

**IMMUNOPATHOLOGY AND INFECTIOUS DISEASES****FOXO1 Deletion Reduces Dendritic Cell Function and Enhances Susceptibility to Periodontitis**Wenmei Xiao,<sup>\*†</sup> Guangyu Dong,<sup>†</sup> Sandra Pacios,<sup>†</sup> Maher Alnammary,<sup>†</sup> Laura A. Barger,<sup>†</sup> Yu Wang,<sup>†‡</sup> Yingying Wu,<sup>†</sup> and Dana T. Graves<sup>†</sup>

From the Department of Periodontology,<sup>\*</sup> School and Hospital of Stomatology, Peking University, Beijing, China; the Department of Periodontics,<sup>†</sup> School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; and the Department of Implantology,<sup>‡</sup> School of Stomatology, Jilin University, Changchun, China

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Address correspondence to  
Dana T. Graves, D.D.S.,  
D.M.Sc., University of Penn-  
sylvania School of Dental  
Medicine, 240 South 40th St.,  
Philadelphia, PA 19104.  
E-mail: [dtgraves@dental.  
upenn.edu](mailto:dtgraves@dental.upenn.edu)

The host response plays both protective and destructive roles in periodontitis. FOXO1 is a transcription factor that is activated in dendritic cells (DCs), but its function *in vivo* has not been examined. We investigated the role of FOXO1 in activating DCs in experimental (CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup>) compared with matched control mice (CD11c.Cre<sup>-</sup>.FOXO1<sup>L/L</sup>) in response to oral pathogens. Lineage-specific FOXO1 deletion reduced the recruitment of DCs to oral mucosal epithelium by approximately 40%. FOXO1 was needed for expression of genes that regulate migration, including integrins  $\alpha$ v and  $\beta$ 3 and matrix metalloproteinase-2. Ablation of FOXO1 in DCs significantly decreased IL-12 produced by DCs in mucosal surfaces. Moreover, FOXO1 deletion reduced migration of DCs to lymph nodes, reduced capacity of DCs to induce formation of plasma cells, and reduced production of bacteria-specific antibody. The decrease in DC function in the experimental mice led to increased susceptibility to periodontitis through a mechanism that involved a compensatory increase in osteoclastogenic factors, IL-1 $\beta$ , IL-17, and RANKL. Thus, we reveal a critical role for FOXO1 in DC recruitment to oral mucosal epithelium and activation of adaptive immunity induced by oral inoculation of bacteria. (*Am J Pathol* 2015, 185: 1085–1093; <http://dx.doi.org/10.1016/j.ajpath.2014.12.006>)

Periodontitis is one of the most prevalent infectious diseases worldwide and the most common cause of inflammatory bone loss.<sup>1,2</sup> Moderate to severe periodontal disease occurs in approximately 30% of adults in the United States<sup>3</sup> and is the most frequent cause of tooth loss.<sup>4</sup> The periodontium is a complex set of tissues that is chronically exposed to large numbers of bacteria that stimulate an inflammatory response, which can induce periodontitis characterized by loss of supporting connective tissue and alveolar bone around the teeth.<sup>5,6</sup> The innate and adaptive immune response induced by infection rather than the direct pathologic effects of the bacteria stimulate periodontal tissue destruction.<sup>7,8</sup> Immune cells, such as polymorphonuclear leukocytes, monocytes or macrophages, lymphocytes, and dendritic cells (DCs), have been linked to periodontal disease in humans and experimental animal models.<sup>2,5</sup> Inflammatory cytokines and prostaglandins play a critical role in promoting breakdown of connective tissue and osteolysis.<sup>8–10</sup>

DCs in the oral mucosa detect bacteria and migrate into regional lymph nodes, where they stimulate antigen-specific

T- and B-cell proliferation, thereby initiating adaptive immune responses,<sup>11</sup> including transformation of B cells to plasma cells that efficiently produce antibodies. Deletion of lymphocyte subsets, such as CD4- and CD8-positive T cells, reduces periodontal bone loss, whereas adoptive transfer of these cells typically increases it.<sup>11,12</sup> Lymphocyte products, particularly IL-17, interferon (IFN)- $\gamma$ , and RANKL, are increased in periodontal disease and functionally linked to periodontal breakdown in animal studies.<sup>13,14</sup> Deletion of Langerhans cells, a unique DC subset, leads to increased periodontal bone loss, suggesting that these cells play a protective role.<sup>15</sup> However, it has also been proposed that the DCs promote periodontitis through activation of the adaptive immune response and as a source of osteoclast precursors that promote bone resorption.<sup>11</sup>

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Disclosures: None declared.

FOXO1, a member of the forkhead box O family of transcription factors, plays an important role in the regulation of many cellular and biological processes, including protection against oxidative stress, promotion of apoptosis, and progression through the cell cycle.<sup>16</sup> FOXO1 is involved in immune responses by controlling cytokine production in a number of cell types.<sup>16–18</sup> We recently reported that FOXO1 mediates lipopolysaccharide-induced cytokine expression in DCs.<sup>18</sup> However, the role of FOXO1 in regulating the local response of DCs to bacterial infection has not been previously investigated.

We examined CD11c.Cre<sup>+/-</sup>.FOXO1<sup>LL</sup> mice using the lineage-specific ablation of FOXO1 to investigate its function in DCs in response to oral pathogens *in vivo*. We hypothesized that the lack of FOXO1 in DCs would reduce activation of the adaptive immune response and reduce periodontal disease. Deletion of FOXO1 in DCs led to a reduced DC function manifested by reduced DC expression of IL-12 and a reduced capacity to stimulate an adaptive immune response. This effect led to an increase in factors that stimulate osteoclast formation, enhancing susceptibility to periodontal bone loss.

## Materials and Methods

### Mice

CD11c-expressing Cre recombinase mice were purchased from Jackson Laboratories (Bar Harbor, ME). FOXO1<sup>LL</sup> mice were generously provided by Dr. Ronald De Pinho (University of Texas MD Anderson Cancer Center, Houston, TX). FOXO1<sup>LL</sup> mice were bred with CD11c.Cre recombinase mice to generate the experimental mice (CD11c.Cre<sup>+</sup>.FOXO1<sup>LL</sup>) and the control mice littermates (CD11c.Cre<sup>-</sup>.FOXO1<sup>LL</sup>). Other than the differences reported here, there were no obvious phenotypic differences between the experimental and control groups. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

### Bacteria and Oral Infection

Broth-grown *Porphyromonas gingivalis* (#33277; ATCC, Manassas, VA) and *Fusobacterium nucleatum* (#25586; ATCC) in logarithmic growth phase were collected and suspended in sterile phosphate-buffered saline. Periodontal infection was induced by oral inoculation, three times per week for 2 weeks, with *P. gingivalis* and *F. nucleatum*, prepared as previously described.<sup>19</sup> To reduce the original oral flora, animals were given sulfamethoxazole-trimethoprim, 10 mL per pint in deionized water, drinking *ad libitum* for 10 days. Experimental animals were given 10<sup>9</sup> colony-forming units of *P. gingivalis* and 10<sup>9</sup> colony-forming units of *F. nucleatum* suspended in 100  $\mu$ L of 2% carboxymethyl cellulose in phosphate-buffered saline (Sigma-Aldrich, St Louis, MO), which was directly inoculated into the cavity. Controls consisted of sham-infected mice that

received the antibiotic pretreatment and the carboxymethyl cellulose oral inoculation without *P. gingivalis* and *F. nucleatum*. Antibody titers generated to *P. gingivalis* are typically higher than those to *F. nucleatum*. Because of limited serum samples, we focused on *P. gingivalis*. Anti-*P. gingivalis* IgG1 was measured by enzyme-linked immunosorbent assay as previously described, and the concentration was determined by reference to a standard curve.<sup>20</sup>

### Flow Cytometry Analysis

Spleens were harvested from experimental mice (CD11c.Cre<sup>+</sup>.FOXO1<sup>LL</sup>) and the control littermates (CD11c.Cre<sup>-</sup>.FOXO1<sup>LL</sup>). Splenocytes were isolated and stained by specific antibodies (all from eBioscience, San Diego, CA). DCs, T cells, and B cells were analyzed and gated as CD11c<sup>+</sup>MHCII<sup>+</sup>, CD3<sup>+</sup>, and B220<sup>+</sup> cells by flow cytometry (FACS LSRII; BD Biosciences, San Jose, CA). Bone marrow-derived DCs from CD11c.Cre<sup>+</sup>.FOXO1<sup>LL</sup> and CD11c.Cre<sup>-</sup>.FOXO1<sup>LL</sup> mice were cultured *in vitro* for 48 hours and then analyzed by flow cytometry with DAPI labeling for the dead cells.

### Alveolar Bone Loss

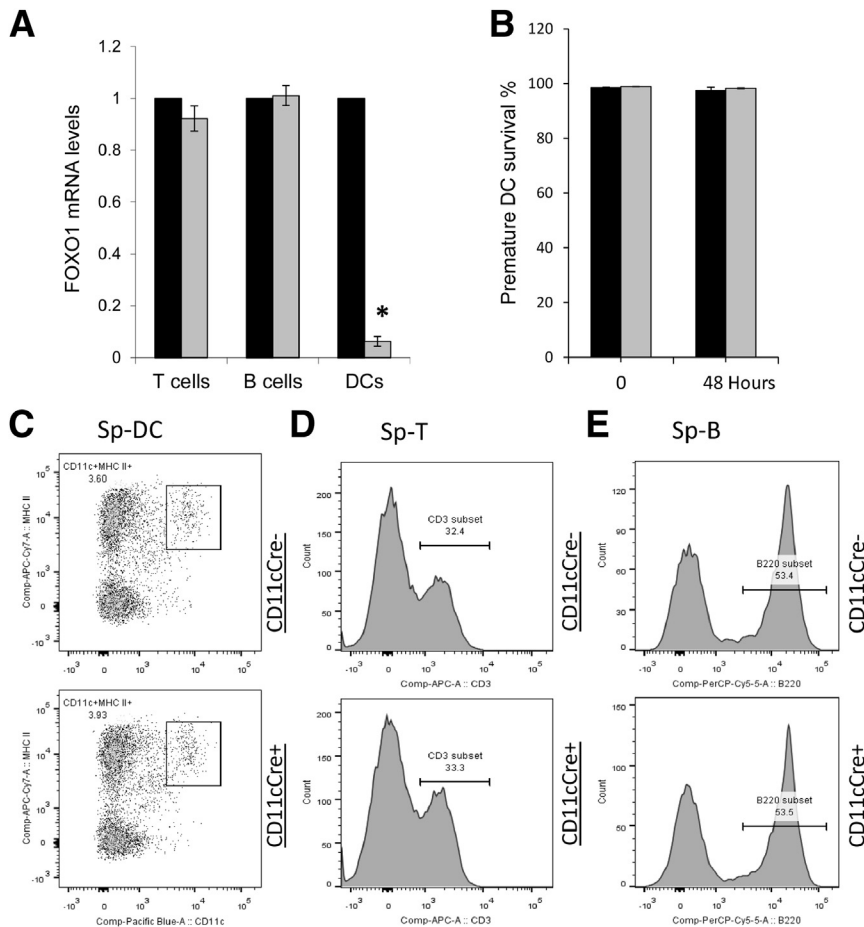
For micro-computed tomography (micro-CT) analysis, the oral cavity was inoculated with bacteria as described above, and mice were euthanized 6 weeks later. The mandible and maxilla were dissected, fixed for 24 hours in cold 10% formalin, and prepared for micro-CT analysis using a micro-CT-40 (Scanco Medical AG, Bassersdorf, Switzerland) to assess periodontal bone levels as previously described.<sup>21</sup> The interproximal bone levels were established using the multi-planar reconstruction tool using OsiriX software version 3.9.4 (Pixmeo, Geneva, Switzerland). For each group there were six to seven mice. Histomorphometric analysis was performed with Image ProPlus software version 7.0 (Media Cybernetics, Silver Spring, MD). The bone loss was measured by the length of bone crest to the cemento-enamel junction in the experimental group minus the distance in the noninfected group. The attachment loss was measured by the length from cemento-enamel junction to the apical extent of epithelium along the root surface.

### Osteoclasts

Osteoclasts were identified and counted as multinucleated tartrate-resistant acid phosphatase-positive bone-lining cells. The region of interest was the coronal 0.35 mm of alveolar bone and gingiva as we have previously described.<sup>22</sup>

### Immunohistochemistry

DCs in the gingiva and lymph nodes *in vivo* were measured using a specific antibody to CD205 (NLDC145; Serotec, Oxford, UK), a marker for DCs. Activated DCs were



**Figure 1** Lineage-specific *FOXO1* deletion in  $CD11c^+$  cells does not affect the population of dendritic cell (DCs), T cells, and B cells in spleen from experimental mice ( $CD11c.Cre^+.FOXO1^{L/L}$ ) compared with the control littermates. **A:** *FOXO1* expression in DCs. RNA was isolated from purified splenic DCs, T cells, and B cells from  $CD11c.Cre^+.FOXO1^{L/L}$  (gray bars) and  $CD11c.Cre^-.FOXO1^{L/L}$  (black bars) mice. *FOXO1* mRNA levels were measured by real-time PCR and normalized to ribosomal protein L32. **B:** Bone marrow–derived DCs from  $CD11c.Cre^+.FOXO1^{L/L}$  and  $CD11c.Cre^-.FOXO1^{L/L}$  mice were cultured *in vitro* for 48 hours and then analyzed by flow cytometry with DAPI labeling for the dead cells. Alive cells designated as DAPI negative cells. **C–E:** Splenocytes from experimental mice ( $CD11c.Cre^+.FOXO1^{L/L}$ ) and the control littermates ( $CD11c.Cre^-.FOXO1^{L/L}$ ) were isolated and stained by specific antibodies. DCs, T cells, and B cells were analyzed and gated as  $CD11c^+MHCII^+$ ,  $CD3^+$ , or  $B220^+$  cells by flow cytometry. The data represent two to three experiments as similar results. \* $P < 0.05$  between experimental  $CD11c.Cre^+.FOXO1^{L/L}$  and littermate  $CD11c.Cre^-.FOXO1^{L/L}$  control mice. Sp-B, splenocyte B cells; Sp-DC, splenocyte DCs; Sp-T, splenocyte T cells.

identified by dual immunofluorescence with an antibody to CD205 and an antibody to IL-12 (R&D Systems, Minneapolis, MN). The numbers of cells with co-localized CD205 and IL-12 in the epithelium or connective tissue adjacent to the epithelium were counted. Cells expressing RANKL (N-19; Santa Cruze Biotechnology, Dallas, TX) near the bone surface were detected by mean fluorescence intensity. The number of plasma cells in lymph nodes was measured using a specific antibody to CD138 (BD Biosciences, San Jose, CA). Primary antibody was detected by biotinylated secondary antibody followed by fluorescein-conjugated avidin (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and mounting with Fluoroshield (Sigma-Aldrich). To enhance the signal-to-noise ratio, citrate (pH 6) antigen retrieval was used along with tyramide signal amplification that enhances the chromogenic signal (PerkinElmer, Waltham, MA). Images were captured with fluorescence microscopy and analyzed using Nikon NIS-Elements software version 3.2 (Nikon, Melville, NY).

### Real-Time PCR

Total mRNA was extracted from gingiva dissected around maxilla molars for the inflammation cytokines test. For *FOXO1* expression, DCs were isolated from mouse spleens and enriched by CD11c microbeads (Miltenyi Biotec, Auburn, CA) followed by fluorescence activated cell sorting (FACS Aria; BD

Biosciences). DC purity was typically  $>95\%$  as  $CD11c^+MHCII^+B220^-NK1.1^-$  cells. T cells were gated as  $CD90.2^+B220^-$  and B cells as  $CD90.2^-B220^+$  and were  $>95\%$  enriched. For DC migration, total RNA was isolated from the bone marrow dendritic cells purified by CD11c microbeads (Miltenyi Biotec).<sup>23</sup> Relative mRNA levels were determined by quantitative real-time PCR using Taq-Man primers and probe sets (Applied Biosystems, Foster City, CA). Results were normalized to a housekeeping gene, ribosomal protein L32. The experiments were performed with six to eight animals per group with triplicate samples and performed two to three times with similar results.

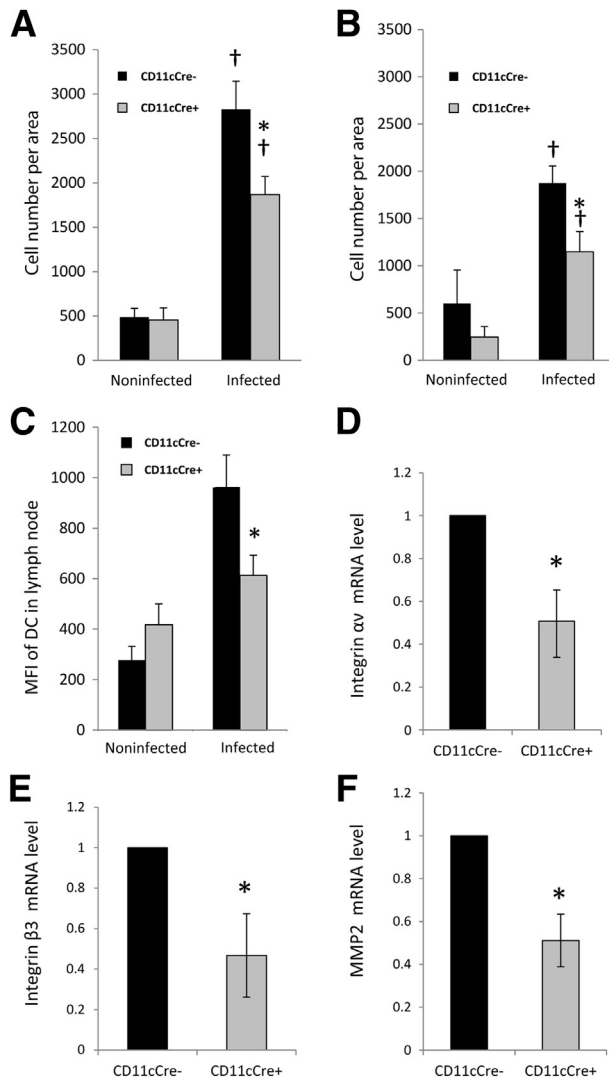
### Statistical Analysis

The individual mouse was chosen as the unit of analysis. Results are presented as the means  $\pm$  SEM of specimens. Statistical tests were performed using one-way analysis of variance and Student's *t*-test. The significance level was set at  $P < 0.05$ .

## Results

### FOXO1 Expression

To establish that CD11c-driven Cre recombinase deletes *FOXO1* in  $CD11c^+$  dendritic cells but not T or B cells,



**Figure 2** *FOXO1* deletion reduces dendritic cell (DC) numbers in bacteria-inoculated tissue and mRNA levels of genes needed for DC migration. Mice were subjected to oral inoculation of the periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* as described in *Materials and Methods*. Six weeks later the periodontal tissue, including the gingival mucosal epithelium, connective tissue, teeth, and bone, was obtained. **A** and **B**: The number of DCs per area of epithelium (**A**) and connective tissue (**B**) was measured as the number of CD205<sup>+</sup> cells using specific antibody CD205 compared with matched IgG control. **C**: Mean fluorescence intensity (MFI) of DCs in lymph nodes. **D–F**: Total RNA was isolated from bone marrow–derived DCs from experimental and control mice. Integrins  $\alpha\nu$  (**D**),  $\beta 3$  (**E**), and matrix metalloproteinase (MMP)-2 (**F**) mRNA levels were measured by real-time PCR normalized to ribosomal protein L32 or 18S. The data are representative of two or three separate experiments. \* $P < 0.05$  between DCs from experimental CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> and littermate CD11c.Cre<sup>-</sup>.*FOXO1*<sup>L/L</sup> control mice; † $P < 0.05$  between noninfected and infected groups in littermate CD11c.Cre<sup>-</sup>.*FOXO1*<sup>L/L</sup> control mice and experimental CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> mice, respectively.

leukocytes were isolated from the spleen and purified by fluorescence activated cell sorting. *FOXO1* mRNA levels were reduced 93% in splenic DCs from experimental CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> mice compared with littermate

controls ( $P < 0.05$ ) but had no effect on mRNA levels in purified T and B cells ( $P > 0.05$ ) (Figure 1A). To establish whether lineage-specific *FOXO1* deletion in CD11c<sup>+</sup> affects the premature DC apoptosis, bone marrow–derived DCs from CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> and CD11c.Cre<sup>-</sup>.*FOXO1*<sup>L/L</sup> mice were cultured *in vitro* for 48 hours and then analyzed by flow cytometry with DAPI labeling for the dead cells. Alive cells dedicated as DAPI-negative cells, which revealed that premature DCs did not undergo apoptosis during 48-hour culture and *FOXO1* deletion did not affect DC survival (Figure 1B). To establish whether lineage-specific *FOXO1* deletion in CD11c<sup>+</sup> cells affects the population of DCs, T cells, and B cells, flow cytometry was performed. Splenocytes from experimental mice (CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup>) and the control littermates (CD11c.Cre<sup>-</sup>.*FOXO1*<sup>L/L</sup>) were isolated and stained by matched specific antibodies. DCs, T cells, and B cells were analyzed and gated as CD11c<sup>+</sup>MHCII<sup>+</sup>, CD3<sup>+</sup>, or B220<sup>+</sup> cells. The data revealed no difference of DC, T-cell, and B-cell populations between experimental and control littermates (Figure 1, C–E). Thus, lineage-specific *FOXO1* deletion in CD11c<sup>+</sup> cells has no effect on splenic DC, T-cell, and B-cell numbers.

### DC Recruitment to Gingiva and Lymph Nodes Is *FOXO1* Dependent

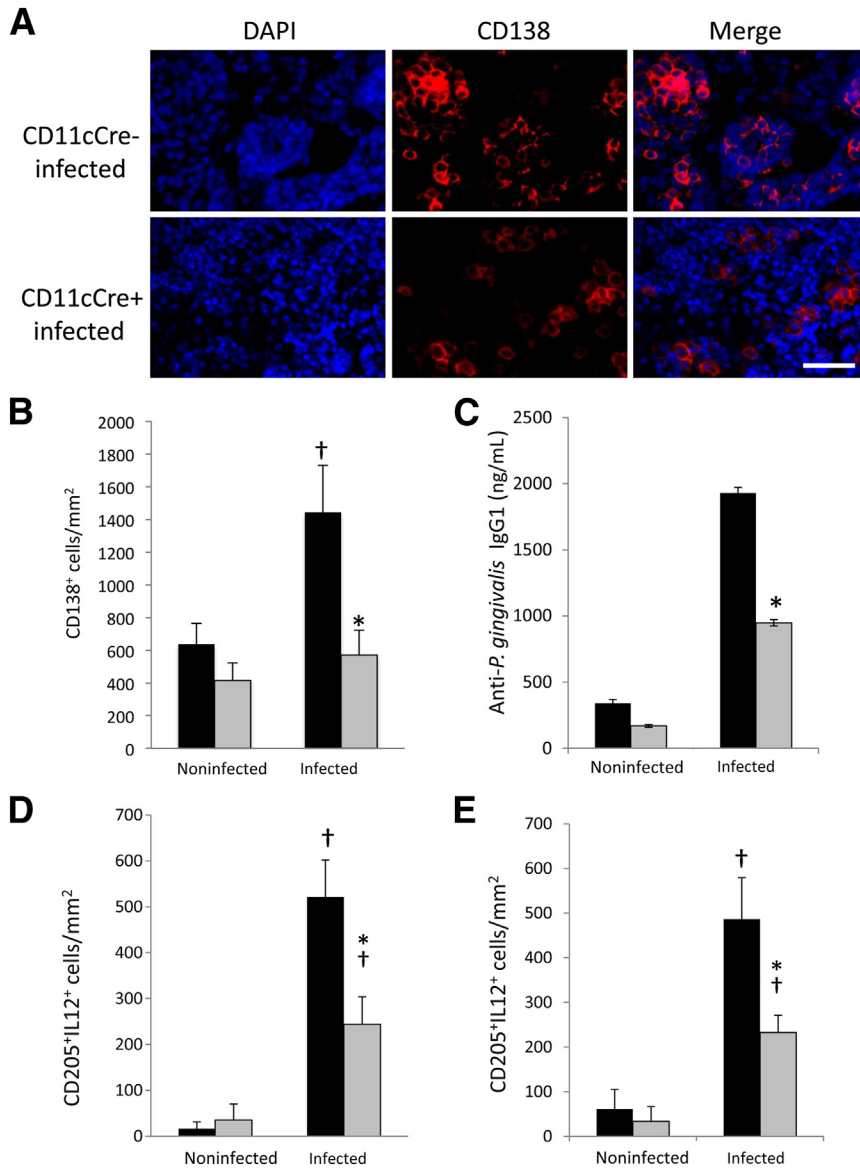
We determined whether deletion of *FOXO1* in DCs would affect the recruitment of DCs to gingiva, the site of oral infection or draining cervical lymph nodes. The number of DCs was measured using specific antibody to CD205, a marker of DCs. Oral infection with periodontal pathogens stimulated a 5.5-fold increase in the number of DCs in the epithelium ( $P < 0.05$ ) and 3.5-fold increase in the connective tissue in control mice (Figure 2, A and B). The number of DCs was reduced by 36% in the epithelium and 32% in the connective tissue of CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> mice compared with control littermates (Figure 2, A and B). In lymph nodes, the number of DCs was reduced by 37% in the CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> mice compared with control littermates (Figure 2C).

DC migration is affected by a number of different genes, including chemokine receptor integrins  $\alpha\nu$  and  $\beta 3$ , and by matrix metalloproteinase-2.<sup>24,25</sup> The effect of *FOXO1* on the mRNA levels of each was assessed. *FOXO1* deletion in experimental mice reduced mRNA levels for these genes by approximately 50% (Figure 2, D–F).

### Infection Stimulated DC Activity *in Vivo* Is *FOXO1* Dependent

To test the capacity of DCs to activate lymphocytes *in vivo*, the number of plasma cells was measured in the draining lymph nodes by immunofluorescence with antibody to CD138 and by the production of anti-*P. gingivalis* antibody. Infection stimulated a 2.3-fold increase in the number of plasma cells in cervical lymph nodes (Figure 3, A and B).





**Figure 3** FOXO1 deletion in dendritic cell (DC) reduces stimulation of an adaptive immune response and activation of DCs in gingival tissue. Mice were infected by oral inoculation of the periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* as described in *Materials and Methods*. **A:** Paraffin sections of neck lymph nodes from CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> mice or control littermate mice were stained for plasma cells by immunofluorescence. CD138 (red) staining decreases in CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> mice relative to control littermate mice after infection. **B:** The number of plasma cells in lymph nodes were quantified in immunofluorescent images as described in **A**. **C:** Experimental CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> (gray bars) and control CD11c.Cre<sup>-</sup>.FOXO1<sup>L/L</sup> (black bars) mice were administered bacteria by oral inoculation. Controls consisted of sham-infected mice that received the antibiotic pretreatment and the carboxymethyl cellulose oral inoculation. The level of *P. gingivalis*-specific IgG1 was measured by enzyme-linked immunosorbent assay. **D** and **E:** The number of activated DCs *in vivo* was measured using specific antibodies (CD205, a marker for DC and IL-12, a cytokine produced by activated DCs) and three-color immunofluorescence. **D:** The number of DCs that produced IL-12 in epithelium. **E:** The number of DCs that produced IL-12 in connective tissue. \**P* < 0.05 between experimental CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> and littermate CD11c.Cre<sup>-</sup>.FOXO1<sup>L/L</sup> control mice; †*P* < 0.05 between noninfected and infected groups in littermate CD11c.Cre<sup>-</sup>.FOXO1<sup>L/L</sup> control mice and experimental CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> mice, respectively. Scale bar = 50 μm. Original magnification: ×400 (**A**).

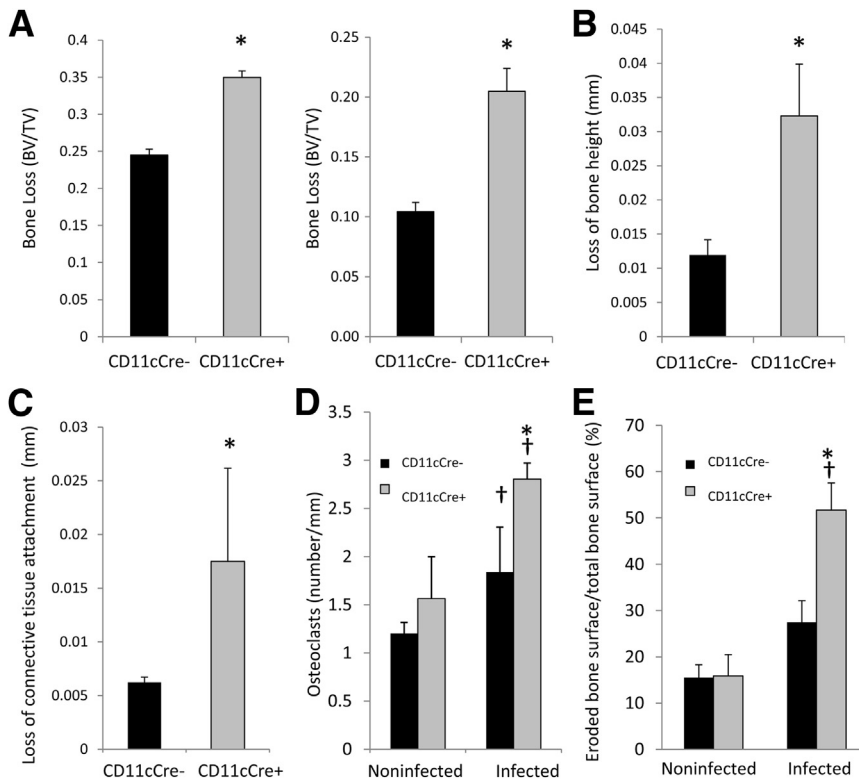
The number of plasma cell was reduced by 57% in the CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> mice compared with control littermates (*P* < 0.05). The level of *P. gingivalis*-specific IgG1 was measured in serum by enzyme-linked immunosorbent assay. Infection stimulated a 4.3-fold increase in anti-*P. gingivalis* IgG1. This production was reduced 50% in the CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> mice compared with littermates that did not have FOXO1 deleted in DCs (Figure 3C).

The effect of FOXO1 on DC activation was tested by examining the number of DCs that expressed IL-12 after infection. At baseline there was no difference between experimental and control mice in IL-12-expressing DCs in the gingival epithelium or connective tissue (Figure 3, D and E). Infection stimulated a fivefold increase of IL-12-expressing DCs in the epithelium of control mice and a similar increase in connective tissue (*P* < 0.05). Lineage-specific FOXO1 deletion reduced the number of CD205<sup>+</sup>IL-12<sup>+</sup> DC cells in the gingival epithelium after

infection by 47% and by 48% in the gingival connective tissue (Figure 3, D and E).

#### Increased Alveolar Bone Loss in CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> Mice

Susceptibility to periodontal disease was measured by micro-CT and in histologic sections by loss of bone height and loss of connective tissue attachment of the gingiva to tooth surface. Infection stimulated 40% (maxilla) and 50% (mandible) more bone loss in experimental CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> mice compared with the control mice littermates, CD11c.Cre<sup>-</sup>.FOXO1<sup>L/L</sup> (Figure 4A). Histologically, lineage-specific deletion of FOXO1 resulted in a 63% greater loss of bone height (Figure 4B) and 64% more loss of connective tissue attachment (Figure 4C) in experimental CD11c.Cre<sup>+</sup>.FOXO1<sup>L/L</sup> mice compared with the control mice littermates, CD11c.Cre<sup>-</sup>.FOXO1<sup>L/L</sup>.



**Figure 4** *FOXO1* deletion in dendritic cell (DC) enhances susceptibility to periodontal disease. Mice were infected by oral inoculation of the periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* as described in *Materials and Methods*. **A:** The micro-computed tomographic analysis of the periodontal bone was examined in both the maxilla (left) and mandible (right). **B:** Loss of bone height. **C:** Loss of connective tissue attachment. **D:** Osteoclast count over bone length. **E:** Eroded bone surface over total bone surface. \* $P < 0.05$  between experimental CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> and littermate CD11c.Cre<sup>-</sup>.*FOXO1*<sup>L/L</sup> control mice; † $P < 0.05$  between noninfected and infected groups in littermate CD11c.Cre<sup>-</sup>.*FOXO1*<sup>L/L</sup> control mice and experimental CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> mice, respectively. BV, bone volume; TV, total volume.

To account for the greater bone loss, we examined the number of osteoclasts that were induced by bacterial infection. Periodontal infection induced twofold increase in the number of osteoclasts compared with baseline (Figure 4D) and a more than threefold increase in osteoclast activity measured by eroded bone surface in mice with DC *FOXO1* deletion (Figure 4E). In control littermates, the increase in osteoclast numbers was reduced by 34% and osteoclast activity by 47%.

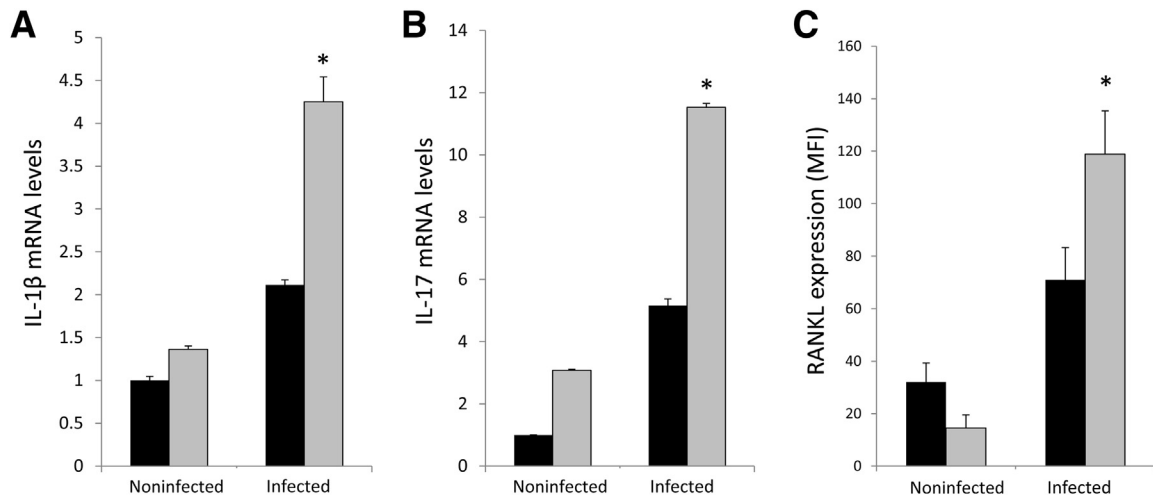
#### Differential Inflammatory Mediator Responses in Periodontal Tissue after Infection

Because osteoclast formation and bone loss are tied to the production of inflammatory mediators, we measured the effect of lineage-specific *FOXO1* ablation on cytokines IL-1 $\beta$  and IL-17 that promote periodontal bone loss.<sup>26,27</sup> Infection stimulated twofold higher levels of IL-1 $\beta$  and fivefold higher levels of IL-17 in control animals with bacterial infection compared with baseline (Figure 5, A and B). The level of these inflammatory cytokines was at least twofold higher in the gingiva of experimental (CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup>) mice compared with control littermates ( $P < 0.05$ ). We also measured RANKL expression in close proximity to bone by immunofluorescence because it is one of the principal osteoclast-inducing factors.<sup>28</sup> The mean fluorescence intensity of RANKL increased to 12-fold higher levels in mice with DC *FOXO1* deletion compared with matched control mice (Figure 5C).

#### Discussion

*FOXO1* is a transcription factor that is needed for expression of inflammatory mediators in cells that regulate inflammation and DCs.<sup>16,18</sup> This may be important in the oral gingiva, where DCs and Langerhans cells have been postulated to play a role in periodontal bone loss.<sup>15,18</sup> Oral inoculation of periodontal pathogens activates the adaptive immune response to stimulate periodontal bone loss.<sup>5</sup> Because some mice are resistant to induction of periodontal disease, we infected mice with both *P. gingivalis* and *F. nucleatum*, which induces periodontal disease more reproducibly than either alone.<sup>29</sup> This model replicates the major events that occur in periodontal disease, including the loss of connective tissue attachment and bone resorption.<sup>5</sup> Using this model, we found that DC deletion of *FOXO1* reduces recruitment of DCs to oral mucosal epithelium and connective tissue. This decreases activation of adaptive immunity induced by oral inoculation of bacteria but leads to a compensatory increase in pro-osteoclastogenic factors that enhance susceptibility to periodontal bone loss.

Deletion of *FOXO1* in DCs of experimental mice induced greater bone loss compared with matched controls. We found that the absence of *FOXO1* in DCs led to 50% more bone loss after infection. Consistent with this observation, *FOXO1* deletion in DCs significantly increased the number of osteoclasts and osteoclast activity after infection with oral pathogens. The level of cytokines that promote bone resorption was significantly elevated by



**Figure 5** *FOXO1* deletion increases the level of pro-osteoclastogenic cytokines. Mice were infected by oral inoculation of the periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* as described in *Materials and Methods*. **A** and **B**: Total RNA was extracted from gingival tissue from experimental (gray bars) and control (black bars) mice. IL-1 $\beta$  (**A**) and IL-17 (**B**) mRNA levels were measured by real-time PCR normalized to ribosomal protein L32. **C**: RANKL expression was assessed by immunofluorescence with anti-RANKL antibody compared with matched control IgG in the gingival connective tissue in close proximity to bone. Data are presented as the mean fluorescence intensity (MFI). \* $P < 0.05$  between experimental CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> and littermate CD11c.Cre<sup>-</sup>.*FOXO1*<sup>L/L</sup> control mice.

lineage-specific deletion of *FOXO1*. IL-1 $\beta$  has been functionally linked to alveolar bone resorption,<sup>26</sup> which has been shown to stimulate osteoclast formation by inducing RANKL.<sup>28</sup> RANKL directly stimulates osteoclastogenesis and bone resorption.<sup>30</sup> The role of IL-17 in periodontal disease is less certain, although recent evidence indicates that inhibition of IL-17 can reduce periodontal bone loss in animal models of periodontal disease and in bone destruction associated with arthritis.<sup>26,31,32</sup> In human studies, IL-1 $\beta$ , IL-17, and RANKL levels are significantly higher in patients with periodontitis than in healthy controls.<sup>2,5,32</sup> These results suggest a mechanism whereby a decrease in DC *FOXO1* leads to up-regulation of the host response and greater expression of proinflammatory cytokines that induce bone resorption, particularly IL-1 $\beta$ , IL-17, and RANKL.

Oral infection with periodontal pathogens significantly increased the number of DCs in the mucosal epithelium and subepithelial connective tissue of the gingiva. Periodontal pathogens, such as *P. gingivalis*, stimulate DC activity.<sup>33</sup> In a human study, the number of DCs in the gingiva increased as dental plaque accumulated and decreased with plaque removal.<sup>34</sup> The number of DCs in the gingival tissue was significantly reduced by DC deletion of *FOXO*<sup>L/L</sup>. This finding is likely significant because DCs, particularly in the gingival epithelium, capture periodontal pathogens to transport them to the lymph nodes and activate a humoral antibody response.<sup>33</sup> *FOXO1* deletion also down-regulated genes that play an important role in DC migration, including integrin  $\alpha\nu$ , integrin  $\beta 3$ , and matrix metalloproteinase-2. This decrease is likely to contribute to the reduced migration of DCs to gingival tissues in *FOXO1*-deleted mice. In this study, *FOXO1* deletion did not affect DC survival *in vitro*; however, it is still a possibility that decreased DC

survival in experimental animals reduced the number of infiltrating DCs.

A critical aspect of DC behavior is homing to regional lymph nodes.<sup>35,36</sup> Oral infection stimulated an increase in the number of DCs that homed to lymph nodes, which was decreased in experimental CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> mice compared with control littermates. Because DCs may directly stimulate B cells, we examined the effect of *FOXO1* deletion on the capacity of DC to stimulate an adaptive immune response after infection of mucosal surfaces. *FOXO1* deletion in DCs decreased stimulation of B cells to form plasma cells in the lymph nodes. Plasma cell number in the lymph nodes was reduced almost 60% in the CD11c.Cre<sup>+</sup>.*FOXO1*<sup>L/L</sup> mice compared with control littermates. In addition to decreased plasma cells, DC *FOXO1* deletion reduced the formation of pathogen-specific antibody. The results suggest that *FOXO1* is important in regulating activity of DC that stimulates an adaptive immune response. They are also consistent with the hypothesis that antibodies against periodontal pathogens protect against periodontal bone loss. In our study, the reduction in an adaptive immune response resulted in a compensatory increase in production of pro-osteoclastogenic cytokines that lead to periodontal bone resorption. Similar results were seen when lymphangiogenesis was blocked.<sup>37</sup> In the latter experiments, transgenic mice that have deficient lymphangiogenesis (K14–vascular endothelial growth receptor-3–immunoglobulin) exhibit a reduced humoral immune response and an increased susceptibility to periodontal disease. The latter is manifested by a compensatory increase in IL-1 $\beta$  and IL-17 and an increase in bacteria-induced bone loss. Our results suggest a similar result with deletion of *FOXO1*. Reduced *FOXO1* in DCs leads to impaired

activation of B cells in lymph nodes, which reduces antibody production, resulting in a compensatory increase in cytokines that increase susceptibility to periodontal disease. Thus, FOXO1 is an important transcription factor expressed in DCs that may be necessary to generate a protective humoral immune response.

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