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Elevated CD14 (cluster of differentiation 14) and toll-like receptor (TLR) 4 signaling deteriorate periapical inflammation in TLR2 deficient mice

Daniel Rider¹, Hisako Furusho², Shuang Xu¹, Alexander J. Trachtenberg³, Winston Patrick Kuo³, Kimito Hirai¹, Mako Sasa⁴, Laila Bahammam⁵, Philip Stashenko^{1,6}, Akira Fujimura⁷, and Hajime Sasaki^{1,6}

¹Department of Immunology and Infectious Diseases, The Forsyth Institute, Cambridge, MA 02142, U.S.A.

²Department of Oral and Maxillofacial Pathobiology, Hiroshima University, Japan

³Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA 02115, U.S.A.

⁴Department of Periodontology and Endodontology, Graduate School of Dentistry, Tohoku University, Sendai, Miyagi, Japan

⁵Department of Endodontics, Faculty of Dentistry, King Abdulaziz University, P.O. Box 80209, Jeddah 21589, Saudi Arabia

⁶Department of Oral Medicine, Infection, and Immunity, Harvard School of Dental Medicine, Boston, MA 02115, U.S.A.

⁷Division of Functional Morphology, Department of Anatomy, Iwate Medical University, 19-1 Uchimarui, Morioka, Iwate 020-8505, Japan

Abstract

Apical periodontitis (periapical lesions) is an infection-induced chronic inflammation in the jaw, ultimately resulting in the destruction of apical periodontal tissue. Toll-like receptors (TLRs) are prominent in the initial recognition of pathogens. Our previous study showed that TLR4 signaling is proinflammatory in periapical lesions induced by a polymicrobial endodontic infection. In contrast, the functional role of TLR2 in regulation of periapical tissue destruction is still not fully understood. Using TLR2 deficient (KO), TLR2/TLR4 double deficient (dKO), and wild-type (WT) mice, we demonstrate that TLR2 KO mice are highly responsive to polymicrobial infection-induced periapical lesion caused by over activation of TLR4 signal transduction pathway that resulted in elevation of NF- κ B (nuclear factor kappa B) and proinflammatory cytokine production. The altered TLR4 signaling is caused by TLR2 deficiency-dependent elevation of CD14 (cluster of differentiation 14), which is a co-receptor of TLR4. Indeed, neutralization of CD14 strikingly

Corresponding author: Hajime Sasaki, D.D.S., Ph.D., Department of Immunology and Infectious Diseases, The Forsyth Institute, 245 First Street, Cambridge, MA 02142, U.S.A., TEL: +1-617-892-8459, FAX: +1-617-262-4021, hsasaki@forsyth.org.
D.R and H.F equally contributed to this project and should be considered as co-first authors.
Current Affiliation of W.P.K.: Predicine, Inc., Hayward, CA 94545, U.S.A.

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suppresses TLR2 deficiency-dependent inflammation and tissue destruction *in vitro* and *in vivo*. Our findings suggest that a network of TLR2, TLR4, and CD14 is a key factor in regulation of polymicrobial dentoalveolar infection and subsequent tissue destruction.

Introduction

A periapical lesion is an inflammatory and immune response caused by anaerobic polymicrobial infection including both Gram-positive and -negative species in the dental root canal system (so called endodontic infection). Endodontic infection elicits immune and inflammatory responses including both innate and adaptive immunity leading to destruction of the alveolar bone surrounding the dental root apex and the formation of granulomatous tissue, which are representative pathologies of this disease. Macrophages is a member of innate immune cells and play a key regulatory role, which is production of the central proinflammatory cytokine IL-1 in the development of periapical lesions (Wang and Stashenko, 1993; Tani-Ishii et al., 1995).

Toll-like receptors (TLRs) play a prominent role in induction of innate immune and inflammatory responses upon recognition of PAMPs (specific pathogen-associated molecular patterns), common structures shared by most pathogens. PAMPs trigger TLR signaling that leads to NF- κ B activation and subsequent pro-inflammatory cytokine production. TLR2 and TLR4 are the components most thoroughly investigated TLRs, because TLR2 and TLR4 are critical in recognizing Gram-positive and -negative bacteria, respectively. TLR2 recognizes Gram-positive PAMPs such as lipoteichoic acid, peptidoglycan, and also lipoprotein, while TLR4 is crucial in recognition of LPS (lipopolysaccharide), a representative PAMP of Gram-negative bacteria.

The actual role of TLRs in host defense against bacterial infections has been extensively studied using TLR deficient (KO) mice and monospecies infection *in vivo* (Gerold et al., 2007). In response to Gram-negative *Salmonella typhimurium*, TLR4 signaling and TLR2 signaling work in a sequential manner. TLR4 signaling induces early cytokine production and killing of *S. typhimurium* by macrophages (6 hours post infection), while TLR2 signaling is important for macrophage bactericidal activity at 24 hours post infection (Weiss et al., 2004). Therefore, C3H/HeJ mice (a congenic hypo-TLR4 signal model) are more susceptible to *S. typhimurium* oral infection compared to C3H/HeOuJ mice exhibiting normal TLR4 signaling (Weiss et al., 2004). In contrast, TLR2 deficient (KO) mice are more susceptible to Gram-positive *Staphylococcus aureus* infection compared to wild-type controls (Takeuchi et al., 2000), demonstrating that recognition of *S. aureus* by TLR2 is critical in prevention of *S. aureus* infection. TLR2 is also important in an early inflammatory response against *Staphylococcus pneumonia* (Knapp et al., 2004).

Regardless of Gram-stainability, endodontic pathogens induce innate and subsequent adaptive immune response during endodontic infection (Hou et al., 2000b, 2000a). TLRs are critical in the development of periapical lesions caused by polymicrobial endodontic infection. The C3H/HeJ mouse, a hypo TLR4 signal model, exhibits less periapical inflammation and reduced bone loss in response to polymicrobial endodontic infection than normal C3H/HeOuJ mice (Hou et al., 2000b). This finding suggests a detrimental role of

TLR4 signal in this disease. By contrast, a recent study demonstrated that the TLR2 signal is unexpectedly protective in the development of periapical lesions (da Silva et al., 2012), although the mechanism of this TLR2-mediated protection is uncertain. Since the extent of periapical lesions in TLR2/TLR4 double deficient mice was similar to that in WT mice in our preliminary study (data not shown), we expected a negative regulatory mechanism between TLR2 and TLR4 signaling in periapical lesions.

Polymicrobial infections including both Gram-positive and negative species cause a variety of disease conditions such as abscesses, respiratory diseases, otitis media, periodontitis, and intestinal disorders via stimulating multiple pattern recognition receptors including TLRs. Such combined activation of TLRs may lead to complementary, synergistic or antagonistic effects that modulate immune response (Trinchieri and Sher, 2007). However, the functional network of TLRs in the regulation of polymicrobial infection and subsequent inflammation is not fully understood. In this report, we demonstrate that the TLR2 signal negatively regulates TLR4-mediated response via suppression of CD14 in periapical lesions induced by polymicrobial infection through morphological/histological and molecular analyses.

Materials and Methods

Animals

Age-matched (6 weeks old) wild-type C57BL/6J (wild-type (WT)), TLR2 KO (B6.129-Tlr2^{tm1Kir/J}; Jackson Laboratory, Bar Harbor, ME), and TLR2/TLR4 double KO mice were employed. The dKO mice were generated by first breeding TLR2 KO and TLR4 KO mice (a generous gift from Dr. Shizuo Akira, Osaka University, Osaka, Japan) to obtain double heterozygote offspring for targeted TLR alleles. Subsequent interbreeding of double-heterozygotes yielded offspring including homozygotes for the targeted alleles. All genetic mutant strains used in this study were on C57BL/6 background. All experimental protocols were approved by The Forsyth Institute's Institutional Animal Care and Use Committee.

Endodontic infection

Polymicrobial endodontic infection was induced as described (Hou et al., 2000b; Sasaki et al., 2000; Sasaki et al., 2004). Briefly, mandibular first molar dental pulps were microsurgically exposed under anesthesia. Common human endodontic pathogens including two Gram-positive bacteria (*Parvimonas micra* (formerly *Peptostreptococcus micros* (ATCC 33270)), and *Streptococcus intermedius* (ATCC 27335)) and two Gram-negative bacteria (*Prevotella intermedia* (ATCC25611), *Fusobacterium nucleatum* (ATCC 25586)) were inoculated into the pulp chamber and root canals. These pathogens were equally mixed to yield 10¹⁰ cells/ml of PBS containing 2% CMC. A total 10 µl of the mixture was inoculated per tooth. Non-exposed/non-infected animals served as negative controls.

Morphological analyses by micro computed tomography (µCT) and histology

On days 10 and 21 post infection, mice were killed. Mandibles were isolated and hemisected. One hemimandible was fixed and subjected to µCT following histological procedures. Quantification of lesion size and subsequent histological examination on same

samples were performed as previously described (AlShwaimi et al., 2013). The lesion size was expressed as square millimeters.

After μ CT scanning, the samples were subjected to histology as we previously described (AlShwaimi et al., 2013). In brief, the samples were decalcified using 10% formic acid and sodium citrate, embedded in paraffin following general histology protocol, sectioned in serial sections (6 μ m thickness). H&E staining was employed for general observation. Pivotal sections containing patent root canal with localized periapical lesion were stained for neutrophils (anti-Ly6G (lymphocyte antigen 6 complex, locus G); dilution 1:200; Abcam), macrophages (anti-F4/80 protein; dilution 1:50; Santa Cruz Biotechnology), ab3523 (anti-inducible NO synthase (iNOS), dilution 1:400; Abcam, Cambridge, MA), N20 (anti-arginase 1 (Arg1), dilution 1:50; Santa Cruz Biotechnology), and endothelial progenitor cells/endothelial cells (anti-CD34 (a hematopoietic progenitor cell antigen); dilution 1:250; Santa Cruz Biotechnology), respectively. At least three specimens were subjected to each primary antibody. Primary antibodies were detected using Vector Elite ABC kits (Vector Laboratories, Burlingame, CA). Omissions of the primary antibodies were used as negative controls. Observations were carried out either at $\times 100$, $\times 200$ or $\times 400$ magnification. Note that controlled counting and comparisons of staining-positive cells between these two strains were impossible due to their histological appearance.

Real-time RT-PCR

Bone blocks containing periapical lesions were isolated from the other hemimandibles. Total RNA extraction following cDNA synthesis was conducted using TRIzol Reagent (Life Technologies, Carlsbad, CA), Fastprep-24 with matrix A (both MP Biomedicals, Solon, OH), and iScript cDNA Synthesis Kit (Bio-Rad, Waltham, MA). Real-time RT-PCR was performed using the Light Cycler 480 II and Light cycler 480 sybr green I master (both Roche, Indianapolis, IN) in triplicates. Genes examined in the present study are listed in Table 1. All primers used in this study were purchased from Real Time Primers (Elkins Park, PA). The primer sequences are available in Supplemental Table 1. The $2^{-\Delta\Delta CT}$ method was used for data analyses.

Cell culture and protein quantifications

Resident peritoneal macrophages were isolated from the mice described above and seeded in 96-well plates (10^5 cells/well) as previously described (Sasaki et al., 2000). The cells were cultured in the presence or absence of highly purified *E. coli* LPS (0111:B4; Sigma-Aldrich, St. Louis, MO; 1 μ g/ml; a TLR4-specific ligand), Pam2CSK4 (InvivoGen, San Diego, CA; 100 ng/ml; a TLR2-specific ligand), and formalin-fixed whole endodontic pathogens (1×10^9 cells/ml) for 24 hrs. The level of IL-1 α , IL-1 β , and soluble CD14 (sCD14) in the culture supernatants was determined by specific ELISA (all R&D Systems, Minneapolis, MN). Cytosolic and membrane CD14 were separately isolated from 3×10^6 cells using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific, Waltham, MA).

CD14 neutralization

We neutralized CD14 either *in vitro* or *in vivo* using anti-mouse CD14 antibody (clone: 4C1; rat IgG2b, k; BD Biosciences, San Jose, CA). For *in vitro* neutralization, TLR2 KO

macrophages were treated with 5 µg/ml of the neutralizing antibody. Infected TLR2 KO mice received i.v. injection of the neutralizing antibody (25 µg/mouse/time) on days -1, 2, 5, 8, 11, 14, and 17 relative to the pulpal infection. An isotype-matched non-specific antibody was used as a control.

Statistics

Statistical analysis was performed using either one-way or two-way ANOVA and Bonferroni post hoc test.

Results

TLR2 and TLR4 play opposing roles in the development of periapical lesions

We determined the functional interaction between TLR2 and TLR4 signaling in a response to polymicrobial infection using a well-established periapical lesion model *in vivo* (Hou et al., 2000b; Sasaki et al., 2000; Sasaki et al., 2004) using wild-type (WT), TLR2 KO and TLR2/TLR4 dKO mice.

Since TLR2 is a major receptor for pathogen-associated molecular patterns derived from Gram-positive bacteria (Medzhitov, 2001), our initial expectation was that TLR2 deficiency attenuates periapical inflammation and bone loss similar to LPS-hyporesponsive C3H/HeJ mice (Hou et al., 2000). However, as shown in Fig. 1, TLR2 KO mice unexpectedly exhibited enhanced periapical lesions on Day 21 post infection, corroborating a previous report describing susceptibility of TLR2 KO mice to this disease (da Silva et al., 2012). In contrast, the extent of lesions in TLR2/TLR4 dKO (double deficient) mice was strikingly lower than that in TLR2 KO mice and was similar to the extent of lesions in WT mice.

Histologically, deficiency of TLR2 and TLR2/TLR4 did not alter normal structure of periapical periodontal tissue compared to wild-type mice (Fig. 2). The level of tissue resident macrophages (F4/80-positive cells) in periodontal ligament was similar in all strains (data not shown). No Ly6G-positive neutrophil infiltration was observed in the region (data not shown). Upon polymicrobial endodontic infection, TLR2 KO mice exhibited a sign of advanced infection and inflammation, which was cells with a foamy appearance (Malcolm et al., 1979; Liu et al., 1984; Gossett et al., 1985), on day 10 post infection (Fig. 3). These cells seemed to be neutrophils, because these cells were mostly Ly6G-positive but F4/80-negative as shown in Fig. 3. Such cytoplasmic vacuolation of neutrophils was not observed in WT and TLR2/TLR4 dKO mice. On day 21 post infection, the vacuolated cells were resolved in TLR2 KO mice. Instead, the lesion was filled with a capillary-rich tissue consisting of CD34-positive cells (Fig. 4), suggesting de novo angiogenesis (Bettencourt et al., 1998). Moderate to severe Ly6G-positive neutrophil infiltration was observed in the capillary-rich tissue (Fig. 3). The proportion of neutrophils in periapical lesions appeared to be elevated at days 21 compared to day 10 in TLR2 KO mice. In addition, the region of neutrophil infiltration in TLR2 KO on day 21 widely overlapped with the area of iNOS-positive cells (Fig. 3), suggesting that periapical inflammation in TLR2 KO was still active at this observation period. By contrast, infected WT mice exhibited chronic inflammation represented by fibrous periapical granuloma with mild Ly6G-positive neutrophil infiltration

on day 21 (Fig. 3). In TLR2/TLR4 dKO mice, Ly6G-positive neutrophil infiltration was notably faint either on day 10 or 21 post infection (Fig. 3), indicating minimal inflammation in the process of wound healing compared to wild-type and TLR2/TLR4 dKO mice. Taken together, these data suggest that the TLR2 signal negatively regulates TLR4-mediated inflammatory responses during the development of periapical lesions.

We, therefore, comprehensively examined whether TLR2 deficiency led to an elevated TLR4 signaling in periapical lesions by quantitative RT-PCR. As shown in Figure 5, overall, infected TLR2 KO mice showed a profile indicating elevated TLR4 signaling and subsequent NF- κ B activation compared to that in infected wild-type mice. Interestingly, the expression of the *TLR4* gene was not affected by either endodontic infection or TLR2 deficiency. In our data, *CD14* gene was strikingly up-regulated in TLR2 KO mice on Day 10 post infection. This data suggests that the TLR2 signal appears to be suppressing CD14 expression in wild-type mice. TLR2 KO mice supposed to be utilizing both *MyD88*- and *TICAM1*-dependent pathways in up-regulation of NF- κ B genes, particularly *NFkB2* and *RELA*. Further, *IL1A* gene was significantly up-regulated in TLR2 KO mice on Day 10, indicating altered pro-inflammatory response in early phase of periapical inflammation. On the other hand, a central type 1 helper T lymphocyte cytokine interferon gamma (IFN γ) seemed not to be involved much in the development of periapical lesion in both strains corroborating a previous study using IFN γ KO mice (Sasaki et al., 2004). The TLR2 signal is involved in expression of an immunosuppressive cytokine interleukin (IL)-10 (Clohisey et al., 2004; Gaddis et al., 2013), which potently suppresses periapical inflammation and bone loss (Sasaki et al., 2000). In the present study, the *IL10* gene expression in periapical lesions has not been modulated by TLR2 deficiency. These data lead us to a hypothesis that the TLR2 signaling negatively regulates the TLR4 signaling via CD14 in polymicrobial endodontic infection.

TLR2 deficiency up-regulates cellular CD14 expression and bacteria-stimulated proinflammatory cytokines by macrophages in vitro

We next examined the impact of TLR2 deficiency on cellular CD14 expression in macrophages, which are prominent cells in the development of periapical lesions (Tani-Ishii et al., 1995). As shown in Fig. 6, membrane-bound form CD14 was largely affected by TLR2 deficiency (4.6-fold higher in TLR2 KO and 3.4-fold higher in TLR2/TLR4 dKO vs. wild-type cells; $p < 0.01$, respectively). The elevation of cytosolic CD14 in TLR2 KO macrophages was 1.9-fold vs. wild-type observed ($p < 0.05$) while cytosolic CD14 was not elevated in TLR2/TLR4 dKO cells. Although CD14 also exists in a soluble form (Kirkland and Viriyakosol, 1998), deficiencies of TLR2 and TLR2/TLR4 did not alter physiological secretion of soluble CD14 in 24-hour observation (data not shown). The elevation of total cellular CD14 (sum of membrane-bound and cytosolic forms) was 3.36-fold in TLR2 KO and 2.2-fold in TLR2/TLR4 KO compared to that in wild-type cells ($p < 0.01$, respectively). There was no statistical significance between TLR2 KO and TLR2/TLR4 dKO cells ($p = 0.099$).

We further assessed the effect of TLR2 deficiency on macrophage-derived proinflammatory cytokines IL-1 production *in vitro* (Tables 2 and 3). Overall, TLR2 KO macrophages

exhibited hyper production of IL-1 α by *E. coli* LPS and fixed whole bacteria including Gram-positive species. In contrast, the production of IL-1 by TLR2/TLR4 dKO cells was generally lower than that by TLR2 KO cells and similar to that by wild-type cells. As expected, TLR2 KO and TLR2/TLR4 dKO cells were hyporesponsive to Pam2CSK4 and both LPS and Pam2CSK4, respectively. These data suggest that TLR2 deficiency compels elevation of cellular CD14 expression and overly up-regulates CD14-TLR4 signaling upon infection.

CD14 neutralization attenuates TLR2 deficiency-dependent inflammation

Finally, we tested whether blocking CD14 attenuates TLR4-mediated inflammation under TLR2 deficiency *in vitro* and *in vivo*. Resident TLR2 KO peritoneal macrophages were stimulated with whole endodontic pathogens or *E. coli* LPS in presence or absence of anti-CD14 antibody. As shown in Figure 7, the anti-CD14 antibody treatment potently suppressed bacteria-stimulated IL-1 α production by TLR2 deficient macrophages compared to control IgG-treated cells (by 60%). According to these data, we neutralized CD14 in infected TLR2 KO mice as described in the materials and methods. The extent of periapical lesion was determined on day 21 post-infection. As shown in Figures 8A and 8B, anti-CD14 treatments strikingly suppressed the extent of periapical lesion by approximately 35% compared to that in the control IgG-treated animals. As shown in Fig. 8C, CD14 neutralization led to down-regulation of *NFKB1* and *RELA* with statistical significance. We also observed a trend of down-regulation of *RELB*. These results proved that CD14 is involved in up-regulation of periapical inflammation and bone destruction under TLR2 deficiency.

Discussion

In the present study, we have investigated a critical immunoregulatory mechanism involving of TLR2, TLR4, and CD14 in response to poly-microbial endodontic infection. As summarized in Fig. 9, we demonstrated that TLR2 signaling down-regulates TLR4 signaling-mediated periapical inflammation via suppression of CD14 using genetic mutant mouse models and functional neutralization of CD14 *in vivo* and *in vitro*.

TLRs are pattern recognition receptors, and each TLR recognizes specific pathogen-associated molecular patterns such as lipoteichoic acids and lipopolysaccharide at infection. According to this specificity, TLR2 and TLR4 are thought to be important in recognition of Gram-positive and negative pathogens, respectively (Hoshino et al., 1999; Takeuchi et al., 1999). However, regardless of Gram stainability, endodontic pathogens have stimulated proinflammatory cytokine production by primary macrophages in all genotypes. Particularly, in TLR2 KO macrophages, IL-1 production induced by Gram-positive *S. intermedius* and *P. micros* was significantly elevated in a range of 3- to 67-fold compared with wild-type cells. In contrast, bacteria-induced hyper cytokine production under TLR2 deficiency became invalid in TLR2/TLR4 dKO cells. Therefore, our data suggest that TLR4 signaling seems to be the most important sensing pathway for both Gram-positive and negative bacteria and subsequent inflammatory responses in polymicrobial endodontic infection.

In our data, the level of IL-1 secreted by TLR2/TLR4 dKO macrophages *in vitro* was in part similar to that in wild-type cells. In addition, the extent of periapical lesions in TLR2/TLR4 dKO mice was similar to that in wild-type controls. We expect that the TLR2/TLR4 double deficiency led to functional compensation by such as other TLRs, resulting in induction of the same level of inflammatory response and bone loss compared to the wild-type. However, details of the functional compensation are unknown and to be determined in follow-up studies.

The result of gene expression analysis (Fig. 5) suggests that the up-regulated TLR4 signaling in infected TLR2 KO mice seems not to be dependent on TLR4 itself. It is widely accepted that the activation of the TLR4 signaling in part depends on other co-receptors including CD14, LBP, and MD2 (Muta and Takeshige, 2001; Gioannini et al., 2004; Akashi-Takamura and Miyake, 2008). In this study, CD14 was strikingly up-regulated by TLR2 deficiency and played a key role in elevation of TLR4 signal activation. The roles of CD14 in TLR4 signaling include: 1) binding with PAMPs; 2) presentation of PAMPs to TLR4; and 3) elevation of TLR4 signaling (Jiang et al., 2000; Muroi et al., 2002). We therefore think that the elevated CD14 expression could make TLR2 KO mice more responsive to polymicrobial infection than wild-type via up-regulation of TLR4 signaling.

Our histological examination revealed that TLR2 deficiency led to toxic change in neutrophils and a pro-angiogenic response. Vacuolization (vacuolation) is a sign of toxic neutrophils, which were frequently observed in acute inflammation and bacterial infections (Malcolm et al., 1979; Liu et al., 1984; Gossett et al., 1985). These toxic neutrophils were iNOS positive cells. In addition, infected TLR2 KO mice exhibited a pro-angiogenic response with continuous accumulation of neutrophils on day 21 post-infection, whereas the importance of TLR2/MyD88 signal in angiogenic response to oxidative stress was previously demonstrated (West et al., 2010). Such TLR2 deficiency-dependent pro-angiogenic response was also observed in an ischemia model (Wagner et al., 2013). In a previous report, ischemia-induced tissue necrosis was observed only in TLR2 KO but not in wild-type and TLR4 KO mice (Sachdev et al., 2012). In the same study, inflammatory response in TLR2 KO mice was mediated by TLR4-MyD88 pathway (Sachdev et al., 2012). On the other hand, *Mycobacterium tuberculosis* and *Toxoplasma gondii* were shown to utilize TLR2/MyD88 pathway to induce arginase 1 (ARG1) leading to inhibition of pro-angiogenic iNOS expression (El Kasmi et al., 2008). Although further investigations are necessary in periapical inflammation, these previous observations suggest that imbalance of TLR-mediated ARG1 and iNOS expressions seems to be a potential mechanism of the elevated myeloid inflammation and pro-angiogenic response in infected TLR2 KO mice. Taken together, these findings suggest two important things: 1) the TLR functional network that consists of TLR2, TLR4, CD14, and MyD88 primarily regulates responses of neutrophils and macrophages; and 2) impaired TLR network, caused by TLR2 deficiency in this study, influences not only myeloid cell responses but also pro-angiogenic response in inflammation.

Intracellular signaling derived from pattern recognition receptors constitutes complex interactions including both synergistic and antagonistic pathways in innate immunity, and a considerable number of pathogens are able to alter innate immune signaling for persistent

infections (Hajishengallis and Lambris, 2011). To date, the importance of TLR2 has been emphasized particularly in induction of periodontitis. A Gram-negative oral pathogen *Porphyromonas gingivalis* induces alveolar bone loss via TLR2 but not TLR4 (Burns et al., 2006; Papadopoulos et al., 2013). More specifically, TLR2-mediated tumor necrosis factor alpha (TNF α) production by macrophages was suggested as an important mechanism of *P. gingivalis*-stimulated marginal periodontitis (Papadopoulos et al., 2013) while *P. gingivalis* potentially antagonize TLR4-mediated antimicrobial responses (Darveau, 2010). Although *P. gingivalis* is involved in human infected root canals and periapical lesions (de Souza et al., 2005; Lin et al., 2007), the impact of *P. gingivalis* as a constituent of poly-microbial endodontic infection vs. a *P. gingivalis* mono-infection has not been precisely examined in endodontic microflora and periapical inflammation.

Since TLR2 naturally expresses on resting/resident macrophages (Trinchieri and Sher, 2007), TLR2 signaling appears to be a key natural endogenous suppressor of cellular CD14 expression. We thus think that this TLR2-mediated suppression of CD14 is an auto-regulatory mechanism that prevents overactive CD14/TLR4-mediated inflammation against poly microbial infection. The mechanism for TLR2-mediated suppression of CD14 needs to be determined by future studies. The proposed immunoregulatory network would impact the understanding of host defense system and the therapeutic strategies not only for oral infections but also for systemic polymicrobial infections such as sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TLR2 KO mice exhibited extended periapical bone destruction compared to wild-type C57BL/6 and TLR2/TLR4 dKO mice

A. Representative μ CT images of periapical lesions in anterior-posterior direction. The effect of TLR2 and TLR2/TLR4 double deficiencies on periapical bone destruction was determined on days 10 and 21 post endodontic infection. Non-infected mice in each strain served as corresponding background controls. The sample number (n) of each group was indicated in each panel. The position of each CT slice was the most central part of mandibular first molar distal root (R). AB: alveolar bone. Arrowheads in the panel of non-infected controls indicate the margin of alveolar bone in periapical region. Arrow points to

enlarged radiolucent area indicating the development of periapical lesion. The original length of the side of each square CT image was 2.4 mm.

B. Size of periapical lesions. The extent of periapical lesions was measured as previously described (AlShwaimi et al., 2013). Although the periapical lesion size on day 10 post-infection was similar in all genotypes, TLR2 KO mice exhibited significantly enlarged periapical bone loss compared to wild-type C57BL/6J and TLR2/TLR4 dKO mice on day 21. **: $p < 0.01$ vs. either C57BL/6J or dKO, vertical bar: standard deviation.

Figure 2. Genotypes did not affect on the structure of normal periapical tissue

Panels are representative histology of normal (non-infected) periapical tissue in wild-type C57BL/6J, TLR2 KO and TLR2/TLR4 dKO mice. The original magnification was $\times 100$. Polygonal orange and green lines outline the margin of dental root and alveolar bone, respectively. The area lying between these two lines is normal periodontal ligament tissue, which anchors tooth in alveolar bone. The structure of periapical region in non-infected animals was similar in all genotypes, and no visible differences were observed. AB: alveolar bone, R: dental root.

Figure 3. Susceptibility of TLR2 KO mice to periapical inflammation was diminished in TLR2/TLR4 dKO mice

Panels are representative histological images of periapical lesions on days 10 and 21 in wild-type C57BL/6J, TLR2 KO and TLR2/TLR4 dKO mice. The original magnification is indicated on the top of each panel. The area enclosed by a polygonal orange line is dental root (R). Polygonal green lines outline the margin of residual alveolar bone (AB). The area lying between these lines is periapical lesion. On day 10 post infection, an accumulation of foamy appearance cells was observed in the area enclosed by a white broken line only in TLR2 KO mice. The square specifies the area for high power ($\times 400$) observation of the foamy appearance cells. To determine the cell type of the foamy cells, neutrophils and

macrophages were visualized using anti-Ly6G and anti-F4/80 antibody, respectively. Most cells in the enclosed area were Ly6G-positive but F4/80-negative, suggesting that these cells were mostly neutrophils. Unlike wild-type and TLR2/TLR4 dKO lesions, on day 21, periapical lesions in TLR2 KO mice were rich in capillaries (C), neutrophils, and iNOS-positive cells.

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Figure 4. Capillaries observed in TLR2 KO mice were in part consisting of CD34-positive cells Panels are representative images of anti-CD34 (a hematopoietic progenitor cell antigen) immunostaining, visualizing cells/capillaries under de novo angiogenesis, in periapical lesion of wild-type C57BL/6J, TLR2 KO and TLR2/TLR4 dKO mice on day 21 post infection. The original magnification was $\times 200$. Polygonal orange and red lines outline the margin of dental root (R) and alveolar bone (B), respectively. The area lying between these two lines is periapical lesion (L). Arrows indicate the area of capillaries with CD34-positive cells. Capillaries with CD34-positive cells were observed only in TLR2 KO mice, suggesting that active angiogenesis occurred in infected TLR2 KO mice.

Figure 5. Infected TLR2 KO mice exhibited elevated TLR4 signaling compared to that in wild-type mice

TLR4 signaling-related gene expression profile in TLR2 KO and wild-type C57BL/6J mice was determined by real-time RT-PCR on days 10 and 21 post infection ($n = 3$, triplicates per sample). The data set was organized based on the TLR4 signal transduction pathway. Non-infected mice (day 0) in each genotype served as baseline controls to determine infection effect (*: $p < 0.05$; **: $p < 0.01$ vs. Day 0). Genotype effect (TLR2 KO vs. C57BL/6J) was determined on days 10 and 21 separately (†: $p < 0.05$). Open dot: C57BL/6J mice, Closed dot: TLR2 KO mice, X-axis: observation periods, Y-axis: fold change value in a semi-

logarithmic format. ND in horizontal orientation: no statistical difference vs. Day 0; ND in vertical orientation: no statistical difference between the genotypes.

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Figure 6. TLR2 deficiency led to elevation of cellular CD14 protein expression in resident peritoneal macrophages

The level of cytosolic and membrane-bounded CD14 protein expression was quantified by ELISA (n=3 per condition) and compared among wild-type C57BL/6J, TLR2 KO and TLR2/TLR4 dKO strains. The results were expressed in nano grams of CD14 protein per 1×10^6 cells. TLR2 deficiency (TLR2 KO or TLR2/TLR4 KO cells) resulted in significant elevation of membrane-bounded CD14 protein compared to wild-type cells. TLR2 single deficiency also up-regulated cytosolic CD14 protein expression compared with wild-type cells. Vertical bar: SD, *: p<0.05, **: p<0.01 vs. wild-type.

Figure 7. CD14 neutralization attenuated IL-1 α production by in TLR2 KO macrophages *in vitro*

Resident peritoneal TLR2 KO macrophages were stimulated with either mixed endodontic pathogens (1×10^9 cells/ml) or *E. coli* LPS (1 μ g/ml) in presence of anti-CD14 antibody or isotype-matched control IgG (5 μ g/ml, respectively) ($n = 3$ per group). Cells treated with control IgG alone served as negative controls ($n = 3$). The level of IL-1 α in culture supernatants was determined by ELISA, and the results were expressed in nano grams of IL-1 α per milliliter supernatant. Vertical bar: SD, *: $p < 0.05$, **: $p < 0.01$.

Figure 8. CD14 neutralization attenuated periapical inflammation in TLR2 KO mice

A. Representative μ CT images of periapical lesions in anterior-posterior direction. The effect of CD14 neutralization on infection-induced periapical bone loss in TLR2 KO mice was determined on day 21 post infection ($n = 5$). Mice that received isotype-matched control IgG served as disease controls ($n = 5$). The treatment regimen was described in the materials and methods. The position of each CT slice was the most central part of mandibular first molar distal root (R). The original length of the side of each square CT image was 2.4 mm. Arrow points to radiolucent periapical lesion. AB: alveolar bone.

B. Size of periapical lesions. CD14 neutralization effectively inhibited the development of periapical lesion in TLR2 KO mice. Anti-CD14 antibody treatment significantly suppressed the extent of periapical lesions in TLR2 KO mice. **: $p < 0.01$ vs. control IgG group.

C. Effect of CD14 neutralization on NF- κ B genes. The treatment effect on NF- κ B gene expressions was determined by real-time RT-PCR ($n = 3$, triplicates per sample). CD14 neutralization suppressed the expression of NF- κ B genes in periapical lesions compared to control IgG group (*: $p < 0.05$; **: $p < 0.01$ vs. control IgG group). Y-axis: relative gene expression level (fold change value) in a semi-logarithmic format.

Figure 9. Graphic summary of present findings

The effect of TLR2 deficiency on the level of TLR4 signaling, subsequent pro-inflammatory response, and bone destruction was graphically summarized. The panel of C3H/HeJ strain, in which TLR4 signaling is missing, is based on a published research article (Hou et al., 2000b). Taken together, our data suggest that TLR2 plays a regulatory role preventing over activation of CD14/TLR4 signaling-mediated inflammatory response in mouse periapical lesions.

Table 1

Genes examined by quantitative RT-PCR

Symbol	Gene name	Type of gene product	Existing domain
<i>CD14</i>	Cluster of differentiation 14	Co-receptor of Toll-like receptor 4	Extracellular/Membrane
<i>TLR4</i>	Toll-like receptor 4	Pattern recognition receptor	Extracellular/Membrane
<i>MYD88</i>	Myeloid differentiation primary response gene 88	Universal TLR adapter protein	Cytoplasmic
<i>IRAK1</i>	Interleukin-1 receptor-associated kinase 1	TLR signal transduction molecule	Cytoplasmic
<i>IRAK2</i>	Interleukin-1 receptor-associated kinase 2	TLR signal transduction molecule	Cytoplasmic
<i>TRAF6</i>	TNF receptor associated factor 6	TLR signal transduction molecule	Cytoplasmic
<i>TICAM1</i>	Toll-Like Receptor Adaptor Molecule 1	TLR signal transduction molecule	Cytoplasmic
<i>NFKB1</i> *	Nuclear factor kappa B p105 subunit	NF- κ B family member	Cytoplasmic/Nucleus
<i>NFKB2</i>	Nuclear factor kappa B p100 subunit	NF- κ B family member	Cytoplasmic/Nucleus
<i>RELA</i>	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	NF- κ B family member	Cytoplasmic/Nucleus
<i>RELB</i> *	Avian reticuloendotheliosis viral (v-rel) oncogene related B	NF- κ B family member	Cytoplasmic/Nucleus
<i>REL</i> *	v-rel avian reticuloendotheliosis viral oncogene homolog	NF- κ B family member	Cytoplasmic/Nucleus
<i>IL1A</i>	Interleukin-1 alpha	Proinflammatory cytokine	Membrane/Extracellular
<i>IL12</i> *	Interleukin-12	Th1 response inducing cytokine	Extracellular
<i>IFNG</i>	Interferon gamma	Th1 type inflammatory cytokine	Extracellular
<i>IL10</i>	Interleukin-10	Anti-inflammatory cytokine	Extracellular
<i>YWHAZ</i>	14-3-3 protein zeta/delta (an internal control gene)		

The sequence of PCR primers is listed in supplemental table 1.

* : Data of these genes are not shown.

Table 2IL-1 α production by TLR2 KO and TLR2/TLR4 dKO primary macrophages *in vitro*.

Stimuli	C57BL/6J	TLR2 KO	TLR2/TLR4 KO
Negative control	8.52 $\pm$ 8.03	42.67 $\pm$ 28.10 *	31.34 $\pm$ 5.12 *
LPS	97.37 $\pm$ 18.22	6544.00 $\pm$ 2225.25 *	25.78 $\pm$ 9.17 * [†]
Pam2CSK4	59.46 $\pm$ 6.87	40.00 $\pm$ 8.00 *	12.67 $\pm$ 6.21 * [†]
<i>P. intermedia</i>	134.80 $\pm$ 22.69	1672.00 $\pm$ 444.49 *	82.32 $\pm$ 16.42 * [†]
<i>F. nucleatum</i>	336.28 $\pm$ 87.84	7272.00 $\pm$ 2056.05 *	372.94 $\pm$ 96.05 [†]
<i>S. intermedius</i>	228.50 $\pm$ 42.54	7293.33 $\pm$ 1610.85 *	350.46 $\pm$ 16.42 * [†]
<i>P. micra</i>	32.33 $\pm$ 1.59	2168.00 $\pm$ 1154.86 *	40.02 $\pm$ 9.50 [†]
Mixed	300.74 $\pm$ 137.01	6850.67 $\pm$ 604.75 *	218.06 $\pm$ 20.09 [†]

Each data indicates Mean value $\pm$ Standard Deviation in pg cytokine/ml culture supernatant.

* : p<0.01 vs. C57BL cells under the same condition.

[†] : p<0.01 vs. TLR2 KO cells under the same condition.

Table 3IL-1 β production by TLR2 KO and TLR2/TLR4 dKO primary macrophages *in vitro*.

Stimuli	C57BL/6J	TLR2 KO	TLR2/TLR4 KO
Negative control	32.27 $\pm$ 6.21	24.03 $\pm$ 0.05	22.48 $\pm$ 3.64
LPS	61.53 $\pm$ 6.70	381.33 $\pm$ 154.78 *	30.55 $\pm$ 23.28 †
Pam2CSK4	31.09 $\pm$ 14.32	8.03 $\pm$ 0.05 *	7.42 $\pm$ 0.73 *
<i>P. intermedia</i>	61.45 $\pm$ 4.09	72.00 $\pm$ 13.86	33.46 $\pm$ 6.76 †
<i>F. nucleatum</i>	150.88 $\pm$ 42.41	957.33 $\pm$ 156.84 *	231.96 $\pm$ 18.52 *†
<i>S. intermedius</i>	179.98 $\pm$ 20.60	579.00 $\pm$ 149.50 *	229.80 $\pm$ 61.55 †
<i>P. micra</i>	44.76 $\pm$ 9.06	213.33 $\pm$ 92.72 *	30.93 $\pm$ 4.37 †
Mixed	190.78 $\pm$ 112.72	922.67 $\pm$ 9.24 *	122.01 $\pm$ 22.32 †

Each data indicates Mean value $\pm$ Standard Deviation in pg cytokine/ml culture supernatant.

* : p<0.01 vs. C57BL cells under the same condition.

† : p<0.01 vs. TLR2 KO cells under the same condition.