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# 18β-Glycyrrhetinic acid inhibits periodontitis via glucocorticoid-independent nuclear factor-κB inactivation in interleukin-10-deficient mice

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*Background and Objective:* 18β-Glycyrrhetinic acid (GA) is a natural anti-inflammatory compound derived from licorice root extract (*Glycyrrhiza glabra*). The effect of GA on experimental periodontitis and its mechanism of action were determined in the present study.

*Material and Methods:* Periodontitis was induced by oral infection with *Porphyromonas gingivalis* W83 in interleukin-10-deficient mice. The effect of GA, which was delivered by subcutaneous injections in either prophylactic or therapeutic regimens, on alveolar bone loss and gingival gene expressions was determined on day 42 after initial infection. The effect of GA on lipopolysaccharide (LPS)-stimulated macrophages, T cell proliferation and osteoclastogenesis was also examined *in vitro*.

*Results:* 18β-Glycyrrhetinic acid administered either prophylactically or therapeutically resulted in a dramatic reduction of infection-induced bone loss in interleukin-10-deficient mice, which are highly disease susceptible. Although GA has been reported to exert its anti-inflammatory activity via downregulation of 11β-hydroxysteroid dehydrogenase-2 (HSD2), which converts active glucocorticoids to their inactive forms, GA did not reduce *HSD2* gene expression in gingival tissue. Rather, in glucocorticoid-free conditions, GA potently inhibited LPS-stimulated proinflammatory cytokine production and RANKL-stimulated osteoclastogenesis, both of which are dependent on nuclear factor-κB. Furthermore, GA suppressed LPS- and RANKL-stimulated phosphorylation of nuclear factor-κB p105 *in vitro*.

*Conclusion:* These findings indicate that GA inhibits periodontitis by inactivation of nuclear factor-κB in an interleukin-10- and glucocorticoid-independent fashion.

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Periodontitis is a chronic inflammatory bone destructive disease that is induced by a complex of oral pathogens, including *Porphyromonas gingivalis* (1). In addition, host immune responses play an important role in the induction and disease progression (2,3). Thus, regulation of the host immune and inflammatory response is a possible therapeutic strategy in ameliorating this disease.

18β-Glycyrrhetinic acid (GA) is an active component of licorice root extract (*Glycyrrhiza glabra*). In traditional medicine, beneficial therapeutic effects of GA have been reported in various diseases, including inflammation, ulcers and cancer (4). Several mechanisms of the GA-mediated anti-inflammatory effect have been proposed, including modulation of glucocorticoid (GC) metabolism, alteration of interleukin-10 (IL-10) production and downregulation of nuclear factor-κB (NF-κB; 5–7). In experimental arthritis, GA reduced inflammatory bone destruction, which correlated with an inhibition of 11β-hydroxysteroid dehydrogenase-2 (HSD2), which converts active corticosteroids to their inactive forms (8). This finding suggests that increased local active GC is a possible mechanism of GA-mediated inhibition of inflammation. However, despite these correlative data, the precise mechanism(s) of action of GA remains unclear.

In the present study, we investigated possible therapeutic effects of GA on infection-stimulated alveolar bone loss in a well-established IL-10-deficient mouse model of periodontitis (9). We have previously reported that IL-10-deficient mice are highly susceptible to *P. gingivalis*-induced periodontal disease, consistent with their hyper-inflammatory response phenotype (10). The hypothesis tested was that GA inhibits periodontitis via local increases in active GCs that is mediated by a downregulation of HSD2. We here demonstrate that GA inhibits periodontal bone loss in this model, but that this is likely to involve modulation of the proinflammatory transcription factor NF-κB rather than inhibition of HSD2 and local GC levels.

## Material and methods

### Reagents and treatments

Reagents, including 18β-glycyrrhetinic acid [molecular weight 470.70 g/mol, purity 97%, insoluble in water and ethanol, and soluble in dimethyl sulfoxide (DMSO)], olive oil (highly refined, low acidity) and DMSO, were purchased from Sigma-Aldrich (St Louis, MO, USA). Mice in GA-treated groups were given an olive oil suspension of GA (30 mg dose/kg body weight) following two treatment regimens. For prophylaxis, GA was delivered by subcutaneous (s.c.) injection on days 0, 2, 4, 13, 20, 27 and 34 relative to infection with *P. gingivalis* W83. To test therapeutic effects, GA was injected s.c. on days 9, 11, 13, 20, 27 and 34 after infection. Control animals received injections of olive oil alone following the prophylactic schedule. For *in vitro* studies, 10 mM GA in DMSO was diluted to 10 or 1 μM in culture medium. Dimethyl sulfoxide alone served as a control.

### Mice

Interleukin-10-deficient (IL-$10^{-/-}$) mice (B6.129P2-Il10$^{tm1Cgn}$/J) on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained under specific pathogen-free conditions in the Forsyth Institute animal facility as previously described (10). The use of animals was approved by the Forsyth Institutional Animal Care and Use Committee.

### Bacterial culture and infection regimen

*P. gingivalis* W83 was grown, harvested, and resuspended at $10^{10}$ cells/ml in phosphate-buffered saline containing 2% carboxymethyl cellulose (CMC; Sigma-Aldrich) as previously described (9). Prior to oral infection, 6- to 7-week-old mice received antibiotic treatment [sulfatrim suspension (concentration of sulfamethoxazole/trimethoprim oral suspension is 200/40 mg/5 mL), 10 mL/100 mL of drinking water] to reduce endogenous oral flora (9). Animals were then orally inoculated with $10^9$ *P. gingivalis* six times, beginning on day 0, at 2 day intervals. Negative control animals received the same volume of CMC without bacteria. Colonization of mice by *P. gingivalis* was determined by RT-PCR as described below.

### Sample preparation

Animals were killed on day 42 after the initial oral infection. Mandibles were isolated, hemisected and defleshed for bone loss measurements as previously described (9). Gingival tissues were isolated for RNA extraction, and were disrupted by FastPrep-24 using Matrix A (both from MP Biomedicals, Solon, OH, USA) and TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

### Bone loss measurements

Alveolar bone loss was determined on digital images of mandibular molar teeth and alveolar bone morphometrically as previously described (9). Results are expressed in millimeters squared.

### Quantitative RT-PCR (qPCR) and RT-PCR

Gene expression of *HSD1*, *HSD2*, IL-12 p40 and β7-integrin in gingival tissue was assessed by qPCR as previously described (10). Predesigned primers for *GAPDH* (catalog number QT00309099, Qiagen, Valencia, CA, USA), *HSD1* (QT00107303), *HSD2* (QT00252609), β7-integrin (QT00105091) and *RANKL* (QT00147385) were used with the QuantiFast SYBR Green PCR Kit (Qiagen) following the manufacturer's instructions. The *GAPDH* gene was used as an internal control. All reactions were carried out in triplicate. The level of gene expression was determined by comparison with standard curves generated with known copy number samples.

To confirm the colonization of *P. gingivalis*, total RNA samples, which contain both host and bacterial RNA, were subjected to RT-PCR to detect the *P. gingivalis* 16S rRNA gene as described previously (10).

### Macrophage cultures

Culture medium RPMI1640 (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Invitrogen) and 10% charcoal–dextran-treated fetal bovine serum (to remove glucocorticoids; HyClone, Waltham, MA, USA) was used for all cell culture experiments. Resident peritoneal macrophages isolated from IL-$10^{-/-}$ mice (11) were plated at $10^5$ cells per well in 96-well plates ($n = 4$), and were stimulated with *E. coli* lipopolysaccharide (LPS, Sigma-Aldrich, 2 μg/mL) for 24 h at 37°C in an atmosphere of 5% $CO_2$–95% air. After the incubation, culture supernatants were subjected to ELISA for cytokines. Adherent macrophages were subjected to protein extraction for western blot.

### T cell proliferation assays

Splenic T cells isolated from IL-$10^{-/-}$ mice (10) were plated at $10^5$ cells per well in 96-well plates coated with anti-mCD3/mCD28 antibodies (both from BD Biosciences, San Jose, CA, USA) and stimulated with concanavalin A (Con A, Sigma-Aldrich, 1 μg/mL) in the presence or absence of GA for 7 days. The proliferation of T cells was determined using the CellTiter 96 Aqueous assay (Promega, Madison, WI, USA).

### Osteoclast differentiation

Osteoclastogenesis was induced in RAW264.7 cells by recombinant mouse RANKL (PeproTech, Rocky Hill, NJ, USA), and TRAP-positive polykaryons with more than three nuclei were enumerated as osteoclast-like cells (12). The cells were also subjected to protein extraction for western blot.

### ELISA

Interleukin-1β, IL-6, IL-12, and interferon-γ (IFNγ) were quantified by ELISA (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Cytokine concentrations were expressed in picograms per milliliter.

### Protein extraction and western blot

Cellular protein was extracted using Tris–Glycine SDS Sample Buffer (Invitrogen). After SDS-PAGE of protein samples (10 μg), NF-κB2 p100/p52, phosphorylated NF-κB2 p100 and phosphorylated NF-κB p105 were detected using specific antibodies (Cell Signaling Technologies, Danvers, MA, USA). Mouse GAPDH served as a control (antibody from Ambion, Austin, TX, USA).

### Statistics

The effect of GA treatment was evaluated statistically by one-way ANOVA with the Bonferroni multiple comparison test.

## Results

### Effect of GA on *P. gingivalis*-induced alveolar bone loss

We have previously characterized IL-10-deficient mice as a hyperinflammatory model of periodontitis, secondary to an increase in the expression of IL-12 and the induction of strong T helper 1 (Th1) responses. The IL-$10^{-/-}$ mice were infected orally with *P. gingivalis* as described in the Material and methods section. Infection was confirmed by the detection of *P. gingivalis* in all infected, but not in non-infected, animals on day 42 as determined by RT-PCR. As shown in Fig. 1, oral infection of IL-$10^{-/-}$ mice with with *P. gingivalis* induced significant alveolar bone loss after 42 days compared with uninfected control animals. Strikingly, GA (30 mg/kg) administered either prophylactically (days 0–32) or therapeutically (days 9–34) completely inhibited *P. gingivalis*-induced bone loss, resulting in bone levels that were indistinguishable from uninfected control animals.

### Effect of GA on gingival gene expression

It has been suggested that GA acts by inhibiting the expression of HSD2, which degrades glucocorticoids. We therefore evaluated gingival gene expression of *HSD1* and *HSD2* by qPCR on day 42. 18β-Glycyrrhetinic acid did not modulate the expression of *HSD1*. Surprisingly, GA also failed to reduce *HSD2* gene expression in gingival tissue (Fig. 2), and instead upregulated *HSD2* gene expression in the treated animals, although this difference was not statistically significant ($p = 0.07$). We also examined the effect of GA on gingival gene expression of IL-12 p40, β7-integrin and *RANKL*, because these genes are crucial in the induction of Th1 immune responses as well as for osteoclastogenesis (10,13,14). However, no significant effect of GA on these genes was observed on day 42 (data not shown).

### Suppressive effect of GA on cell functions in IL-$10^{-/-}$ mice

Since macrophages and T cells play key roles in the induction of periodontitis in IL-$10^{-/-}$ mice (10), we determined whether GA inhibits pro-inflammatory cytokine production by *E. coli* LPS-stimulated IL-$10^{-/-}$ macrophages *in vitro*. As shown in Fig. 3A, GA significantly suppressed the production of IL-1β and IL-12 p70 by macrophages in a dose-dependent fashion, whereas there was no effect on IL-6, indicating a lack of toxicity of this compound.

Next, we examined the effect of GA on T cell proliferation and IFNγ production. 18β-Glycyrrhetinic acid modestly upregulated T cell proliferation compared with positive controls, again indicating that it is not toxic. 18β-Glycyrrhetinic acid also failed to inhibit IFNγ production by T cells (Fig. 3B).

Finally, we determined the effect of GA on RANKL-stimulated osteoclastogenesis. As shown in Fig. 3C, GA profoundly inhibited the formation of TRAP-positive osteoclast-like polykaryons in a RAW264.7 cell model. Essentially no TRAP-positive cells were observed in GA-treated cultures.

### 18β-Glycyrrhetinic acid suppresses phosphorylation of NF-κB

To further explore the mechanism of GA action, we determined the

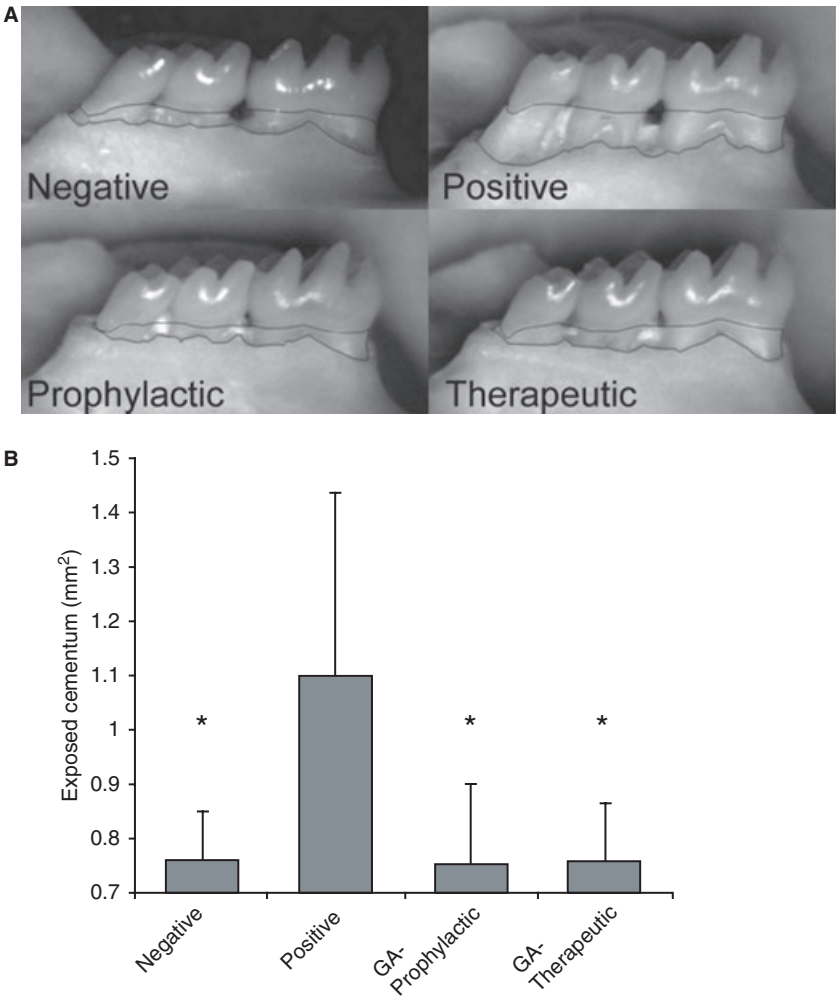


*Fig. 1.* 18β-Glycyrrhetinic acid inhibits the induction and progression of infection-induced alveolar bone loss in interleukin-10-deficient mice. (A) Representative images of defleshed hemimandibles. Negative: non-infected, vehicle alone, $n = 9$. Positive: *P. gingivalis* infected, vehicle alone, $n = 8$. Prophylactic: *P. gingivalis* infected, GA 30 mg/kg, day 0–34, $n = 9$. Therapeutic: *P. gingivalis* infected, GA 30 mg/kg, day 9–34, $n = 8$. The area enclosed by black line is the area of exposed cementum. (B) Effect of GA on alveolar bone loss. Vertical bars represent SD; $*p < 0.05$ by ANOVA/Bonferroni test.

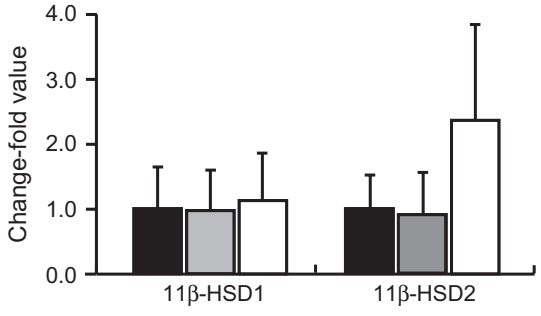


*Fig. 2.* 18β-Glycyrrhetinic acid does not inhibit gingival gene expression of *HSD1* or *HSD2*. Gingival gene expression of HSDs was determined by qPCR on day 42. Values are expressed as multiples of the positive control (change-fold values). Filled columns: positive control (infected, no treatment), Shaded columns: negative control (no infection, no treatment). Open columns: infected, treated with prophylactic regimen. Vertical bars represent SD. No significant differences were detected by ANOVA/Bonferroni test.

effects of GA on the activation of NF-κB *in vitro*. Lipopolysaccaride-induced phosphorylation of NF-κB p105 in IL-$10^{-/-}$ macrophages was significantly downregulated by 1 μM of GA (Fig. 4A). In addition, GA inhibited RANKL-induced phosphorylation of NF-κB p105 (Fig. 4B). There was no effect of GA on the phosphorylation of NF-κB2 p100/p52 or NF-κB2 p100 (data not shown). These data suggest that inhibition of NF-κB phosphorylation may underlie the protective anti-inflammatory effect of GA on *P. gingivalis*-induced periodontitis in IL-$10^{-/-}$ mice.

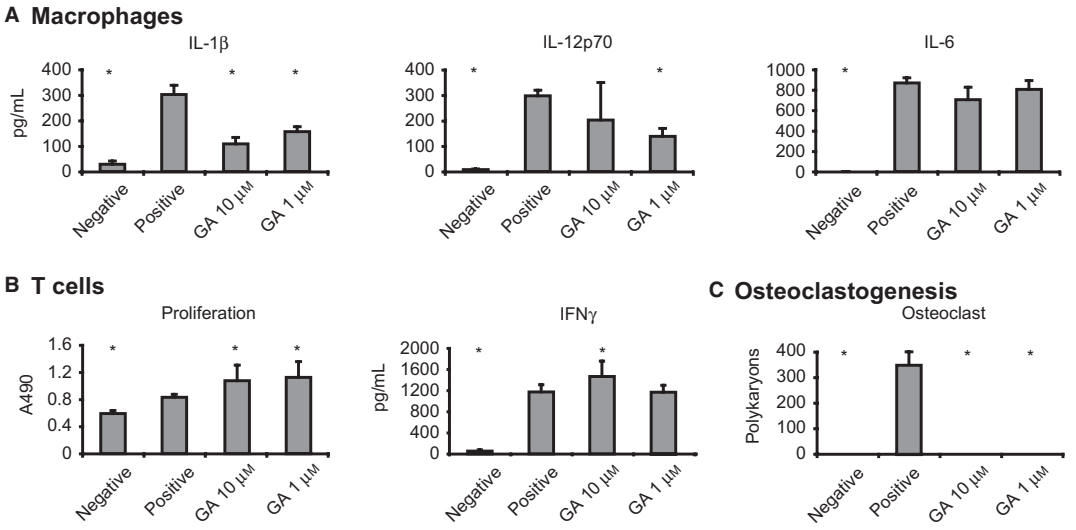


*Fig. 3.* 18β-Glycyrrhetinic acid suppresses proinflammatory cytokine production and osteoclastogenesis *in vitro*. (A) The effect of GA on cytokine production by LPS-stimulated IL-$10^{-/-}$ macrophages. Negative: no LPS, no GA. Positive: LPS, no GA. GA: LPS, GA 10 or 1 μM. $n = 4$ per group. Vertical bars represent SD; $^*p < 0.05$ by ANOVA/Bonferroni test. (B) The effect of GA on T cell proliferation and IFNγ production. Negative: no ConA, no GA. Positive: Con A, no GA. GA: Con A, GA 10 or 1 μM. $n = 4$ per group, Vertical bars represent SD; $^*p < 0.05$ by ANOVA/Bonferroni test. (C) The effect of GA on RANKL-stimulated osteoclastogenesis by RAW264.7 cells. Negative: no RANKL, no GA. Positive: RANKL, no GA. GA: RANKL, GA 10 or 1 μM. $n = 4$ per group. Vertical bars represent SD; $^*p < 0.0005$ by ANOVA/Bonferroni test.

## Discussion

18β-Glycyrrhetinic acid has been used as an anti-inflammatory agent for more than 2000 years (4), and in recent studies inhibited joint destruction in experimental adjuvant-induced autoimmune arthritis (8). The protective effect of GA on arthritis has been attributed to local increases in GC levels, via inhibition of HSD2, which mediates GC inactivation.

IL-$10^{-/-}$ mice have a hyperinflammatory phenotype and are highly susceptible to *P. gingivalis*-induced alveolar bone destruction (9). Oral infection of *P. gingivalis* induces five times more alveolar bone loss in IL-$10^{-/-}$ than in wild-type C57BL/6J mice that are resistant to periodontitis (10). Hence, we chose IL-$10^{-/-}$ mice as a stringent model to test the therapeutic potential and mechanism of action of GA. As we previously reported, the susceptibility of IL-$10^{-/-}$ mice to *P. gingivalis*-induced periodontitis is dependent on a hyperinflammatory state established via IL-12-mediated polarized Th1 immune responses (10).

In the present studies, we demonstrate that GA completely inhibits *P. gingivalis*-induced bone loss when delivered in either prophylactic or therapeutic regimens, suggesting a mode of action of GA that is exerted at the time of, as well as following, periodontal disease initiation. 18β-Glycyrrhetinic acid also has therapeutic

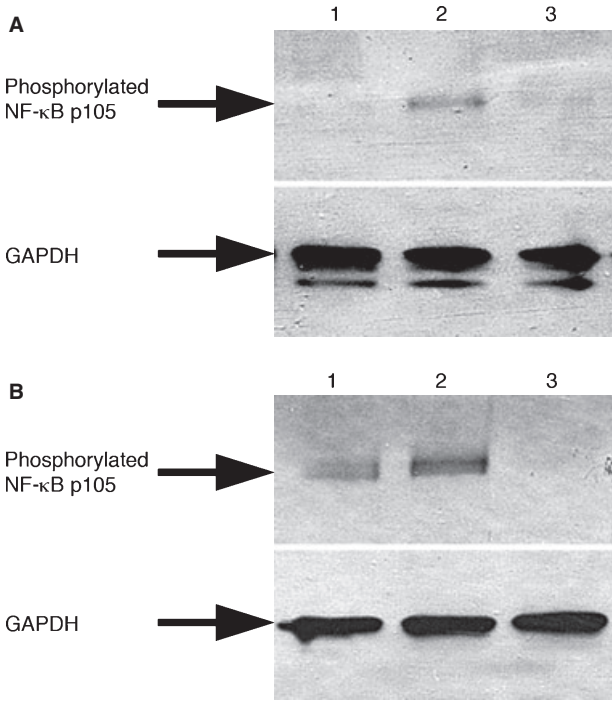


*Fig. 4.* 18β-Glycyrrhetinic acid inactivates the phosphorylation of NF-κB p105. (A) Inactivation of NF-κB in LPS-stimulated IL-$10^{-/-}$ macrophages. Lane 1: negative (vehicle alone). Lane 2: positive (LPS, vehicle). Lane 3: GA (LPS, 1 μM GA). (B) Inactivation of NF-κB in RANKL-stimulated RAW264.7 cells. Lane 1: negative (vehicle alone). Lane 2: positive (RANKL, vehicle). Lane 3: GA (RANKL, 1 μM GA).

impact in wild-type animals, since it significantly suppressed ligature- and *P. gingivalis* infection-induced alveolar bone loss in wild-type Lewis rats (H.S., unpublished observations). The observation that GA inhibits bone loss in both IL-$10^{-/-}$ and wild-type animals clearly indicates that its mode of action is IL-10 independent.

18β-Glycyrrhetinic acid has been reported to mediate anti-inflammatory effects in target tissues by increasing active GCs via downregulation of HSDs (15). In contrast to autoimmune arthritis (8), we found that GA failed to downregulate gingival expression of HSDs, indicating that the HSD–GC axis appears not to be a key mechanism in GA-mediated suppression of alveolar bone loss.

In contrast, GA significantly suppressed a number of NF-κB-dependent events, including the production of IL-1β and IL-12 p70 by LPS-stimulated macrophages (16,17). *P. gingivalis* LPS was not employed in this study owing to its weak and inconsistent effects in this assay system (H.S., unpublished observations). However, IL-6, IFNγ and T cell proliferation, all of which are less NF-κB dependent (18–21), were unaffected. Consistent with NF-κB as a primary point of regulation (22), GA completely inhibited RANKL-stimulated osteoclastogenesis and LPS-induced phosphorylation of NF-κB p105 (NF-κB1), but not NF-κB2 p100/p52 or NF-κB2 p100, in GC-free conditions *in vitro*. Taken together, these data strongly indicate that GA-mediated blockade of infection-stimulated alveolar bone loss is dependent on its ability to inhibit the activation of NF-κB and does not involve modulation of GCs or IL-10.

Nuclear factor-κB1 represents the canonical NF-κB pathway, whereas NF-κB2 is involved in the non-canonical pathway. The canonical pathway is activated by various stimuli, such as cytokines and microbial components. Nuclear factor-κB1 was also upregulated in bone marrow cells derived from tail-suspended mice, a model of microgravity in which animals rapidly become osteoporotic (23). Although Rel-A [v-rel reticuloendotheliosis viral oncogene homolog A (avian)] complexes (Rel-A/c-Rel/p50) will be released from the IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) in the canonical pathway (24), Rel-A was reported as a key molecule in inhibition of osteolytic osteomyelitis (25). Nuclear factor-κB p50/p50 homodimers mediate reactive arthritis, which is an infection-induced inflammation that also involves urethra and eyes (26). Of interest, in a sepsis arthritis model, systemic inhibition of NF-κB2 using antisense oligonucleotides failed to suppress bone loss (27). In contrast, non-canonical pathway activation closely relates to autoimmunity, and is not activated by most of the classical NF-κB inducers, such as proinflammatory cytokines (28). In addition to rheumatoid arthritis, NF-κB2 appears to be important in induction of juvenile idiopathic arthritis and psoriatic arthritis (29). These findings suggest that NF-κB1, but not NF-κB2, may play an important role in infection-stimulated bone loss.

The apparent lack of effect of GA on GC metabolism in periodontitis compared with arthritis models may reflect inherent differences in the pathways activated by infection compared with an autoimmune disease. 11β-Hydroxysteroid dehydrogenases are key regulators of GC pre-receptor metabolism (15), by interconverting active and inactive GCs. 18β-Glycyrrhetinic acid preferentially inhibits HSD2, which converts active GCs to their inactive form, but does not affect HSD1, which converts inactive GCs to their active form (5). 11β-Hydroxysteroid dehydrogenase-2 was elevated in autoimmune arthritis (30) and was also significantly upregulated in synovial macrophages of rheumatoid arthritis patients compared with normal synovium (31). These correlations are consistent with a key role of HSD2 and local GCs in the protective effect of GA in autoimmune arthritis, which is a sterile inflammatory condition. However, direct evidence for the role of this mechanism is still lacking and, as noted earlier, other mechanisms of action have been reported (6,7). In contrast, proinflammatory TNFα and IL-1β significantly downregulated HSD2 in placental trophoblasts (32), suggesting a GA-independent upregulatory pathway of active GCs. In contrast, we demonstrate that GA can exert its inhibitory effects via an HSD- and GC-independent mechanism, indicating the complexity of the GA–HSD2–GC axis. Thus, further studies are essential to examine precisely the effect of GA on the NF-κB pathways and HSD–GC axis using NF-κB$1^{-/-}$ and HSD$2^{-/-}$ mice (33,34).

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