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Mouse Strain-Dependent Osteoclastogenesis in Response to Lipopolysaccharide

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Bacterial lipopolysaccharide (LPS) is a potent stimulator of bone resorption in periodontitis. Co-culture systems of mouse calvaria-derived osteoblasts and bone marrow-derived preosteoclasts were used as an *in vitro* osteoclast differentiation. This study revealed that co-cultures using ddY or ICR mouse strain responded differently to LPS while responded equally to 1α,25(OH)$_2$D$_3$. Thus, the different response to LPS indicates dissimilarity of two mouse stains in their capacity for generating osteoclasts while the two mouse strains share the similarity in response to 1α,25(OH)$_2$D$_3$. To identify which cells between osteoblasts and preosteoclasts in the co-culture are responsible for the dissimilarity, the reciprocal co-cultures were performed between ddY and ICR mouse strains. The treatment of 1,25(OH)$_2$D$_3$ to ddY/ICR (osteoblasts from ddY/preosteoclasts from ICR) and ICR/ddY reciprocal co-cultures also showed the similarity. In case of LPS treatment, the results of ddY/ICR were similar to ddY/ddY and the results of the other reciprocal co-culture, ICR/ddY combination, were consistent with those of ICR/ICR. It suggests that the dissimilarity between the two mouse strains may resident in osteoblasts but not in preosteoclasts. Therefore, the osteoblast is responsible for mouse strain-dependent osteoclastogenesis in response to LPS. Although mouse models will continue to provide insights into molecular mechanisms of osteoclastogenesis, caution should be exercised when using different mouse strains, especially ddY and ICR strains as models for osteoclast differentiation.

***Keywords*:** osteoclast, osteoblast, LPS, osteoclastogenesis

Osteoclasts are multinucleated cells (MNCs) with a bone-resorbing activity. They are derived from hematopoietic cells through multiple steps including proliferation, expression of tartrate resistant acid phosphatase (TRAP), and fusion of cells (Takahashi *et al.*, 1999). Osteoblasts or stromal cells express the receptor activator of nuclear factor-κB (RANK) ligand (RANKL, also known as an osteoclast differentiation factor and osteoprotegerin ligand). The osteoclast precursor (preosteoclast) expresses RANK which interacts with RANKL, and the cell then differentiates into the osteoclast (Hsu *et al.*, 1999). RANKL expression in osteoblasts is up-regulated by pro-resorptive hormones, cytokines and mediators, such as 1α, 25-dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$ or VitD$_3$], parathyroid hormone (PTH), prostaglandin E$_2$ (PGE$_2$), and interleukin (IL)-1 (Yasuda, *et al.*, 1998; Hofbauer *et al.*, 1999; Hofbauer *et al.*, 2000; Walsh and Choi, 2003). Administration of 1α,25(OH)$_2$D$_3$, PTH, or IL-1 to a normal mouse led to an elevation of bone resorption (Li *et al.*, 2000). The 1α,25(OH)$_2$D$_3$ is often used as a positive control for the research of osteoclastogenesis.

Alveolar bone resorption is one of the significant clinical characteristics in periodontitis (Schwartz *et al.*, 1997). A number of bacteria harbored in periodontal pockets stimulate osteoclast formation, and thus the bacteria play a major part in bone resorption. Periodontitis is a mixed bacterial infection and it is important to identify common pathways of bone destruction induced by the periodontopathogens. Based on the understanding of osteoclastogenesis, the pathogenesis of lipopolysaccharide (LPS) in periodontitis appears to be one of common pathways of bone destruction induced by Gram-negative periodontopathogens (Sismey-Durrant and Hopps, 1987; Miyata *et al.*, 1997; Chen and Yan, 2001; Chiang *et al.*, 2003; Chung *et al.*, 2006; Cheng *et al.*, 2007; Kang *et al.*, 2007; Rogers *et al.*, 2007). Thereby, *Escherichia coli* LPS is commonly used as a positive control for the study of osteoclastogenesis in periodontitis.

An *in vitro* osteoclast differentiation system, a co-culture system of mouse calvaria-derived osteoblasts and bone marrow-derived preosteoclasts, was established in the late (Takahashi *et al.*, 1988; Suda *et al.*, 1992). It has been appreciated through this co-culture system that direct and indirect cell-cell contacts between preosteoclasts and osteoblasts are critical for the osteoclast differentiation. Since then, the co-culture system is commonly used in the research of osteoclastogenesis to evaluate osteoclast differentiation. This system provided not only the insight into the RANKL expressed by osteoblasts which supports osteoclastogenesis but also fundamental knowledge about the effects of osteoclastogenic factors such as 1α,25(OH)$_2$D$_3$ and LPS on osteoblasts (Suda *et al.*, 1992). Recently, mouse strains of ddY and ICR are used in the co-culture system to isolate the calvaria-derived osteoblasts and bone marrow-derived preosteoclasts. However, the differences between the two mouse strains, ddY and ICR, have not been investigated in terms of osteoclastogenesis. To characterize mouse strain-dependent osteoclastogenesis,

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the responses of osteoclastogenesis *in vitro* in ddY and ICR mouse strains to 1α,25$(OH)_2D_3$ or LPS were compared and evaluated.

Materials and Methods

Isolation of osteoblasts from calvaria

The osteoblasts were isolated from the calvaria of 1- to 2-day old ddY (SLC Inc., Japan) or ICR (Harlan, USA) mice. The calvaria were digested in 10 ml of α-minimum essential medium (MEM) containing 0.2% collagenase (Waco Pure Chemicals, Japan) and 0.1% dispase (GIBCO BRL, USA) for 20 min at 37°C with vigorous shaking and then centrifuged at 250×g for 5 min. The first supernatant was discarded, and another 10 ml of the collagenase-dispase enzyme solution was added and incubated for 20 min. The digestion procedure was repeated four times, and the cells isolated by the last three digestions were combined as osteoblasts. Isolated cells were cultured for 3 days in α-MEM containing 10% FBS in 10 cm culture dishes and cells are detached by trypsin-EDTA, centrifuged and suspended in α-MEM containing 20% FBS and 10% DMSO, and stored at -80°C until use.

Isolation of bone marrow-derived preosteoclasts

The bone marrow cells were collected from 5 to 8-week old ddY or ICR mice. The ends of the tibiae and femurs were removed, and each marrow cavity was flushed by slowly injecting α-MEM at one end with a 25-gauge needle. The marrow cells suspended in α-MEM containing 10% FBS were cultured with 100 ng/ml M-CSF in 6 cm culture dishes for 16 h. Non-adherent cells were gently collected and used for co-cultures.

Co-cultures of osteoblasts and preosteoclasts

Osteoblasts were seeded at a concentration of 10^6 cells per 10 cm culture dish and grown to confluence. The cells were them detached from the culture dishes with trypsin-EDTA (GIBCO BRL). Subsequently, osteoblasts (8×10^3 cells/well) were co-cultured with preosteoclasts (8×10^4 cells/well) in α-MEM containing 10% FBS in 96 well plates (Corning Inc., USA). The culture volume was adjusted to 200 μl per well with α-MEM containing 10% FBS. The 10^{-8} M 1α,25$(OH)_2D_3$ (Waco Pure Chemicals, Japan) or 1 μg/ml LPS (Sigma, USA) was added to each co-culture. Cultures were fed every 3 days by replacing with the fresh medium. The co-cultures were then maintained for additional 6 days and stained for tartrate-resistant acid phosphatase (TRAP) as described following.

TRAP staining

Adherent cells were then fixed with 10% formaldehyde in phosphate-buffered saline (PBS), treated with ethanol-acetone (50:50), and stained for TRAP as described preciously (Suda *et al.,* 1997). TRAP-positive multinucleated cells (MNCs) containing more than three nuclei were counted as osteoclasts. The osteoclast cells per well were counted for osteoclast formation activity. The stained cells were photographed using

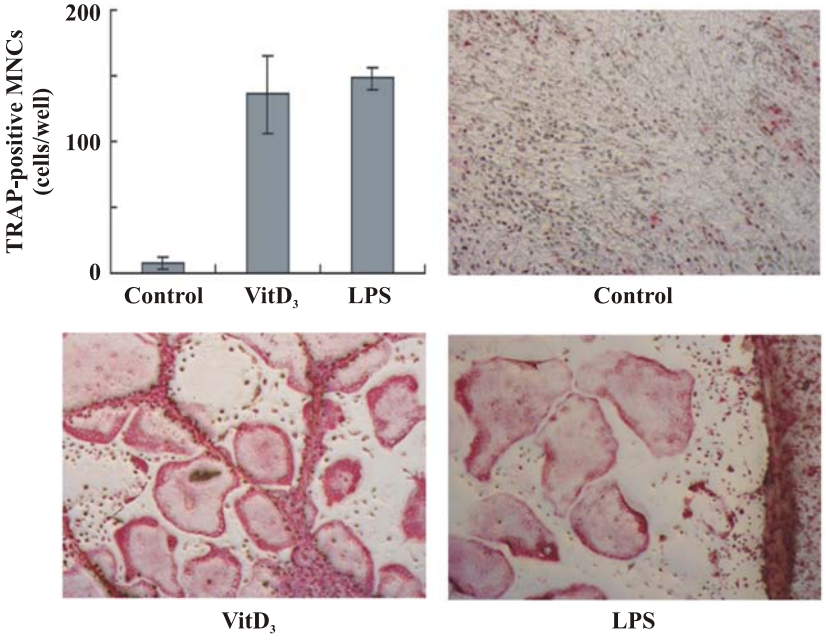

Fig. 1. Effect of 1α,25$(OH)_2D_3$ ($VitD_3$) or LPS on the osteoclast differentiation in the co-culture of ddY/ddY combination. The induction of osteoclastogenesis by $VitD_3$ or LPS was determined in a co-culture system of mouse calvaria-derived osteoblasts and bone marrow cells isolated from ddY mouse. The co-culture in a 96-well plate was incubated for 6 days in the absence or presence of $VitD_3$ or LPS and stained for TRAP. TRAP-positive MNCs possessing more than three nuclei were defined as osteoclasts. The osteoclast cells per well were counted for osteoclast formation activity. A p value of less than 0.05 was considered to indicate a statistically significant difference. There was no significant difference between the values of $VitD_3$ and LPS treatments. The stained cells were photographed with the 100× magnification.

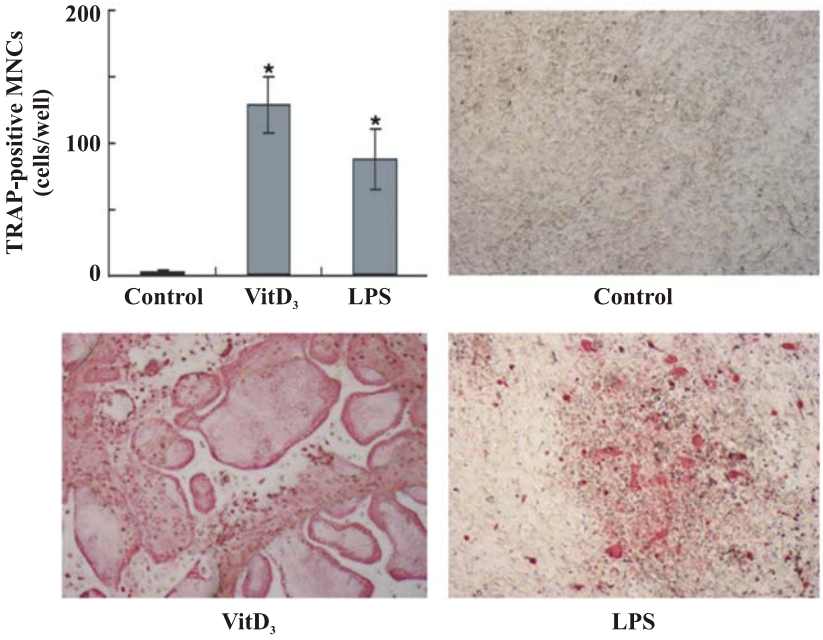

Fig. 2. Effect of 1α,25(OH)$_2$D$_3$ (VitD$_3$) or LPS on the osteoclast differentiation in the co-culture of ICR/ICR combination. The induction of osteoclastogenesis by VitD$_3$ or LPS was determined in a co-culture system of mouse calvaria-derived osteoblasts and bone marrow cells isolated from ICR mouse strain. The symbol of * indicates significant differences at $p<0.05$ between the values of VitD$_3$ and LPS treatments.

Olympus CKX41 inverted microscope system (Olympus, Japan). All assays were performed in triplicate on two separate experiments.

Statistical analysis

Statistical analysis was performed by using SPSS 12.0 statistical package program (SPSS Inc., USA). Paired two-tailed Student *t* tests were used to assess significant differences. A *p* value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Osteoclast formation of ddY mouse strain in response to 1α,25(OH)2D3 or LPS

The induction of osteoclastogenesis by 1α,25(OH)$_2$D$_3$ or LPS was determined in a co-culture system of mouse calvaria-derived osteoblasts and bone marrow-derived preosteoclasts isolated from ddY mouse strain (Osteoblast origin strain/preosteoclast origin strain=ddY/ddY combination) (Fig. 1). TRAP-positive MNCs possessing more than three nuclei were defined as differentiated osteoclasts. In the absence of 1α,25(OH)$_2$D$_3$ or LPS, less than eight TRAP-positive MNCs were observed per well. Administration of 1α,25(OH)$_2$D$_3$ increased the TRAP-positive MNCs to ~136 per well. In addition to the increased number, it is worth noting that most TRAP-positive cells possessed more than 50 nucleuses and therefore the size of the TRAP-positive MNCs is large, suggesting that numerous fusions of preosteoclasts occurred to differentiate into osteoclasts. Administration of LPS increased the similar number (~148) and size of TRAP-positive MNCs to those induced by the 1α,25(OH)$_2$D$_3$ and therefore the values of VitD$_3$ and LPS treatments showed no statistically significant difference.

Osteoclast formation of ICR mouse strain in response to 1α,25(OH)2D3 or LPS

The induction of osteoclastogenesis by 1α,25(OH)$_2$D$_3$ or LPS was determined by the number of TRAP-positive MNCs in a co-culture system of osteoblasts and preosteoclasts isolated from ICR mouse strain (ICR/ICR combination) (Fig. 2). In the absence of 1α,25(OH)$_2$D$_3$ or LPS, only two TRAP-positive MNCs were observed per well. Administration of 1α,25(OH)$_2$D$_3$ increased the number of TRAP-positive MNCs to ~128 per well and the size of the TRAP-positive MNCs similar to those of ddY mouse strain. In contrast to 1α,25(OH)$_2$D$_3$, administration of LPS induced ~87 TRAP-positive MNCs, which was decreased to 0.7 fold when compared with ddY mouse strain, and furthermore TRAP-positive MNCs induced by LPS were significantly less ($p<0.05$) and much smaller than TRAP-positive MNCs induced by the 1α,25(OH)$_2$D$_3$. Most of the small osteoclasts possessed less than 10 nucleuses.

Mouse strain-dependent osteoclastogenesis in response to LPS is not due to preosteoclast

The different osteoclastogenesis in response to LPS was observed between ddY and ICR mouse strains. To find out which cells between osteoblasts and preosteoclasts in the co-culture system are responsible for the mouse strain-dependent osteoclastogenesis in response, reciprocal co-cultures were performed between ddY and ICR mouse strains. First of all, osteoblasts from the ddY and preosteoclasts from the ICR were isolated (ddY/ICR combination) (Fig. 3). The ddY/ICR combination was co-cultured in absence or presence of 1α,25(OH)$_2$D$_3$ or LPS. Interestingly, in the absence of 1α,25(OH)$_2$D$_3$ or LPS, over 50 TRAP-positive MNCs

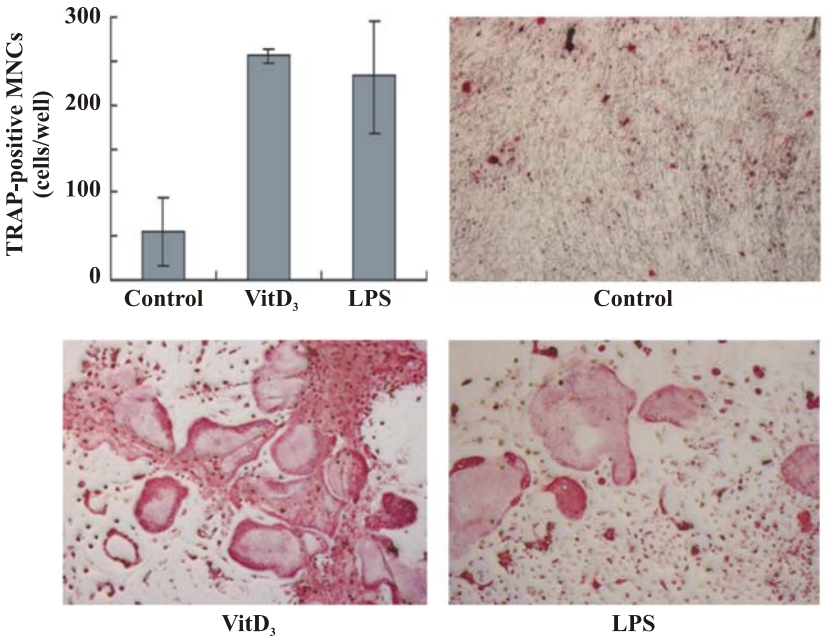

Fig. 3. Effect of LPS on the osteoclast differentiation in the co-culture of ddY/ICR combination. The induction of osteoclastogenesis by $VitD_3$ or LPS was determined in a co-culture system of mouse calvaria-derived osteoblasts isolated from ddY and bone marrow cells isolated from ICR. There was no statically significant difference between the values of $VitD_3$ and LPS treatments.

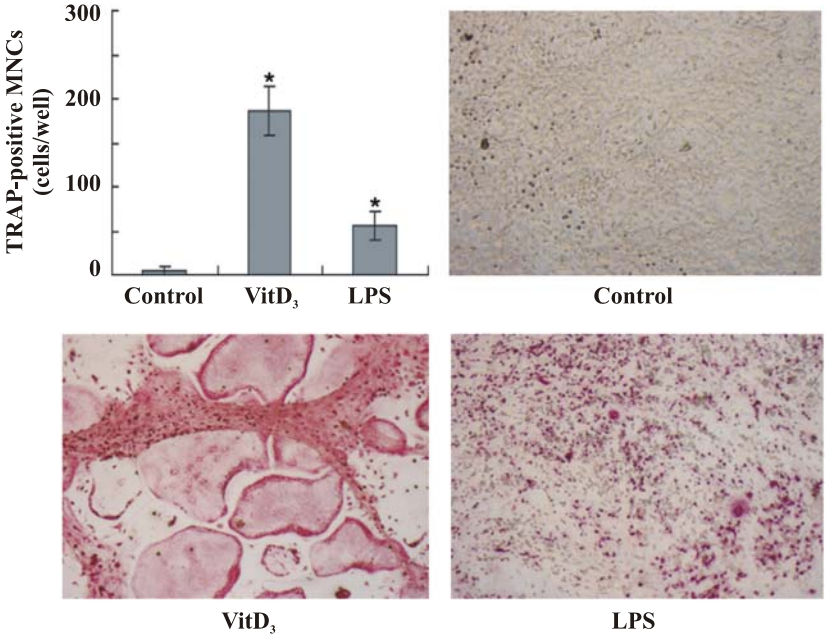

Fig. 4. Effect of LPS on the osteoclast differentiation in the co-culture of ICR/ddY combination. The induction of osteoclastogenesis by $VitD_3$ or LPS was determined in a co-culture system of mouse calvaria-derived osteoblasts isolated from ICR and bone marrow cells isolated from ddY. The symbol of * indicates significant differences at $p<0.05$ between the values of $VitD_3$ and LPS treatments.

were observed per well, indicating that certain background appears in ddY/ICR co-culture. Most of TRAP-positive MNCs contained less than 10 nucleuses, which was much smaller than TRAP-positive MNCs induced by $1\alpha,25(OH)_2D_3$. Administration of $1\alpha,25(OH)_2D_3$ as a positive control increased the number of TRAP-positive MNCs to ~256 per well and, in terms of size, the heterogeneous populations of the large and small TRAP-positive MNCs were observed, even though the large MNCs were dominant, and similar to those observed in ddY/ddY or ICR/ICR co-culture with induction of $1\alpha,25(OH)_2D_3$. The administration of LPS increased the number of TRAP-positive MNCs to ~232 per well, and the

heterogeneous populations of the large and small TRAP-positive MNCs were also observed. However, in case of LPS, the large MNCs were less dominant than those treated with $1\alpha,25(OH)_2D_3$. The large TRAP-positive MNCs were similar to the cells induced by the $1\alpha,25(OH)_2D_3$ and the small cells were similar to the cells of negative control. As statistical analysis, the values of $VitD_3$ and LPS treatments showed no significant difference. These results of ddY/ICR combination were similar to ddY/ddY combination except the background. Therefore, when the preosteoclast of ddY/ddY combination was switched with the preosteoclast of ICR, the result was still similar to ddY/ddY and thus the preosteoclast of ICR didn't change the osteoclastogenesis in response to LPS. It suggests that mouse strain-dependent osteoclastogenesis in response to LPS is not due to preosteoclast.

Osteoblast is responsible for mouse strain-dependent osteoclastogenesis in response to LPS

To continue discovering which cells between osteoblasts and preosteoclasts in the co-culture are responsible for the mouse strain-dependent osteoclastogenesis in response to LPS, the other reciprocal co-culture was performed. The osteoblasts from ICR and preosteoclasts from ddY were isolated (ICR/ddY combination) (Fig. 4). The ICR/ddY combination was co-cultured in absence or presence of $1\alpha,25(OH)_2D_3$ or LPS. In the absence of $1\alpha,25(OH)_2D_3$ or LPS, less than five TRAP-positive MNCs were observed per well, indicating that no background appears in ICR/ddY reciprocal co-culture. Administration of $1\alpha,25(OH)_2D_3$ as a positive control increased the number of TRAP-positive MNCs to ~187 per well and the size of the TRAP-positive MNCs similar to those of ddY/ddY and ICR/ICR combinations induced by $1\alpha,25(OH)_2D_3$. In contrast to $1\alpha,25(OH)_2D_3$, LPS induced significantly less TRAP-positive MNCs ($p<0.05$) and furthermore TRAP-positive MNCs were much smaller than those induced by the $1\alpha,25(OH)_2D_3$. Most of TRAP-positive MNCs possessed less than 10 nucleuses. The results of the ICR/ddY combination were consistent with those of the ICR/ICR. Therefore, when the osteoblast of ddY/ddY combination was switched with the osteoblast of ICR, the osteoclastogenesis in response to LPS was similar to the ICR/ICR in stead of the ddY/ddY. It suggests that mouse strain-dependent osteoclastogenesis in response to LPS is due to the osteoblast.

Discussion

Co-culture systems of ddY/ddY and ICR/ICR combinations responded equally to $1,25(OH)_2D_3$, indicating similarity between the two mouse strains in their capacity for generating osteoclasts. However, the co-cultures from ddY/ddY and ICR/ICR responded differently to *E. coli* LPS, indicating dissimilarity between the two mouse strains. To identify which cells between osteoblasts and preosteoclasts in the co-culture are responsible for the dissimilarity, the reciprocal co-cultures were performed between ddY and ICR mouse strains. First of all, the treatment of $1,25(OH)_2D_3$ to ddY/ICR and ICR/ddY also induced the osteoclastogenesis similar to ddY/ddY or ICR/ICR in terms of the number and the size of TRAP-positive MNCs. Thus, the results indicate the similarity in response of $1,25(OH)_2D_3$ between the two mouse strains in their capacity for osteoclastogenesis. Secondly, in case of LPS treatment, the results of ddY/ICR were similar to ddY/ddY. It suggests that preosteoclast isolated from ICR may not determine the osteoclastogenesis in response to LPS and probably osteoblast isolated from ddY mouse strain may be responsible for the osteoclastogenesis in response to LPS. In addition, the results of the other reciprocal co-culture, ICR/ddY combination, with LPS treatment were consistent with those of ICR/ICR. It suggests that preosteoclast isolated from ddY may not determine the osteoclastogenesis in response to LPS but probably osteoblast isolated from ICR mouse strain may be responsible for the osteoclastogenesis in response to LPS. Thus, the dissimilarity between the two mouse strains may not be resident in preosteoclast cells but in osteoblasts. Therefore, the osteoblast is responsible for mouse strain-dependent osteoclastogenesis in response to LPS.

LPS is a potent stimulator of bone resorption in inflammatory diseases. The mechanism by which LPS induces osteoclastogenesis was investigated in the co-culture system and mice *in vivo*. LPS up-regulates RANKL mRNA expression but down-regulates osteoprotegerin expression in osteoblasts (Suda *et al.*, 2004), which explain the induction of osteoclast formation by LPS. Recently, using toll-like receptor 4 (TLR4) knock-out mice, Zhuang *et al.* (2007) demonstrated that LPS is a potent activator of bone resorption *in vivo* and that this effect is mediated through TLR4 expression primarily on the cells of osteoblastic lineage. Kikuchi *et al.* (2001) also reported using C3H/HeJ mice containing a nonfunctional mutation in the TLR4 gene that TLR4 is involved in the induction of RANKL expression in osteoblasts and thus TLR4 plays an essential role in the osteoclast differentiation. Interestingly, Sakuma *et al.* (2000a and 2000b) reported using the co-culture and EP4, one of PGE receptor four subtypes, knock-out mice that PGE_2 and EP4 are crucially involved for the induction of osteoclastogenesis by LPS but not by $1,25(OH)_2D_3$. The previous studies suggest that the levels of TLR4 or EP4 expression in osteoblasts of ddY and ICR mouse strains may be responsible for the mouse stain-dependent response to LPS, which needs to be investigated in a comprehensive manner due to the importance of understanding LPS-induced inflammation and bone resorption.

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