were significant only at 5 days p.i., supportive of a role for IL-10 in balancing proinflammatory cytokine levels in the cornea.

These data suggest that macrophage production of IL-10 is an important regulator of the resistance phenotype in BALB/c mice. Other studies support this conclusion and have shown that IL-10 therapy leads to beneficial effects in experimental animals with sepsis syndrome. Survival was improved in animals made bactereemic with cecal ligation and puncture (49) or in mice infected with Trypanosoma cruzi (50). For the lung, other laboratories (51) showed that the efficacy of IL-10 treatment in experimental P. aeruginosa-induced pneumonia depended on whether the bacterial strain was cytotoxic or invasive. Disease from cytotoxic strains (like strain 19660 used in this study) was ameliorated by recombinant IL-10 treatment.

In summary, our results demonstrate that the macrophage is important in the control of bacterial growth, PMN influx, and regulation of a balance of pro (IL-10) vs anti-inflammatory (IFN-γ) cytokines and that in the absence of this cell, resistant mice are converted to the susceptible phenotype.

References


