G(−) Anaerobes–Reactive CD4$^+$ T-Cells Trigger RANKL-Mediated Enhanced Alveolar Bone Loss in Diabetic NOD Mice

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Diabetic patients experience a higher risk for severe periodontitis; however, the underlying mechanism remains unclear. We investigated the contribution of antibacterial T-cell–mediated immunity to enhanced alveolar bone loss during periodontal infection in nonobese diabetic (NOD) mice by oral inoculation with *Actinobacillus actinomycetemcomitans*, a G(−) anaerobe responsible for juvenile and severe periodontitis. The results show that *1*) inoculation with *A. actinomycetemcomitans* in pre-diabetic NOD mice does not alter the onset, incidence, and severity of diabetes; *2*) after *A. actinomycetemcomitans* inoculation, diabetic NOD mice (blood glucose >200 mg/dl and with severe insulitis) exhibit significantly higher alveolar bone loss compared with pre-diabetic and nondiabetic NOD mice; and *3*) *A. actinomycetemcomitans*–reactive CD4$^+$ T-cells in diabetic mice exhibit significantly higher proliferation and receptor activator of nuclear factor κB ligand (RANKL) expression. When diabetic mice are treated with the RANKL antagonist osteoprotegerin (OPG), there is a significant reversal of alveolar bone loss, as well as reduced RANKL expression in *A. actinomycetemcomitans*–reactive CD4$^+$ T-cells. This study clearly describes the impact of autoimmunity to anaerobic infection in an experimental periodontitis model of type 1 diabetes. Thus, microorganism-reactive CD4$^+$ T-cells and the RANKL-OPG axis provide the molecular basis of the advanced periodontal breakdown in diabetes and, therefore, OPG may hold therapeutic potential for treating bone loss in diabetic subjects at high risk. *Diabetes* 54:1477–1486, 2005

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CLN, cervical lymph node; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; IL, interleukin; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; s-RANKL, soluble RANKL; STZ, streptozocin; TNF, tumor necrosis factor.

Human periodontal disease is a chronic inflammatory disease triggered by infection with specific subgingival microorganisms. Initially, inflammation and bleeding of the gingival tissue (gingivitis) prevails, followed by destruction of the supporting connective and bone tissues around the tooth (periodontitis), eventually leading to tooth loss. Microbial species such as *Actinobacillus actinomycetemcomitans*, *Porphorymonas gingivalis*, *Bacteriodes forsythus*, and mixed spirochetes have been shown to be involved in the disease pathogenesis, the development of which is further influenced by the host's immune system, diet, oral hygiene, and any genetic predisposition (1,2). The G(−) anaerobe *A. actinomycetemcomitans* is responsible for >90% juvenile periodontitis (or aggressive periodontitis [3]). *A. actinomycetemcomitans*–associated periodontitis is marked by rapid destruction of bone and connective tissues (4–6), and this species has also been implicated in life-threatening diseases like endocarditis and pneumonia (3,5). To date, many virulence factors (e.g., leukotoxin, adhesins, toxins, and immunosuppressive factors [4–6]) have been identified and studied.

T-cells, especially CD4$^+$ T-cells, are implicated in periodontal inflammation and tissue destruction (7–9) and may secrete proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α (2,7–9). Receptor activator of nuclear factor-κB (RANK) ligand (RANKL), a member of the TNF ligand super-family, is a recently discovered molecule that links T-cell–mediated immunity and bone remodeling and is expressed by osteoblasts and bone stromal cells (10–14). Its biological activity involves interactions with RANK on osteoclasts and osteoclast precursors for their differentiation, activation, and survival (10–12). Another player is osteoprotegerin (OPG), secreted by osteoblasts and bone marrow stromal cells and functioning by interrupting RANK-RANKL signaling by binding to membrane-bound and soluble RANKL (s-RANKL [11–13]). OPG transgenic mice are osteopetrotic with defective osteoclast activity, and OPG-deficient mice are severely osteoporotic (12). Moreover, the RANK-RANKL interaction also occurs in the immune system, including lymph node organogenesis and B-cell development as elucidated by the study of RANKL$^{-/-}$ mice (14,15). In addition, activated and memory T-cells express RANKL in response to bacterial stimulation and thereby

promote osteoclast differentiation and maturation (2,8,11, 15,16). Genetic mutations of RANKL and RANK demonstrate similar phenotypes in osteoclast development with severe osteopetrosis, suggesting that they are essential for osteoclastogenesis during bone remodeling (10,12,15). Thus, RANKL, RANK, and OPG are essential for controlling osteoclast development and functions in bone remodeling.

Type 1 diabetes is an autoimmune disease in which insulin-producing pancreatic β-cells are destroyed by infiltrating autoreactive T-cells. Both $CD4^+$ and $CD8^+$ T-cells are involved in disease pathogenesis (17,18). Diabetogenic T-cells react to autoantigens such as insulin, GAD65, and ICA512 (insulinoma-associated protein 2) in diabetic patients and experimental animal models (19). Islet-specific $CD4^+$ T-cells isolated from the pancreas and draining lymph nodes secrete predominantly Th1 cytokines (19). Clinical studies suggest that periodontitis can rapidly progress to advanced stages in type 1 and type 2 diabetic patients and is even more adversely exacerbated among poorly controlled diabetic individuals (20–22). It is well documented that severe periodontitis is the sixth most common complication among diabetic individuals (20–22). In addition, Lalla et al. (23) reported an increased alveolar bone loss in streptozotocin (STZ)-induced diabetic mice and attributed this to a higher expression of receptor for advanced glycation end products via the advanced glycation end products signaling pathway. However, the molecular mechanism of antibacterial cell–mediated immunity, by which diabetes exacerbates or enhances periodontal breakdown, are not well understood.

We investigated the contribution of the host's antibacterial T-cell–mediated immunity to the enhanced periodontal breakdown in type 1 diabetic patients by using the nonobese diabetic (NOD) mouse, the analog of human type 1 diabetes. We found that when orally infected with *A. actinomycetemcomitans*, diabetic NOD mice manifested significantly higher alveolar bone loss than nondiabetic and pre-diabetic NOD mice, which was associated with increased pathogen-specific proliferation and RANKL expression in local $CD4^+$ T-cells at the population but not at the single-cell level. Moreover, treatment of diabetic NOD mice with OPG significantly reduced alveolar bone loss to its baseline, suggesting that RANK-RANKL/OPG interactions are the critical mediators for the enhanced osteoclastogenesis that typifies type 1 diabetes when mounting anaerobic infection in vivo.

RESEARCH DESIGN AND METHODS

Female NOD/LtJ mice aged 4–6 weeks ($H\text{-}2^{g7} = K^d, Aa^d, Ab^{g7}, E^{null}, D^b$) were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained in a specific pathogen-free environment at the animal facilities of the University of Western Ontario and the University of Rochester. A total of 200 NOD mice and 18 BALB/c mice ($H\text{-}2^d$) were used in this study. All mice were monitored for type 1 diabetes by daily monitoring of urine ketone and glucose levels by Diastix strips and a Glucometer Elite XL meter (Bayer, Toronto, Canada). Mice were considered diabetic when whole-blood glucose levels exceeded 200 mg/dl on 2–3 consecutive days, with the histological evidence of severe lymphocytic infiltration in the pancreatic β-islets (insulitis). Typically, 70–80% female NOD mice developed diabetes by age 16–20 weeks. Keto-Diastix strips (Bayer) were used for daily monitoring and diagnosis. Diabetic mice were treated for hyperglycemia with Humulin U insulin (1–4 units/day; Eli Lilly, Indianapolis, IN) to maintain urine ketones (0–0.5 unit) and glucose <2 units on the Keto-Diastix strips. All mice were categorized based on their age and the state of diabetes with random pancreatic histology as follows: mice 6–8 weeks considered pre-diabetic with peri-insulitis, mice >12–16 weeks with blood glucose levels <140 mg/dl that did not develop diabetes considered nondiabetic, and mice >16–20 weeks old with blood glucose levels >200 mg/dl and insulitis considered diabetic. In addition, serum HbA_{1c} levels were also monitored. The incidence and age of disease onset in *A. actinomycetemcomitans*–infected versus –noninfected control NOD mice were compared throughout the entire experiment. All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the University Councils on Animal Care and Use Subcommittee.

Bacterial oral inoculation and adoptive transfer. *A. actinomycetemcomitans* JP2 (ATCC-29523) strain was purchased from ATCC (Rockville, MD) and grown anaerobically (80% N_2, 10% H_2, 10% CO_2) in tryptic soy broth-yeast extract culture broth (TSBYE; Sigma Chemical, St. Louis, MO) supplemented with 0.75% glucose and 0.4% $NaHCO_3$. Six different groups of pre-diabetic, nondiabetic, and diabetic NOD mice (n = 10/group) were orally inoculated with *A. actinomycetemcomitans* (10^9 cfu/100-μl broth mixed with 2% carboxymethylcellulose in PBS) two times per week for 3 consecutive weeks as described (8–9). The mice were then killed at weeks 4 and 8. Age-matched NOD mice without bacterial inoculation served as controls (n = 10/group) and were kept under the same conditions. To monitor the incidence of diabetes, a separate group of 4- to 6-week-old pre-diabetic NOD mice were inoculated with *A. actinomycetemcomitans* two times per week (in 2% carboxymethylcellulose) for 3 weeks and monitored for the incidence of diabetes until age 25 weeks. The adoptive transfer experiment was conducted as described (8). Briefly, total splenocytes from the diabetic NOD mice were cocultured in vitro with 25Gy-irradiated syngeneic splenocytes as antigen-presenting cells and sonicated *A. actinomycetemcomitans* antigens (10 μg/ml) for 72 h, and subsequently, $25–30 \times 10^6$ cells in PBS (per recipient) were used for tail vein injection into 4- to 6-week-old pre-diabetic NOD mice. Then, the recipients were immediately inoculated with *A. actinomycetemcomitans* orally two times per week from weeks 1–3. All mice were killed between weeks 7 and 8 (typically 13–14 weeks old) and did not develop type 1 diabetes or hyperglycemia. Later, the jaw samples were harvested and prepared for studying alveolar bone loss.

STZ-induced hyperglycemia in NOD mice. To assess the contribution of hyperglycemia to bone loss in *A. actinomycetemcomitans*–infected NOD mice, pre-diabetic NOD mice were injected with STZ to induce hyperglycemia. Briefly, 5- to 6-week-old pre-diabetic NOD mice (n = 6/group) were injected intraperitoneally with 40–50 mg/kg STZ twice (Sigma), at a 2-day interval, to induce pancreatic β-cell death. All injected pre-diabetic NOD mice developed hyperglycemia in 5–6 days, as determined by a Glucometer Elite XL meter (>200 mg/dl). Then, the mice were subjected to *A. actinomycetemcomitans* oral inoculation from weeks 1 to 3 as described above. All mice were killed at week 6, after which the jaw samples were harvested for the assessment of alveolar bone loss by digital histomorphometry as described below.

OPG treatment in mice. A separate group of female diabetic NOD mice (n = 8), 16–20 weeks old, were injected intraperitoneally with 2.5 μg hu-OPG-Fc/100 μl PBS (8,9,11,15) three times per week from 1 week before the start of *A. actinomycetemcomitans* oral inoculation and throughout the entire 8-week experimental period, as described (8,11). The controls included diabetic NOD mice with and without *A. actinomycetemcomitans* inoculation (n = 6 and n = 5, respectively), and the effect of PBS injection as the control has been described (8).

T-cell proliferation in vitro. All tissue cultures were performed using RPMI-1640 (GIBCO-BRL), supplemented with 10% FCS (GIBCO-BRL), 50 μmol/l β-2-mercaptoethanol, 100 μg/ml streptomycin sulfate, and 100 U/ml penicillin. Anti-mouse CD4 monoclonal antibodies were prepared from GK1.5 hybridoma cell line. To generate >95% pure $CD4^+$ T-cells, single cell suspensions of cervical lymph node (CLN), mesenteric lymph node, and splenic cells were treated with ACK lysis buffer (0.15 mmol/l NH_4Cl, 1 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA, pH 7.3), passed through nylon wool columns, and then panned on Petri dishes coated with anti–mCD4 monoclonal antibody prepared from GK1.5 cell line (8,9,24). Then, 5×10^5 $CD4^+$ T-cells were dispensed in triplicate in 96-well U-bottom plates in complete medium with 25Gy-irradiated syngeneic splenocytes as antigen-presenting cells (1:2 ratio) and serially diluted *A. actinomycetemcomitans* sonicate antigens at 1.25, 2.5, 5, 10, and 20 μg/ml (8,9, 24). The assays included $CD4^+$ T-cells activated with 10 μg/ml Con-A (Pharmacia, MI) and unstimulated $CD4^+$ T-cells as the positive and the negative controls, respectively. All cells were incubated in a 5% CO_2 incubator at 37°C for 72 h. For the final 18 h, cells were pulsed with 1 μCi/ml [^{3}H]-thymidine and then harvested onto 96-well filter paper and read in a Wallac Micro-β liquid scintillation counter (Packard Instrument, Boston, MA). The results of proliferation are shown as the mean of triplicate value ± SE.

FACS and quantitative immunofluorescence study. $CD4^+$ T-cells activated in vitro as described above were harvested and subjected to immunostaining for RANKL expressions at 72 h as described (8–9). To block nonspecific binding, cells were first incubated with anti–mFcR-IgG (all anti-

bodies from BD-Pharmingen, San Jose, CA) and then followed by incubation with OPG-hu-Fc to label RANKL molecules and PerCP-Cy5.5–conjugated rat anti–mCD4 monoclonal antibodies (a True-Red fluorochrome conjugate for flow cytometry analysis with excitation at 490 and 675 nm and emission at 695 nm) to label CD4 molecules on T-cells, respectively. Then, samples were washed twice and incubated with goat anti–hFc-IgG-FITC (fluorescein isothiocyanate) 2° conjugate. The isotypic control was incubated with goat anti–hFc-IgG-FITC. For FACS (fluorescence-activated cell sorter) scanning, cells were gated on live lymphocytes and analyzed for RANKL expression in $CD4^+$ T-cells using FACS-Calibur and CellQuest software (BD Biosciences). For quantitative immunofluorescence assay, 10^5 $CD4^+$ T-cells were distributed into 96-well flat-bottom plates in replicates and then scanned under a Leica DM-IRBE IF-microscope. The images of the fluorescent intensity (in pixels) were captured via a digital camera over a motorized staging facility equipped with an Openlab software for automated quantitation of fluorescent intensity per single PerCP-Cy5.5–expressing $CD4^+$ T-cell to generate a series of 17 scanned fields per well under 100× magnification. All of the optical parameters (e.g., exposure time, light source, shutter, focus) were kept constant in all experiments. Quantification of the mean fluorescent intensity per cell was performed using the automated density slice features of OpenLab software (v.3.1.4; ImproVision, Toronto, Canada) loaded onto a Macintosh-G4 computer with Macintosh OS X, by enumerating the numbers and signal intensities of individual cells in each well. The sum fluorescent intensity was calculated by adding the total fluorescent signals per well and then subtracting the background intensities. The results are shown as the average values of fluorescent intensity (mean ± SE) by at least 0.5–1 × 10^6 countable $CD4^+$ T-cells after 72-h cultures from two to three independent experiments. Furthermore, immunofluorescence images showing the individually stained RANKL-expressing $CD4^+$ T-cells from various groups of *A. actinomycetemcomitans*–inoculated NOD mice were scanned and photographed under 400× magnification. Then, the resulting images were also enumerated for full quantitation using the same automated density slice features of the Open-Lab software described above.

Assessment of alveolar bone loss by digital histomorphometry. The mouse jaw samples were de-fleshed and stained with methylene blue to define the area between the cementum-enamel junction and the alveolar bone crest (8,9). The surface areas measured represent the total amount of alveolar bone loss on the jaws (in millimeters squared), which was carried out with a calibrated Leica MZ_{95} stereo microscope and a Hamamatsu Orca digital camera (8,9). The jaw images were captured under 16× magnification, where the right and left maxillary first two molars (i.e., M1 and M2) were scanned and automatically enumerated by using the density slice features of the OpenLab for full quantitation. The resulting values are expressed as the mean of the surface areas ± SE (in millimeters squared) of M1 and M2 from each mouse in each group.

Statistical analysis. Statistical analysis was performed using the two-sided Student's *t* test, and the difference between various groups was considered statistically significant when the *P* value was <0.05.

RESULTS

Oral inoculation with *A. actinomycetemcomitans* does not alter type 1 diabetes development in NOD mice. It has been shown that infections by viruses, bacteria, or helminthes could alter type 1 diabetes development in NOD mice (25–29). To investigate whether oral inoculation with *A. actinomyctemcomitans* has any impact on developing type 1 diabetes, the incidence and severity of type 1 diabetes was studied by assessing 6-week-old pre-diabetic NOD mice inoculated with *A. actinomycetemcomitans* two times per week for 3 weeks and then their blood and urinary glucose levels were monitored until 25 weeks of age. The results showed that the incidence, age of disease onset, and severity in *A. actinomycetemcomitans*–inoculated NOD mice were comparable to those of the control, noninoculated mice (diabetes: ~80% in females by 25 weeks of age, $n = 24$ mice per group; Fig. 1*A*). Histological studies of the pancreas revealed compatible levels of lymphocytic infiltration in β-islets when comparing *A. actinomycetemcomitans*–inoculated with non–*A. actinomycetemcomitans*–inoculated control NOD mice during the entire period (from peri-insulitis to severe insulitis at 8 and 18 weeks of age; Fig. 1*B*), and blood glucose and ketone levels were also not affected (data not shown). Thus, *A. actinomycetemcomitans* oral inoculation did not alter the diabetic incidence, onset age, and severity of type 1 diabetes in NOD mice.

Higher alveolar bone loss detected in diabetic NOD mice after *A. actinomycetemcomitans* inoculation. To assess the effect of microbial infection on periodontal inflammation and tissue destruction associated with type 1 diabetes, NOD mice of different ages (5–6 weeks old pre-diabetic, 14–16 weeks old nondiabetic, and 16–20 weeks old diabetic) were inoculated with live *A. actinomycetemcomitans* for the first 3 weeks and then followed for another 5 weeks, after which the mice were killed to measure alveolar bone loss (CEJ-ABC [8,9]), at weeks 4 and 8. The mice were assessed for diabetes status by blood glucose and histological study of the pancreas (Fig. 2*B*). The results show that while diabetic NOD mice showed no significantly different alveolar bone loss compared with nondiabetic and pre-diabetic mice by weeks 7–8 (Fig. 2*A*), after *A. actinomycetemcomitans* inoculation, only diabetic NOD exhibited significantly robust alveolar bone loss ($P = 6.8 \times 10^{-4}$), not pre-diabetic or nondiabetic NOD mice (Fig. 2*A* and *B*). This difference remained statistically significant after adjusting the age difference for physiological remodeling of the alveolar bone in periodontium over time ($P < 0.05$) and normalizing to the alveolar bone loss detected in naive immunocompetent BALB/c mice orally inoculated with *A. actinomycetemcomitans* ($P < 0.03$ data not shown; [8,9]), consistent with our previous findings using humanized mice, in which significant alveolar bone loss did not occur until 6–8 weeks' postinoculation (8,9,24). In addition, oral inoculation with *A. actinomycetemcomitans* did not change the course and severity of diabetes, based on the histological studies (Figs. 1*B* and 2*B*). These data are in accordance with experimental and clinical studies that type 1 diabetes is significantly associated with severe periodontal breakdown (2,20–23,30,31).

To investigate whether the enhanced alveolar bone loss detected here (Fig. 2*A* and *B*) is associated with the diabetic autoimmune environment, total splenocytes from fully diabetic NOD mice (18–20 weeks old) were primed with total *A. actinomycetemcomitans* antigens in vitro and then adoptively transferred into pre-diabetic NOD mice, after which they were inoculated with live *A. actinomycetemcomitans* orally. At the time of death, those pre-diabetic NOD mice postadoptive transfer were only 13–14 weeks old and had not developed spontaneous type 1 diabetes, whereby contribution of hyperglycemia in this experiment was minimal. The results showed that there was a significantly higher alveolar bone loss in pre-diabetic NOD after adoptive transfer with *A. actinomycetemcomitans*–stimulated diabetic splenocytes compared with the control pre-diabetic NOD mice ($P < 0.02$; Fig. 2*C*). Furthermore, oral inoculation with *A. actinomyctemcomitans* in pre-diabetic NOD mice after receiving adoptive transfer yielded significantly more alveolar bone loss than *A. actinomycetemcomitans*–inoculated pre-diabetic NOD mice ($P < 0.03$; Fig. 2*C*). These results were further confirmed by treating pre-diabetic NOD with STZ to induce hyperglycemia. STZ-induced hyperglycemic mice did not exhibit significantly higher amounts of alveolar bone

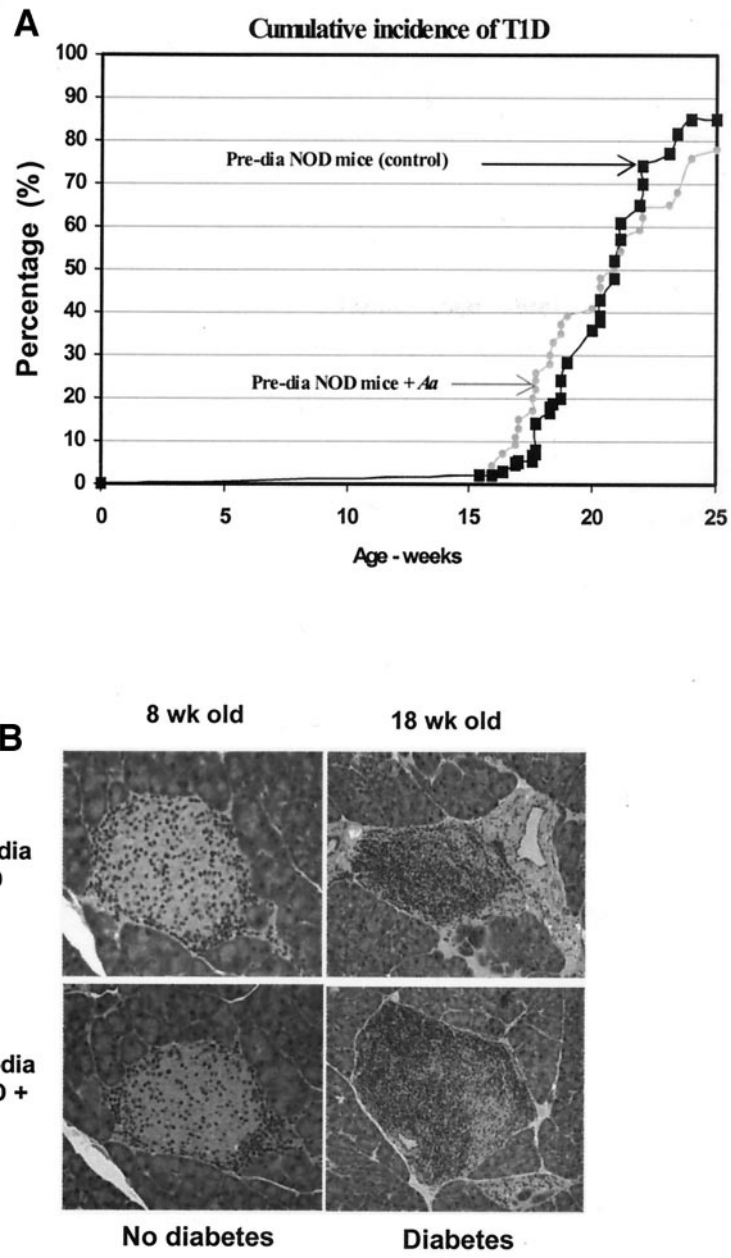
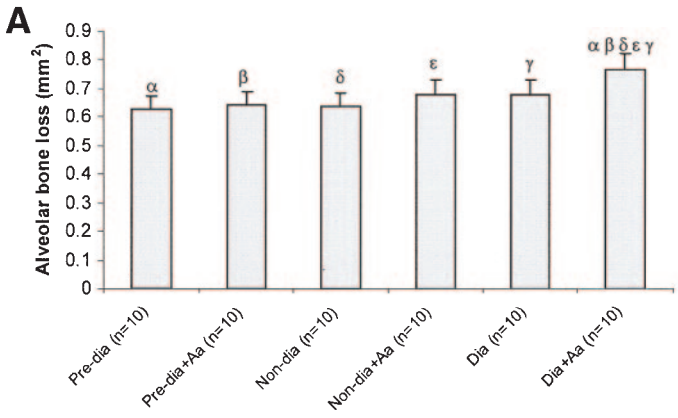
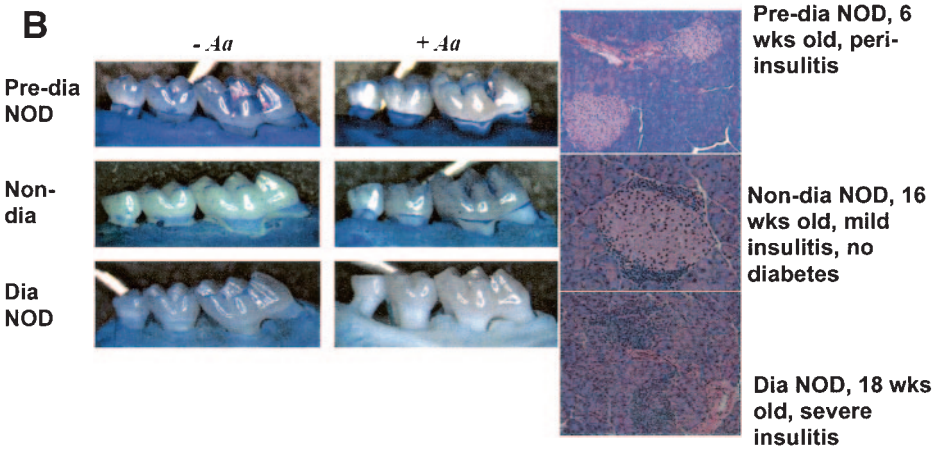


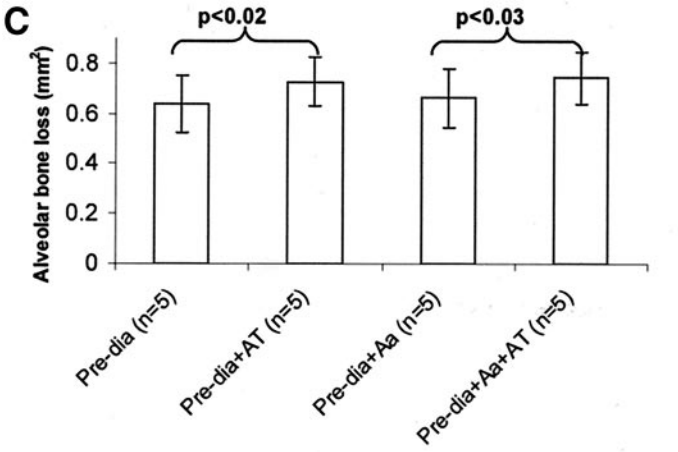
FIG. 1. No significant difference in the diabetic incidence, the age of disease onset, and severity in *A. actinomycetemcomitans*–inoculated versus control NOD mice. *A*: Six-week-old pre-diabetic (Pre-dia) NOD mice were orally inoculated either with *A. actinomycetemcomitans* or vehicle alone (n = 24/group) and then monitored for the accumulative incidence of diabetes until 25 weeks of age. The results showed that there were no significant differences between *A. actinomycetemcomitans*–inoculated pre-diabetic (light-gray dots) and non–*A. actinomycetemcomitans*–inoculated control NOD mice (black squares) regarding their diabetic incidences and the age of disease onset in the entire experimental period. *B*: Representative photographs of the histological sections of pancreases from pre-diabetic NOD with or without *A. actinomycetemcomitans*. There were comparable levels of lymphocytic infiltration in the pancreatic β-islets in both groups of the pre-diabetic NOD mice. T1D, type 1 diabetes.

loss compared with normoglycemic pre-diabetic control NOD mice with and without *A. actinomycetemcomitans* inoculation ($0.54 < P < 0.81$; Fig. 2*D*). In parallel, the results of separate experiments also showed that there was no significant difference regarding alveolar bone loss between STZ-induced hyperglycemic versus normoglycemic BALB/c mice after microbial challenge (data not shown). These results suggest that the overall enhanced alveolar bone loss observed here was more likely associated with diabetic autoimmunity than hyperglycemia. Together, our data indicate that autoimmunity can significantly contribute to enhancing alveolar bone loss in the diabetic host when mounting anaerobic *A. actinomycetemcomitans* infection in vivo.

Increased *A. actinomycetemcomitans*–specific T-cell proliferation in diabetic NOD mice. We had shown previously that *A. actinomycetemcomitans*–specific cell-mediated immunity can be measured by using purified $CD4^+$ T-cells from periodontal tissue, CLNs, or splenocytes after oral challenge with *A. actinomycetemcomitans* (8,9,24), where the first two cellular sources gave comparable immune responses and the same specificity. To study cell-mediated immunity to *A. actinomycetemcomitans* infection in NOD mice, $CD4^+$ T-cells were purified from the mice CLN and spleens after microbial inoculation, as well as the same source of cells from naive NOD mice. The results showed that the proliferation to *A. actinomycetemcomitans* antigens was much greater from the inoculated than the noninoculated NOD mice ($P < 0.05$; Fig. 3). Specifically, T-cell proliferation was significantly higher in $CD4^+$ T-cells from *A. actinomycetemcomitans*–inoculated diabetic when compared with that in *A. actinomycetemcomitans*–inoculated nondiabetic ($P < 0.05$) and pre-diabetic ($P < 0.01$) NOD mice (Fig. 3). The proliferation detected was concentration dependent, peaking around 10 μg/ml, which was significantly higher than that from the







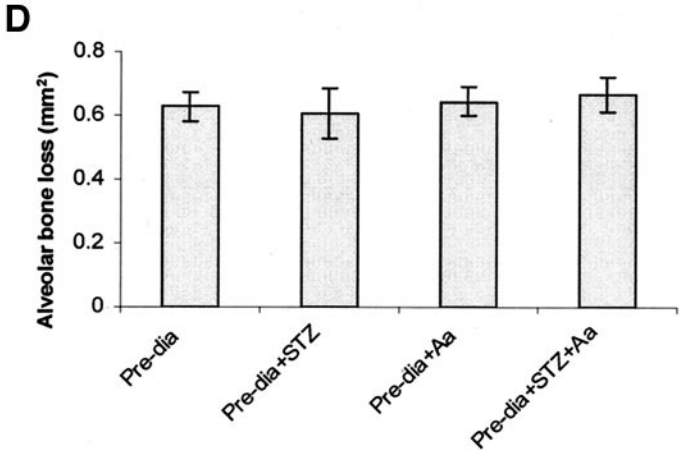


FIG. 2. Significantly higher alveolar bone loss in diabetic mice in comparison with pre-diabetic and nondiabetic NOD mice. *A*: Pre-diabetic (Pre-dia), nondiabetic (Non-dia), and diabetic (Dia) NOD mice (n = 10/group) inoculated with live *A. actinomycetemcomitans* (see RESEARCH DESIGN AND METHODS) for 3 weeks. The jaw samples were collected and assessed for their alveolar bone loss by digital histomorphometry under 16× magnification for the maxillary molars. P values from the statistical analyses are $\alpha = 1.7 \times 10^{-5}$, $\beta = 3.2 \times 10^{-6}$, $\delta = 4.4 \times 10^{-5}$, $\epsilon = 4.2 \times 10^{-3}$, $\gamma = 6.8 \times 10^{-4}$. *B*: Representative jaw photographs from each group of mice are shown and accompanied by the representative histological sections of the pancreases from each group. *C*: Alveolar bone loss of pre-diabetic NOD mice (n = 5 each) after receiving adoptive transfer (AT) of total splenocytes from diabetic NOD mice, followed by oral inoculation with *A. actinomycetemcomitans*. Results are shown as the mean of the alveolar bone loss ± SE. There are statistically significant differences between Pre-dia and Pre-dia+AT mice (0.637 ± 0.114 and 0.728 ± 0.099, respectively, $P < 0.020$) and between Predia+*A. actinomycetemcomitans* (*Aa*) and Pre-dia+*Aa*+AT (0.662 ± 0.116 and 0.743 ± 0.105, respectively, $P < 0.030$). *D*: No significant differences in the alveolar bone loss among the pre-diabetic NOD mice that were sham treated or treated with STZ alone in the presence or absence of *A. actinomycetemcomitans* oral inoculation (n = 6 mice/group). The results shown above are from one of the two representative experiments. Note: The P value was 0.81 for comparing pre-diabetic NOD (0.628 ± 0.047) vs. pre-diabetic NOD plus STZ (0.604 ± 0.080) and 0.54 for *A. actinomycetemcomitans*–inoculated pre-diabetic NOD (0.645 ± 0.045) vs. *A. actinomycetemcomitans*–inoculated pre-diabetic NOD plus STZ (0.666 ± 0.055).

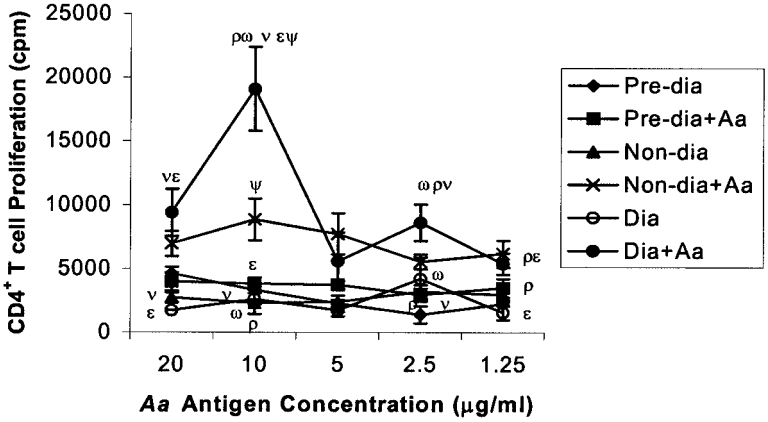


FIG. 3. Significantly higher CD4$^+$ T-cell proliferation in diabetic NOD mice after *A. actinomycetemcomitans* challenge. T-cell proliferation assay of CLN CD4$^+$ T-cells purified from various groups of NOD mice with and without *A. actinomycetemcomitans* (Aa) inoculation and restimulated with *A. actinomycetemcomitans*–sonicated antigens in vitro. The results shown above are from one of the three representative experiments as the average values of the triplicates ± SE (three mice per group per experiment). The *P* values are ω = 0.0499 and ψ = 0.010. Note: The background levels of proliferation in the control cultures containing medium-only or irradiated antigen-presenting cells with *A. actinomycetemcomitans* antigens gave rise to cpm counts ranging from 285 to 750 (at 20 μg/ml). Dia, diabetic; Non-dia, nondiabetic; Pre-dia, prediabetic.

other groups (Fig. 3). In addition, there were no significant differences in the overall proliferation of purified CD4$^+$ T-cells from different groups of NOD mice when cocultured with ConA (e.g., $0.38 < P < 0.95$) or third-party antigens (*P. gingivalis*) or when splenic CD4$^+$ T-cells from different NOD mice were used (data not shown). These data suggest that the resulting proliferation was specific to oral/periodontal CD4$^+$ T-cells in the cervical region when mounting microbial challenge, not from remote tissues/sources. Thus, it appears that there may be a link between *A. actinomycetemcomitans*–specific local cell-mediated immune response and enhanced alveolar bone loss detected in the diabetic NOD mice (Figs. 2 and 3).

Increased RANKL expression in *A. actinomycetemcomitans*–reactive CD4$^+$ T-cells in diabetic NOD mice. To study the interactions between T-cell–mediated immunity and the enhanced alveolar bone loss, CLN CD4$^+$ T-cells were purified from pre-diabetic, nondiabetic, and diabetic NOD mice followed by stimulation with total *A. actinomycetemcomitans* antigens in vitro for their cell surface RANKL expressions by flow cytometry. The results showed that CLN CD4$^+$ T-cells of the diabetic mice expressed significantly higher membrane-bound RANKL than those of the pre-diabetic and nondiabetic NOD mice (Fig. 4*A*). A significantly higher percentage of *A. actinomycetemcomitans*–reactive RANKL-expressing CD4$^+$ T-cells was noted in diabetic (mean 68.1%) compared with nondiabetic (47.5%, $P < 0.018$) and pre-diabetic (35.7%, $P < 0.003$) NOD mice (Fig. 4*A*). An example of their expressions is shown in Fig. 4*B*. On average, despite a 31% increased RANKL expression in the pre-diabetic *A. actinomycetemcomitans* mice compared with the control pre-diabetic mice, no significant difference was noted ($P = 0.089$). This was also not the case regarding any different RANKL expression between nondiabetic and diabetic NOD mice ($P = 0.24$). Furthermore, by using an immunofluorescent microscope for quantitation, we found that there was no statistically significant difference for RANKL expressions between different groups of *A. actinomycetemcomitans*–inoculated NOD mice at the single *A. actinomycetemcomitans*–reactive CD4$^+$ T-cell level (Fig. 5*A* and *B*), suggesting that the intensity of RANKL staining (mean FITC–fluorescent intensity over CD4$^+$ T-cells–Cy5.5) was comparable among all groups. These RANKL-expressing CD4$^+$ T-cells were fully activated and competent as assessed by the significant upregulation of CD25 and CD69 (data not shown). Collectively, these data suggest that higher RANKL expression (Fig. 4*A* and *B*) was due to increased expansion or frequency of *A. actinomycetemcomitans*–reactive CD4$^+$ T-cells in diabetic NOD mice than those in pre-diabetic and nondiabetic NOD mice.

Significant reversal of *A. actinomycetemcomitans*–induced alveolar bone loss by OPG treatment in vivo. To study the overall contribution and clinical significance of RANKL-RANK signaling to the enhanced alveolar bone loss in vivo, diabetic NOD mice were pretreated with OPG-Fc fusion protein 1 week (three times per week) before the start of *A. actinomycetemcomitans* inoculation until week 8 (total 27 injections per mouse). The results clearly showed that when *A. actinomycetemcomitans*–inoculated diabetic mice were pretreated with OPG, there was a significant reduction of alveolar bone loss to its baseline (mean 81 ± 10%, $P < 0.03$; Fig. 6) compared with that of diabetic NOD mice. In addition, diabetic NOD mice receiving OPG treatment without *A. actinomycetemcomitans* inoculation showed a slight reduction of bone loss comparable to that of the noninoculated pre-diabetic mice at baseline (data not shown). This is consistent with the above findings in Fig. 2*A* that *A. actinomycetemcomitans* was required for the enhanced alveolar bone loss. Furthermore, the frequency of *A. actinomycetemcomitans*–reactive RANKL-expressing CD4$^+$ T- cells from OPG-treated diabetic mice was also significantly reduced (~31.9%, $P < 0.0176$) to the level comparable to that of pre-diabetic and nondiabetic NOD mice, respectively (Fig. 4*A* and *B*). These data strongly suggest that the RANK-RANKL signaling controls the major switch associated with the enhanced alveolar bone loss in type 1 diabetes, consistent with the results of our previous study using human leukocytes (8,9).

DISCUSSION

The present study is the first that clearly describes the impact of diabetic autoimmunity on anaerobic infection in an experimental periodontitis model of type 1 diabetes and that indicates that RANKL-RANK signaling is involved in controlling the enhanced alveolar bone loss in the local environment. Although *A. actinomycetemcomitans* infection does not alter the development of type 1 diabetes (Figs. 1 and 2), the results show that diabetic NOD mice exhibit higher alveolar bone loss compared with pre-diabetic and nondiabetic NOD mice when infected by the G(−) anaerobe (Fig. 2*A*) that is associated with a higher

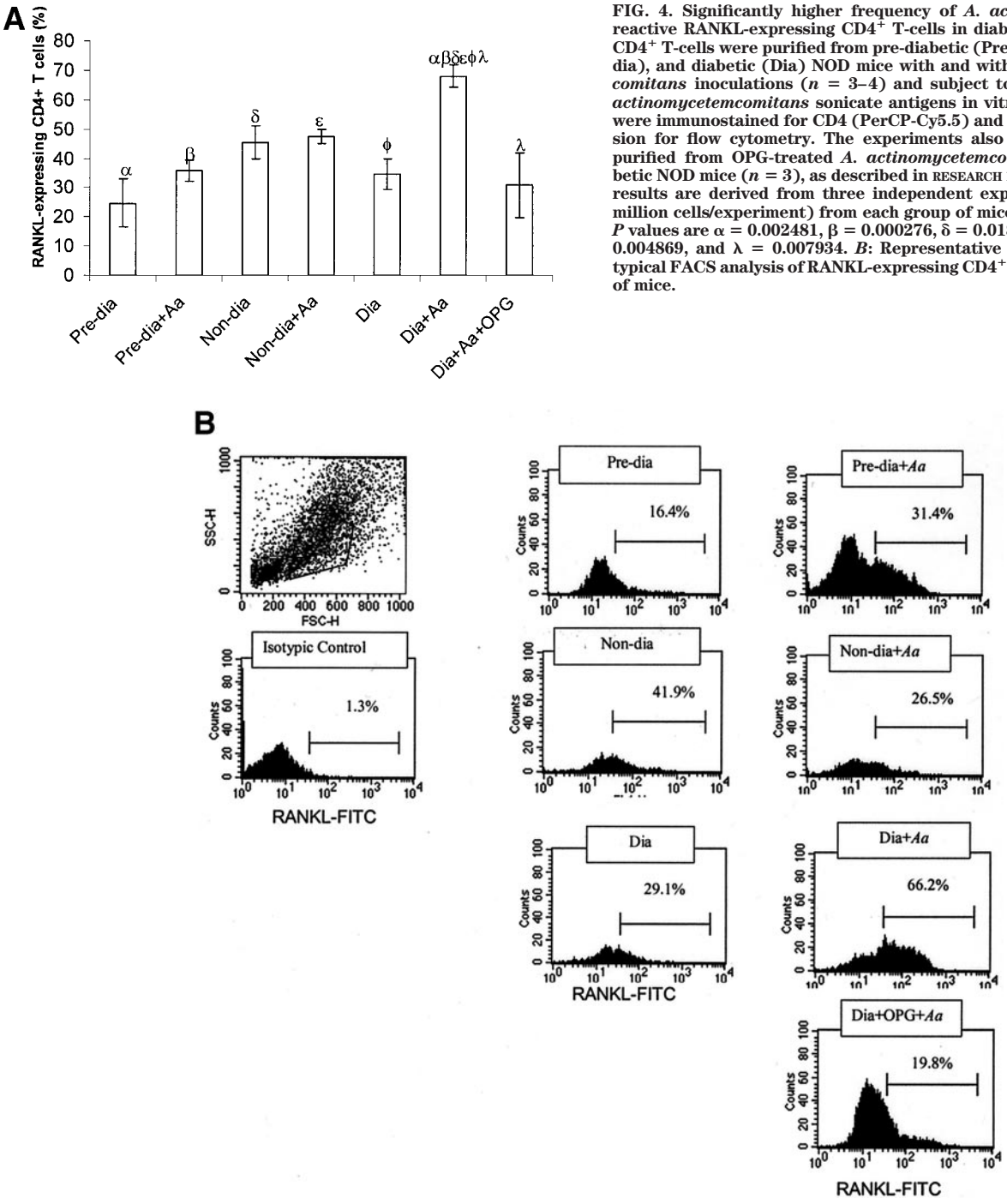


FIG. 4. Significantly higher frequency of *A. actinomycetemcomitans*–reactive RANKL-expressing CD4$^+$ T-cells in diabetic NOD mice. *A*: CLN CD4$^+$ T-cells were purified from pre-diabetic (Predia), nondiabetic (Non-dia), and diabetic (Dia) NOD mice with and without *A. actinomycetemcomitans* inoculations (n = 3–4) and subject to restimulation with *A. actinomycetemcomitans* sonicate antigens in vitro. After 72 h, the cells were immunostained for CD4 (PerCP-Cy5.5) and RANKL (FITC) expression for flow cytometry. The experiments also included CD4$^+$ T-cells purified from OPG-treated *A. actinomycetemcomitans*–inoculated diabetic NOD mice (n = 3), as described in RESEARCH DESIGN AND METHODS. The results are derived from three independent experiments (±SE 0.5–1.0 million cells/experiment) from each group of mice studied. The resulting P values are $\alpha = 0.002481$, $\beta = 0.000276$, $\delta = 0.018915$, $\epsilon = 0.013195$, $\phi = 0.004869$, and $\lambda = 0.007934$. *B*: Representative histograms depicting a typical FACS analysis of RANKL-expressing CD4$^+$ T-cells from each group of mice.

T-cell proliferation (Fig. 3) and a higher percent of RANKL-expressing CD4$^+$ T-cells (Fig. 4*A* and *B*) and is contributed by diabetic autoimmunity (Fig. 2*C*). Moreover, OPG treatment protects diabetic NOD mice from microbe-induced alveolar bone loss to nearly its baseline in vivo (Figs. 4*A* and *B* and 6*A* and *B*). We have previously shown that injection of OPG-Fc into mice blocked RANKL activity and reduced the numbers of TRAP$^+$ osteoclast (also RANK$^+$) in the periodontal crestal bone after *A. actinomycetemcomitans* inoculation (8). Ourselves and other researchers have also reported that osteoclasts are the key and predominant cell type in the periodontal and mesenchymal tissues expressing RANK and responding to RANKL (8,9,12,13,15,16). It remains to be studied whether OPG-Fc administration may also affect other RANK$^+$ subsets in alveolar bone or mucosae resident cells. Collectively, these data strongly suggest that RANK-RANKL/OPG interactions are the key factors controlling alveolar bone loss and remodeling in vivo and that OPG holds the potential to prevent or treat active bone lesions associated with microbial infections in autoimmune disorders such as type 1 diabetes.

Diabetes incidence and severity can be modulated by bacterial and viral infections or helminthes, as well as by bacterial and viral products such as LPS and CpG-nucleotides (25–29). However, our present study shows that oral infection with *A. actinomycetemcomitans* does not lead to any change in diabetes incidence or disease onset or

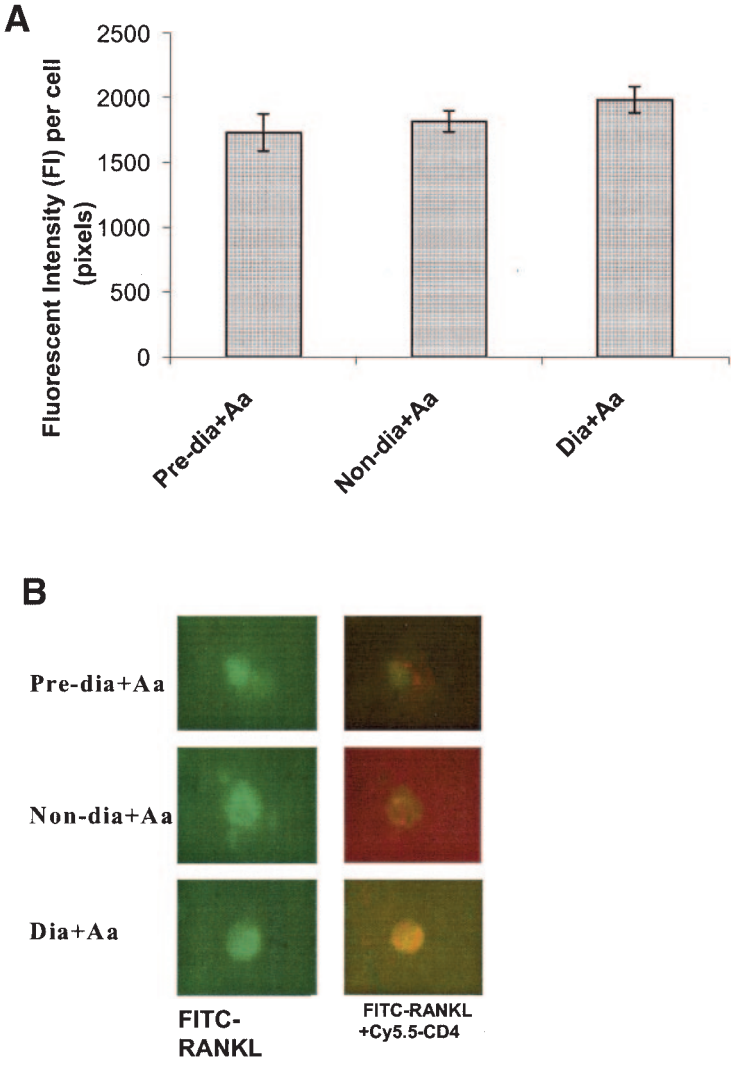


FIG. 5. There is a comparable RANKL expression at the single-cell level of CD4$^+$ T-cells from all groups of *A. actinomycetemcomitans*–inoculated NOD mice. *A*: CLN CD4$^+$ T-cells from *A. actinomycetemcomitans*–inoculated pre-diabetic (Pre-dia), non-diabetic (Non-dia), and diabetic (Dia) NOD mice were purified and restimulated with *A. actinomycetemcomitans* antigens before they were subject to immunostaining for CD4 and RANKL. The resulting cells were seeded onto 96-well plates (100,000 CD4$^+$ T-cells/well), and the images of fluorescent intensity (in pixels) were quantitatively analyzed, as described in RESEARCH DESIGN AND METHODS. Results are shown as the average values of fluorescent intensity (mean ± SE) from at least 10^5 to 10^6 activated CD4$^+$ T-cells from three independent experiments. *B*: Fluorescent images showing the RANKL-expressing (in FITC green) CD4$^+$ T-cells (combined FITC in green and PerCP-Cy5.5 in red) from various groups of *A. actinomycetemcomitans*–inoculated NOD mice photographed under 400× magnification.

severity (Figs. 1*A* and *B* and 2*B*), suggesting that local immune-inflammatory response to *A. actinomycetemcomitans* cannot alter the systemic outcomes in type 1 diabetes. This discrepancy (25–29,32) is currently unclear and requires further investigation. One possibility is that different microorganisms may have differential effects on the systemic outcomes (33). The link between diabetes and periodontitis is well established, such that a higher risk for severe periodontitis is associated with both type 1 and type 2 diabetes (20–22,31,34). Sims et al. (30) observed an association among periodontal pocket depth changes, serum IgG titers to *P. gingivalis*, and autoantibodies to GAD65. Lagervall et al. (31) reported a correlation between teeth lost and diabetes incidence. Although recent studies suggest that periodontitis may be associated with certain systemic disorders, including cardiovascular diseases, diabetes, and pneumonia (31,34), conflicting data also exist (35).

It is now clear that CD4$^+$ T-cells play a crucial role in alveolar bone loss (7–9,24) and that this local immune response is specific to the invading pathogen (8 and Figs. 1–3). Recent studies have shown that activated T-cells induce osteoclastogenesis by interacting with osteoclast through the RANKL-RANK pathway (8,9,11,36). Other pro-inflammatory cytokines play important roles in periodontitis (2). Deficiencies in interferon-γ (7), IL-6 (7), IL-1, and TNF-α (37) were shown to result in reduced alveolar bone loss. It is known that TNF-α promotes osteoclast development (38) and IL-6 has a proinflammatory effect by promoting osteoclastogenesis in arthritis-associated bone loss (39). In diabetes, activated T-cells produce predominantly Th1 cytokines such as interferon-γ and TNF-α (19), which can exacerbate immunity-mediated alveolar bone loss (2,32,33). Additionally, they produce less protective Th2 cytokines such as IL-4 and -5 (19,40). We have previously shown that *A. actinomycetemcomitans*–specific CD4$^+$ T-cells can be detected in and retrieved from periodontal tissues or marginal oral mucosa and CLN, all of which showed the same immune specificity and activation profiles (CD69 and CD25 [8,9,24]). Further purification of the CD4$^+$ T-cells was studied here (Figs. 3 and 4) by sorting CD69- or CD25-positive cells followed by labeling with carboxyfluorescein ester, a FITC-based dye for a 3-day restimulation assay in the presence of 25Gy-irradiated splenocytes, and *A. actinomycetemcomitans* antigens rendered ≥85–90% carboxyfluorescein ester–positive T-cells in active division, indicating their specific reactivity to *A. actinomycetemcomitans* antigens (data not shown). Moreover, the results of our separate analyses also show that *A. actinomycetemcomitans*–reactive periodontal CD4$^+$ T-cells in NOD mice react to *A. actinomycetemcomitans*–specific viru-

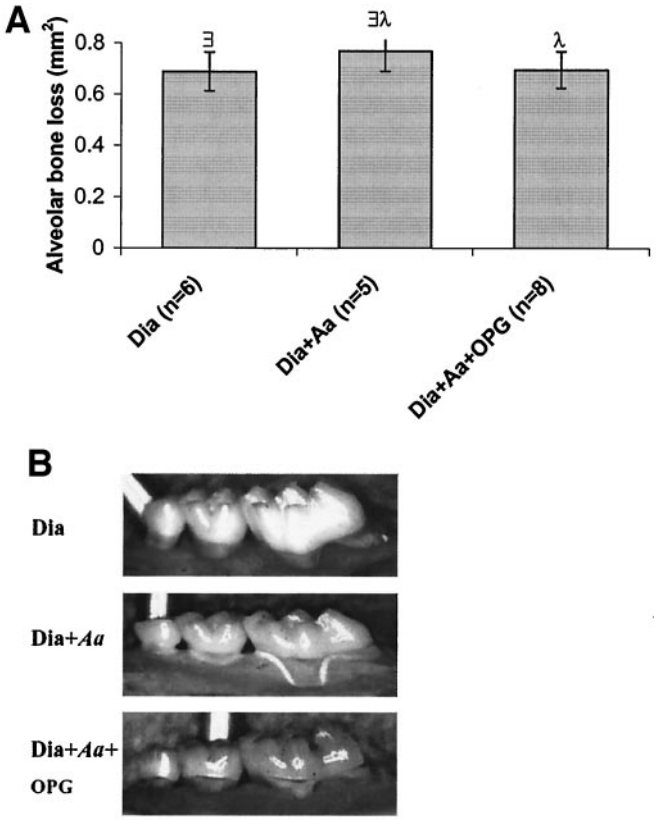


FIG. 6. Significant reduction of the alveolar bone loss in *A. actinomycetemcomitans*–inoculated diabetic NOD mice after OPG treatment in vivo. *A*: Diabetic NOD mice were injected with OPG-Fc protein 1 week before the start of *A. actinomycetemcomitans* (Aa) inoculations until the end of the experimental period (Dia+*Aa*+OPG, $n = 8$); diabetic NOD were left untreated as the negative control (diabetic, $n = 6$), or orally inoculated with *A. actinomycetemcomitans* as the positive control (diabetic+*Aa*; $n = 5$). Data represent mean alveolar bone loss in each group ± SE. The resulting *P* values for the statistical analyses are 0.0293 and 0.0217, respectively. *B*: Representative jaw samples from each group of mice.

lence antigens (CagE homologue and OMP1) (6) and express similar cytokine profiles described above and that interferon-γ is significantly upregulated in *A. actinomycetemcomitans*–specific RANKL$^+$ CD4$^+$ T-cells in diabetic NOD mice (Y.-T.A.T., D.A.M., B.S., personal communication). Despite the presence of circulating diabetognic T-cells, CD4$^+$ T-cells prepared from distant lymphoid tissues (i.e., spleen and mesenteric lymph nodes) yielded comparable levels of cellular proliferation and activation profiles after in vitro *A. actinomycetemcomitans* antigens stimulation (or ConA, data not shown) without the significant differences that CLN CD4$^+$ T-cells exhibited (Fig. 4*A*), suggesting specific local immunity to *A. actinomycetemcomitans* infection. In parallel, *A. actinomycetemcomitans*–specific hCD4$^+$ T-cells express both RANKL$^+$ Th1 and Th2 profiles in situ with significantly elevated interferon-γ/TNF-α and IL-10/transforming growth factor-β associated with active alveolar bone loss in vivo (8,9,24), representing >83–91% of the T-cell receptor repertoire in juvenile periodontitis patients (41).

In general, NOD T-cells are hyporesponsive to T-cell receptor/CD3 stimulation as well as to mitogen (42–43). These mice have defects in CD4$^+$ CD25$^+$ Treg cells (44) as well as deficient Th2 cells (38), a lower frequency and function of autoreactive CD1d$^+$ natural killer T-cells (45), and/or inefficient deletion of autoreactive immature thymocytes (46). In our present study, there were significantly increased proliferation and RANKL expressions in *A. actinomycetemcomitans*–reactive CD4$^+$ T-cells rather than hyporesponsiveness in diabetic mice compared with those from nondiabetic and pre-diabetic NOD mice. Further study will determine whether the differences are primarily due to rapidly expanding T-cells or change of immune frequency (i.e., repertoire) related to diabetes when encountering anaerobic infection. It is clear that the higher RANKL expression in T-cells (Fig. 4) cannot be simply explained by any changes at the single-cell level (Fig. 5). In addition, our recent limiting dilution analysis and quantitative comparison of all *A. actinomycetemcomitans*–reactive CD4$^+$ T-cell populations suggest that this higher RANKL expression in diabetic NOD mice is likely associated with an increased immune frequency to microbial infection (data not shown). It remains to be determined whether T-cells under diabetic situations may become more resistant to activation-induced cell death (47), thus contributing to an enhanced survival of *A. actinomycetemcomitans*–reactive RANKL-expressing CD4$^+$ T-cells.

The mechanism of severe alveolar bone loss in diabetic mice may be due to a direct effect of RANKL-RANK signaling between activated CD4$^+$ T-cells and osteoclast (8,11,12), coupled with local recruitment of inflammatory cells. In STZ-induced diabetes, C57BL6 mice were more prone to alveolar bone loss than nondiabetic controls (23); however, our study showed that there was no significant difference between STZ-induced hyperglycemic versus normoglycemic control NOD (Fig. 2*D*) and BALB/c mice (data not shown). This further suggests that enhanced alveolar bone loss is likely more associated with the diabetic autoimmunity than hyperglycemia, at least in NOD background, since different mouse strains were used in different studies (23).

In summary, this study clearly demonstrates for the first time that diabetic NOD mice manifest enhanced alveolar bone loss associated with the increased T-cell proliferation and RANKL expressions during anaerobic infection by *A. actinomycetemcomitans*, much more than that of pre-diabetic and nondiabetic NOD mice. Importantly, OPG treatment blocks this enhanced alveolar bone loss triggered by G(−) *A. actinomycetemcomitans*–reactive CD4$^+$ T-cells in vivo. Therefore, inhibition of specific RANKL-RANK pathway(s) has therapeutic value to treat inflammatory bone disorders such as human periodontitis, bone loss associated with diabetes or systemic autoimmune diseases, and even diabetes-associated tooth loss in the future.

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