**รายงานวิจัยฉบับสมบูรณ์**

# โครงการ ปฏิกิริยาระหว่างเซลล์เดนดริทิกกับแอลพีเอสจาก*พอร์ไฟโรโมแนส จินจิวาลิส* และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน*

**โดย**

**รังสินี มหานนท์ และคณะ**

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## สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)



(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

# กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณ สกว.ที่ได้ให้การสนับสนุนในการทำงานอย่างต่อเนื่อง และคณะผู้วิจัยขอขอบคุณ สถาบันวิจัยวิทยาศาสตร์การแพทย์ทหารที่ให้ความร่วมมือในการทำวิจัย และสุดท้ายนี้ขอขอบคุณ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยที่เล็งเห็นความสำคัญในงานวิจัยและสนับสนุนคณะผู้วิจัยเป็นอย่างดียิ่งมาโดยตลอด

# ABSTRACT

The development of T helper1 (Th1)- versus Th2-type response is known as fundamental important in determining whether the response to infectious pathogens will be protective or no protective. The Th1 or Th2 response, on the other hand that continues unabated may cause overproduction of cytokines leading to damage of host normal tissues. High levels of inflammatory mediators as well as numerous infiltrated T and B cells are the characteristics of periodontitis, the advanced form of periodontal disease. Accumulating data regarding Th1- and Th2-type response in periodontitis are however in conflict. This inconclusive piece of information is crucial in the pathogenesis of periodontitis and still lacking. To date, dendritic cells (DCs) have been well recognized for the critical role not only in the initiation of naïve T cell priming but also in controlling the type of Th response. The mechanisms of how DCs induce Th1 and Th2-type response have been the subject of intense investigation. Several determined factors in Th differentiation include interleukin (IL)-12, IL-10, ICAM-1 expression, dose of antigen, Notch ligands and DC subsets.

In periodontitis, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are key pathogens. They habitat as microbial plaque biofilms adjacent to gingiva which are found to be enriched by antigen presenting cells (APCs). Langerhans cells are presence in gingival epithelium while tissue DCs are presence in lamina propria. Significant up-regulation of CD83 (mature DC antigen) and co-stimulatory molecule expression on DCs and gingival B cells was detected in periodontitis tissues. These $CD83^+$ cells were associated with clusters of $CD4^+$ T cells, thus indicating close interaction of T cells and APCs in gingival microenvironment. 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 regards to co-stimulatory molecule expression, cytokine production and the induction of Th cell differentiation. Human mixed-leukocyte reaction (MLR) and *in vivo* ovalbumin (OVA)-specific T cell response in mouse model were used to explore the type of Th response induced by LPS of the two plaque bacterial pathogens in comparison with *Escherichia coli* LPS.

DCs were generated from flow cytometrically-sorted $CD14^+$ monocytes from healthy donors. The monocytes were differentiated into immature DCs by culturing in RPMI1640 medium containing 10% heat-inactivated autologous serum and the cytokines, GM-CSF and IL-4. The phenotype of immature DCs was found to be $CD1a^+$, $CD14^-$, $CD40^+$, $CD80^+$, $CD83^+$ and $HLA\text{-}DR^+$. Immature Mo-DCs were stimulated with different concentrations of LPS from *P. gingivalis*, *A. actinomycetemcomitans*, and *E. coli* (0, 100, 300, 1000 ng/mL) for 24 hrs. Culture supernatants were analyzed for tumor necrosis factor (TNF)-$\alpha$ , IL-10 and IL-12 p70 production using ELISA. The cells were harvested to measure CD40, CD80, CD83 and HLA-DR by flow cytometry. Unlike *E. coli* LPS and *A. actinomycetemcomitans* LPS, *P. gingivalis* LPS induced low levels of co-stimulatory molecule

(CD40, CD80), HLA-DR and maturation marker (CD83) expression. Both *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS induced low levels of TNF-$\alpha$ and negligible amounts of IL-10 as compared with *E. coli* LPS. All the bacterial LPS induced minimal or no IL-12 p70 production. 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. In line with the weak cytokine induction, *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-$\gamma$ production were detected in MLR cultures with *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS-stimulated Mo-DCs as compared with higher IFN-$\gamma$ levels in those MLR cultures with *E. coli* LPS -stimulated Mo-DCs. Similar low levels of IL-5 production were detected in all MLR cultures.

Due to the low frequency of antigen-specific T cells in human peripheral blood lymphocytes, the *in vivo* antigen-specific T cell experiment in mouse model was set up in order to investigate the ability of LPS from *P. gingivalis*, *A. actinomycetemcomitans* and *E. coli* in the induction of OVA-specific Th response. As expected, the splenocytes from Th2 control group (Montanide ISA720 + OVA) produced low amount of antigen-specific IFN-$\gamma$ and high amount of antigen-specific IL-5 as compared to the positive Th1 control group; Montanide ISA720 + CpG ODN 1826 + OVA. Mice that immunized with LPS from *P. gingivalis* or *A. actinomycetemcomitans* combined with Montanide ISA720 + OVA showed lower levels of antigen-specific IFN-$\gamma$ production and higher levels of antigen-specific IL-5 production than the positive Th1 control group. It should be noted that mice that immunized with Montanide ISA720 + *E. coli* LPS + OVA showed to produce low amount of both antigen-specific IFN-$\gamma$ and IL-5. In conclusion, our study showed that *P. gingivalis* and *A. actinomycetemcomitans* LPS poorly 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 seem to drive Th2-bias.

# บทคัดย่อ

การพัฒนาการของการตอบสนองของทีเฮลพเพอร์ (T helper) แบบที่ 1 (Th1) กับ ทีเฮลพเพอร์แบบที่ 2 (Th2) เป็นที่ทราบว่าเป็นรากฐานสำคัญในการกำหนดว่าการตอบสนองต่อเชื้อที่ก่อให้เกิดโรคนั้นจะเป็นไปในทางการป้องกันหรือไม่ป้องกัน ในทางตรงกันข้ามหากการตอบสนองของทีเฮลพเพอร์แบบที่ 1 หรือ ทีเฮลพเพอร์แบบที่ 2 ดำเนินต่อไปอย่างไม่สิ้นสุดอาจเป็นสาเหตุของการผลิตไซโตคายส์ (cytokines) มากเกิน อันจะนำไปสู่การเสียหายของเนื้อเยื่อปกติได้ โดยที่ปริมาณของสารสื่อกลางการอักเสบ (inflammatory mediators) ที่มาก เช่นเดียวกับทีเซลล์ (T cells) และบีเซลล์ (B cells) ที่แทรกซึมมาจำนวนมากเป็นลักษณะของโรคปริทันต์อักเสบขั้นรุนแรง การรวบรวมข้อมูลเกี่ยวกับการตอบสนองของทีเฮลพเพอร์แบบที่ 1 กับ ทีเฮลพเพอร์แบบที่ 2 ในโรคปริทันต์อักเสบยังมีความขัดแย้งกันอยู่ ส่วนของข้อมูลที่ยังไม่มีผลสรุปที่ชัดเจนเหล่านี้มีความสำคัญในพยาธิสภาพของโรคปริทันต์อักเสบและยังคงไม่เพียงพอ ปัจจุบัน เซลล์เดนดริทิก (dendritic cells, DCs) ได้รับการยอมรับเป็นอย่างดีว่ามีบทบาทที่สำคัญไม่เฉพาะในการเริ่มการกระตุ้นนาอีฝ ทีเซลล์ (naïve T cells) นอกจากนั้นยังควบคุมชนิดของการตอบสนองของทีเฮลพเพอร์ด้วย กระบวนการที่เดนดริทิกเซลล์ไปชักนำการตอบสนองของทีเฮลพเพอร์แบบที่ 1 และ ทีเฮลพเพอร์แบบที่ 2 อย่างไรนั้น เป็นหัวข้อที่ได้รับความสนใจอย่างมาก มีหลายปัจจัยที่เป็นตัวกำหนดการเปลี่ยนแปลงของทีเฮลพเพอร์อันรวมถึง อินเตอร์ลูคิน-12, อินเตอร์ลูคิน-10, การแสดงออกของไอแคม-1 (ICAM-1), ปริมาณของแอนติเจน, น็อทช ไลแกน (Notch ligands) และชนิดของเดนดริทิกเซลล์

*พอร์ไฟโรโมแนส จินจิวาลิส* (*Porphyromonas gingivalis*) และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* (*Actinobacillus actinomycetemcomitans*) เป็นกุญแจสำคัญในการเกิดโรคปริทันต์อักเสบ แบคทีเรียพวกนี้อาศัยอยู่ในรูปของคราบจุลินทรีย์ในไบโอฟิล์ม (microbial plaque biofilms) ที่อยู่ชิดกับเหงือกซึ่งพบว่าเต็มไปด้วยแอนติเจนพรีเซ็นติงเซลล์ (antigen presenting cells (APCs)) พบแลงเกอฮานส์เซลล์ (langerhans cells) ในชั้นเยื่อบุผิวเหงือก ขณะที่เดนดริทิกเซลล์ในเนื้อเยื่อถูกพบในลามิน่า โพรเพรีย (lamina propria) มีการเพิ่มขึ้นอย่างมีนัยสำคัญของ ซีดี83 (CD83) (แอนจิเจนของเดนดริทิกเซลล์ที่เจริญเต็มที่) และการแสดงออกของโค-สติมูลาโทรีโมเลกุล (co-stimulatory molecules) บนเซลล์เดนดริทิกและบีเซลล์ในเนื้อเยื่อปริทันต์ เซลล์ซีดี83 (CD83+) เหล่านี้เชื่อมโยงอยู่กับกลุ่มของทีเซลล์ที่แสดงซีดี4 (CD4+) ดังนั้นจึงเป็นการชี้ให้เห็นการทำงานร่วมกันอย่างใกล้ชิดของทีเซลล์และแอนติเจนพรีเซ็นติงเซลล์ในสภาพแวดล้อมของเหงือก ในการศึกษานี้คณะผู้วิจัยได้ทำการศึกษาเกี่ยวกับผลของไลโปโปลีแซคคารายด์ (แอลพีเอส) (lipopolysaccharide, LPS) จาก *พอร์ไฟโรโมแนส จินจิวาลิส* และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* ต่อเซลล์เดนดริทิกที่เตรียมมาจากโมโนไซท์ของมนุษย์ (human monocyte-derived dendritic cells, Mo-DCs) โดยพิจารณาที่การแสดงออกของโค-สติมูลาโทรีโมเลกุล, การผลิตไซโตคายส์ และการชักนำของการเปลี่ยนแปลงของทีเฮลพเพอร์ การทดสอบ mixed-leukocyte reaction (MLR) ในมนุษย์ และการทดลองในสิ่งมีชีวิตของการตอบสนองของทีเซลล์ที่จำเพาะเจาะจงต่อ ovalbumin (OVA) ในหนูทดลอง (mouse model) ซึ่งถูกใช้ในการตรวจสอบชนิดของการตอบสนองของทีเฮลพเพอร์ที่ชักนำโดย LPS จากแบคทีเรียที่ก่อโรคทั้งสองชนิดในคราบจุลินทรีย์เปรียบเทียบกับไลโบโฟลีแซคคารายด์ของ *เอสเชอร์รีเชีย คอไล* (*Escherichia coli* LPS)

ทำการเพาะเลี้ยงเซลล์เดนดริทิกจาก ซีดี14 โมโนไซท์ (CD14+ monocytes) ที่แยกโดยวิธีทางโฟลไซโตมิเตอร์ (flow cytometrically-sorted) ที่ได้มาจากผู้บริจาคที่มีสุขภาพสมบูรณ์ โมโนไซท์จะถูกทำให้เปลี่ยนแปลงไปเป็นเดนดริทิกเซลล์ที่ยังไม่เจริญเต็มที่ (immature DCs) โดยการเพาะเลี้ยงในอาหารเลี้ยงเซลล์ (RPMI-1640) ที่ประกอบด้วย 10 % ของซีรัมของผู้

บริจาค และไซโตคายส์ GM-CSF และอินเตอร์ลูคิน-4 พบว่าลักษณะภายนอกของเดนดริทิกเซลล์ที่ยังไม่เจริญเต็มที่มี CD1a+, CD14+, CD40+, CD80+, CD83+ และ HLA-DR+ เดนดริทิกเซลล์ที่ยังไม่เจริญเต็มที่ซึ่งเตรียมมาจากโมโนไซท์ (immature Mo-DCs) จะถูกกระตุ้นด้วย LPS ที่มีความเข้มข้นที่แตกต่างกันของ *พอร์ไฟโรโมแนส จินจิวาลิส*, *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* และ *เอสเชอร์รีเชีย คอไล* (0, 100, 300, 1000 นาโนกรัมต่อมิลลิลิตร) เป็นเวลา 24 ชั่วโมง ตรวจสอบการผลิตของ ทูเมอร์ เนโครซิซ แฟคเตอร์ (TNF)-อัลฟ่า, อินเตอร์ลูคิน-10 และ อินเตอร์ลูคิน-12 พี 70 (IL-12 p70) ในสารละลายที่หลั่งออกมาในอาหารเลี้ยงเซลล์ (culture supernatants) ด้วยวิธีอีไลซ่า (ELISA) ส่วนเซลล์จะถูกนำไปวัด CD40, CD80, CD83 และ HLA-DR โดยโฟลไซโตมิเตอร์ (flow cytometry) *พอร์ไฟโรโมแนส จินจิวาลิส* LPS ชักนำการแสดงออกของโค-สติมูลาโทรีโมเลกุล (CD40, CD80), HLA-DR และ CD83 ในระดับต่ำ ซึ่งแตกต่างกับ *เอสเชอร์รีเชีย คอไล* LPS และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* LPS ทั้ง *พอร์ไฟโรโมแนส จินจิวาลิส* LPS และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* LPS ชักนำการผลิต ทีเอ็นเอฟ-อัลฟ่า ในปริมาณน้อย และแทบไม่พบ อินเตอร์ลูคิน-10 เมื่อเปรียบเทียบกับ *เอสเชอร์รีเชีย คอไล* LPS ซึ่ง LPS จากแบคทีเรียทั้ง 3 ชักนำการผลิต อินเตอร์ลูคิน-12 พี70 เพียงเล็กน้อย หรือไม่มีเลย คณะผู้วิจัยทำการประเมินผลต่อไปถึงความสามารถของ *พอร์ไฟโรโมแนส จินจิวาลิส* LPS และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* LPS ในการกระตุ้นเดนดริทิกเซลล์ที่ยังไม่เจริญเต็มที่ซึ่งเตรียมมาจากโมโนไซท์เพื่อเริ่มการกระตุ้นเนอีฝ ซีดี4+ ทีเซลล์ (naïve CD4+ T cells) ของมนุษย์ และมีส่วนช่วยในการพัฒนาของการตอบสนองของทีเฮลพเพอร์แบบที่ 1 หรือ ทีเฮลพเพอร์แบบที่ 2 ที่เกี่ยวกับการชักนำการผลิตไซโตคายส์ที่ไม่ดีนั้น ในการกระตุ้นที่เซลล์เดนดริทิกยังไม่เจริญเต็มที่ซึ่งเตรียมมาจากโมโนไซท์ของ *พอร์ไฟโรโมแนส จินจิวาลิส* LPS และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* LPS จะชักนำการตอบสนองของเนอีฝ ทีเซลล์ที่มาจากต่างคนกันได้ไม่ดี (allogeneic naïve CD4+ T cells) เมื่อเปรียบเทียบกับ *เอสเชอร์รีเชีย คอไล* LPS ในการกระตุ้นเซลล์เดนดริทิกที่ยังไม่เจริญเต็มที่ซึ่งเตรียมมาจากโมโนไซท์ พบการผลิตอินเทอเฟรอน แกมม่า (IFN-$\gamma$) ในระดับต่ำในการทดลอง MLR ของ *พอร์ไฟโรโมแนส จินจิวาลิส* LPS และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* LPS ในการกระตุ้นเซลล์เดนดริทิกที่ยังไม่เจริญเต็มที่ซึ่งเตรียมมาจากโมโนไซท์ เมื่อเทียบกับการทดลอง MLR ของ *เอสเชอร์รีเชีย คอไล* LPS ในการกระตุ้นเซลล์เดนดริทิกที่ยังไม่เจริญเต็มที่ซึ่งเตรียมมาจากโมโนไซท์ ซึ่งพบการผลิตอินเทอเฟรอน แกมม่า ในปริมาณที่สูง ส่วนการผลิต อินเตอร์ลูคิน-5 ถูกตรวจพบในปริมาณต่ำเหมือนๆ กันในทุกการทดลอง MLR

เนื่องจาก การมีทีเซลล์ที่มีความจำเพาะต่อแอนติเจนในอัตราที่ต่ำในเซลล์เม็ดเลือดขาวในกระแสเลือดของมนุษย์ การทดลองในสิ่งมีชีวิตเกี่ยวกับทีเซลล์ที่มีความจำเพาะต่อแอนติเจนจึงถูกสร้างขึ้นเพื่อตรวจสอบความสามารถของ LPS จาก *พอร์ไฟโรโมแนส จินจิวาลิส* และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* และ *เอสเชอร์รีเชีย คอไล* ในการชักนำของการตอบสนองของทีเซลล์ที่จำเพาะเจาะจงต่อ OVA โดยทำการทดลองในหนู ดังที่คาดไว้ splenocytes จากกลุ่มควบคุมที่เป็นทีเฮลพเพอร์แบบที่ 2 (Montanide ISA720 + OVA) จะผลิตแอนติเจนที่จำเพาะต่ออินเทอเฟรอน แกมม่าจำนวนน้อย และแอนติเจนที่จำเพาะต่ออินเตอร์ลูคิน -5 จำนวนมาก เมื่อเปรียบเทียบกับกลุ่มควบคุมที่เป็นทีเฮลพเพอร์แบบที่ 1; Montanide ISA720 + CpG ODN 1826 + OVA. หนูทดลองที่ได้รับการฉีดด้วย LPS จาก *พอร์ไฟโรโมแนส จินจิวาลิส หรือ แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* กับ Montanide ISA720 + OVA แสดงการมีแอนติเจนที่จำเพาะต่ออินเทอเฟรอน แกมม่าจำนวนน้อย และแอนติเจนที่จำเพาะต่ออินเตอร์ลูคิน -5 จำนวนมากกว่ากลุ่มควบคุมที่เป็นทีเฮลพเพอร์แบบที่ 1 เป็นที่น่าสังเกตว่าหนูทดลองซึ่งฉีดด้วย Montanide ISA720 + *เอสเชอร์รีเชีย คอไล* LPS + OVA นั้นผลิตแอนติเจนที่จำเพาะต่ออินเทอเฟรอน แกมม่าและอินเตอร์ลูคิน -5 ในปริมาณ ต่ำ โดยสรุป งานของคณะผู้วิจัยได้แสดงว่า *พอร์ไฟโรโมแนส จินจิวาลิส* และ

*แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน* LPS เป็นตัวกระตุ้นที่ไม่ดีในเซลล์เดนดริทิกที่ยังไม่เจริญเต็มที่ซึ่งเตรียมมาจากโมโนไซท์ที่ได้จากมนุษย์ ข้อมูลจากการทดลอง MLR ในมนุษย์ และการตอบสนองของทีเซลล์ที่มีความจำเพาะต่อแอนติเจนในหนูทดลอง แสดงให้เห็นว่า LPS จากแบคทีเรียในคราบจุลินทรีย์ทั้ง 2 ชนิด ดูเหมือนจะผลักดันการแสดงออกทีเฮลพเพอร์ไปในแบบที่ 2

# EXECUTIVE SUMMARY

TITLE : Interaction between dendritic cells with LPS derived from *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*

ชื่อโครงการ : ปฏิกิริยาระหว่างเซลล์เดนดริทิกกับแอลพีเอสจาก*พอร์ไฟโรโมแนส จินจิวาลิส* และ *แอคติโนเบซิลัส แอคติโนไมซิเตมคอมมิแทน*


RESEARCH TEAM

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## RATIONALE

The development of T helper1 (Th1)- versus Th2-type response is known as fundamental important in determining whether the response to infectious pathogens will be protective or not. The Th1 or Th2 response that continues unabated may cause overproduction of cytokines leading to damage of normal tissues. High levels of inflammatory mediators and cytokines as well as numerous infiltrated T and B cells are the characteristics of periodontitis, the advanced form of periodontal disease. Accumulating data regarding Th1- and Th2-type response in periodontitis are however in conflict. This inconclusive piece of information is crucial in the pathogenesis of periodontitis and still lacking. Recent studies have suggested that the specific type of T cell-mediated immune response is governed by the interactions between dendritic cells (DCs) and infectious agents. We therefore investigated the mechanisms of DC activation by periodontopathic bacteria such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. This model may represent how the primary immune response is initiated in periodontitis and how the bacteria activated DCs subsequently influence the development of the specific type of Th response. It's anticipated that the results obtained from this line of investigation will provide a better understanding into the immunoregulation and immunopathogenesis of periodontitis.

## OBJECTIVES

1. To investigate the interaction between DCs and plaque bacterial products such as *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS with regards to expression of co-stimulatory molecule and cytokine production
2. To determine how bacterial plaque LPS-stimulated DCs influence the type of Th response both *in vitro* and *in vivo* and how such a response could explain some of the pathophysiology of periodontitis.

## RESEARCH METHODOLOGIES

1. Peripheral blood samples were collected from healthy adult volunteers with no signs and symptoms of periodontal disease.
2. LPS from *P. gingivalis* and *A. actinomycetemcomitans* were used to stimulate cells.
3. DCs were derived from human $CD14^+$ monocytes by culturing in GM-CSF plus IL-4.
4. Cell surface marker analysis were determined by flow cytometry.
5. Cytokine production were analyzed by ELISA.
6. Th differentiation in mixed leukocyte reaction and *in vivo* ovalbumin (OVA)-specific T cell response were determined by measuring IFN-γ and IL-5 production.

**แผนการดำเนินงานตลอดโครงการ 2 ปี 6 เดือน**

| ปีที่ | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| **แผนงาน** | 1-6 เดือน | 7-12 เดือน | 13-18 เดือน | 19-24 เดือน | 25-30 เดือน | |
| จัดซื้อเตรียมวัสดุอุปกรณ์, เตรียมแบคทีเรีย | 6 เดือน ⟷ | | | | | |
| เตรียม monocyte-derived DCs | | 8 เดือน ⟷ | | | | |
| DC activation study | | 12 เดือน ⟷ | ⟷ | | | |
| Mixed leukocyte reaction study | | | 12 เดือน ⟷ | ⟷ | | |
| *in vivo* antigen-specific T cell study | | | | 12 เดือน ⟷ | ⟷ | |
| Report & publication | | | | 12 เดือน ⟷ | ⟷ | |

# BACKGROUND OF THE STUDY

*Periodontitis and plaque bacteria*

Periodontitis is an advanced form of periodontal disease which involves destruction of tooth supporting structures including gingiva, connective tissue and alveolar bone . The disease is manifested by chronic gingival inflammation, loss of periodontal attachment and bone support which may eventually lead to tooth loss (Williams, 1990). It is caused by the complex interaction between host defenses and microorganisms in dental plaque (Seymour, 1991).

Plaque microorganisms and their products, particularly lipopolysaccharide (LPS) has long been recognized as etiologic agents of periodontal disease. The composition of plaque bacteria associated with periodontitis are different from those associated with healthy gingiva or gingivitis (a gingivally confined inflammatory lesion with no periodontal attachment / bone loss) (Slots, 1977a; Slots, 1977b; Socransky and Haffajee, 1991). The majority of plaque at healthy and gingivitis sites are Gram positive aerobes or facultative anaerobes, such as *Streptococcus* and *Actinomyces* species. On the other hand, subgingival plaque at periodontitis sites consists of a group of Gram negative anaerobes or facultative anaerobes (Darveau *et al.*, 1997). Indeed, at the 1996 World Workshop on Clinical Periodontics, the relevant working group concluded that most human periodontitis is caused by *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* and *Bacteroides forsythus*.

*Periodontitis lesion*

Histologically periodontitis lesion is characterized by densed infiltration of T and B cells in the extravascular gingival connective tissues (Seymour *et al.*, 1993). It's suggested that inappropriate production of cytokines particularly from infiltrated T cells plays a significant role in the pathogenesis of the disease (Seymour *et al.*, 1993). Study of cytokines in periodontitis during the past 10 years provides variable results with regard to the type of cytokines, whether T helper1 (Th1) or Th2, detected. For example, the expression of mRNA of IL-2, IFN-$\gamma$ and IL-5 was detected from patients' tissues by one group (Takeichi *et al.*, 2000), while the other group reported the detection of IFN-$\gamma$ and IL-5 but little or no IL-2 or IL-4 mRNA (Fujihashi *et al.*, 1993). As for the protein detection, IL-4 and IL-6 producing memory T cells detected by immunochemistry were observed in periodontal tissues (Aoyagi *et al.*, 1995). The difference in findings among these observations may relate to the technique used to process tissues as well as to detect cytokine production. So far, the issue of Th1 and Th2-type response

in periodontitis is still inconclusive. Why and how the specific type of Th response is so crucial in the disease process will be discussed.

*Th1 and Th2-type of response*

It is now known that CD4+ T cells or Th cells can be divided into, at least 2 subsets i.e. Th1 and Th2 according to their cytokine production. The first Th cell types reported were mouse Th1 and Th2 cells. Mouse Th1 cells were found to secrete IFN-$\gamma$, while Th2 cells secreted IL-4 (Mosmann *et al*., 1986). In human, Th1 cells were also identified that secrete IFN-$\gamma$ and TNF-$\beta$ while Th2 cells produce IL-4, IL-5, IL-6 and IL-13 (Seder and Paul, 1994; Romagnani, 1997). IL-10 is also classified as a Th2-type cytokine in the mouse, but in humans, both Th1 and Th2 subsets secrete IL-10 (Ferrick *et al*., 1995; Katsikis *et al*., 1995). The ensuing Th1- and Th2-type immune responses both include potent humoral and cell-mediated components, but the effector cells and antibodody (Ab) isotypes involved are quite distinct (Abbas *et al*., 1996). Th1 cells are responsible for the activation of macrophages to a microbicidal state, the induction of IgG Abs that mediate opsonization and phagocytosis, and the support of CD8+ antiviral effector T cells. By contrast, Th2 cells stimulate the growth and differentiation of mast cells and eosinophils, as well as the production of Ab isotypes, including IgE, which can mediate the activation of these cells. Th1 and Th2 cells are not precommitted phenotypes but rather, represent endpoints of a multistep differentiative process whereby a common precursor population acquires a distinct cytokine secretion profile (Romagnani, 1997). A key question is how this differentiation decision is made. This will be discussed later.

Study of the numerous animal models, together with the few studies in humans, have revealed that the ability of a host to eradicate effectively an invading pathogen depend on the type of effector-specific immune response (Th1- or Th2-type response) that is mounted (Romagnani, 1997). This has been suggested to be a major branch point in the immune response since it is an important determinant of whether the response to infectious pathogen will lead to protection of host or dissemination of the disease. In general, Th1 cells are more suitable for protection against intracellular parasites including bacteria, protozoa, fungi and viruses such as *Mycobaterium tuberculosis* (Brightbill *et al*., 1999), *Toxoplasma gondii* (Gazzinelli *et al*., 1994), whereas Th2 cells protect against extracellular parasites (most helminthic parasites) such as *Schistosoma mansoni* (Velupillai and Harn, 1994), *Acanthoceilonema vitae* (Whelan *et al*., 2000). On the other hand, generating the inappropriate type of immune response can induce pathogenesis or even the matter of life and death such as a lethal Th2-type response found in *Leishmania major* infection in mouse model (Liew, 1989; Locksley *et al*., 1991).

*Dendritic cells*

T cells play important role in adaptive immunity as they respond to antigens with high specificity, however, these two parties alone do not always lead to immunity. T cells need antigen to be processed and presented to them by a third party, antigen presenting cells (APCs). DCs are professional APCs which has been well recognized for being the most potent APCs with an extraordinary capacity to stimulate naïve T cells and initiate primary immune response (Liu *et al*., 2001). They play critical role as the initiator and modulator of the immune response (Steinman, 1991).

DCs are bone marrow-derived cells. They are scattered throughout the body, including the various portals of microbe, where they reside in an immature form (Steinman, 1991). Immature DCs can be considered "immunological sensors," alert for potentially dangerous pathogens (Pulendran *et al*., 2001). When a microbe infects a tissue, resident immature DCs sense the microbe by recognizing specific molecular patterns. Those pathogen-associated molecular patterns, or PAMPs are intergral to microbial carbohydrates, lipids, and nucleic acids. And as different classes of microbe carry different PAMPs, DCs can use so-called pattern recognition receptor (PRR), of which the recently recognized Toll-like receptors (TLRs) are prime examples (Akira *et al*., 2001), to sense and classify the pathogens and respond appropriately. The binding of PAMPs to TLRs, triggers a cascade of cellular signals resulting in transcription of genes that modulate and mediate immune response. Some results of such reaction are the increased expression of co-stimulatory molecule and the release of T cell immunoregulatory ctyokines such as IL-12 and IFN-$\alpha$.

It appears that TLRs offer DCs a means of discriminating between different stimuli. For example, *Escherichia coli* LPS binds to TLR4; peptidoglycans from *Staphylococcus aureus* and zymosan bind to TLR2; CpG bacterial DNA binds to TLR9; and bacterial flagellin binds to TLR5 (Medzhitov, 2000; Hoffmann *et al*., 1999; Aderem and Ulevitch, 2000). As can be seen that TLRs on DCs play a critical role in triggering innate immune responses and subsequently to activate naïve T cells.

Once a DC has detected a specific microbe, information about the pathogen is then relayed to naïve T cells in the draining lymph nodes, in a sequence of events (Steinman, 1991). First immature DCs capture the microbe or its products, exit the site of infection and migrate toward the T cell areas of proximal lymph nodes via afferent lymphatics. The DC migration is guided by the chemokines e.g. 6Ckine and MIP-3$\beta$ , which are expressed in the lymphatics and T cell areas of lymphoid organs (Banchereau and Steinman, 1998; Banchereau *et al*., 2000). These are ligands for CCR7 receptor, which is up-regulated on DCs as they migrate. During the journey, DC become mature, losing their antigen-capture activities but acquiring the capacity to process and display peptide antigens on their surface, in conjunction with molecules of the major histocompatibility complex (MHC) (Banchereau and Steinman, 1998; Banchereau *et al*., 2000). Here, for productive immunity to occur, DCs

must present not only peptide-MHC complexes but also additional co-stimulatory signals (such as B7 family, including CD80 and CD86) to T cells. The interaction between CD86 and its corresponding ligand CD28 on T cells results in the up-regulation of CD40 ligand (CD40L) on T cells. The T cells may then engage CD40 on DCs and trigger a burst of cytokine expression, including IL-12, which induces IFN-γ in T cells (Banchereau and Steinman, 1998; Banchereau *et al*., 2000). Signaling through CD40 also up-regulates numerous other co-stimulatory molecules, which may play distinctive roles in tuning the immune response.

*Tuning the response by DCs*

From early studies, we know that the cytokines produced in the local environment are determined factors that control the type of Th response. For example, IL-12 and IL-4 induce Th1 and Th2 cells respectively (Seder and Paul, 1994; Trinchieri, 1993). Recent evidence suggest that DCs have the capacity not only to initiate naïve T cell priming but also to control the type of Th response.

It was first proposed that human myeloid DCs which produce IL-12, induce Th1 response and plasmacytoid DCs do not produce IL-12, thus induce Th2 response (Rissoan *et al*., 1999). However, his concept has now been challenged by several studies; for example, plasmacytoid DCs have the ability to induce Th2 responses (IL-4, IL-5 and IL-10) when stimulated with IL-3, in contrast to the virus stimulated plasmacytoid DCs which induce $CD4^+$ T cells to produce IFN-γ and IL-10 (Cella *et al*., 2000; Kodawaki *et al*., 2000). More recently, it was demonstrated that plasmacytoid DCs activated by influenza virus and CD40L drive a strong Th1 response (Cella *et al*., 2000).

Different pathogens seem to interact with DCs differently. Several pathogens such as bacteria, viruses, mycoplasma and *T. gondii* induce DC maturation and IL-12 production (Reis e Sousa *et al*., 1999). Malaria blood stage parasites suppress the maturation of DCs and their capacity to priming T cells (Urban *et al*., 1999). Measles virus inhibits IL-12 production while leaves other aspects of DCs maturation process unaffected (Karp *et al*., 1999). Other virus such as HIV can infect DCs and vigorously replicate in DC-derived syncytia *in vitro* (Pope *et al*, 1997). Canarypox viruses infect DCs and induce a rapid apoptotic death (Tortorella *et al*., 2001).

The mechanisms of how DCs induce Th1 and Th2-type responses have been the subject of intense investigation. Whatever, the control mechanisms of DCs might be, the interaction between DCs and pathogens seems to be significant factors in determining the effective of the immune response and the outcome of disease. A great need remains to decipher the mechanisms how plaque bacteria stimulate DCs and how these stimulated DCs influence the specific type of T cell-mediated responses. Preliminary observations from our laboratory demonstrate that LPS from *A. actinomycetemcomitans* is much more potent than LPS from *P. gingivalis* with regard to cytokine induction. This suggests that LPS from these two bacteria may act on immune cells differently.

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# 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 was used throughout this study. Ficoll-Hypaque (Histopaque 1.077) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were obtained from R & D System Inc. (Minneapolis, MA, USA).

Monoclonal antibodies (mAbs) against CD1a (FITC), CD40 (APC), CD80 (PE ), CD83 (PE) and CD86 (FITC) were obtained from PharMingen (San Jose, CA, USA). mAbs 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). mAbs and their specificity are listed in table 1.

OVA and *E. coli* LPS was 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).

| Monoclonal antibodies | Specificity |
|---|---|
| Anti-CD1a | Dendritic cells, Langerhans cells |
| Anti-CD4 | T cell subset |
| Anti-CD8 | T cell subset |
| Anti-CD14 | Monocytes |
| Anti-CD20 | B cells |
| Anti-CD40 | Co-stimulatory molecule |
| Anti-CD45RA | Resting / naïve T cells |
| Anti-CD45RO | Activated / memory T cells |
| Anti-CD56 | NK cells |
| Anti-CD80 | Co-stimulatory molecule |
| Anti-CD83 | Mature antigen on dendritic cell |
| Anti-CD86 | Co-stimulatory molecule |
| Anti-HLA-DR | Major histocompatibility complex class II |
| Anti-TCR-γδ | T cell subset |

**Table 1.** List of mAbs used in the study.

Preparation of bacteria and bacterial LPS

*P. gingivalis* strain 381 were grown in trypticase soybroth at 37°C under anaerobic chamber (Forma Scientific, USA). *A. actinomycetemcomitans* (Y4) were grown in trypticase soybroth at 37°C in 5% $CO_2$ incubator (Ishii *et al*., 1992). The purity of bacterial species was examined by colony morphology on trypticase soy blood agar for *P. gingivalis* and trypticase soy agar for *A. actinomycetemcomitans* as well as by Gram's staining. The bacteria were harvested by centrifugation (Beckman Instruments, USA) at 2060xg for 15 minutes, washed twice in sterile PBS (0.15M: pH 7.2-7.4) and lyophilized.

*P. gingivalis* LPS and *A. actinomycetemcomitans* LPS was extracted from the lyophilized cells by the hot phenol-water procedure and subsequent treatment with DNase I, RNase A, and proteinase K (for *P. gingivalis* LPS) and nuclease P1 (for *A. actinomycetemcomitans*), followed by chromatographic purification. The purity of both LPS preparation was confirmed by immunodiffusion analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining (Schifferle *et al*., 1998; Preshaw *et al*., 1999; Morozumi *et al*., 2001).

Generation of Mo-DCs

Under a protocol approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, subjects signed a consent form after being advised of the nature of the study. Heparinized peripheral blood was obtained from healthy adult volunteers. These subjects had clinically healthy periodontal conditions, with < 4 mm. probing depth and no bleeding upon probing. They had not taken antibiotics or anti-inflammatory drugs within the past 3 months. None of them had any symptoms of infection.

DCs were generated from peripheral blood mononuclear cells (PBMC) prepared from these volunteers. PBMC were separated over Ficoll-Hypaque (Sigma Chemical Co.), as previously described (Boyum, 1968). T cells were depleted from PBMC by rosetting with neuraminidase-treated sheep red blood cells. T cell depleted population was stained with mAbs against CD14 and then they were positively sorted using fluorescence-activated cell sorter (FACSVantage, BD Biosciences, Mountain View, CA). Immature DCs were generated by culturing $1x10^5$ $CD14^+$ monocytes in 200 μL of RPMI1640 medium containing 10% heat-inactivated autologous serum, 50 ng/mL GM-CSF and 50 ng/mL IL-4 in 96-well flat microtiterplates (Costar, Cambridge, MA). The culture plates were incubated at 37°C in 5% $CO_2$ atmosphere for 5-7 days to allow differentiation into immature DCs. Approximately half of the spent culture medium was removed and replaced with equal volume of fresh culture medium together with cytokines every other day.

To evaluate the efficiency of the generation of the Mo-DCs, we also cultured $CD14^+$ monocytes in the RPMI1640 medium containing 10% heat-inactivated autologous serum without the above cytokine. The phenotype of monocytes and immature DCs was routinely monitored by flow cytometry and they were typically found to be $CD1a^+$, $CD14^-$, $CD40^+$, $CD80^+$, $CD83^+$ and $HLA\text{-}DR^+$.

Stimulation of DC cultures with bacterial LPS

Immature Mo-DCs ($5x10^5$ cells/ml) were stimulated with different concentrations of LPS from *P. gingivalis*, *A. actinomycetemcomitans*, and *E. coli* (0, 100, 300, 1000 ng/mL) for 24 hrs. Culture supernatants were analyzed for tumor necrosis factor (TNF)-$\alpha$ , IL-10 and IL-12 p70 production using ELISA. The cells were harvested to measure CD40, CD80, CD83 and HLA-DR by flow cytometry.

Mixed leukocyte reaction

Enriched T cell population were prepared from PBMC by rosetting with neuraminidase-treated sheep red blood cells (RBCs). Sheep RBCs were lysed with $NH_4Cl$-Tris. Allogeneic naïve $CD4^+$ T cells were obtained by negative sorting of enriched T cell population that had been stained with mAbs against CD8 (FITC), CD20 (FITC), CD56 (FITC), CD45RO (FITC), and TCR-$\gamma\delta$ (PE) using FACSVantage. This technique provides $CD4^+CD45RA^+$ naïve T cells with a purity of >90%. Flow cytometrically sorted allogeneic naïve T cells ($5x10^5$ cells/mL) were added in cultured wells that contained control and LPS-stimulated Mo-DCs ($5x10^4$ cells/mL). Culture supernatants were harvested on day 5 and then analyzed for IFN-$\gamma$ and IL-5 production using ELISA.

Antigen-specific T cell response

Female BALB/c mice, 6-8 weeks were obtained from National Laboratory Animal Centre, Thailand. Mice were divided into 4 groups (n = 4 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 ISA 720 + OVA + *A. actinomycetemcomitans* LPS. Fifty microgram of OVA in phosphate-buffered saline (PBS) was emulsified with Montanide ISA720 at the ratio of 3/7 parts in the presence of 50 $\mu$g of CpG ODN 1826, 50 $\mu$g of *E. coli* LPS, 50 $\mu$g of *P. gingivalis* LPS, or 50 $\mu$g of *A. actinomycetemcomitans* LPS. Mice were immunized with 100 $\mu$L of each vaccine at day 0, 14 and 28 via subcutaneous, subcutaneous and intraperitoneal

routes respectively. Splenocytes were harvested on day 38. They ($5x10^6$cells/mL) were stimulated with OVA (40 μg/mL) for 5 days. Culture supernatants were harvested and then assayed for IFN-γ and IL-5 production.

Flow cytometric analysis

To study the effect of *P. gingivalis*, *A. actinomycetemcomitans* and *E.coli* LPS on DC activation, both control DCs and LPS-treated DCs were stained with mAbs anti-CD40 (APC), anti-CD80 (PE), anti-CD83 (FITC) and HLA-DR (PerCP). Mouse isotype mAbs conjugated with APC, PE, FITC and Per CP were used as control.

Mo-DCs were stained at $4^oC$ for 30 min, washed in PBS and then reconstituted with 1% paraformaldehyde. Normally, 5,000-10,000 cells were analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA). The levels of surface molecule expression were presented by mean fluorescence intensity (MFI).

Cytokine determination

Cytokine production levels in supernatant were measured using commercially available ELISA kits for IL-10, IL-12 p70, IL-5, IFN-γ and TNF-α (R&D Systems, Minneapolis, MN, USA). The detection limits for IL-10, IL-12 p70, IL-5, IFN-γ and TNF-α were 3.9, 4.0, 3.0, 8.0 and 4.5 pg/mL respectively.

# RESULTS

## Morphology and phenotypes of monocyte-derived DCs

In the present study we isolated monocyte from human blood by positive sorting of $CD14^+$ cells using flow cytometry. The purity of the sorted cells was always > 95%. Fig. 1 shows flow cytometrically sorted $CD14^+$ monocytes. These sorted $CD14^+$ monocytes were cultured in medium containing 10% heat inactivated autologous serum and 50 ng/ml of GM-CSF and 50 ng/ml of IL-4. After 5-7 day incubation in enriched medium, the small round $CD14^+$ monocytes differentiated into monocyte-derived DCs with a morphology of irregular shape cell as shown in Fig. 2. Differentiation of monocytes to immature DCs was analyzed by cell morphology and cell phenotypes. These cells became non-adherent, clustered, displayed different extracts of veiled and dendritic morphology. Phenotypic analysis by flow cytometry indicated that monocyte-derived DCs up-regulated several co-stimulatory molecules including HLA-DR, CD40, CD80. Dendritic cell marker (CD1a) was found to be

expressed on monocyte-derived DCs but the monocyte marker (CD14) and CD83 were negligibly expressed suggesting that monocytes cultured with GM-CSF and IL-4 had acquired typical characteristics of immature DCs (Fig. 3).

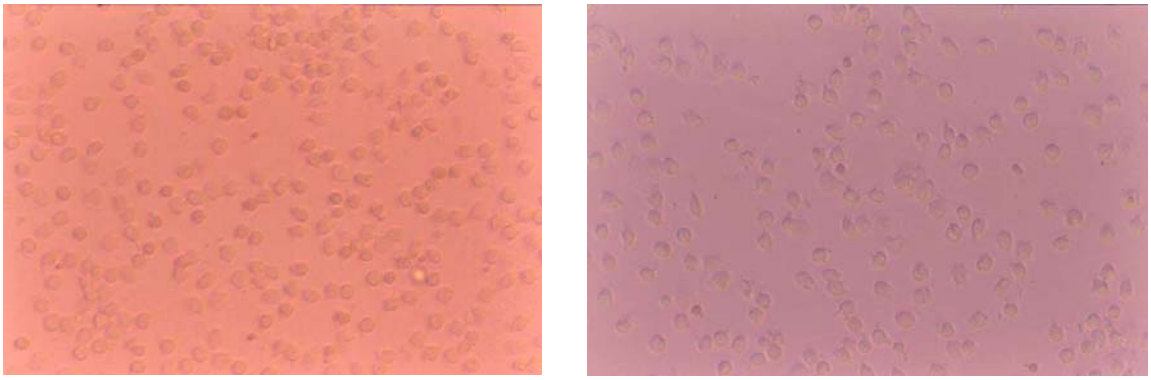

**Figure 1.** Flow cytometrically sorted $CD14^{+}$ monocytes.

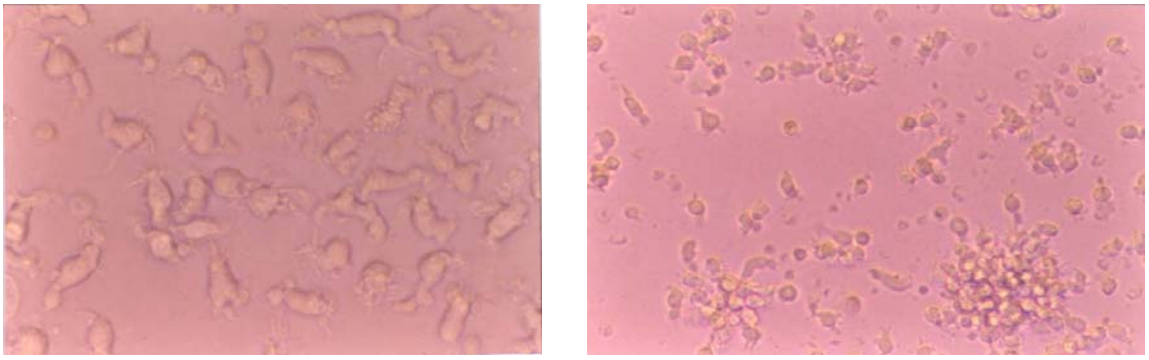

**Figure 2.** Immature Mo-DCs.

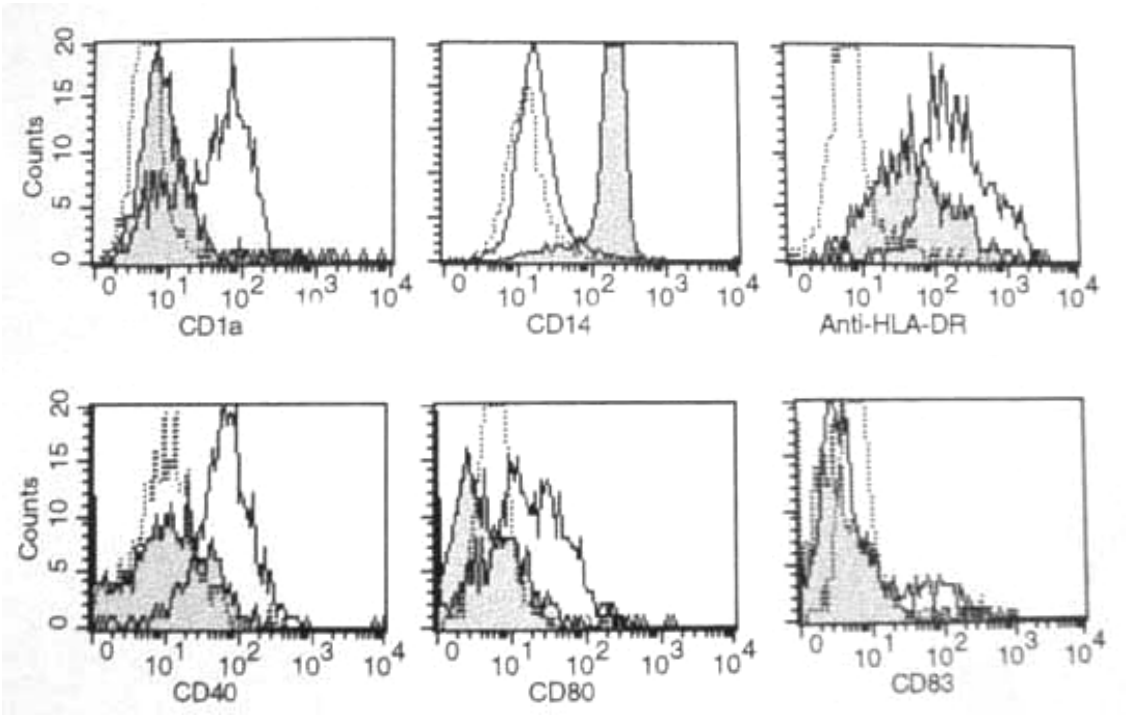


**Figure 3.** Phenotypic analysis of Mo-DCs in comparison with monocytes. (dot line : IgG, gray area : monocyte, solid line : monocyte-derived dendritic cell)

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

We investigated the ability of two different LPS derived from plaque bacteria, *P. gingivalis* and *A. actinomycetemcomitans* to induce DC maturation in comparison with a well known *E. coli* LPS. Expression of HLA-DR, CD40, CD80, and CD83 and their cytokine production (TNF-$\alpha$, IL-10, and IL-12 p70) were measured after 24 hr stimulation of Mo-DCs with different bacterial LPS. Fig. 4A, 4B and 4C are the representative histograms, which showed the expression of HLA-DR, CD40, CD80, and CD83 on Mo-DCs after stimulation with different doses of *P. gingivalis* LPS, *A. actinomycetemcomitans* LPS and *E. coli* LPS, respectively. The expression of HLA-DR, CD40, CD80, and CD83 on Mo-DCs was up-regulated in a dose dependent manner upon all the bacterial LPS stimulation. Fig. 4D demonstrated the comparison between the two bacterial plaque LPS with *E. coli* LPS for their ability to up-regulation of HLA-DR, CD40, CD80, and CD83 expression on Mo-DCs. *A.*

*actinomycetemcomitans* LPS and *E. coli* LPS enhanced similar expression levels of these molecules and the levels were higher than *P. gingivalis* LPS at each corresponding concentration. The results of cytokine production from bacterial LPS-stimulated Mo-DCs were shown in Table 2. Both bacterial plaque LPS induced low levels of TNF-$\alpha$ and negligible amounts of IL-10 from Mo-DCs. On the contrary, *E. coli* LPS appeared to be a potent inducer for TNF-$\alpha$ production and induced moderate amounts of IL-10. Little or no IL-12 p70 production was detected in all LPS-stimulated Mo-DC cultures (Fig. 5).

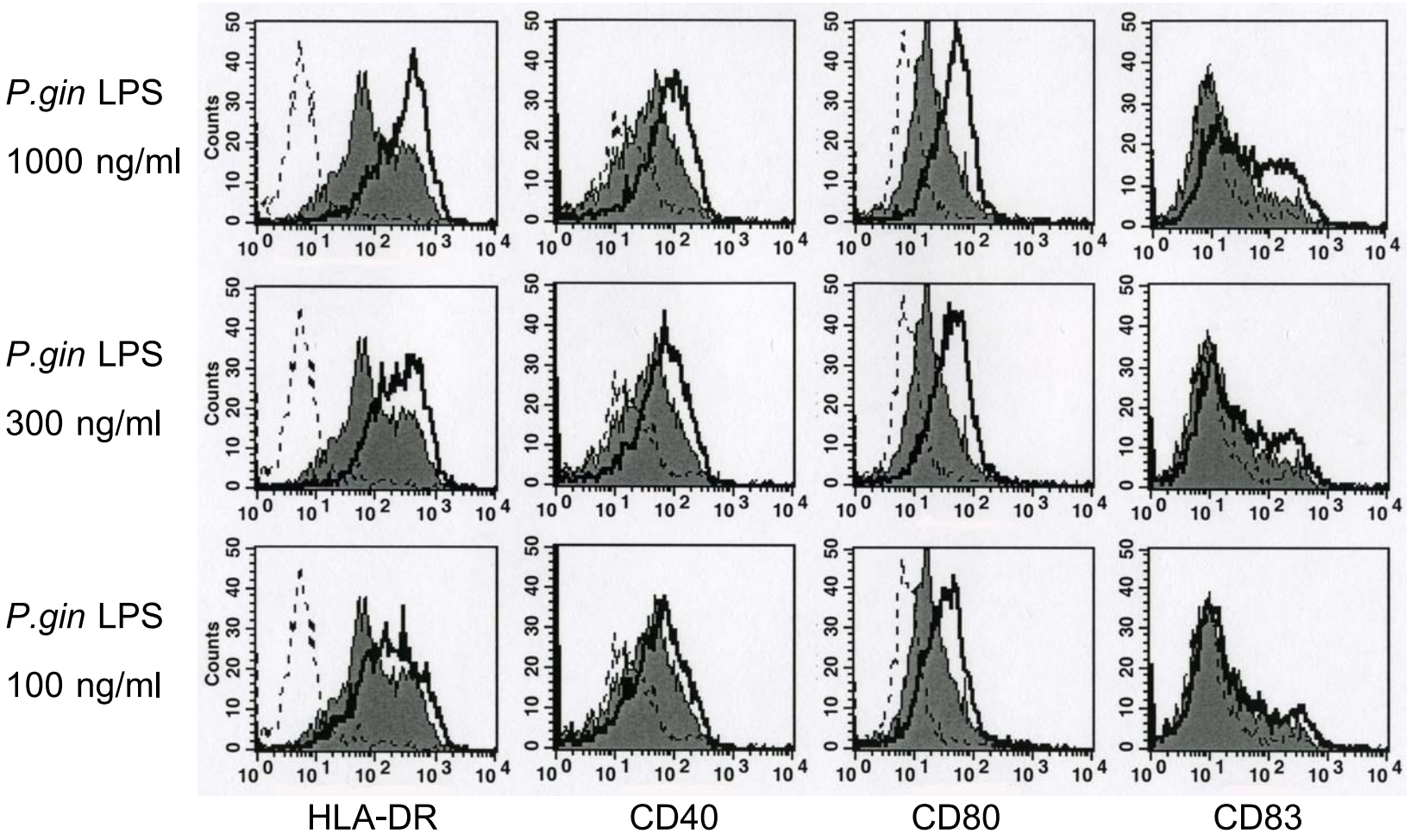


Figure 4A. LPS from *P. gingivalis* induced up-regulation of HLA-DR, CD40, CD80 and CD83 on Mo-DCs. Representative histograms (n=4) of immature Mo-DCs which were stimulated with different concentrations of LPS (0, 100, 300, 1000 ng/ml) for 24 hrs. Cell surface expression of HLA-DR, CD40, CD80, and CD83 was analyzed by flow cytometry. Dotted lines are isotype controls; the shaded areas are media controls; the solid lines are cultures incubated with *P. gingivalis* LPS.

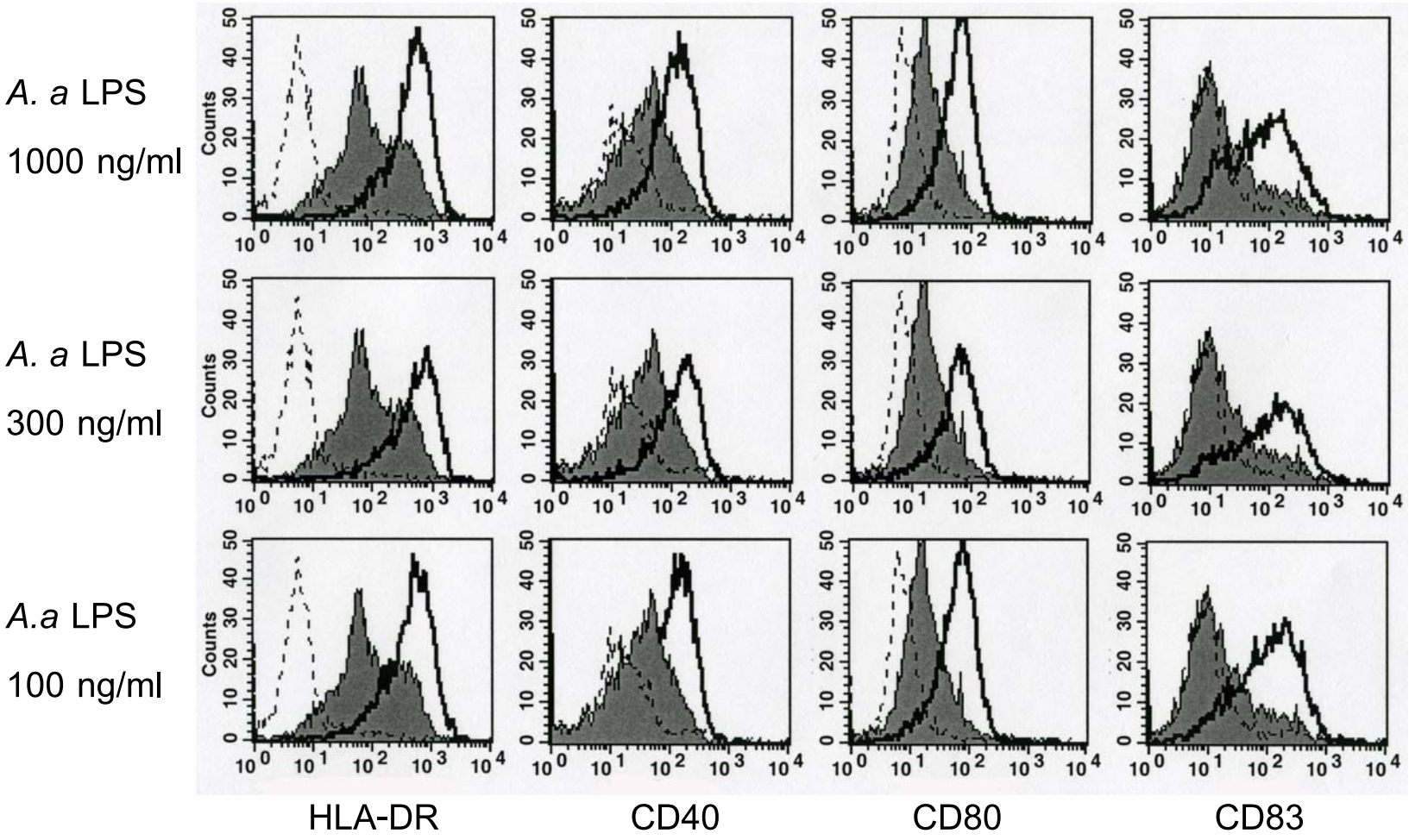


**Figure 4B.** LPS from *A. actinomycetemcomitans* induced up-regulation of HLA-DR, CD40, CD80 and CD83 on Mo-DCs. Representative histograms (n=4) of immature Mo-DCs which were stimulated with different concentrations of LPS (0, 100, 300, 1000 ng/ml;) for 24 hrs. Cell surface expression of HLA-DR, CD40, CD80, and CD83 was analyzed by flow cytometry. Dotted lines are isotype controls; the shaded areas are media controls; the solid lines are cultures incubated with *A. actinomycetemcomitans* LPS.

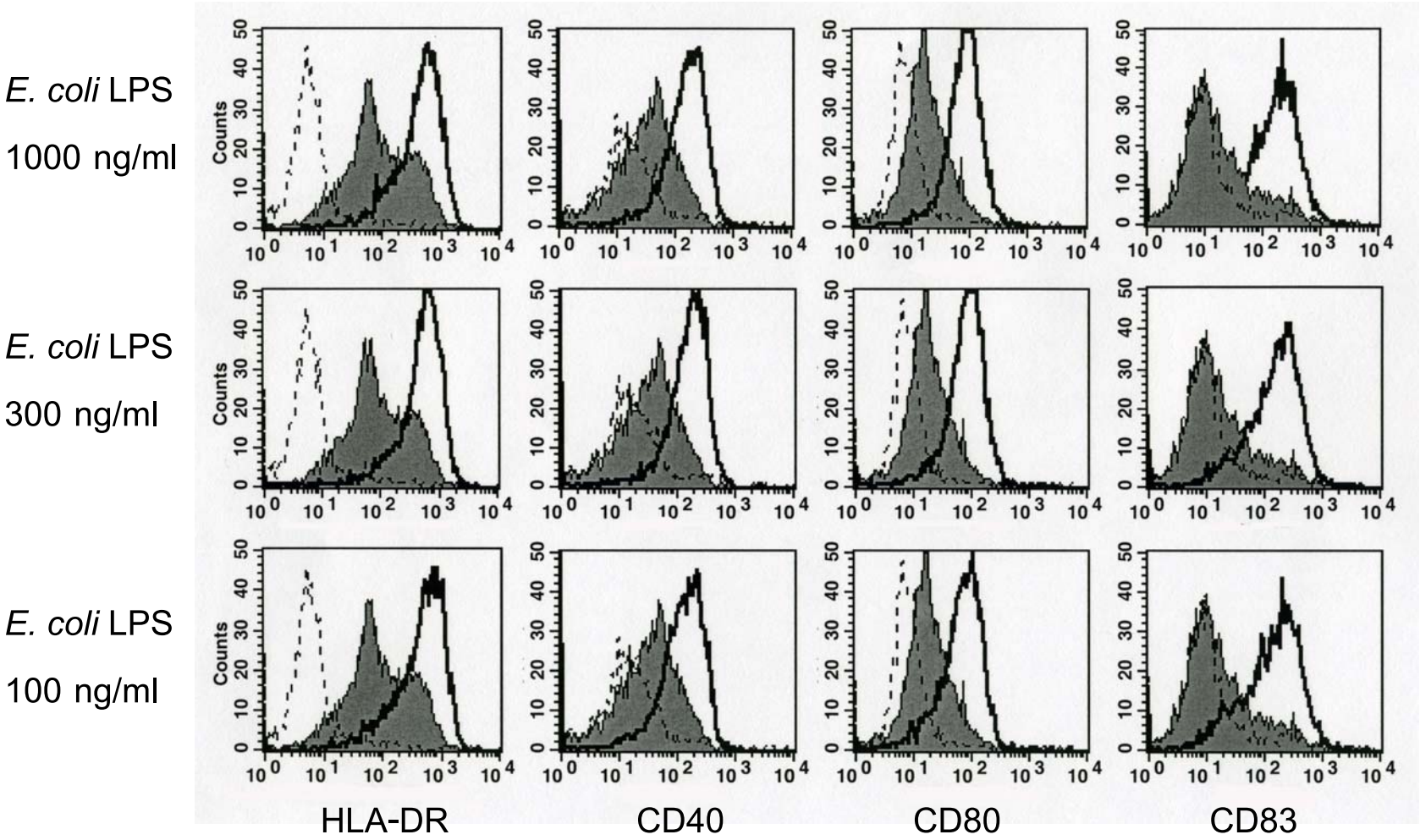


**Figure 4C.** LPS from *E. coli* induced up-regulation of HLA-DR, CD40, CD80 and CD83 on Mo-DCs. Representative histograms (n=4) of immature Mo-DCs which were stimulated with different concentrations of LPS (0, 100, 300, 1000 ng/ml) for 24 hrs. Cell surface expression of HLA-DR, CD40, CD80, and CD83 was analyzed by flow cytometry. Dotted lines are isotype controls; the shaded areas are media controls; the solid lines are cultures incubated with *E. coli* LPS.

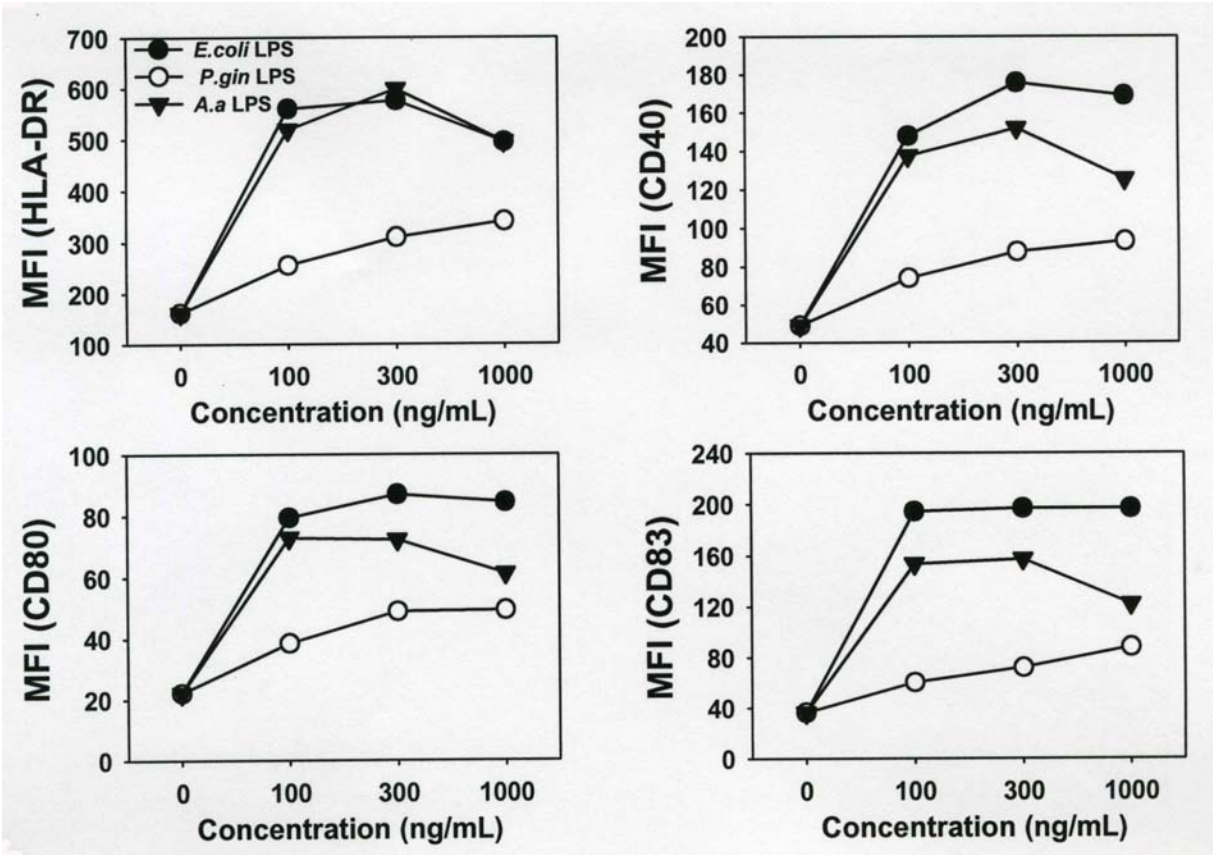


Figure 4D. Comparison between the bacterial plaque LPS and *E. coli* LPS for the ability to induce up-regulation of HLA-DR, CD40, CD80 and CD83 on Mo-DCs. Immature Mo-DCs were stimulated with different concentrations of LPS (0, 100, 300, 1000 ng/ml) for 24 hrs. Cell surface expression of HLA-DR, CD40, CD80, and CD83 was analyzed by flow cytometry. Data on molecule surface expression are representatives of 4 independent experiments and shown as MFI.

| Bacterial LPS-stimulated Mo-DCs | | Human IL-10 Conc. (pg/mL) | Human IL-12 Conc. (pg/mL) | Human TNF-α Conc. (pg/mL) |
|---|---|---|---|---|
| 1 | Control | - | 4.76 | 26.54 |
| | *E.coli* LPS | 128.43 | 14.07 | 535.94 |
| | *A.a* LPS | 79.63 | - | 205.48 |
| | *P.gin* LPS | - | - | 82.26 |
| 2 | Control | - | - | 5.99 |
| | *E.coli* LPS | 16.23 | 14.07 | 795.37 |
| | *A.a* LPS | - | - | 64.05 |
| | *P.gin* LPS | - | - | 107.29 |
| 3 | Control | - | 10.66 | 29.76 |
| | *E.coli* LPS | 527.10 | 20.10 | 1715.55 |
| | *A.a* LPS | - | 6.43 | 374.66 |
| | *P.gin* LPS | 8.67 | 8.12 | 258.63 |
| 4 | Control | - | 5.60 | 10.65 |
| | *E.coli* LPS | 24.40 | 31.45 | 982.27 |
| | *A.a* LPS | 4.41 | 12.36 | 149.37 |
| | *P.gin* LPS | - | 11.51 | 39.48 |
| Mean ± SE | Control | 0.00 ± 0.00 | 5.25 ± 2.18 | 18.24 ± 5.84 |
| | *E.coli* LPS | 174.04 ± 120.43 | 19.92 ± 4.10 | 1007.28 ± 253.20 |
| | *A.a* LPS | 21.01 ± 19.57 | 4.70 ± 2.98 | 198.39 ± 65.56 |
| | *P.gin* LPS | 2.16 ± 2.16 | 4.91 ± 2.92 | 121.92 ± 47.67 |

**Table 2.** Cytokine production from bacterial LPS-stimulated Mo-DCs

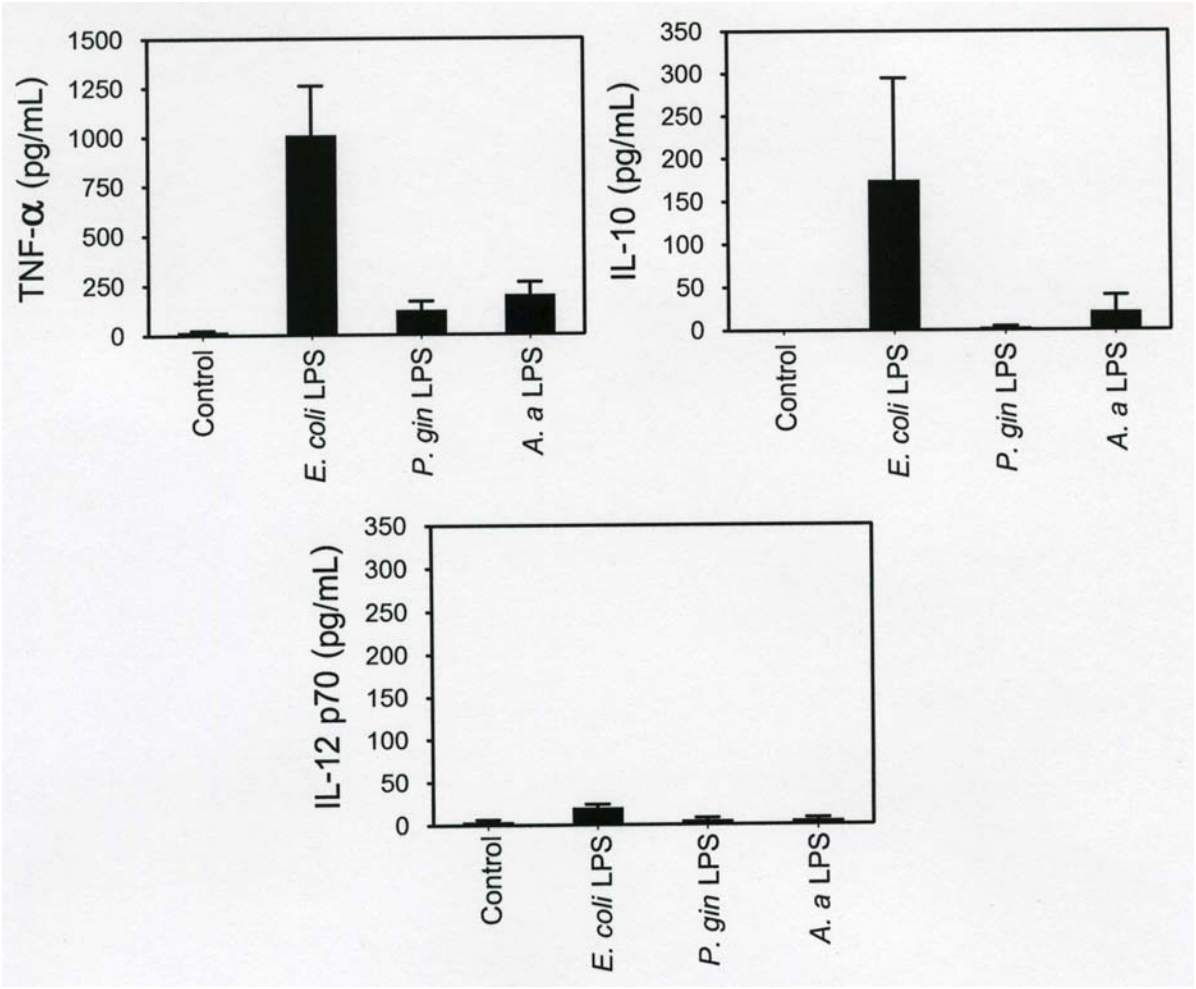


**Figure 5D.** Cytokine production from *P. gingivalis*, *A. actinomycetemcomitans*, and *E. coli* LPS-stimulated Mo-DCs. Immature Mo-DCs were stimulated with different concentrations of LPS (0, 100, 300, 1000 ng/mL) for 24 hrs. TNF-α, IL-10 and IL-12 p70 production was analyzed by ELISA. Data on cytokines are mean ± SE.

## *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. In line with the weak cytokine induction, *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) as compared with higher IFN-γ levels in those MLR cultures with *E. coli* LPS -stimulated Mo-DCs (1778 ± 612 pg/mL). Similar low levels of IL-5 production were detected in all MLR cultures (mean values ranged from 216 ± 345 pg/mL) (Table 3, Fig. 6).

| *Donor 1* | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| Mo-DC+naïve T | 18.00 | 115.00 |
| *E.coli* LPS-stimulated Mo-DCs+ naïve T | 3998.00 | 207.00 |
| *P.gin* LPS-stimulated Mo-DCs+ naïve T | 62.00 | 182.00 |
| *A.a* LPS-stimulated Mo-DCs+ naïve T | 259.00 | 205.00 |

| *Donor 2* | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| Mo-DC+naïve T | 19.00 | 76.00 |
| *E.coli* LPS-stimulated Mo-DCs+ naïve T | 1906.00 | 211.00 |
| *P.gin* LPS-stimulated Mo-DCs+ naïve T | 18.00 | 115.00 |
| *A.a* LPS-stimulated Mo-DCs+ naïve T | 64.00 | 163.00 |

| *Donor 3* | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| Mo-DC+naïve T | 20.00 | 37.00 |
| *E.coli* LPS-stimulated Mo-DCs+ naïve T | 1442.00 | 47.00 |
| *P.gin* LPS-stimulated Mo-DCs+ naïve T | 180.00 | 66.00 |
| *A.a* LPS-stimulated Mo-DCs+ naïve T | 630.00 | 85.00 |

| *Donor 4* | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| Mo-DC+naïve T | 0.00 | 22.00 |
| *E.coli* LPS-stimulated Mo-DCs+ naïve T | 309.00 | 80.00 |
| *P.gin* LPS-stimulated Mo-DCs+ naïve T | 0.00 | 20.00 |
| *A.a* LPS-stimulated Mo-DCs+ naïve T | 0.00 | 50.00 |

| *Donor 5* | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| Mo-DC+naïve T | 20.20 | 1320.90 |
| *E.coli* LPS-stimulated Mo-DCs+ naïve T | 1235.40 | 757.10 |
| *P.gin* LPS-stimulated Mo-DCs+ naïve T | 358.30 | 696.20 |
| *A.a* LPS-stimulated Mo-DCs+ naïve T | 769.50 | 1222.40 |

| Mean ± S.E (n=5) | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| Mo-DC+naïve T | 15.44 ± 3.88 | 314.18 ± 252.20 |
| *E.coli* LPS-stimulated Mo-DCs+ naïve T | 1778.08 ± 612.78 | 260.42 ± 128.47 |
| *P.gin* LPS-stimulated Mo-DCs+ naïve T | 123.66 ± 66.52 | 215.84 ± 123.06 |
| *A.a* LPS-stimulated Mo-DCs+ naïve T | 344.50 ± 152.78 | 345.08 ± 221.04 |

**Table 3.** Cytokine production in human mixed-leukocyte reaction

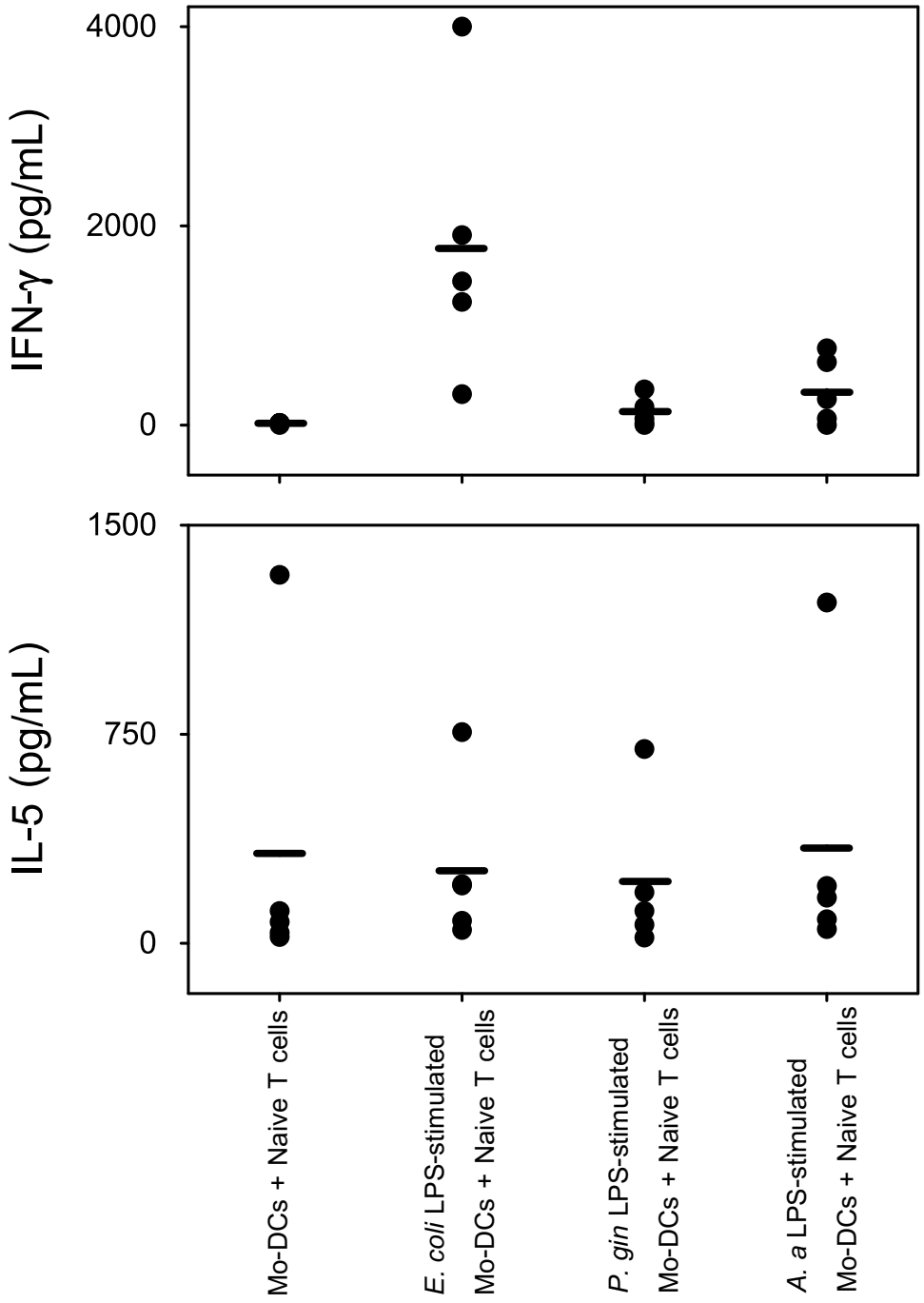


Figure 6. The stimulatory capacity of bacterial LPS-stimulated Mo-DCs on allogeneic naïve $CD4^+$ T cells in MLR. Control Mo-DCs and *P. gingivalis*, *A. actinomycetemcomitans*, or *E. coli* LPS stimulated-MoDCs were used to prime naïve $CD4^+$ T cells for 5 days, and then cultured supernatant were assayed for IFN-γ and IL-5 production. Each spot represents the cytokine levels of individual experiment (n = 5) and horizontal lines are mean values.

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

Recently we have established OVA-specific Th differentiation in 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) (data not shown). Therefore, in this experiment we examined the ability of LPS from *P. gingivalis*, *A. actinomycetemcomitans* and *E. coli* in the induction of OVA-specific Th response *in vivo* mouse model. 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=1810 ± 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 showed to produce low amount of both antigen-specific IFN-γ and IL-5 (102 ± 44 pg/ml, and 106 ± 29 pg/mL, respectively) (Table4, Fig. 7).

| Ova + ISA720 | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| a | 63.80 | 706.90 |
| b | 453.20 | 1757.20 |
| c | 324.00 | 933.20 |
| d | 150.30 | 517.50 |
| Mean ± SE | 247.83 ± 87.25 | 978.70 ± 273.06 |

| OVA + ISA720 + E. coli LPS | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| a | 225.00 | 161.30 |
| b | 11.80 | 28.30 |
| c | 88.60 | 99.90 |
| d | 84.50 | 133.30 |
| Mean ± SE | 102.48 ± 44.49 | 105.70 ± 28.69 |

| OVA + ISA720 + CpG | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| a | 2253.30 | 11.60 |
| b | 847.50 | 7.00 |
| c | 3368.60 | 27.50 |
| d | 771.30 | 7.00 |
| Mean ± SE | 1810.18 ± 621.23 | 13.28 ± 4.86 |

| OVA + ISA720 + P. gin LPS | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| a | 254.80 | 445.10 |
| b | 629.10 | 1308.90 |
| c | 588.00 | 833.90 |
| d | 226.00 | 309.40 |
| Mean ± SE | 424.48 ± 106.77 | 724.33 ± 224.33 |

| OVA + ISA720 + A. a LPS | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| a | 72.70 | 121.00 |
| b | 148.60 | 199.30 |
| c | 153.80 | 302.00 |
| Mean ± SE | 125.03 ± 26.21 | 207.43 ± 52.41 |

| Mean ± SE | IFN-γ (pg/mL) | IL-5 (pg/mL) |
|---|---|---|
| Ova++ISA720 | 247.83 ± 87.25 | 978.70 ± 273.06 |
| OVA+ISA720+E.coli LPS | 102.48 ± 44.49 | 105.70 ± 28.69 |
| OVA+ISA720+CpG | 1810.18 ± 621.23 | 13.28 ± 4.86 |
| OVA+ISA720+P. gin LPS | 424.48 ± 106.77 | 724.33 ± 224.33 |
| OVA+ISA720+A. a LPS | 125.03 ± 26.21 | 207.43 ± 52.41 |

**Table 4.** Cytokine production from antigen-specific mouse model *in vivo*.

Note: One mouse died in OVA + ISA720 + Aa LPS before the completion of the experiment.

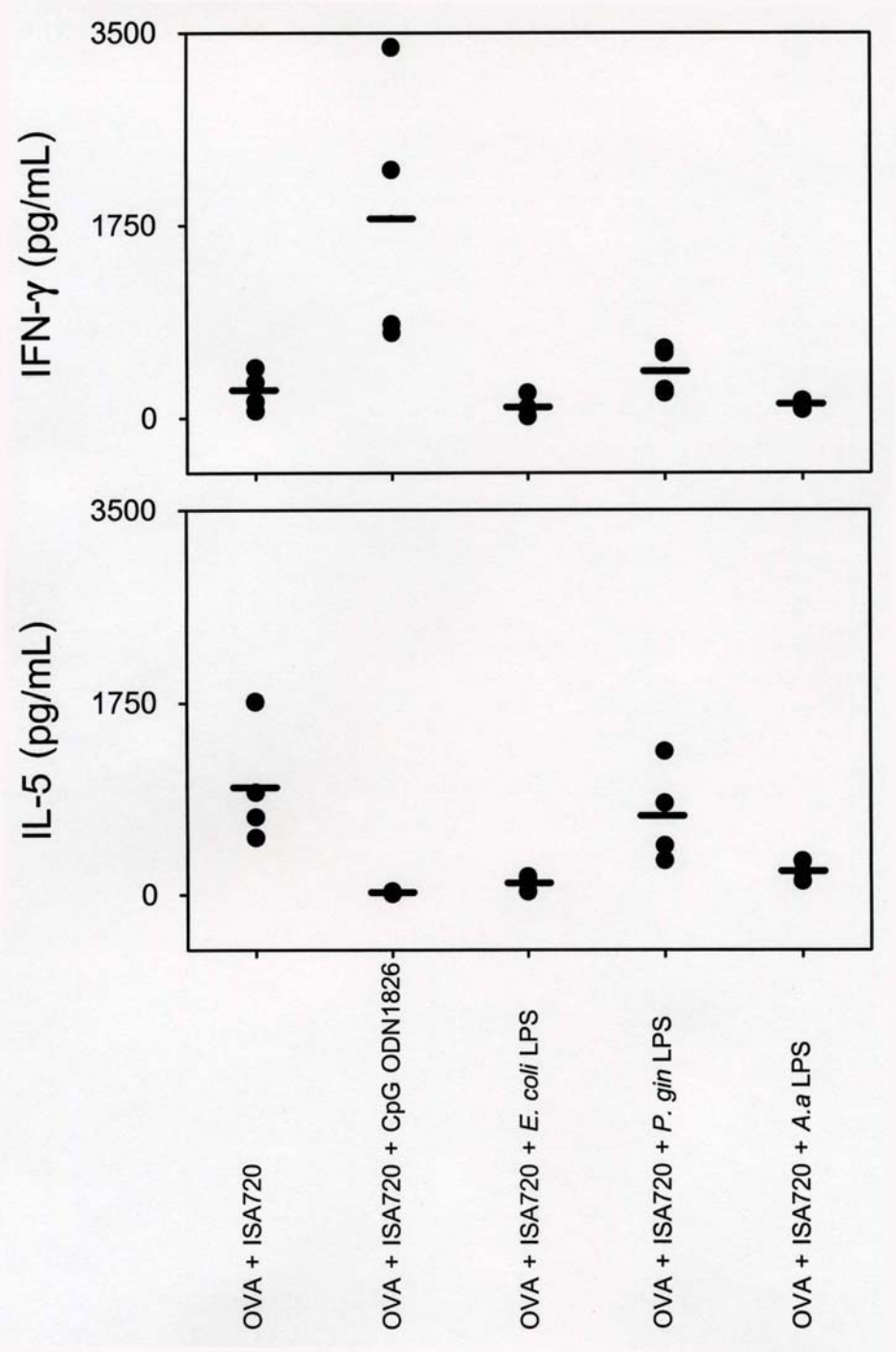


**Figure 7.** Induction of OVA-specific T cell response. Splenocytes collected from four groups of mice were evaluated for antigen-specific IFN-γ and IL-5 production. Each spot represents the levels of cytokine produced from each individual animal and horizontal lines are mean values.

## 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 (Bainbridge *et al*., 2001; Martin *et al*., 2001). Recently *A. actinomycetemcomitans* LPS has been reported to bind to TLR4 (Yoshimura *et al*., 2002; Mochizuki *et al*., 2004). Binding between certain TLRs on DCs with different PAMPS in DC-pathogen interaction is crucial and contribute to different types of adaptive immunity (Th1/Th2). In this study we evaluated the effects of LPS derived from *P. gingivalis* and *A. actinomycetemcomitans* on Mo-DCs with regards 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 low levels of co-stimulatory molecule (CD40, CD80), HLA-DR and maturation marker (CD83) expression. Both *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS induced low levels of TNF-$\alpha$ and negligible amounts of IL-10 as compared with *E. coli* LPS. All the 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 weak stimulator for DCs agree with the study of Kanaya *et al.* (2004) with regard to the induction of co-stimulatory molecule expression and cytokine production. The results on the effect of *A. actinomycetemcomitans* LPS on DCs are different from the recent study of Kikuchi *et al*. (2004) which demonstrates 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-$\gamma$ 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-$\gamma$ and high IL-5 response). Pulendran *et al*. (2001) 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. We do not have good explanation for this discrepancy. The conflicting results could be due to the different concentrations of OVA used. We administered 40-fold less amount of the antigen. The concentration of antigen is known to influence Th1 and Th2 differentiation (Boonstra *et al*., 2003).

In conclusion, our study showed that *P. gingivalis* and *A. actinomycetemcomitans* LPS poorly 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 seem to drive Th2-bias.

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# ภาคผนวก

1. Research report submitted to Journal of Dental Research

# The Effects of *Porphyromonas gingivalis* LPS and *Actinobacillus actinomycetemcomitans* LPS on Human Dendritic Cells.

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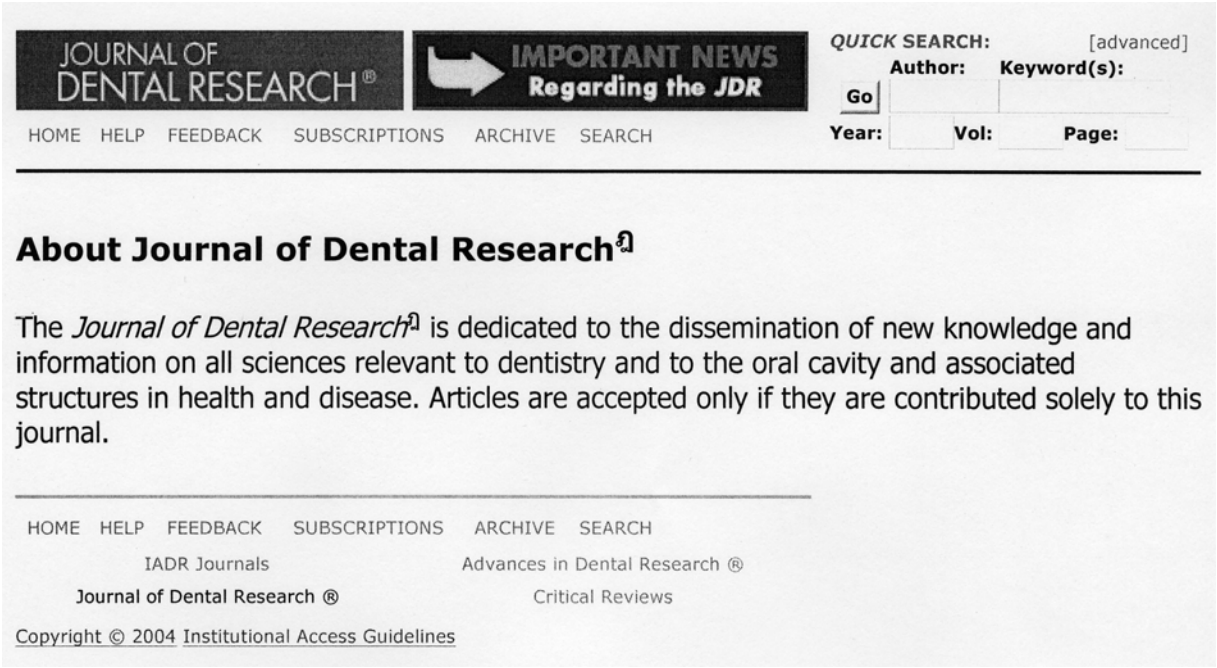


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The Effects of *Porphyromonas gingivalis* LPS and *Actinobacillus actinomycetemcomitans* LPS on Human Dendritic Cells

***Key findings***: *Porphyromonas gingivalis* LPS and *Actinobacillus actinomycetemcomitans* LPS poorly activated human monocyte-derived dendritic cells with regards to co-stimulatory molecule expression and cytokine production. Data from mixed leukocyte reaction and antigen-specific T cell response in mouse model suggest that LPS from two bacterial plaque LPS seem to drive Th2-bias.

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**KEY WORDS:** Dendritic cells, *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans*, LPS, Th response

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Abstract

Interaction between different plaque pathogens with 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 regard to co-stimulatory molecule expression, cytokine production and induction of Th cell differentiation. Unlike *Escherichia coli* and *A. actinomycetemcomitans* LPS, *P. gingivalis* LPS induced low levels of CD40, CD80, HLA-DR and CD83 expression on Mo-DCs. Both bacterial plaque LPS-stimulated Mo-DCs produced low TNF-$\alpha$ and minimal IL-10 as compared with *E. coli* LPS. All LPS induced negligible or no IL-12 production. Human mixed-leukocyte reaction and ovalbumin-specific T cell response in mouse model demonstrated that both bacterial plaque LPS induced suppressed levels of IFN-$\gamma$ production. In conclusion, *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS seem to poorly induce Mo-DC maturation and drive Th2-bias.

Introduction

The development of T helper1 (Th1)- versus Th2-type response is known as fundamental important in determining whether the response to infectious pathogens will be protective or no protective (Romagnani, 1997). The Th1 or Th2 response, on the other hand that continues unabated may cause overproduction of cytokines leading to damage of host normal tissues. High levels of inflammatory mediators as well as numerous infiltrated T and B cells are the characteristics of periodontitis, the advanced form of periodontal disease (Gemmell and Seymour, 2004). Accumulating data regarding Th1- and Th2-type response in periodontitis are however in conflict (Gemmell and Seymour, 2004). This inconclusive piece of information is crucial in the pathogenesis of periodontitis and still lacking. To date, dendritic cells (DCs) have been well recognized for the critical role not only in the initiation of naïve T cell priming but also in controlling the type of Th response. The mechanisms of how DCs induce Th1 and Th2-type response have been the subject of intense investigation. Several determined factors in Th differentiation include interleukin (IL)-12 (Trinchieri *et al*., 2003), IL- 10 (Liu *et al*., 1998), ICAM1 expression (Salomon and Bluestone, 1998), dose of antigen (Boonstra *et al*., 2003), Notch ligands (Amsen *et al*., 2004) and DC subsets (Kalinski *et al*., 1999 ).

In periodontitis, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are key pathogens (Slots, 1999). They habitat as microbial plaque biofilms adjacent to gingiva which are found to be enriched by antigen presenting cells (APCs). Langerhans cells are presence in gingival epithelium while tissue DCs are presence in lamina propria (Cirrincione *et al*., 2002; Jotwani and Cutler, 2003). Significant up-regulation of CD83 (mature DC antigen) and co-stimulatory molecule expression on DCs and gingival B cells was detected

in periodontitis tissues (Mahanonda *et al*., 2002; Jotwani and Cutler, 2003). These $CD83^+$ cells were associated with clusters of $CD4^+$ T cells, thus indicating close interaction of T cells and APCs in gingival microenvironment (Jotwani and Cutler, 2004). 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 regards to co-stimulatory molecule expression, cytokine production and the induction of Th cell differentiation. Human mixed-leukocyte reaction (MLR) and *in vivo* ovalbumin (OVA)-specific T cell response in mouse model were used to explore the type of Th response induced by LPS of the two plaque bacterial pathogens in comparison with *Escherichia coli* LPS.

## Materials and Methods

### Antibodies and Reagents

RPMI 1640 medium supplemented with 2 mM L-glutamine, 80 ug/mL of gentamycin (Gibco Laboratory, Grand Island, NY, USA), and 10% heat-inactivated autologous serum was 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 System 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-$\gamma\delta$ (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 (Schifferle *et al*., 1998; Preshaw *et al*., 1999; Morozumi *et al*., 2001). 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 Mo-DCs and stimulation with bacterial LPS

Under a protocol approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, subjects signed a consent form after being advised of the nature of the study. Peripheral

blood was obtained from healthy donor. 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 ($5x10^5$ cells/mL) were stimulated with different concentrations of LPS from *P. gingivalis*, *A. actinomycetemcomitans*, and *E. coli* (0, 100, 300, 1000 ng/mL) for 24 hrs. Culture supernatants were analyzed for tumor necrosis factor (TNF)-$\alpha$, IL-10 and IL-12 p70 production using ELISA. The cells were harvested and stained with different 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). The levels of expression were presented by mean fluorescence intensity (MFI).

**Mixed leukocyte reaction**

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-$\gamma\delta$ (PE). This technique provides $CD4^+CD45RA^+$ naïve T cells with a purity of >90%. Flow cytometrically sorted allogeneic naïve T cells ($5x10^5$ cells/mL) were added in cultured wells that contained control and LPS-stimulated Mo-DCs ($5x10^4$ cells/mL). Culture supernatants were harvested on day 5 and then analyzed for IFN-$\gamma$ and IL-5 production using ELISA.

**Antigen-specific T cell response**

Female BALB/c mice, 6-8 weeks were obtained from National Laboratory Animal Centre, Thailand. Mice were divided into 4 groups (n = 4 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 microgram of OVA in phosphate-buffered saline (PBS) was emulsified with Montanide ISA720 at the ratio of 3/7 parts in the presence of 50 $\mu$g of CpG ODN 1826, 50 $\mu$g of *E. coli* LPS, 50 $\mu$g of *P. gingivalis* LPS, or 50 $\mu$g of *A. actinomycetemcomitans* LPS. Mice were immunized with 100 $\mu$L of each vaccine at day 0, 14 and 28 via subcutaneous, subcutaneous and intraperitoneal routes respectively. Splenocytes were harvested on day 38. They ($5x10^6$cells/mL) were stimulated with OVA (40 $\mu$g/mL) for 5 days. Culture supernatants were harvested and then assayed for OVA-specific IFN-$\gamma$ and IL-5 production.

**Detection of 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 plaque bacteria, *P. gingivalis* and *A. actinomycetemcomitans* to induce DC maturation in comparison with a well known *E. coli* LPS. Expression of HLA-DR, CD40, CD80, and CD83 and their cytokine production (TNF-α, IL-10, and IL-12 p70) were measured after 24 hr stimulation of Mo-DCs with different bacterial LPS. Fig. 1A showed that the expression of HLA-DR, CD40, CD80, and CD83 on Mo- DCs was up-regulated in a dose dependent manner upon bacterial LPS stimulation. *A. actinomycetemcomitans* LPS and *E. coli* LPS enhanced similar expression levels of these molecules and the levels were higher than *P. gingivalis* LPS at each corresponding concentration. With regard to LPS-induced cytokine production, both bacterial plaque LPS induced low levels of TNF-α and negligible amounts of IL-10 from Mo-DCs. On the contrary, *E. coli* LPS appeared to be a potent inducer for TNF-α production and induced moderate amounts of IL-10. Little or no IL-12 p70 production was detected in all 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. In line with the weak cytokine induction, *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 $\pm$ 67 and 344 $\pm$ 153 pg/mlL, respectively) as compared with higher IFN-γ levels in those MLR cultures with *E. coli* LPS -stimulated Mo-DCs (1778 $\pm$ 612 pg/mL). Similar low levels of IL-5 production were detected in all MLR cultures (mean values ranged from 216 – 345 pg/mL) (Fig. 2).

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

Recently we have established OVA-specific Th differentiation in 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) (data not shown). Therefore, in this experiment we examined the ability of LPS from *P. gingivalis*, *A. actinomycetemcomitans* and *E. coli* in the induction of OVA-specific Th response *in vivo* mouse model. 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=1810 ± 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 both antigen-specific IFN-γ and IL-5 (102 ± 44 pg/mL, and 106 ± 29 pg/mL, respectively) (Fig. 3).

**Figure legends**

**Figure 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 hrs. 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 representatives of 4 independent experiments and shown as MFI. Data on cytokines are mean ± SE.

**Figure 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 5 days, and then cultured supernatant were assayed for IFN-γ and IL-5 production. Each spot represents the cytokine levels of individual experiment (n = 5) and horizontal lines are mean values.

**Figure 3.** Induction of OVA-specific T cell response. Splenocytes collected from four groups of mice were evaluated for antigen-specific IFN-γ and IL-5 production. Each spot represents the levels of cytokine produced from 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 (Bainbridge *et al*., 2001; Martin *et al*., 2001). Recently *A. actinomycetemcomitans* LPS has been reported to bind to TLR4 (Yoshimura *et al*., 2002; Mochizuki *et al*., 2004). Binding between certain TLRs on DCs with different PAMPS in DC-pathogen interaction is crucial and contribute to different types of adaptive immunity (Th1/Th2). In this study we evaluated the effects of LPS derived from *P. gingivalis* and *A. actinomycetemcomitans* on human Mo-DCs with regards 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 low levels of co-stimulatory molecule (CD40, CD80), HLA-DR and maturation marker (CD83) expression. Both *P. gingivalis* LPS and *A. actinomycetemcomitans* LPS induced low levels of TNF-$\alpha$ and negligible amounts of IL-10 as compared with *E. coli* LPS. All the 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 weak stimulator for DCs agree with the study of Kanaya *et al*. (2004) with regard to the induction of co-stimulatory molecule expression and cytokine production. The results on the effect of *A. actinomycetemcomitans* LPS on DCs are different from the recent study of Kikuchi *et al*. (2004) which demonstrates 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-$\gamma$ 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-$\gamma$ and high IL-5 response). Pulendran *et al*. (2001) 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. We do not have good explanation for this discrepancy. The conflicting results could be due to the different concentrations of OVA used. We administered 40-fold less amount of the antigen. The concentration of antigen is known to influence Th1 and Th2 differentiation (Boonstra *et al*., 2003).

In conclusion, our study showed that *P. gingivalis* and *A. actinomycetemcomitans* LPS poorly 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 seem to drive Th2-bias.

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