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The Effects of *Porphyromonas gingivalis* LPS and *Actinobacillus actinomycetemcomitans* LPS on Human Dendritic Cells *in vitro*, and in a Mouse Model *in vivo*

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SUMMARY Interaction between different bacterial plaque pathogens and dendritic cells may induce different types of T helper (Th) cell response, which is critical in the pathogenesis of periodontitis. In this study we investigated the effects of lipopolysaccharide (LPS) from *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* on human monocyte-derived dendritic cells (Mo-DCs) with respect to co-stimulatory molecule expression, cytokine production and Th cell differentiation. Unlike *Escherichia coli* and *A. actinomycetemcomitans* LPS, *P. gingivalis* LPS induced only low levels of CD40, CD80, HLA-DR and CD83 expression on Mo-DCs. LPS from both bacteria induced considerably lower TNF-α and IL-10 than did *E. coli* LPS. LPS from all three bacteria induced only negligible IL-12 production. In a human mixed-leukocyte reaction, and in an ovalbumin-specific T cell response assay in mice, both types of LPS suppressed IFN-γ production. In conclusion, stimulation by *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS appears to bias Mo-DCs towards Th2 production.

The balance of T helper1 (Th1)-*versus* T helper 2 (Th2)-type response is fundamentally important in determining whether the immune response to infectious pathogens will be protective.[1] Also, both the Th1 and Th2 responses, if unduly prolonged, may cause overproduction of cytokines leading to tissue damage. High levels of inflammatory mediators, and large numbers of infiltrated T and B cells, are cardinal characteristics of periodontitis, the advanced form of periodontal disease.[2] Available data regarding Th1- and Th2-type responses in periodontitis are, however, in conflict.[2] Advancing knowledge in this area is crucial to understanding the pathogenesis of periodontitis. Dendritic cells (DCs) are well recognized to have a vital role not only in initiating naïve T cell priming, but also in controlling the type of Th response. The mechanisms through which DCs induce Th1 and Th2-type responses are

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the subject of intense investigation. Factors influencing Th differentiation include interleukin (IL)-12,[3] IL-10,[4] ICAM-1 expression,[5] dose of antigen,[6] Notch ligands[7] and DC subsets.[8]

In periodontitis, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are key pathogens.[9] They form microbial plaque biofilms adjacent to gingiva, which are rich in antigen presenting cells (APCs). Langerhans cells are present in gingival epithelium, while tissue DCs are present in lamina propria.[10,11] Significant up-regulation of CD83 (mature DC antigen) and co-stimulatory molecule expression on DCs and gingival B cells was detected in periodontitis tissues.[11,12] These $CD83^+$ cells were associated with clusters of $CD4^+$ T cells, thus suggesting close interaction of T cells and APCs in the gingival microenvironment.[13]

In this study we investigated the effects of lipopolysaccharide (LPS) from *P. gingivalis* and *A. actinomycetemcomitans* on human monocyte-derived dendritic cells (Mo-DCs) with respect to co-stimulatory molecule expression, cytokine production and Th cell differentiation. In an *in vivo* mouse model, we also characterized antigen-specific Th cell responses to treatment with these two types of LPS, and compared these responses to those induced by *Escherichia coli* LPS.

MATERIALS AND METHODS

Antibodies and reagents

RPMI 1640 medium supplemented with 2 mM L-glutamine, 80 μg/ml of gentamycin (Gibco Laboratory, Grand Island, NY, USA), and 10% heat-inactivated autologous serum were used throughout this study. Ficoll-Hypaque (Histopaque 1.077) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were obtained from R & D Systems Inc. (Minneapolis, MA, USA).

Monoclonal antibodies against CD1a (FITC), CD40 (APC), CD80 (PE), CD83 (PE) and CD86 (FITC) were obtained from BD PharMingen (San Diego, CA, USA). Monoclonal antibodies against CD4 (FITC), CD8 (FITC), CD14 (FITC), CD20 (FITC), CD45RA (PE), CD45RO (FITC), HLA-DR (PerCP), CD56 (FITC) and TCR-γδ (PE) were obtained from Becton Dickinson (San Jose, CA, USA).

OVA and *E. coli* LPS were obtained from Sigma Chemical Co. Montanide ISA720 was obtained from SEPPIC (Paris, France). CpG ODN 1826 (TCCATGACGTTCCTGACGTT) was obtained from Coley Pharmaceutical Group (Wellesley, MA, USA).

Highly purified LPS from *P. gingivalis* strain 381 and *A. actinomycetemcomitans* strain Y4 was prepared as previously described.[14-16] Briefly, the LPS was purified by phenol-water extraction and subsequent treatment with DNase I, RNase A, and proteinase K (for *P. gingivalis* LPS) and nuclease P1 (for *A. actinomycetemcomitans*). The purity of both LPS preparations was confirmed by immunodiffusion analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining.

Preparation of human Mo-DCs and stimulation with bacterial LPS

Under a protocol approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, healthy subjects signed a consent form after being informed of the nature of the study. Peripheral blood was obtained from these subjects. Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Hypaque. Mo-DCs were prepared from flow cytometrically sorted $CD14^+$ monocytes (FACSVantage, BD Biosciences, Mountain View, CA, USA) and were cultured with 50 ng/ml of GM-CSF and IL-4 for 5-7 days. The cultured cells showed phenotypes of immature DCs associated with $CD1a^+$, $CD14^-$ and minimal or no expression of CD83. Immature Mo-DCs (5×10^5 cells/ml) were stimulated with different concentrations of LPS from *P. gingivalis*, *A. actinomycetemcomitans*, and *E. coli* (0, 100, 300, 1,000 ng/ml) for 24 hours. Culture supernatants were analyzed for tumor necrosis factor (TNF)-α, IL-10 and IL-12 p70 production using ELISA. The cells were also harvested and stained with monoclonal antibodies against CD40, CD80, CD83 and HLA-DR. Mouse isotype monoclonal antibodies were used as controls. Normally, 5,000-10,000 cells were analyzed by FACSCalibur (Becton Dickinson). Levels of expres-

sion were quantified by mean fluorescence intensity (MFI).

Mixed leukocyte reaction (MLR) in human cells

T cells were enriched from PBMC by rosetting with neuraminidase-treated sheep red blood cells. Allogeneic naïve $CD4^+$ T cells were obtained by negative sorting of enriched T cell populations that had been stained with monoclonal antibodies against CD8 (FITC), CD20 (FITC), CD56 (FITC), CD45RO (FITC), and TCR-γδ (PE). This technique provides $CD4^+CD45RA^+$ naïve T cells with a purity of > 90%. Flow cytometrically sorted allogeneic naïve T cells (5×10^5 cells/ml) were added in culture wells that contained control and LPS-stimulated Mo-DCs (5×10^4 cells/ml). Culture supernatants were harvested on day 5 and then analyzed for IFN-γ and IL-5 production.

Antigen-specific T cell response in mice

Female BALB/c mice, 6-8 weeks were obtained from National Laboratory Animal Center, Thailand. Mice were divided into 5 groups (n = 5 per group): 1). Montanide ISA720 + OVA; 2) Montanide ISA720 + OVA + CpG ODN 1826; 3) Montanide ISA720 + OVA + *E. coli* LPS; 4) Montanide ISA720 + OVA + *P. gingivalis* LPS; 5) Montanide ISA720 + OVA + *A. actinomycetemcomitans* LPS. Fifty micrograms of OVA in phosphate-buffered saline (PBS) was emulsified with Montanide ISA720 at the ratio of 3:7 parts in the presence of 50 μg of CpG ODN 1826, 50 μg of *E. coli* LPS, 50 μg of *P. gingivalis* LPS, or 50 μg of *A. actinomycetemcomitans* LPS. Mice were immunized with 100 μl of each vaccine at days 0, 14 and 28 via subcutaneous, subcutaneous and intraperitoneal routes, respectively. Splenocytes were harvested on day 38. They (5×10^6cells/ml) were stimulated with OVA (40 μg/ml) for 5 days. Culture supernatants were harvested and then assayed for OVA-specific IFN-γ and IL-5 production.

Detection of human and mouse intracellular cytokines

The production of human and mouse cytokines (TNF-α, IFN-γ, IL-5**,** IL-10 and IL-12 p70) was measured by ELISA (R&D Systems, Minneapolis, MN, USA).

RESULTS

LPS from *P. gingivalis* and *A. actinomycetemcomitans* activates human Mo-DCs

We investigated the ability of two different LPS, derived from *P. gingivalis* and *A. actinomycetemcomitans*, to induce DC maturation in comparison with a well characterized *E. coli* LPS. Expression of HLA-DR, CD40, CD80, and CD83 and cytokine production (TNF-α, IL-10, and IL-12 p70) were measured after 24 hours stimulation of Mo-DCs with different bacterial LPS. Fig. 1A shows that all three types of LPS up-regulated expression of HLA-DR, CD40, CD80, and CD83 on Mo- DCs in an essentially dose dependent manner. *A. actinomycetemcomitans* LPS and *E. coli* LPS induced similar levels of these molecules, and these levels were consistently higher than those induced by *P. gingivalis* LPS. With regard to LPS-induced cytokine production, both bacterial plaque LPS induced only low levels of TNF-α and negligible amounts of IL-10 from Mo-DCs. In contrast, *E. coli* LPS induced substantial levels of TNF-α and moderate amounts of IL-10. Little or no IL-12 p70 was detected in any LPS-stimulated Mo-DC cultures (Fig. 1B).

***P. gingivalis* and *A. actinomycetemcomitans* LPS-stimulated Mo-DCs induce Th response in human MLR**

We next evaluated the ability of *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS-stimulated Mo-DCs to prime human naïve $CD4^+$ T cells and to promote the development of Th1 or Th2 response. Un-stimulated Mo-DCs were used as a baseline control. In line with observed cytokine induction patterns, *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS-stimulated Mo-DCs induced a poor allogeneic naïve $CD4^+$ T cell response as compared with *E. coli* LPS-stimulated Mo-DCs. Lower levels of IFN-γ production were detected in MLR cultures with *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS-stimulated Mo-DCs (123 ± 67 and 344 ± 153 pg/ml, respectively) than in corresponding cultures with *E. coli* LPS (1,778 ± 612 pg/ml). Furthermore, IL-5

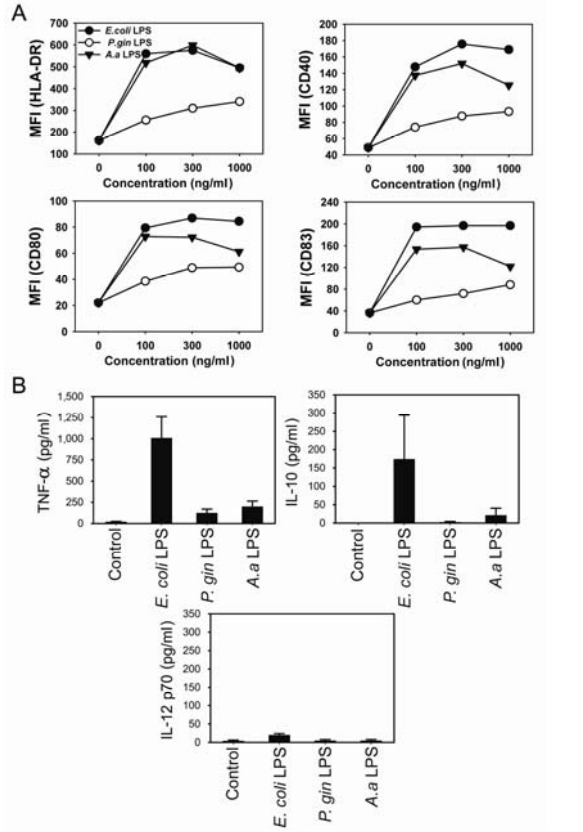

Fig. 1 LPS from *P. gingivalis*, *A. actinomycetemcomitans*, and *E. coli* induced Mo-DC maturation. Immature Mo-DCs were stimulated with different concentrations of LPS (0, 100, 300, 1000 ng/ml) for 24 hours. Cell surface expression of HLA-DR, CD40, CD80, and CD83 (A) and cytokine production of TNF-α, IL-10 and IL-12 p70 (B) were analyzed by flow cytometry and ELISA, respectively. Data on molecule surface expression are representative of four independent experiments and shown as MFI. Data on cytokines are mean ± SE.

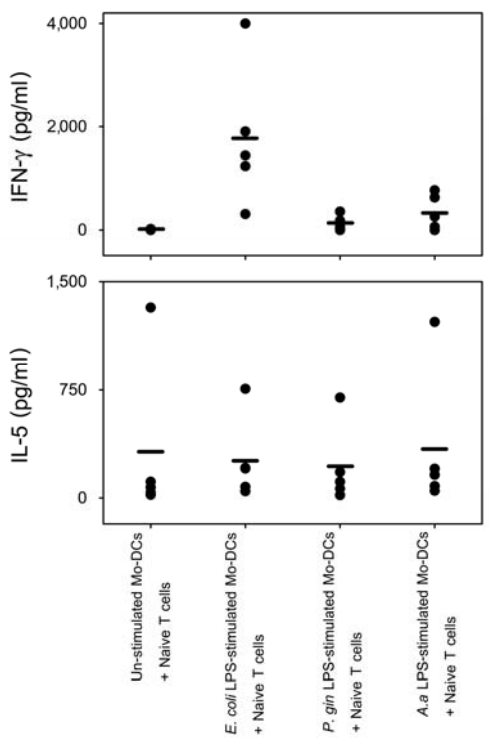

Fig. 2 The stimulatory capacity of bacterial LPS-stimulated Mo-DCs on allogeneic naïve $CD4^+$ T cells in MLR. Control Mo-DCs and *P. gingivalis*, *A. actinomycetemcomitans*, or *E. coli* LPS stimulated-MoDCs were used to prime naïve $CD4^+$ T cells for five days, and then cultured supernatant were assayed for IFN-γ and IL-5 production. Each data point represents the cytokine level in an individual experiment (n = 5) and horizontal lines are mean values.

levels were generally low in all MLR cultures (mean 216-345 pg/ml)(Fig. 2).

P. gingivalis and *A. actinomycetemcomitans* LPS induced antigen-specific Th response *in vivo*

Recently we have developed OVA-specific Th differentiation in an *in vivo* mouse model. Administration of Montanide ISA720 + OVA induces Th2 bias (low antigen-specific IFN-γ and high IL-5 production). On the other hand, administration of Montanide ISA720 + CpG ODN 1826 + OVA induces Th1 bias (high antigen-specific IFN-γ and low IL-5 production) (unpublished data). In this mouse model, we characterized and compared the OVA-specific T cell response after treatment with LPS from *P. gingivalis*, *A. actinomycetemcomitans* and *E. coli*. As expected, the splenocytes from Th2 control group (Montanide ISA720 + OVA) produced low amount of antigen-specific IFN-γ (248 ± 87 pg/ml) and high amount of antigen-specific IL-5 (988 ± 273 pg/ml) as compared to the positive Th1 control group (Montanide ISA720 + CpG ODN 1826 + OVA; IFN-γ = 1,810 ± 621 pg/ml and IL-5 = 13 ± 5 pg/ml). Mice that immunized with LPS from *P. gingivalis* or *A. actinomycetemcomitans* combined with Montanide ISA720 + OVA showed lower levels of antigen-specific IFN-γ production (*P. gingivalis*

group = 424 ± 106 pg/ml and *A. actinomycetemcomitans* group = 125 ± 26 pg/ml) and higher levels of antigen-specific IL-5 production (*P. gingivalis* group = 724 ± 224 pg/ml, and *A. actinomycetemcomitans* group = 207 ± 52 pg/ml) than the positive Th1 control group. It should be noted that mice that immunized with Montanide ISA720 + *E. coli* LPS + OVA produced low amount of antigen-specific IFN-γ and IL-5 (102 ± 44 pg/ml, and 106 ± 29 pg/ml, respectively) (Fig. 3).

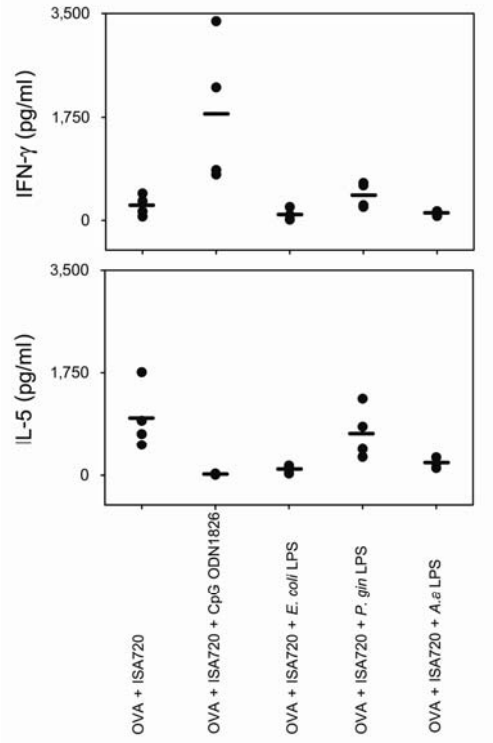

Fig. 3 Induction of OVA-specific T cell response in mice. Splenocytes collected from five groups of mice were evaluated for antigen-specific IFN-γ and IL-5 production. Each data point represents the levels of cytokine produced in each individual animal and horizontal lines are mean values.

DISCUSSION

DCs use Toll like receptors (TLRs) to discriminate between different pathogen-associated molecular patterns (PAMPS). *P. gingivalis* and *E. coli* LPS is known to have different LPS structures and bind to TLR2 and TLR4, respectively.[17-18] Recently *A. actinomycetemcomitans* LPS has been reported to bind to TLR4.[19,20] Binding of certain TLRs on DCs with different PAMPS in DC-pathogen interaction is crucial, and contributes to different types of adaptive immunity (e.g., Th1/Th2 balance). In this study we evaluated the effects of LPS derived from *P. gingivalis* and *A. actinomycetemcomitans* on human Mo-DCs with respect to co-stimulatory molecule expression, cytokine production and the induction of Th cell differentiation. Unlike *E. coli* LPS and *A. actinomycetemcomitans* LPS, *P. gingivalis* LPS induced only low levels of the co-stimulatory molecules CD40 and CD80, and of the maturation marker CD83. Both *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS induced low levels of TNF-α and negligible amounts of IL-10 as compared with *E. coli* LPS. All three bacterial LPS induced minimal or no IL-12 p70 production. Data of human MLR suggest that both *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS-stimulated Mo-DCs appeared to induce Th2 differentiation of allogeneic $CD4^+$ T cells. Our findings of *P. gingivalis* LPS being a weak stimulator for DCs agree with the study of Kanaya *et al.*[21] with regard to the induction of co-stimulatory molecule expression and cytokine production. However, our findings regarding the effect of *A. actinomycetemcomitans* LPS on DCs are different from the recent study of Kikuchi *et al.*[22] which reported that *A. actinomycetemcomitans* LPS is a stronger DC stimulator than *E. coli* LPS. This discrepancy needs further investigation.

In vivo antigen-specific mouse model clearly demonstrated that the CpG ODN 1826 group which was injected together with Montanide ISA720 + OVA, induced a strong antigen-specific Th1 response (high IFN-γ and low IL-5 response). Whereas, the *P. gingivalis*, and *A. actinomycetemcomitans* group which was injected together with Montanide ISA720 + OVA induced antigen-specific Th2 response (low IFN-γ and high IL-5 response). Pulendran *et al.*[23] demonstrated that *E. coli* LPS induced OVA-specific Th1 response in mouse model. Surprisingly, in our study, *E. coli* LPS administered together with Montanide ISA720 + OVA induced poor antigen–specific T cell response with Th2 bias. The inconsistent results could conceivably reflect the different types of mice and the different experiment conditions. We used normal BALB/c mice, whereas Pulendran *et al.*[23] used mice that reconstituted with transgenic OVA-specific T cells. These mice had

much higher frequency of OVA-specific T cells than normal mice. In addition, the different concentrations of OVA used may affect the outcome. We administered 40-fold less amount of the antigen. The concentration of antigen is known to influence Th1 and Th2 differentiation.[6]

In conclusion, our study showed that *P. gingivalis* and *A. actinomycetemcomitans* LPS activated human Mo-DCs. Data from human MLR and antigen-specific T cell response in mouse model suggest that LPS from the two plaque bacteria drive Th2-bias.

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