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J Immunol 2014; 193:2349-2362; Prepublished online 28 July 2014;
doi: 10.4049/jimmunol.1400853
http://www.jimmunol.org/content/193/5/2349

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The role of the macrophage in the immunopathology of periodontitis has not been well defined. In this study, we show that intraoral inoculation of mice with Porphyromonas gingivalis resulted in infection, alveolar bone resorption, and a significant increase in F4/80+ macrophages in gingival and submandibular lymph node tissues. Macrophage depletion using clodronate-liposomes resulted in a significant reduction in F4/80+ macrophage infiltration of gingival and submandibular lymph node tissues and significantly (p < 0.01) less P. gingivalis–induced bone resorption compared with controls in BALB/c and C57BL/6 mice. In both mouse strains, the P. gingivalis–specific IgG Ab subclass and serum cytokine [IL-4, IL-10, IFN-γ, and IL-12 (p70)] responses were significantly (p < 0.01) lower in the macrophage-depleted groups. Macrophage depletion resulted in a significant reduction in the level of P. gingivalis infection, and the level of P. gingivalis infection was significantly correlated with the level of alveolar bone resorption. M1 macrophages (CD86+), rather than M2 macrophages (CD206+), were the dominant macrophage phenotype of the gingival infiltrate in response to P. gingivalis infection. P. gingivalis induced a significant (p < 0.01) increase in NO production and a small increase in urea concentration, as well as a significant increase in the secretion of IL-1β, IL-6, IL-10, IL-12 (p70), eotaxin, G-CSF, GM-CSF, macrophage chemoattractant protein-1, macrophage inflammatory protein-α and -β, and TNF-α in isolated murine macrophages. In conclusion, P. gingivalis infection induced infiltration of functional/inflammatory M1 macrophages into gingival tissue and alveolar bone resorption. Macrophage depletion reduced P. gingivalis infection and alveolar bone resorption by modulating the host immune response. The Journal of Immunology, 2014, 193: 2349–2362.

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth that is characterized by the accumulation of immune cells in gingival connective tissue, resorption of alveolar bone, and the degradation of periodontal connective tissues, which lead to increased tooth mobility and eventual tooth loss (1, 2). Moderate to severe forms of the disease affect ~38% of the adult dentate population, such that it is a major public health problem (3). Chronic periodontitis is strongly associated with the presence of Gram-negative anaerobic bacteria in subgingival plaque, in particular Porphyromonas gingivalis, Tannerella forsythus, and Treponema denticola (4, 5). Of these three periodontal pathogens, P. gingivalis is the most strongly associated with clinical indicators of periodontitis, including increased pocket depth and bleeding on probing (6, 7). Recently, P. gingivalis was described as a keystone pathogen or pathobiont because of its ability to cause a dysbiosis between host and plaque, resulting in disease initiation and progression (8, 9). The disease also may have an impact on systemic health, because it was reported to be a risk factor for cardiovascular diseases, diabetes, spontaneous preterm birth and low birth weight infants, pancreatic cancer, and rheumatoid arthritis (10–14).

Macrophages are known to have a major role in mediating the immune response, and their cell numbers are higher in gingival tissue biopsies from chronic periodontitis patients in comparison with tissue from healthy subjects (15–17). The macrophage is a primary producer of proinflammatory cytokines, such as IL-1, IL-12, and TNF-α (16, 18). A number of studies demonstrated the elevation of proinflammatory cytokines, including IL-1 and TNF-α, in gingival tissue and gingival crevicular fluid of patients with chronic periodontitis compared with those of healthy subjects (19–21). P. gingivalis LPS, in the presence of IL-1 and TNF-α, was shown to induce cultured human fibroblasts and epithelial cells to release PGE2. PGE2 is associated with periodontal bone resorption and was shown to intensify the proinflammatory activity of macrophages (2, 19, 22–25). IL-1 and TNF-α not only enhance inflammation, they also promote bone resorption (26–28). Compared with healthy subjects, the gingival tissue and crevicular fluid of patients with chronic periodontitis also were shown to contain significantly increased amounts of macrophage inflammatory protein (MIP)-1α and IL-8 (29, 30). Macrophages are a major source of chemokines, including MIP-1α and IL-8, which are involved in the migration and activation of phagocytic cells and lymphocytes during inflammatory responses (31–33). In addition, macrophage lineages are recognized as precursors to osteoclasts, the primary cells responsible for bone resorption (34–36). The macrophage is considered essential for the degradation of organic bone matrix, providing attachment sites for osteoclasts that differentiate to form multinucleated cells and resorb bone (36–38).

Macrophages can be classified into two main phenotypes, M1 and M2, depending on the microenvironment that they encounter at
an infected site (39). M1 macrophages are termed “classically activated,” with differentiation occurring in the presence of IFN-γ, LPS, or TNF-α; they are involved in inflammation and host defense (40, 41). Following activation, M1 macrophages increase surface CD86 but decrease CD206 expression, as well as increase the production of IL-1, IL-12, IL-23, TNF-α, reactive oxygen intermediates, and NO. The M1 phenotype is suggested to mirror a Th1 response (40, 41). M2 macrophages are termed “alternatively activated,” and differentiation occurs in the presence of IL-4, IL-10, or IL-13 (a homolog of IL-4). They have an immunoregulatory and tissue repair role. The M2 phenotype is characterized by low production of NO, an increase in CD206, a decrease in CD86 surface expression, and an increase in secretion of urea and L-ornithine (the precursor for collagen formation) (40, 42–44).

The emergence of P. gingivalis in subgingival plaque and the release of one of its major virulence factors, the Arg- and Lys-specific cysteine proteinase–adhesin complexes (RgpA–Kgp complexes), into gingival tissue was suggested to be a major factor in inducing a chronic host inflammatory response, resulting in the cumulative tissue destruction and alveolar bone resorption observed in chronic periodontitis patients (5, 45–51). In addition to P. gingivalis proteins and LPS penetrating gingival tissue, P. gingivalis phosphorylated dihydrocarnosamide lipids have been isolated from gingival tissue and subgingival plaque of chronic periodontitis patients (52–54). Whole P. gingivalis cells and certain components, including the RgpA–Kgp complexes and lipids, induce secretion of a number of proinflammatory cytokines from epithelial and fibroblast cells in vitro, such as IL-1, IL-6, IL-8, PGE2, macrophage chemotactic protein (MCP)-1, and ICAM-1 (50, 54, 55). The elevation in levels of the proinflammatory cytokines IL-1, IL-6, IL-8, IL-12, and TNF-α is commonly found in diseased gingival tissue of chronic periodontitis (19–21, 30). An increase in the level of these cytokines is also detected in other chronic inflammatory diseases, such as rheumatoid arthritis and neuropsychiatric hyperalgesia, where macrophages play a major role in the immunopathology (56–59).

In the current study, the role of the macrophage in periodontitis was investigated using a mouse periodontitis model by depleting macrophages using s.c. and i.v. administration of liposome-encapsulated dichloromethylene-bisphosphonate (clodronate liposomes). Clodronate liposomes have been used to study the role of macrophages when it is delivered encapsulated in a phospholipid bilayer liposome. The clodronate liposomes are cytotoxic compound that induces apoptotic cell death of macrophages in a number of chronic diseases (60, 61). Clodronate liposomes were injected s.c. over the masseter muscle below the zygomatic bone (25 μl each to the left and right side of the face) and over the triangularis muscle along the mandible (25 μl each to the left and right facial) or i.v. via the tail vein (100 μl). The control groups (sham treated) were managed liposomes encapsulating PBS (PBS liposomes) s.c. or i.v., as above. The clodronate liposomes and PBS liposomes were kindly provided by Prof. Nico Van Rooijen (Vrije Universiteit, Amsterdam, the Netherlands) (65).

Mouse periodontitis model and measurement of alveolar bone resorption in mouse maxillae

The mouse periodontitis experimental protocol was modified from Baker et al. (73) and performed as described previously by O’Brien-Simpson et al. (48). Mice were given clodronate liposomes or sham treated on days 0 and 20 prior to oral inoculation with P. gingivalis on days 4 and 24. Each oral inoculation consisted of four doses of P. gingivalis W50 (1 × 1011 viable P. gingivalis W50 cells suspended in 20 μl PGB buffer [50 mM Tris-HCl, 150 mM NaCl, 10 mM MgSO4, and 14.3 mM 2-ME, pH 7.4] containing 2% w/v carboxymethylcellulose (Sigma-Aldrich, Castle Hills, NSW, Australia) given 2 d apart. On day 58, mice were bled from the retrobulbar plexus and killed. Maxillae were removed and halved through the midline, with 10 halves used to determine alveolar bone resorption, 5 halves used to enumerate P. gingivalis W50 colonization of gingival plaque by real-time PCR, and 5 halves used for immunohistochemical analysis. Submandibular lymph nodes were also removed for immunohistochemical analysis. Sera were used to determine the Ab and cytokine profile using ELISA and Bio-Plex assays, respectively.

Immunohistochemical analysis

All procedures were carried out at room temperature (RT), unless stated otherwise. Isolated SMLNs and maxillae (with supporting tissues) were fixed overnight in 0.25% w/v periodate–lysin–paraformaldehyde buffer (0.01 M NaH2PO4, 0.075 M lysine in 0.05 M phosphate buffer [pH 7.2]; Sigma–Aldrich). The teeth of the maxillae were decalcified in 0.1 M PB (0.01 M Na2HPO4, 1.5 mM KH2PO4, 0.15 M NaCl [pH 7.2]) containing 10% w/v EDTA (24 h). Both the lymph nodes and maxillae were rinsed with 0.1 M PBS and incubated in ethanol (30 min, three times), followed by histolene (30 min, three times) (Francine, Sydney, NSW, Australia). After incubation, the samples were embedded at 55°C in paraffin (low temperature technique; 54–56°C setting paraffin; Sigma–Aldrich). Serial sections of 3 μm thickness were cut using a rotary microtome (Leitz Rotary Microtome, Frankfurt, Germany) and were used to stain for macrophages using an anti-F4/80 mAb (MCAP497; AbD Serotec, Oxford, U.K.).

Identification of macrophages in cross-sections of lymph nodes and decalcified maxillae using anti-F4/80 mAb

Sample sections were dewaxed by serially washing in histolene (30 s, three times), ethanol (20 s, two times), and running water (5 min). The sections were subjected to trypsin digestion (0.1% w/v trypsin in 0.05 M Tris saline...
containing 0.1% CaCl2 (pH 7.6) for 20 min. The sections were washed (5 min) under running water and immersed in 0.1 M PBS (5 min). The sections were incubated in 10% v/v normal swine serum (NSS; Commonwealth Serum Laboratory, Melbourne, VIC, Australia) in PBS (30 min), drained, and incubated overnight with a 1/10 dilution of rat anti-mouse F4/80 mAb (MCA497; AbD Serotec) in 0.1 M PBS containing 1% v/v NSS. Following incubation, the sections were washed in 0.1 M PBS (5 min) prior to incubation (30 min) with 1/200 dilution of rabbit anti-rat IgG secondary Ab (R3756; Sigma-Alrich) in 0.1 M PBS containing 1% v/v NSS and 5% v/v normal mouse serum (Sigma-Alrich) (30 min). The sections were washed in 0.1 M PBS (5 min), followed by a series of graded 70–100% ethanol washes (30 s each). The sections were incubated in 3% v/v H2O2 in methanol (20 min) to block endogenous peroxidase. The sections were reverse washed through the graded (100–70% ethanol washes (30 s each), followed by immersion in 0.1 M PBS (5 min)). The sections were incubated (30 min) with a 1/300 dilution of biotinylated swine anti-rabbit IgG tertiary Ab (E0353; Dako, Denmark) in 0.1 M PBS containing 1% v/v NSS and 5% normal mouse serum, followed by 0.1 M PBS wash (5 min). Sections were then incubated (30 min) with a 1/10 dilution of Avidin-Biotin Complex/Horseradish Peroxidase (Dako) in 0.05 M Tris-HCl (pH 7.6). After washing with 0.1 M PBS (5 min), the immunoreactivity was visualized using diamino benzidine tetrahydrochloride (Sigma-Alrich; one 50-mg tablet dissolved in 100 ml 0.1 M PBS containing 0.3% v/v H2O2). After visualization, the sections were counterstained with Harris' Hematoxylin (Sigma-Alrich; 30 s) and dehydrated with 100% analytical-grade ethanol (1 min/wash, six times). Sections were Washed in histolene (1 min) and mounted using Gurr DePeX mounting medium (BDH, Dorset, UK). To confirm immunoreactivity of the primary Ab, 0.1 M PBS containing 1% v/v NSS was applied, instead of the primary Abs, as controls to serial cut sections. Photographic images of lymph nodes and maxillae were captured using a Spot 32 digital microscope–linked camera (Scitech, Melbourne, VIC, Australia) at 40× magnification. The F4/80+ cells in the lymph node and maxillae were counted at 40× magnification. The surface area of measurement was 1.0 × 10⁻⁷ mm². For the maxillary samples, the counting area was positioned adjacent to the tooth root under the cement–enamel junction. Serial paraffin sections were stained with H&E using standard methods (74). Images were taken using a scanning microscope (Aperio Scanscope T3) and analyzed using Aperio Image Analysis 2008 (both from Aperio Technologies, Vista, CA).

**Determination of P. gingivalis infection using real-time PCR**

Maxillae obtained at the termination of the mouse periodontitis experiments for real-time PCR analysis were stored at −70°C until processing, using a similar methodology as described by Pathirana et al. (49). The cell numbers of P. gingivalis and total oral bacteria/maxilla were quantified using Taqman gene expression assays for universal and universal oral bacteria. The universal primers were specific for the 16S rRNA gene (Life Technologies Australia, North Ryde, NSW, Australia), and plates were read on a Bio-Plex 200 System and Bio-Plex Manager Version 4.0; Bio-Rad Laboratories, according to the manufacturer’s instructions. The concentrations of the following 10 cytokines were determined in sera from mice that were used in the mouse periodontitis model: IL-2, IL-4, IL-5, IL-7, IL-12 (p70), IL-13, GM-CSF, IFN-γ, TNF-α, and M-CSF. The concentrations of 24 cytokines [IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, etoxin, G-CSF GM-CSF, IFN-γ, KC, MCP-1 (MCAF), M-CSF, MIP-1α, MIP-1β, RANTES, and TNF-α] were determined in real-time PCR stromatopore IFR B cell lines, P. gingivalis and C57BL/6 peritoneal macrophages (CD11b+, F4/80+, CD11c+, TCR β/γ, Ly-6C+FITC conjugate [clone IA8], and CD11b+PE conjugate [clone M1/70]); or eBio-science (San Diego, CA; TCR β/γ-FITC conjugate (clone H5-S07), CD115 (CSF-1R)-PE conjugate (clone AF508), and F4/80-PECy7 conjugate (clone BMB5). All cell culture reagents were obtained from Sigma-Aldrich, unless otherwise specified.

**Reagents used in flow cytometry and cell culture assays**

Abs to mouse Ags were purchased from AbD Serotec (CD206-PE conjugate [clone C66682]); BD Biosciences, Franklin Lakes, NJ, CD86-PE-Cy7 conjugate [clone G1L], CD11b-allophycocyanin conjugate [clone M1/70], CD45R/B220-FITC conjugate [clone RA3-6B2], Ly-6C+FITC conjugate [clone IA8], and CD11b+PE conjugate [clone M1/70]; or eBio-science (San Diego, CA; TCR β/γ-FITC conjugate (clone H5-S07), CD115 (CSF-1R)-PE conjugate (clone AF508), and F4/80-PECy7 conjugate (clone BMB5). All cell culture reagents were obtained from Sigma-Aldrich, unless otherwise specified.

**Determination of M1 and M2 macrophage phenotypes in gingival tissues from mice inoculated intraorally with P. gingivalis W50**

Mice (female BALB/c or C57BL/6, 6–8 wk old, n = 6 mice/group) were inoculated intraorally with either four or eight doses of P. gingivalis, with each dose consisting of 1 × 10⁹ viable P. gingivalis W50 cells suspended in 20 μl PG buffer, as described above. Three days following the last oral inoculation, mice were killed, and maxillae (with gingival tissue still attached) were removed. The whole maxillae were placed in digestion buffer (1 mg/ml collagenase II, 1 mg/ml collagenase IV, and 25 μ U Dnase I in complete DMEM supplemented with 10% v/v heat-inactivated FBS (30 min, 56°C), 2 mM L-glutamine, 2 mM sodium pyruvate, 0.1 mM 2-ME, 30 g/ml gentamicin, and 100 IU/ml penicillin) (1 h, 37°C). Bones were removed from the digestion, and RBCs were removed from the suspension by treatment with RBC lysis buffer (Sigma-Alrich) (2 min, RT) and washed twice in Dulbecco’s PBS (800 × g, 5 min). All cell suspensions and Ab solutions (1:200, unless otherwise stated) were resuspended in FACS buffer (24% w/v BSA, 0.1% w/v sodium azide, 20 μM EDTA in Dulbecco’s PBS), and all incubations were performed on ice for 20 min. Cells were resuspended at 1.0 × 10⁶ cells/10⁵ μl in FACS buffer in duplicate and incubated with 1:400 Fc Block (BD Pharmingen, San Diego, CA). Cells were washed (800 × g, 5 min) and stained with Abs TCR β/γ/B220/Ly6G-FITC, CD11c+PE, and CD86-PE-Cy7 (1:500) for surface cellular phenotypic analysis (M1 macrophages) or TCR β/γ/B220/Ly6G-FITC, CD11b-allophycocyanin, and CD206-FITC for intracellular phenotypic analysis (M2 macrophages) using a BD Cytometric/Cytoperm Kit (BD Biosciences). Appropriate isotype controls were used in all cases. Cells were washed twice (800 × g, 5 min) and resuspended in FACS buffer. For flow cytometric analysis, a typical forward and side-scatter gate was set to exclude dead cells and aggregates; a minimum of 1 × 10⁵ events in the gate was collected and analyzed using an FC500 flow cytometer and Kaluza Flow Cytometry software V1.1 (Beckman Coulter Australia, Gladstone, NSW, Australia). The phenotypic analysis was carried out by negative gating on T cells, B cells, and neutrophils (TCR β/γ, B220, and Ly6G markers, respectively), followed by positive gating identification of M1 macrophages (CD11b and CD86 markers) and M2 macrophages (CD11b and CD206 markers).

**Determination of M1 and M2 peritoneal macrophage phenotypes from mice injected i.p. with HK-W50**

On day 0, eight groups of mice (female BALB/c or C57BL/6, 8–10 wk old, n = 6 mice/group) were injected i.p. with HK-W50 (100 μg in 100 μl PBS/mouse) and a control group of mice was injected i.p. with PBS (controls). Peritoneal cells from the PBS-injected control mice (day 0) and from mice injected i.p. with HK-W50 were washed from the peritoneal cavity by lavage with 5 ml ice-cold, sterile PBS (Sigma-Alrich) at days 0, 1, 2, 3, 4, 5, 7, 10, and 15 after injection. The peritoneal cells collected from each mouse were treated as an individual sample and washed once in Dulbecco’s PBS (800 × g, 5 min). Cells were resuspended to 2.0 × 10⁶ cells/10⁵ μl in PBS buffer and used in duplicate. The procedure to stain surface cellular and intracellular phenotypic analysis for M1 and M2 macrophages, as well as flow cytometric analysis of the macrophage phenotypes, was performed as described above.
Isolation of mouse peritoneal macrophages for functional assays

Peritoneal cells elicited on day 6 post-i.p. injection with HK-W50 (100 μg in 100 μl PBS/mouse) from two groups of mice (female BALB/c or C57BL/6, 8–10 wk old, n = 6 mice/group) were resuspended to 2.0 × 10^6 cells/180 μl in FACS buffer in duplicate and incubated with 1:400 Fe Block (BD Pharmingen), as described above. Cells were washed (800 × g, 5 min) and resuspended with Alexafluor-conjugated antibodies (T905, T908, and CD115) for 15 min. The cell lysate reaction solution (1 mg/ml) and 10 μl sulfanilic acid (1.0 mM). A standard curve was generated using 2-fold serial dilutions of a 100–1.56 μl sulfanilic acid (1.0 mM). A standard curve was generated using 2-fold serial dilutions of a 100–1.56 μM standard solution (100–1.56 μM). The reaction was allowed to proceed (30 min, RT), and the absorbance was measured at 550 nm using a VICTOR3 1420 Multilabel Counter (PerkinElmer, Waltham, MA).

NO assay

NO concentration was determined using the Greiss reagent kit, according to the manufacturer’s instructions. Briefly, 150 μl peritoneal macrophage (CD11b^+/, F4/80^+/, CD115^+/, TCRβ^+/B220^+/Ly6g^-/) culture supernatant from either unstimulated or HK-W50–stimulated cultures was collected after 24 or 48 h of incubation and combined with 130 μl deionized water, 10 μl N-(1-naphthyl)ethylenediamine dihydrochloride (1 mg/ml), and 10 μl sulfanilic acid (1.0 mM). A standard curve was generated using 2-fold serial dilutions of a 100-μM nitrite standard solution (100–1.56 μM). The level of NO was measured using the Pearson correlation (SPSS for Windows, version 12). The data for alveolar bone resorption (mm2) and cytokine measurement assays, and the cell lysates were used in the NO assay.

Arginase assay

Peritoneal macrophage (CD11b^+/, F4/80^+/, CD115^+/, TCRβ^+/B220^+/Ly6g^-/) culture supernatant from either unstimulated or HK-W50–stimulated cells was removed and 24 or 48 h of incubation, and the cells were lysed with the addition of 100 μl 1% v/v Triton X-100 in PBS. The cell lysate was heated with 100 μl 25 mM Tris and 1 mM MnCl2 (10 min, 55°C) and then cooled and incubated with 200 μl 0.5 M arginine in PBS (1 h, 37°C) (Sigma-Aldrich). The arginase reaction was stopped by the addition of 900 μl 4.46 N H2PO4, 3.6NH2SO4 solution. The cell lysate reaction solution was incubated with 40 μl 9% v/v isomitrospirophenone in ethanol (30 min, 100°C). Values were quantified from a standard curve calibrated using 200 mg/ml urea and 2-fold serial dilutions of 20 μg/ml urea (Sigma-Aldrich). The absorbance was measured at 540 nm using a VICTOR3 1420 Multilabel Counter (PerkinElmer).

Statistical analysis

The choice of a parametric or nonparametric statistical test was made based on normal distribution of the data, determined using Levene’s method (SPSS for Windows, version 12). The data for alveolar bone resorption (mm2) and real-time PCR were analyzed using one-way ANOVA, with a Dunnett T post hoc test. The level of P. gingivalis infection, measured as a percentage of total bacterial cells/half maxilla, was correlated with the level of alveolar bone resorption for all BALB/c and C57BL/6 animals inoculated intraorally with P. gingivalis using the Pearson correlation (SPSS for Windows, version 12). The F4/80^-/macrophage immunohistochemical staining counts were analyzed using the Kruskal–Wallis test and the Mann–Whitney U-Wilcoxon rank sum test (SPSS for Windows, version 12). The IgM and IgG subclass Ab titer analysis, the concentration of cytokines in serum and peritoneal macrophage culture supernatants, urea, and NO concentrations in peritoneal macrophage culture supernatants, and the number of M1 and M2 macrophages in gingival tissue were analyzed statistically using the Student t test (SPSS for Windows, version 12). Flow cytometric data were analyzed using Kaluza Flow Cytometry software V1.1 (Beckman Coulter Australia).

Results

P. gingivalis W50 increases macrophage infiltration into SMLNs and gingival tissue and induces alveolar bone resorption in BALB/c mice

To evaluate the effect of P. gingivalis W50 intraoral infection on macrophage infiltration into SMLNs and gingival tissue, BALB/c mice were inoculated intraorally with viable P. gingivalis W50 in the mouse periodontitis model, and the presence of macrophages in SMLNs and maxilla connective tissue was evaluated by immunohistochemical staining. To conduct this analysis of macrophages, it was first essential to develop a method to cross-section the maxilla while retaining bone and soft tissue morphology and antigenicity. It was found that fixation of the maxilla in periodate–lysine–paraformaldehyde buffer and decalcification with EDTA, followed by low-temperature embedding in paraffin, provided the optimal conditions for cross sectioning. Ag recovery, and staining. Serial paraffin sections of decalcified maxillae were probed with macrophage-specific anti-F4/80 mAb, counterstained with hematoxylin, and counted. Fig. 1A is a representative cross-section of gingival tissue stained with anti-F4/80 Ab from mice inoculated intraorally with P. gingivalis showing the presence of macrophages. Staining of a serial section of these samples with Ab controls indicated that there was no nonspecific immunohistochemical staining of maxillae from the P. gingivalis–infected group (Fig. 1B). The number of macrophages (F4/80^-/) in gingival connective tissue and SMLNs was significantly (p < 0.001) higher in the P. gingivalis–infected mice compared with the uninoculated animals (Table I, Experiment A). The level of alveolar bone resorption was also significantly higher in the P. gingivalis–infected mice (0.58 ± 0.04 mm^2) compared with uninoculated mice (0.35 ± 0.04 mm^2, p < 0.001).

Intravenous and s.c. clodronate treatment reduces macrophage infiltration into SMLNs and gingival tissue and prevents P. gingivalis W50–induced alveolar bone resorption in BALB/c mice

To evaluate the ability of clodronate-liposomes to deplete macrophages in SMLNs and gingival tissue, BALB/c mice were injected either s.c. or i.v. with clodronate-liposomes prior to intraoral inoculation with viable P. gingivalis W50 in the mouse periodontitis model. The presence and number of macrophages in SMLNs and gingival tissue after P. gingivalis inoculation were evaluated by immunohistochemical staining. Table I (Experiment B) shows the number of macrophages (F4/80^-/) in gingival tissue and SMLNs. In BALB/c mice, there was a significant (p < 0.001) increase in macrophages (F4/80^-/) in SMLNs and gingival tissue of the mice inoculated intraorally with P. gingivalis compared with uninoculated mice. A similar increase in macrophages (F4/80^-/) in SMLNs and gingival tissue was detected in mice that received s.c. PBS liposomes and were inoculated with P. gingivalis compared with uninoculated mice. In the s.c. and i.v. clodronate liposomes–treated/injected mice, there were significantly (p < 0.001) fewer macrophages (F4/80^-/) detected in SMLNs and gingival tissue compared with inoculated and uninoculated mice. No significant differences in the numbers of macrophages (F4/80^-/) were ob-
served in SMLNs and gingival tissue of BALB/c mice that were administered clodronate liposomes via the s.c. or i.v. route.

A representative profile of alveolar bone resorption induced by intraoral infection with \textit{P. gingivalis} W50 is shown in Fig. 2A and 2B. \textit{P. gingivalis}–induced alveolar bone resorption was significantly (\(p < 0.001\)) reduced in mice treated s.c. or i.v. with clodronate liposomes compared with mice treated s.c. or i.v. with PBS liposomes and inoculated with \textit{P. gingivalis} (Fig. 2C). There was no significant difference in the level of bone resorption in mice administered clodronate liposomes s.c. or s.c. compared with uninoculated mice. The levels of bone resorption in PBS liposome–treated/infected mice (s.c. and i.v.) were not significantly different from those in the untreated/infected mice (Fig. 2C). In all subsequent experiments, clodronate liposomes and PBS liposomes were administered s.c.

Subcutaneous clodronate treatment reduces macrophage numbers and decreases \textit{P. gingivalis} W50–induced alveolar bone resorption in BALB/c and C57BL/6 mice

The mouse periodontitis model was used to evaluate the effect of macrophage depletion using s.c. clodronate treatment on alveolar bone resorption induced by \textit{P. gingivalis} W50 in BALB/c and C57BL/6 mice. Fig. 3A shows that there was significantly (\(p < 0.001\)) less alveolar bone resorption in the clodronate liposome–treated/infected BALB/c and C57BL/6 mice compared with PBS liposome–treated/infected and untreated/infected mice. Further, in BALB/c mice, there was no significant difference in the level of bone resorption between s.c. clodronate liposome–treated/infected mice and uninoculated mice. The level of bone resorption in BALB/c mice treated with PBS liposomes and inoculated intraorally with \textit{P. gingivalis} was not significantly different from that of the untreated/infected mice. Similarly, C57BL/6 mice treated with clodronate liposomes (s.c.) and inoculated intraorally with \textit{P. gingivalis} exhibited significantly (\(p < 0.001\)) less alveolar bone resorption compared with both the PBS liposome (s.c.)–treated/infected mice and untreated/infected mice (Fig. 3B). The mean bone resorption for PBS liposome–treated C57BL/6 mice was not significantly lower than that of the untreated/infected animals (Fig. 3B). There was no significant difference in the level of bone resorption in C57BL/6 mice when clodronate liposome–treated/infected mice were compared with uninoculated mice.

The number of macrophages (F4/80\(^+\)) in SMLNs and gingival tissue of PBS liposome (s.c.)–treated/infected and untreated/infected BALB/c and C57BL/6 mice was significantly (\(p < 0.001\)) higher than the number seen in uninoculated mice (Table I, Experiment C). Clodronate liposome–treated/infected BALB/c and C57BL/6 mice had significantly (\(p < 0.001\)) fewer gingival and submandibular macrophages compared with PBS liposome–treated mice. There was no significant difference in macrophage numbers between clodronate liposome–treated/infected C57BL/6 mice and uninoculated mice.

Determination of \textit{P. gingivalis} infection by real-time PCR in BALB/c and C57BL/6 mice after \textit{P. gingivalis} intraoral inoculation in the mouse periodontitis model

At the termination of the mouse periodontitis model experiments, DNA was extracted from the half maxillae of BALB/c and C57BL/6 mice; \textit{P. gingivalis} infection, expressed as a percentage of total bacterial cell numbers recovered, was determined by real-time PCR. Both BALB/c and C57BL/6 mice inoculated intraorally with \textit{P. gingivalis}, irrespective of pretreatment (clodronate liposomes, PBS liposomes, or untreated), were positive for the presence of \textit{P. gingivalis}, and all exhibited significant (\(p < 0.001\)) levels of infection relative to the uninoculated animals (Fig. 4A, 4B). However, BALB/c and C57BL/6 mice inoculated intraorally with \textit{P. gingivalis}, but untreated in terms of liposome administration, had significantly (\(p < 0.05\)) higher levels of infection compared with the inoculated, but liposome–treated (clodronate liposomes and PBS liposomes), animals (Fig. 4A, 4B). Clodronate liposome–treated, intraorally inoculated C57BL/6 mice exhibited a significantly (\(p < 0.05\)) lower level of infection with \textit{P. gingivalis} compared with the PBS liposome–treated, inoculated animals (Fig. 4B). The level of \textit{P. gingivalis} infection was significantly correlated with the level of alveolar bone resorption for both the BALB/c (\(p < 0.01\)) and C57BL/6 (\(p < 0.01\)) mice (Fig. 4).

Ab subclass of sera from mice inoculated intraorally with \textit{P. gingivalis} W50 in the mouse periodontitis model

BALB/c and C57BL/6 mice were bled at the termination of the mouse periodontitis model experiment, and serum IgG, IgG1, IgG2a/IgG2c, IgG2b, IgG3, and IgM titers were determined by ELISA using FK W50 and RgpA–Kgp complexes as the adsorbed Ags (Fig. 5).
BALB/c mice, there were significantly ($p$, 0.001) higher Ab titers to FK W50 and RgpA–Kgp complexes detected in both the PBS liposome–and untreated/infected mouse sera compared with uninoculated mouse sera (Fig. 5A, 5B). The dominant Ab subclass to FK W50 and RgpA–Kgp complexes was IgGl for the untreated/infected BALB/c mice. BALB/c mice treated with s.c. clodronate liposomes and inoculated intraorally with $P$. gingivalis exhibited a significantly reduced Ab response to FK W50 and RgpA–Kgp complexes.

In C57BL/6 mice, the groups inoculated intraorally with $P$. gingivalis W50 (PBS liposomes or untreated) had specific and significantly ($p$, 0.001) higher Ab responses to FK W50 and RgpA–Kgp complexes compared with the uninoculated group (Fig. 5C, 5D). IgG3 and IgG2b were the predominant serum IgG subclasses directed to FK W50, followed by IgG2c for each intraorally inoculated group. The specific Ab response to FK W50 and RgpA–Kgp complexes in the clodronate liposome–treated animals was significantly ($p < 0.05$) lower than that observed for the PBS liposome–treated/infected or untreated/infected mice. No IgG- or IgM-specific Abs to FK W50 or RgpA–Kgp complexes were detected in the uninoculated mice. The IgG Ab–specific titers to FK W50 and RgpA–Kgp complexes in the sera from C57BL/6 mice (Fig. 5C, 5D) were 10–100-fold higher compared with the titers induced in BALB/c mice (Fig. 5A, 5B).

Serum cytokine levels in mice inoculated intraorally with $P$. gingivalis W50 in the mouse periodontitis model

BALB/c and C57BL/6 mice were bled at the termination of the mouse periodontitis model experiment, and sera were analyzed by Bio-Plex assay for the presence and concentration of 10 cytokines [IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, GM-CSF, IFN-γ, TNF-α, and M-CSF] (Fig. 6). In BALB/c mice, the PBS liposome and untreated/infected groups, as well as the uninoculated group, had detectable serum concentrations (with no significant difference between each group) of IL-4, IL-10, and IL-12 (p70) among the 10 cytokines examined (Fig. 6A). None of the 10 cytokines

FIGURE 2. Subcutaneous and i.v. administration of clodronate liposomes prevents $P$. gingivalis–induced alveolar bone resorption in BALB/c mice. Representative images of maxillae taken from an uninoculated mouse (A) and a mouse inoculated intraorally with $P$. gingivalis showing alveolar bone resorption (B). The alveolar bone crest (ABC) and cemento-enamel junction (CEJ) are marked. (C) BALB/c mice were pretreated with either i.v. or s.c. clodronate liposomes (Clo-lip) or PBS liposomes (PBS-lip) or were left untreated; subsequently, they were inoculated intraorally with viable $P$. gingivalis W50. An uninoculated group was included as a negative control. $P$. gingivalis–induced bone resorption was determined as described in Materials and Methods for each group ($n$ = 10). Data are mean ± SD (mm²) and were analyzed using a one-way ANOVA and Dunnett T3 post hoc test. *$p < 0.001$ versus unmarked groups.

FIGURE 3. Macrophage depletion reduces $P$. gingivalis–induced alveolar bone resorption in BALB/c and C57BL/6 mice. BALB/c (A) and C57BL/6 mice (B) were pretreated s.c. with clodronate liposomes (Clo-lip) or PBS liposomes (PBS-lip) or were left untreated; subsequently, they were inoculated intraorally with viable $P$. gingivalis W50. An uninoculated group was included as a negative control. $P$. gingivalis–induced bone resorption was determined as described in Materials and Methods for each group ($n$ = 10). Data are expressed as the mean ± SD (mm²) and were analyzed using one-way ANOVA and a Dunnett T3 post hoc test. *$p < 0.001$, versus unmarked groups.
examined could be detected in BALB/c mice treated with clodronate liposomes and inoculated intraorally with *P. gingivalis*

In C57BL/6 mice, the infected clodronate liposome–treated, PBS liposome–treated, and untreated groups, as well as the uninoculated group, had detectable serum concentrations of IL-10 and IFN-γ among the 10 cytokines examined (Fig. 6B). The PBS liposome and untreated/infected mouse sera had significantly (p < 0.05) higher IL-12 (p70) levels than did the uninoculated and clodronate liposome–treated/infected mouse sera. There was no significant difference in the sera levels of IL-10 and IFN-γ between PBS liposome and untreated/infected mice and the uninoculated mice. The levels of IFN-γ, IL-10, and IL-12 (p70) in the sera of clodronate liposome–treated/infected mice were significantly (p < 0.01) lower than were those in the sera from the untreated/infected and uninoculated mice (Fig. 6B).

The expression of M1 and M2 macrophage phenotypes in gingival tissue from mice inoculated intraorally with *P. gingivalis* W50

Flow cytometry data for M1 and M2 macrophage phenotypes detected in BALB/c and C57BL/6 mouse gingival tissue obtained 2 d following the last intraoral inoculation of four doses of *P. gingivalis* W50 (1 × 10^10 viable cells) are shown in Fig. 7. BALB/c and C57BL/6 mice inoculated intraorally with *P. gingivalis* exhibited a significant (p < 0.01) increase in M1 macrophages (CD11b^+^, CD86^+^, [TCRβ/B220/Ly6G^-]) in gingival tissue com-
The objective of this study was to investigate the role of macrophages in *P. gingivalis*-induced alveolar bone resorption in the mouse periodontitis model by selectively depleting macrophages using s.c. or i.v. administration of clodronate liposomes. To enu-

P. gingivalis activation of M1 and M2 macrophage phenotypes

To investigate the activation of M1 and M2 macrophages by *P. gingivalis* and to acquire sufficient cell numbers for in vitro assays, BALB/c and C57BL/6 mice were injected (i.p.) with HK-W50, and the proportion of M1 and M2 macrophages over time (days 0, 1, 2, 3, 4, 5, 7, 10, and 15) was analyzed by flow cytometry (Fig. 8A). The cell numbers of M1 macrophages were significantly higher than those of M2 macrophages at all points measured. In both BALB/c and C57BL/6 mice, there was an initial reduction in M1 ([TCR²¹⁺, B220⁻, Ly6G⁻] (CD11b⁺, CD86⁺) and M2 ([TCR²¹⁺, B220⁺, Ly6G⁺] (CD11b⁺, CD206⁺)) macrophage cell numbers in the first 24 h, which then increased, reaching a maximum on day 6 after HK-W50 injection (Fig. 8A). In subsequent assays, periodontal macrophages (CD11b⁺, F4/80⁺, CD11b⁺, CD206⁺, [TCR²¹⁺, B220⁺, Ly6G⁺]) and C57BL/6 (Fig. 8C). The supernatant from peritoneal macrophages that were stimulated with *P. gingivalis* and to acquire sufficient macromolecules compared with unstimulated macrophages from both BALB/c and C57BL/6 mice. There was a significant (p < 0.01) increase in NO production in the peritoneal macrophages that were stimulated with *P. gingivalis* compared with the unstimulated macrophages from BALB/c and C57BL/6 mice (Fig. 8C). The supernatant from periodontal macrophages, incubated in the presence or absence of HK-W50, was used in a microbead array (Bio-Plex) to assess the presence and concentration of 24 cytokines and chemokines [IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1 (MCAF), M-CSF, MIP-1α, MIP-1β, RANTES, and TNF-α] (Fig. 8D). Of the 24 cytokines and chemokines analyzed, 11 cytokines [IL-1β, IL-6, IL-10, IL-12 (p70), eotaxin, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β, and TNF-α] were detected in BALB/c and C57BL/6 peritoneal macrophage supernatants that were incubated or not with HK-W50. The concentrations of detectable cytokines were significantly (p < 0.01) higher in supernatants from macrophages stimulated with *P. gingivalis* than in those from unstimulated macrophages for both BALB/c and C57BL/6 mice, with the exception of eotaxin in BALB/c mice and TNF-α in BALB/c and C57BL/6 mice.

Discussion

The objective of this study was to investigate the role of macrophages in *P. gingivalis*-induced alveolar bone resorption in the mouse periodontitis model by selectively depleting macrophages using s.c. or i.v. administration of clodronate liposomes. To enu-
erate macrophages in the maxillary gingival tissue of mice, we developed a protocol so that sections could be cut through gingival, tooth, and bone tissue while retaining tissue morphology and F4/80 marker antigenicity. F4/80 is a cell surface glycoprotein specifically expressed on the cell surface of mature tissue macrophages, which are derived from the myeloid lineage (80, 81). In both BALB/c and C57BL/6 mice, the SMLN and gingival tissue from \( P. \) gingivalis inoculated intraorally (sham-treated/untreated-infected) mice exhibited increased macrophages (F4/80+) compared with uninoculated mice. This indicates that oral infection with \( P. \) gingivalis in the mouse periodontitis model initiated the innate immune response, resulting in an influx of macrophages into SMLNs and gingival tissue. These results from our animal model agree with the findings of Lappin et al. (17), who reported an increase in inflammatory macrophages in gingival tissue biopsies from periodontitis patients compared with samples from healthy individuals. In our study, the marked reduction in macrophages (F4/80+) observed in SMLNs and gingival tissue from the clodronate liposome–treated mice compared with control (sham-treated/infected and uninoculated) animals showed that macrophages could be depleted in these tissues using clodronate liposomes injected either s.c. or i.v. Mice that were pretreated with s.c. clodronate liposomes and inoculated intraorally with \( P. \) gingivalis had fewer macrophages in the SMLN and gingival tissues compared with uninoculated, PBS liposome ( sham)-treated/infected and untreated/infected mice. Our data are in agreement with previous studies that showed, using immunohistochemistry and flow cytometry, that the administration of clodronate liposomes depletes macrophage (F4/80+) populations in tissues. These previous studies showed that tissue macrophages in the cornea and lung play a significant role in HSV-1–induced stromal keratitis and measles infection, respectively (61, 82).

Oral infection with \( P. \) gingivalis in BALB/c and C57BL/6 mice not only resulted in the influx of macrophages into the SMLNs and gingival tissue, it also led to an increase in alveolar bone resorption compared with uninoculated mice in the periodontitis model. The amount of alveolar bone resorption induced by oral infection with \( P. \) gingivalis measured in the untreated/infected groups of both strains of mice is consistent with the findings of previous studies, indicating that the \( P. \) gingivalis–induced alveolar bone resorption is independent of mouse strain (48, 49). The significant decrease in alveolar bone resorption by clodronate liposome treatment indicates that macrophages play an important role in mediating bone resorption in the mouse periodontitis model. This finding is consistent with previous studies indicating that macrophages have a role in osteoclast development and differentiation, as well as in helping to provide sites for osteoclast attachment and bone resorption (34–36, 38).

During bone resorption, macrophages are required to degrade the organic bone matrix, thus providing the attachment sites and chemotactic factors for osteoclasts (36, 37). Once the osteoclasts attach to the degraded bone matrix, they form multinucleated cells and begin to resorb bone (38). Hence, the depletion of macrophages (by clodronate liposomes) in this study would lead to the reduction in osteoclast activation and differentiation. However, although liposome-encapsulated bisphosphonates (clodronate) selectively target macrophages, “free” bisphosphonates were shown to

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Serum cytokine levels in macrophage-depleted mice in the periodontitis model. Cytokine levels in sera from BALB/c mice (A) and C57BL/6 mice (B) from the periodontitis model were determined using a 10-plex kit for Bio-Plex analysis. Data are representative of two separate experiments (mean ± SD; \( n = 3 \)). *\( p < 0.05 \), versus uninoculated group, †\( p < 0.01 \), versus PBS liposome and untreated groups, Student t test.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** The expression of M1 and M2 macrophage phenotypes in gingival tissue from mice inoculated intraorally with \( P. \) gingivalis W50. BALB/c and C57BL/6 mice were inoculated intraorally with \( P. \) gingivalis W50, and the M1 and M2 macrophage infiltrates were determined by flow cytometry as \((\text{TCR}^\beta/B220/Ly6G^2), \text{CD}11b^+, \text{CD}86^+\) and \((\text{TCR}^\beta/B220/Ly6G^2), \text{CD}11b^+, \text{CD}206^+\), respectively. Data are mean ± SD (\( n = 6 \)) and are from two separate experiments. *\( p < 0.01 \), versus uninoculated group, †\( p < 0.05 \), versus uninoculated group, Student t test.
directly affect osteoclasts (83); hence, it is possible that, although liposome-encapsulated clodronate was used, there was some direct affect on osteoclasts that also contributed to the reduction in alveolar bone resorption.

Serum IgG and IgM Ab–specific responses to *P. gingivalis* Ags were detected in BALB/c and C57BL/6 mice from the PBS liposome infected and untreated/infected groups. The predominant IgG subclasses detected in the PBS liposome infected and untreated/infected C57BL/6 mice were IgG2b and IgG3, whereas IgG1 was the predominant subclass in the untreated/infected BALB/c mice. The contrasting IgG Ab responses to *P. gingivalis* infection seen between the BALB/c and C57BL/6 mouse strains may reflect differences in cytokine immune responses for each strain of mouse, because numerous studies reported that

**FIGURE 8.** Activation profile of *P. gingivalis*–primed peritoneal macrophages. (A) BALB/c and C57BL/6 mice were injected i.p. with HK-W50, and M1 ([TCRβ/B220/Ly6G<sup>−</sup>, CD11b<sup>+</sup>, CD86<sup>+</sup>]) and M2 ([TCRβ/B220/Ly6G<sup>−</sup>, CD11b<sup>−</sup>, CD206<sup>+</sup>]) macrophage infiltrations were monitored over time by flow cytometry. Data are mean ± SD (n = 6) and are from two separate experiments. Peritoneal macrophages ([TCRβ/B220/Ly6G<sup>−</sup>, CD11b<sup>+</sup>, F4/80<sup>+</sup>, CD115<sup>+</sup>]) isolated on day 6 post-i.p. injection with HK-W50 were incubated for 24 h in the presence or absence of HK-W50, and the levels of NO (B), urea (C), and cytokines (D) were determined. Data are mean ± SD (n = 3) and are from two separate experiments. *p < 0.01 versus unstimulated group.
BALB/c mice are predisposed to a Th2 anti-inflammatory cytokine phenotype, whereas C57BL/6 mice are predisposed to a Th1 proinflammatory cytokine phenotype (84, 85). There was no \textit{P. gingivalis}–specific IgG Ab detected in the unoinculated groups in either mouse strain. Therefore, the study shows that intraoral inoculation with \textit{P. gingivalis} resulted in the production of \textit{P. gingivalis}–specific Abs. This is consistent with other murine periodontitis studies in which IgG Abs were detected in mice infected with \textit{P. gingivalis}, and no specific Ab was detected in unoinculated mice (48, 73, 86). Further, this is also consistent with human studies in which \textit{P. gingivalis}–specific IgG Abs were detected in sera and gingival crevicular fluid from periodontitis patients, and the Ab titer correlated with disease severity (47, 87). In both C57BL/6 and BALB/c mice, treatment with clodronate liposomes significantly reduced the specific Ab response to \textit{P. gingivalis}. This is consistent with the depletion of macrophages resulting in significantly less Ag presentation in the clodronate-treated animals. However, because clodronate liposomes also can affect phagocytic dendritic cells (88), a reduction in dendritic cell numbers also may help to explain the decrease in Ab response.

Sera from BALB/c and C57BL/6 mice obtained at the termination of the periodontitis model experiment also were analyzed using the Bio-Plex assay for 10 cytokines. BALB/c mice infected with \textit{P. gingivalis} had detectable levels of serum IL-4, IL-10, and IL-12 (p70), whereas C57BL/6 mice infected with \textit{P. gingivalis} had detectable serum levels of IFN-\(\gamma\), IL-10, and IL-12 (p70). The overall serum cytokine levels in macrophage-depleted BALB/c and C57BL/6 mice inoculated intraorally with \textit{P. gingivalis} were undetectable or significantly reduced compared with sham-treated (PBS liposomes) and untreated mice infected with \textit{P. gingivalis}. There was no IL-12 (p70) detected in BALB/c and C57BL/6 mice depleted of macrophages and inoculated intraorally with \textit{P. gingivalis}; this similarity between the mouse strains could be attributed to the absence of macrophages, which are reported to be the primary cellular producers of IL-12 (16). Interestingly, macrophage-depleted C57BL/6 mice inoculated intraorally with \textit{P. gingivalis} had significantly reduced levels of detectable IFN-\(\gamma\) and IL-10 compared with sham-treated/infected mice. In the absence of macrophages, the low levels of IFN-\(\gamma\) and IL-10 may be attributed to dendritic cells in C57BL/6 mice, which, in response to bacterial Ags, primarily secrete proinflammatory cytokines promoting a Th1 cytokine phenotype, whereas dendritic cells derived from BALB/c mice secrete anti-inflammatory cytokines promoting a Th2 cytokine phenotype (89–94). The predisposition of C57BL/6 mice toward a Th1 cytokine immune response and that of BALB/c mice toward a Th2 cytokine immune response could explain the presence of IFN-\(\gamma\) in C57BL/6 mice and IL-4 in BALB/c mice. Furthermore, this also may explain the significantly (\(p < 0.01\)) higher level of serum IL-12 (p70) detected in C57BL/6 mice, but not in BALB/c mice, infected with \textit{P. gingivalis} compared with unoinculated mice.

Macrophages can be generally classified as M1 or M2 macrophages, which have an inflammatory or immunoregulatory/tissue repair role, respectively (40, 41). M1 and M2 macrophages are distinguished by their expression of CD86 (M1) and CD206 (M2) cell surface receptors and their functional activities based on the arginine-metabolism pathway (40, 42–44, 95). M1 macrophages metabolize arginine to form bactericidal NO via the inducible NO synthase pathway, whereas M2 macrophages break down arginine into urea and L-ornithine (collagen precursor) using the arginase-1 enzyme (96).

In the mouse periodontitis model, we found that there was a significant increase in M1 macrophages (defined as CD11b\(^+\), CD206\(^−\), [TCR\(\beta/\)B220/Ly6G\(^−\)]) and a relatively smaller M2 macrophage population (defined as CD11b\(^+\), CD206\(^+\), [TCR\(\beta/\)B220/Ly6G\(^−\)]) in gingival tissue of C57BL/6 and BALB/c mice infected with \textit{P. gingivalis}. This staining protocol was reported previously as a reproducible method for the identification of macrophage populations; however, it does not exclude some subpopulations of dendritic cells (97, 98). Intrapерitoneal injection of \textit{P. gingivalis} (HK-W50) induced a similar M1/M2 macrophage ratio in the peri- toneal cavity as that found in gingival tissue. Hence, in subsequent assays, peritoneal macrophages (CD11b\(^+\), F4/80\(^+\), CD115\(^+\), [TCR\(\beta/\)B220/Ly6G\(^−\)]) were used to determine M1/M2 macrophage functionality upon \textit{P. gingivalis} activation. Purified \textit{P. gingivalis} peritoneal macrophages produced NO and urea, indicating that the mixed population of M1 and M2 macrophages infiltrating into the \textit{P. gingivalis}–infected sites were functionally active. The reduced numbers of macrophages initially seen in the first 24 h after injection of HK-W50 reflects a well-documented “macrophage disappearance reaction” seen during an inflammatory reaction in the peritoneum (99).

\textit{P. gingivalis} infection enhanced the secretion of the cytokines IL-1\(\beta\), IL-6, IL-12 (p70), TNF-\(\alpha\), G-CSF, and GM-CSF, in addition to the chemokines eotaxin, MCP-1, MIP-1\(\alpha\), and MIP-\(\beta\) from macrophages, reflecting a proinflammatory response. These cytokines and chemokines are reported to act as proinflammatory mediators, induce monocytes to migrate from the bloodstream into gingival tissue, and act synergistically to further stimulate proinflammatory cytokine production (22, 25, 31, 33, 50, 54, 100). In addition, IL-10, which is produced primarily by macrophages, was detected among the vast array of cytokines induced by \textit{P. gingivalis} infection. IL-10 tends to upregulate the M2 macrophage phenotype, which potentially could lead to an increase in arginase production, resulting in collagen accumulation and fibrosis of gingival tissue, which is a common clinical characteristic of chronic periodontitis (101–104).

A significant reduction in the level of \textit{P. gingivalis} infection was observed in the mice treated with clodronate liposomes relative to the untreated, but infected, animals (Fig. 4). Interestingly, the treatment of mice with PBS liposomes also resulted in a significant reduction in the level of \textit{P. gingivalis} infection, possibly mediated by a modulation of macrophage function (Fig. 4). In both BALB/c and C57BL/6 mice inoculated intraorally with \textit{P. gingivalis}, a positive and significant correlation was found between the level of \textit{P. gingivalis} infection and alveolar bone resorption (Fig. 4). This result highlights the importance of the level of pathogen infection in alveolar bone resorption and is consistent with studies in humans that showed a correlation between the level of \textit{P. gingivalis} infection in subgingival plaque and periodontal attachment loss (7, 45, 105). The final level of \textit{P. gingivalis} infection in the LPS liposome–treated animals was ∼9–14% of the recoverable bacterial cell load, and this level still resulted in significant alveolar bone resorption (Fig. 3B). This is consistent with the result of Byrne et al. (45), who showed that this level of \textit{P. gingivalis} infection in subgingival plaque was associated with the risk for imminent periodontal attachment loss at that site in humans. The level of \textit{P. gingivalis} infection in the mice treated with clodronate liposomes was decreased to 7–12%, and these animals exhibited the lowest amount of alveolar bone resorption. Hence, these results help to explain the important nexus between pathogen and inflammation, because they suggest that inflammation is an important component of the host–biofilm interaction for the emergence of the pathobiont that dysregulates the host response to produce dysbiosis and alveolar bone resorption (8, 9, 106). The predominance of the M1 macrophage phenotype in gingival tissue after \textit{P. gingivalis} infection in the current study suggests that \textit{P. gingivalis} may promote a dysregulated inflammatory response orchestrated by M1 macrophages.
In conclusion, the results of this study suggest that M1 macrophages play an important role in P. gingivalis-induced alveolar bone resorption by being a central component of the immune network that contributes to a proinflammatory immune response to the periodontal pathogen, allowing infection, dysbiosis, and alveolar bone resorption.

Acknowledgments

We thank Julie Macguire (Monash Medical School, Monash Medical Centre, Prahran, VIC, Australia), Dr. Glenn Walker, and Emily Szyman for assistance with the real-time PCR methodology. Jenny Davis and Ben Radford are gratefully acknowledged for assistance with mouse husbandry. Yan Tan is thanked for assistance with the real-time PCR methodology.

Disclosures

The authors have no financial conflicts of interest.

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