

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 6
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
JUAN PABLO CADENA SARMIENTO

Asunto: Radicación: 8-70809- -11
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 2509
 Folios: 6

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2007/060222				
Fecha de Presentación Internacional	08/01/2007				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 08-070809-00000-0000	9/Julio/2008
Reivindicaciones:	Radicado No. 08-070809-00000-0000	9/Julio/2008
	Radicado No. 08-070809-00005-0000	8/Octubre/2012
Dibujos:	Radicado No. 08-070809-00000-0000	9/Julio/2008
Prioridad:	US 61/026,207	5/Febrero/2008
	US 61/095,429	9/Septiembre/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso, se tiene en cuenta la totalidad del nuevo capítulo reivindicatorio (Radicado No. 08-070809-00005-0000) para el examen de patentabilidad.

Título de la solicitud: “MÉTODO PARA MODULAR RECEPTORES DE LA SUPERFICIE CELULAR PARA PREVENIR O REDUCIR LA INFLAMACIÓN”.

El problema técnico consiste en proporcionar métodos para el diagnóstico y tratamiento de la inflamación.

Para solucionar este problema, la solicitud estudiada proporciona un método para determinar el efecto antiinflamatorio de un agente que incluye al menos una bacteria o porción de bacteria y una célula gingival, y un indicador que detecte la presencia de dichas bacterias. El método se basa en la detección del indicador, una ausencia sustancial del material indicador significa que la especie de bacteria es deletérea.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12Q 1/04; G01N 33/50, 33/569.

4. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 carece de claridad y concisión porque utiliza las expresiones “célula”, “agente”, “una bacteria deletérea”, “porción de dicha bacteria”, “un compuesto indicador”, “un receptor tipo toll”, “agentes que exhiben una concentración inhibitoria mínima (CIM) de menos de 20%”, lo cual dificulta determinar la extensión del objeto que se busca proteger. El método reclamado comprende las etapas de 1) poner en contacto la célula con el agente en presencia de una bacteria deletérea y 2) detectar la presencia de un compuesto indicador. Sin embargo, no se describen las condiciones de las etapas, indicando, por ejemplo, las condiciones para la detección del “compuesto indicador”. Igualmente, según la descripción, la etapa de poner en contacto la célula con el agente puede llevarse a cabo *in vivo* (Fl. 24), por lo que el método no sería patentable, por tratarse de un método de tratamiento o de diagnóstico, de acuerdo al Art 20, lit. d) de la D 486. De la misma forma, es preciso indicar que la invención no está suficientemente descrita, pues no comprende ejemplos que permitan indicar que la misma puede ponerse en práctica por la persona del oficio normalmente versada en la materia. La descripción se limita a establecer las condiciones generales por las que se puede detectar la presencia del compuesto o el tipo de bacterias deletéreas que pueden utilizarse. Sin embargo, no se muestra un ejemplo por medio del cual se determine efectivamente el efecto antiinflamatorio de un agente en especial, a través del uso de alguna técnica para detectar directamente una molécula generada por la modulación del receptor tipo toll.

Se requiere al solicitante hacer las aclaraciones necesarias.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: enero 10 de 2006, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D2	Clinical Chemistry, Vol. 51, Págs. 2252-2256	"Development of an in vitro screening assay to test the anti-inflammatory properties of dietary supplements and pharmacological agents"	Diciembre/2005
D3	Journal of Medical Microbiology, Vol. 51, Págs. 626-634	"Priming of human oral epithelial cells by interferón-gamma to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans"	Agosto/2002

D2¹ divulga un método para determinar el efecto antiinflamatorio de un agente caracterizado porque comprende poner en contacto la célula con el agente en presencia de lipopolisacárido bacteriano (LPS (Pág. 2253).

D3² enseña un método para determinar el nivel de moléculas generadas por la modulación de un receptor tipo toll caracterizado porque comprende poner en contacto la célula con el agente con una bacteria deletérea, o una porción de dicha bacteria, y detectar la presencia del compuesto indicador (Pág. 628, Col. izquierda, Párr. 1).

6. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en un método para determinar el efecto antiinflamatorio de un agente caracterizado porque comprende poner en contacto la célula con el agente en presencia de una bacteria deletérea, o una porción de dicha bacteria, y detectar la presencia de un compuesto indicador, en donde la ausencia sustancial de un material indicador significa que el agente es antiinflamatorio, donde el material indicador es seleccionado del grupo que consiste en moléculas generadas por la modulación de un receptor tipo toll, el agente antiinflamatorio se selecciona de agentes que exhiben una concentración inhibitoria mínima (CIM) de menos de 20% y el material indicador es detectado directamente.

Características esenciales	D2	D3
Un método para determinar el efecto antiinflamatorio de un agente caracterizado porque comprende poner en contacto la célula con el agente en presencia de una bacteria deletérea, o una porción de dicha bacteria, y detectar la presencia de un compuesto indicador, en donde la ausencia sustancial de un material indicador significa que el agente es antiinflamatorio, donde el	Un método para determinar el efecto antiinflamatorio de un agente caracterizado porque comprende poner en contacto la célula con el agente en presencia de lipopolisacárido bacteriano (LPS), y detectar la presencia de un compuesto indicador, en donde la ausencia sustancial de un material indicador significa que el agente es antiinflamatorio, donde el	Un método para determinar el nivel de moléculas generadas por la modulación de un receptor tipo toll caracterizado porque comprende poner en contacto la célula con el agente con una bacteria deletérea, o una porción de dicha bacteria, y detectar la presencia del compuesto indicador. (Pág. 628, Col. izquierda,

1 <http://www.ncbi.nlm.nih.gov/pubmed/16166164>

2 <http://www.ncbi.nlm.nih.gov/pubmed/12171292>

material indicador es seleccionado del grupo que consiste en moléculas generadas por la modulación de un receptor tipo toll, el agente antiinflamatorio se selecciona de agentes que exhiben una concentración inhibitoria mínima (CIM) de menos de 20% y el material indicador es detectado directamente.	material indicador es seleccionado del grupo que consiste en moléculas generadas por la modulación de un receptor tipo toll. (Pág. 2253)	Párr. 1)
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El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga un método para determinar el efecto antiinflamatorio de un agente caracterizado porque comprende poner en contacto la célula con el agente en presencia de lipolisacárido bacteriano (LPS), y detectar la presencia de un compuesto indicador, en donde la ausencia sustancial de un material indicador significa que el agente es antiinflamatorio, donde el material indicador es seleccionado del grupo que consiste en moléculas generadas por la modulación de un receptor tipo toll, como IL-1beta, TNF-alfa y IL-6 (Pág. 2253).

La diferencia entre la invención, definida en la reivindicación 1 y el método del documento D2 consiste en que el método de la solicitud pone en contacto con la célula una bacteria deletérea, o una porción de dicha bacteria.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja al método reclamado en esta solicitud porque no hay evidencia del tipo de bacteria o de la porción de dicha bacteria que pueda utilizarse efectivamente en un método para determinar el efecto antiinflamatorio de un agente, según lo establecido en la descripción.

El problema técnico objetivo de esta solicitud consiste en la necesidad de un método basado en la determinación de moléculas generadas por la modulación de un receptor tipo toll alternativo al ya conocido en el documento D2.

Teniendo en cuenta que el documento D2 ya divulgaba un método para determinar el efecto antiinflamatorio de un agente caracterizado porque comprende poner en contacto la célula con el agente en presencia de lipolisacárido bacteriano (LPS), y detectar la presencia de un compuesto indicador, en donde la ausencia sustancial de un material indicador significa que el agente es antiinflamatorio, donde el material indicador es seleccionado del grupo que consiste en moléculas generadas por la modulación de un receptor tipo toll, como IL-1beta, TNF-alfa y IL-6 (Pág. 2253), y que el documento D3 enseña igualmente un método para determinar el nivel de moléculas generadas por la modulación de un receptor tipo toll caracterizado porque comprende poner en contacto la célula con el agente con LPS, y detectar la presencia del compuesto indicador (Pág. 628, Col. izquierda, Párr. 1), donde el LPS es purificado de una bacteria (Pág. 627, Col. izquierda, Párr. 3), la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, utilizar el método del documento D2, considerando que el LPS es una porción de una bacteria, para determinar el efecto antiinflamatorio de un agente para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y en las reivindicaciones 2 – 11 es obvia y no tiene nivel inventivo.

7. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D2	Clinical Chemistry, Vol. 51, Págs. 2252-2256	1 – 11	Nivel inventivo
D3	Journal of Medical Microbiology, Vol. 51, Págs. 626-634	1 – 11	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-03-04

Elaboró: Moncaleano Prado Juan David

Revisó: Consuelo Leguizamon Leguizamon

Development of an In Vitro Screening Assay to Test the Antiinflammatory Properties of Dietary Supplements and Pharmacologic Agents

UMA SINGH, JAMES TABIBIAN, SENTHIL K. VENUGOPAL, SRIDEVI DEVARAJ, and
ISHWARLAL JIALAL*

Background: Monocytes and macrophages are critical in atherosclerosis and on stimulation secrete proinflammatory, proatherogenic cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , which have been shown to be present in atherosclerotic lesions. The aim of this study was to develop a rapid in vitro screening assay to test the antiinflammatory effects of different compounds.

Methods and Results: THP-1 cells (human monocytic cell line) were stimulated with different concentrations of lipopolysaccharide (LPS; 0 to 1000 $\mu\text{g/L}$) and for different times (4, 12, and 24 h), and the secretion of proinflammatory cytokines (IL-1, IL-6, and TNF- α) was assessed. TNF- α secretion was maximum at the lowest LPS concentration (100 $\mu\text{g/L}$) and at shortest duration of incubation (4 h). Maximum secretion of IL-1 β and IL-6 was achieved at 24 h with higher doses of LPS. Treatment of THP-1 with various test compounds such as dietary supplements (α -tocopherol, N-acetylcysteine, catechin and epigallocatechin gallate) as well as pharmacologic agents (statins, peroxisome proliferator-activated receptor- γ agonists, and an angiotensin II receptor blocker) significantly inhibited LPS-stimulated TNF- α release.

Conclusions: The release of TNF- α after stimulation of THP-1 cells with LPS is a valid model system to test novel compounds for potential antiinflammatory effects.

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Atherosclerosis is a complex inflammatory process that is characterized by the presence of monocytes/macrophages and T lymphocytes in the atheroma (1). Fatty streaks, the earliest detectable lesions in atherosclerosis, contain macrophage-derived foam cells that differentiate from recruited blood monocytes. In addition, more advanced atherosclerotic lesions such as fibro-fatty plaques are the result of monocyte recruitment together with smooth muscle cell migration and proliferation. Moreover, macrophage density is greater in the shoulder region of plaques that rupture. Monocytes/macrophages are pivotal in atherosclerosis and are present at all stages of atherosclerosis, from nascent fatty streak lesions to culmination in acute coronary syndromes (2).

Macrophages promote atherosclerosis via production of various key biomediators (3), including cytokines such as interleukin (IL)-1 β ,¹ tumor necrosis factor- α (TNF- α), and IL-6; chemokines such as IL-8 and monocyte chemoattractant protein-1 (MCP-1); matrix metalloproteinases; and integrins (CD11b and VLA4). Although concentrations of the cytokines IL-1, IL-6, and TNF- α are increased in patients with increased risk of atherosclerosis (3–5), monocytic concentrations of these cytokines appear to be a more sensitive indicator of inflammatory status (6). Importantly, monocytic function has been reported to be altered in patients with increased cardiovascular risk, including dyslipidemia, diabetes, smoking, and endstage renal disease (7–9).

Therapies that have been shown to reduce cardiovascular events appear to have antiinflammatory properties (10–17). This has been well documented with the hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibi-

Laboratory for Atherosclerosis and Metabolic Research, Department of Pathology & Laboratory Medicine, University of California, Davis Medical Center, Sacramento, CA.

* Address correspondence to this author at: Laboratory for Atherosclerosis and Metabolic Research, Department of Pathology & Laboratory Medicine, University of California, Davis Medical Center, 4635 IInd Ave., Res Bldg. 1, Rm 3000, Sacramento, CA 95817. Fax 916-734-6593; e-mail ishwarlal.jialal@ucdmc.ucdavis.edu.

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¹ Nonstandard abbreviations: IL, interleukin; TNF- α , tumor necrosis factor- α ; MCP-1, monocyte chemoattractant protein-1; HMG-CoA, hydroxymethylglutaryl-CoA; NAC, N-acetylcysteine; AT, α -tocopherol; EGCG, epigallocatechin gallate; LPS, lipopolysaccharide; ARB, angiotensin receptor type I blocker; CRP, C-reactive protein; and PPAR- γ , peroxisome proliferator-activated receptor- γ .

tors (statins) (10–12). Newer, specific antiinflammatory therapies are also being developed; it therefore is essential to have a simple in vitro screening assay to test their antiinflammatory effects. Because inflammation is pivotal to atherosclerosis and the monocyte/macrophage is a critical participant, we developed an in vitro model system of monocytic cells to test the antiinflammatory properties of various compounds, i.e., dietary supplements and pharmacologic agents.

Materials and Methods

Human monocytic leukemia THP-1 cells were obtained from the American Type Culture Collection. The cells were maintained in endotoxin-free RPMI-1640 containing 5.5 mmol/L glucose, 50 μ mol/L mercaptoethanol, 100 mL/L fetal bovine serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 10 mmol/L HEPES and were used for experiments between the third and fifth passages. The various cytokine ELISAs used were obtained from R&D systems. *N*-Acetylcysteine (NAC), vitamin E [α -tocopherol (AT)], catechin, epigallocatechin gallate (EGCG), ciglitazone, and lovastatin were obtained from Sigma Chemical Company. Candesartan was obtained from AstraZeneca (Wilmington, DE) as a kind gift. All of these compounds were dissolved in either dimethyl sulfoxide or ethanol. The concentration of vehicle control never exceeded 1 mL/L in culture medium. A vehicle control was run in all experiments. Cell viability, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, was >95% in all experiments.

LIPOPOLYSACCHARIDE STIMULATION OF THP-1 CELLS

The human monocytic leukemia cell line THP-1 was chosen for this study because it is a highly differentiated monocytic cell line with phagocytic properties and has Fc as well as C3b receptors. Indeed, it is the most commonly used model to study the biology of foam cell formation because it can be easily induced to a macrophage phenotype after phorbol ester treatment (18). Furthermore, these cells have been reported to produce proinflammatory cytokines (IL-1, IL-6, and TNF) and chemokines (IL-8 and MCP-1) in response to lipopolysaccharide (LPS) stimulation (19, 20). The THP-1 cell line rather than human monocytes was used in this in vitro model system to minimize variability and to allow for high throughput. Using the THP-1 cells, we have demonstrated that under hyperglycemic conditions, superoxide anion and IL-6 release are increased, as observed in diabetic monocytes, and have elucidated the molecular mechanisms that mediate the increased superoxide anion and cytokine release from diabetic monocytes (21–23). Thus, like other investigators, we have found that the THP-1 cell line is the best in vitro model system to understand monocyte/macrophage biology as it relates to human disease.

The cells were grown in 75-mm² flasks in RPMI supplemented with fetal bovine serum until they attained 70% confluency. On reaching confluency, the cells were

plated in 12-well tissue culture plates ($\sim 5 \times 10^5$ cells/mL) in serum-free medium at 37 °C in 5% CO₂. The cells were challenged with different concentrations (0–1000 μ g/L) of LPS for different times (4, 12, and 24 h). The supernatants were harvested after each time point and stored frozen at –20 °C until analysis. IL-1 β , TNF- α , and IL-6 were quantified in all supernatants. The cells were lysed in 0.1 mol/L NaOH. The results for release of each cytokine are reported as per milligram of protein. The intra- and interassay CVs for the cytokine assays were <10%. The specific time points at which maximum cytokine stimulation was achieved were noted.

TESTING THE ANTIINFLAMMATORY ACTIVITY OF VARIOUS COMPOUNDS

The various compounds selected were divided into 2 categories: dietary supplements and pharmacologic agents. From our preliminary experiments, we found that TNF- α was the cytokine released at the earliest time point (4 h as opposed to 24 h for IL-1 β and IL-6) and also at the lowest LPS concentration (half maximum ~ 50 μ g/L) as discussed in *Results*. Therefore, for all of our future experiments testing the antiinflammatory effects of these compounds, THP-1 cells were incubated with LPS (50 μ g/L) for a duration of 4 h and TNF- α concentrations were assayed in supernatants. THP-1 cells were pretreated with different concentrations of various compounds at biologically relevant concentrations. After 1 h of pretreatment with these compounds, the cells were challenged with LPS (50 μ g/L) for 4 h. The supernatants were used for measurement of TNF- α . The range of TNF- α calibrators was 0–1000 ng/L.

STATISTICAL ANALYSIS

All experiments were repeated at least 3 times. The Student unpaired *t*-test was used to compute the differences, with significance set at 5%. ANOVA was used to assess dose-response effects.

Results

DOSE RESPONSE FOR LPS-STIMULATED THP-1 CELLS

THP-1 cells revealed maximum stimulation of TNF- α at an LPS concentration of 100 μ g/L after a 4-h incubation (Fig. 1); however, both IL-1 β and IL-6 continued to increase with maximum release after 24 h at the highest LPS concentration (≥ 500 μ g/L; Fig. 2). Whereas LPS induced the release of all 3 cytokines, TNF- α secretion was maximum at the lowest LPS concentration and shortest duration of incubation. The calculated half-maximum dose of LPS required for TNF- α secretion at 4 h was 50 μ g/L. Thus, all further experiments used TNF- α as an endpoint with incubation for 4 h at an LPS concentration of 50 μ g/L.

EFFECT OF VARIOUS DIETARY SUPPLEMENTS ON TNF- α RELEASE

THP-1 cells were pretreated for 1 h with various compounds or vehicle control. The results for TNF- α release from THP-1 cells pretreated with various dietary supple-

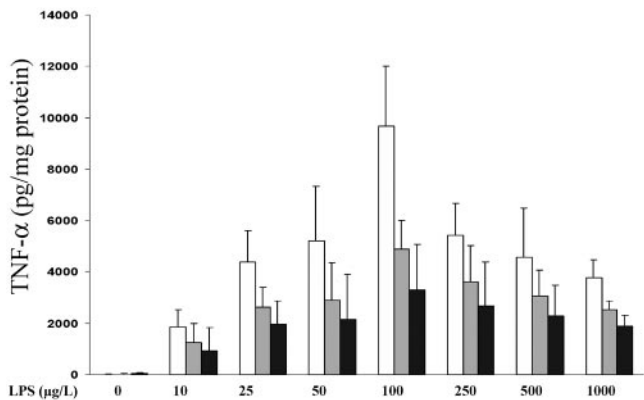


Fig. 1. Dose response of LPS stimulation on TNF- α secretion in THP-1 cells. Cells were stimulated with different concentrations of LPS (0–1000 $\mu\text{g/L}$) for 4 ($\square$), 12 ($\blacksquare$), and 24 h ($\blacksquare$). TNF- α was measured in culture supernatants as described in the *Materials and Methods*. Data are the mean (SD; error bars) of 3 experiments performed in duplicate.

ments, AT (25, 50, and 100 $\mu\text{mol/L}$), catechin (0.25, 0.5, and 1 $\mu\text{mol/L}$), and EGCG (10–50 $\mu\text{mol/L}$) are shown in Table 1. The results revealed that AT inhibited TNF- α

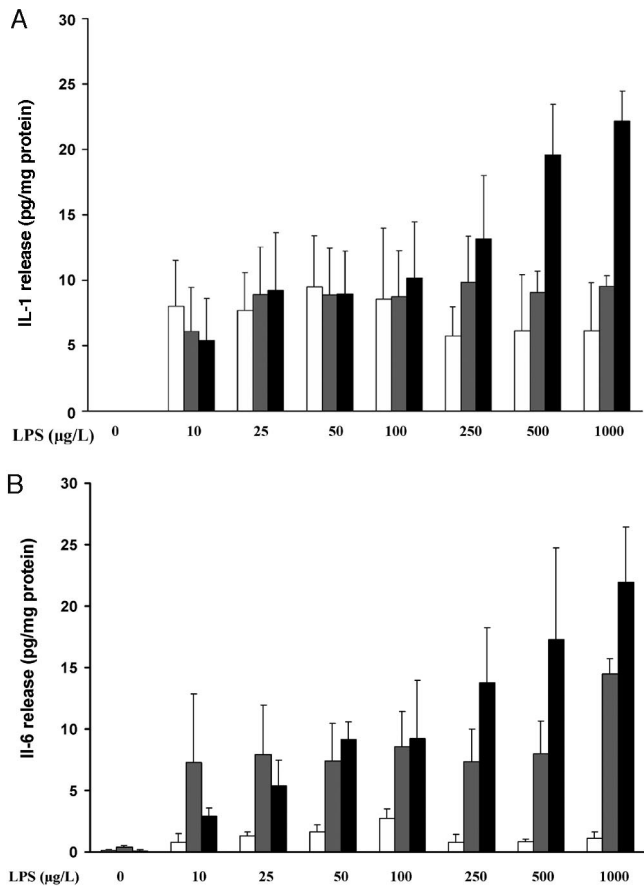


Fig. 2. Dose response of LPS stimulation on IL-1 β (A) and IL-6 (B) secretion in THP-1 cells. Cells were stimulated with different concentrations of LPS (0–1000 $\mu\text{g/L}$) for 4 ($\square$), 12 ($\blacksquare$), and 24 h ($\blacksquare$). IL-1 β or IL-6 was measured in culture supernatants as described in the *Materials and Methods*. Data are the mean (SD; error bars) of 3 experiments performed in duplicate.

release significantly at all concentrations used in the study: 45%, 57%, and 62% inhibition at 25, 50, and 100 $\mu\text{mol/L}$ AT, respectively. EGCG also dose-dependently inhibited TNF- α secretion (47%, 52%, and 65% inhibition at 10, 25, and 50 $\mu\text{mol/L}$ EGCG, respectively). However, for NAC, TNF- α secretion was significantly inhibited only at concentrations ≥ 3 mmol/L ($\geq 28\%$ inhibition), and for catechin, TNF- α secretion was significantly inhibited only at concentrations ≥ 0.5 $\mu\text{mol/L}$ ($\geq 42\%$ inhibition).

EFFECTS OF VARIOUS PHARMACOLOGIC AGENTS ON TNF- α RELEASE

The results for LPS-stimulated TNF- α release from THP-1 cells pretreated with various pharmacologic agents are shown in Table 2. All 3 drugs—lovastatin, ciglitazone, and candesartan—caused significant inhibition of TNF- α release. Lovastatin (a well-known HMG-CoA reductase inhibitor with pleiotropic effects) significantly inhibited TNF- α release at concentrations ≥ 1 $\mu\text{mol/L}$ (43% and 49% inhibition at 1 and 5 $\mu\text{mol/L}$, respectively; $P < 0.05$). For ciglitazone, doses ≥ 2.5 $\mu\text{mol/L}$ significantly inhibited TNF- α release (31% and 43% inhibition at 2.5 and 5 $\mu\text{mol/L}$, respectively; $P < 0.05$). Candesartan [an angiotensin receptor type I blocker (ARB)] at concentrations ≥ 1.0 $\mu\text{mol/L}$ significantly inhibited TNF- α release (45% and 49% at 1 and 5 $\mu\text{mol/L}$, respectively; $P < 0.05$).

Discussion

Inflammatory processes are recognized to play a central role in the pathogenesis of atherosclerosis and its complications. The monocyte/macrophage is a crucial cell in atherosclerosis and is present in all stages of atherosclerosis. The importance of monocytes/macrophages in atherosclerosis is underscored by knock-out studies, which showed that loss of MCP-1 or its cognate receptors leads

Table 1. Effects of various dietary supplements on TNF- α secretion in LPS-stimulated THP1 cells.^a

Treatment	TNF- α secretion, pg/mg of protein
Control	82 (38)
LPS (50 $\mu\text{g/L}$)	4772 (875) ^b
LPS + 25 $\mu\text{mol/L}$ AT	2668 (465) ^c
LPS + 50 $\mu\text{mol/L}$ AT	2057 (622) ^c
LPS + 100 $\mu\text{mol/L}$ AT	1844 (331) ^d
LPS + 0.25 $\mu\text{mol/L}$ catechin	3243 (894)
LPS + 0.5 $\mu\text{mol/L}$ catechin	2736 (734) ^e
LPS + 1.0 $\mu\text{mol/L}$ catechin	2026 (423) ^c
LPS + 10 $\mu\text{mol/L}$ EGCG	2515 (469) ^c
LPS + 25 $\mu\text{mol/L}$ EGCG	2296 (731) ^c
LPS + 50 $\mu\text{mol/L}$ EGCG	1655 (336) ^d
LPS + 1 mmol/L NAC	3725 (923)
LPS + 3 mmol/L NAC	2979 (835) ^e
LPS + 6 mmol/L NAC	1264 (125) ^c

^a The results are the mean (SD) of 3 different experiments run in duplicate.
^b $P < 0.001$, compared with control.
^{c-e} Compared with LPS stimulation alone: ^c $P < 0.01$; ^d $P < 0.001$; ^e $P < 0.05$.

Table 2. Effects of various therapeutic drugs on TNF- α secretion in LPS-stimulated THP1 cells.^a

Treatment	TNF- α secretion, pg/mg of protein
Control	69 (29)
LPS (50 μ g/L)	4310 (1409) ^b
LPS + 0.5 μ mol/L ciglitazone	4208 (1085)
LPS + 1.0 μ mol/L ciglitazone	3578 (670)
LPS + 2.5 μ mol/L ciglitazone	2973 (271) ^c
LPS + 5.0 μ mol/L ciglitazone	2476 (429) ^c
LPS + 0.5 μ mol/L lovastatin	3349 (803)
LPS + 1.0 μ mol/L lovastatin	2497 (350) ^c
LPS + 5.0 μ mol/L lovastatin	2211 (64) ^c
LPS + 0.5 μ mol/L candesartan	2880 (741)
LPS + 1.0 μ mol/L candesartan	2372 (514) ^c
LPS + 5.0 μ mol/L candesartan	2228 (464) ^c

^a The results are the mean (SD) of 3 different experiments run in duplicate.

^b $P < 0.001$ compared with control.

^c $P < 0.05$ compared with LPS stimulation alone.

to a decrease in atherosclerosis. Monocytes/macrophages secrete the proinflammatory cytokines IL-1, IL-6, and TNF- α . The plasma concentrations of several markers of inflammation have been found to be associated with future cardiovascular risk in a variety of clinical settings (4, 5, 9). The plasma half-life of cytokines is relatively short, and the monocytic release of cytokines is a more sensitive and reliable indicator of inflammatory status (3).

Thus, considering the critical role of monocytes/macrophages in the inflammatory process and atherosclerosis, we developed an in vitro cell-based system using THP-1 cells after LPS stimulation to test the antiinflammatory effects of different compounds. The rationale for using LPS as the chosen agonist is based on recent data suggesting that the LPS receptors (toll-like receptor-4) are pathogenically related to atherosclerosis (24). Such a model system depicted increased secretion of IL-1, IL-6, and TNF- α after LPS stimulation. It is pertinent here to state that all of these inflammatory cytokines are reported to be increased in atherosclerotic lesions (3). In the current study, our aim was to develop a rapid, high-throughput assay as a first-line in vitro screen to provide a cost-effective strategy to test novel antiinflammatory compounds. We therefore focused only on TNF- α secretion because secretion reached maximum at the lowest concentration of LPS and at the shortest duration of incubation.

Dietary micronutrients such as AT and flavonoids have been shown to have, in addition to their antioxidant activity, antiinflammatory properties. In this system, we tested the effects of AT, catechin, EGCG, and NAC, a potent provider of sulfhydryl groups and an inhibitor of nuclear factor- κ B. Catechins are flavonoid compounds that appear predominantly in green tea. Smaller amounts of catechins are also present in black tea, grapes, wine, and chocolate. The polyphenol catechins in green tea include galliccatechin, epigallocatechin, epicatechin, and EGCG. In experimental models, catechins showed a wide

range of protective effects, including cardioprotective and chemoprotective properties, particularly, the tea catechin EGCG (25, 26). Furthermore, high-dose AT supplementation has been shown previously to have antiinflammatory effects (27) in diabetic patients.

In preventive cardiology, one of the major breakthroughs has been the reduction in cardiovascular events as a result of the introduction of HMG-CoA reductase inhibitors (10–12), commonly known as statins. The reduction in cardiovascular endpoints with statin use could be partly ascribed to their antiinflammatory effects (27). Numerous studies have shown that statins lower C-reactive protein (CRP) (10–12), TNF- α , IL-1, IL-6, IL-8, and MCP-1 concentrations (10). In this study, we used lovastatin, which in the AFCAPS-TEXCAPS study was shown to reduce the number of cardiovascular events and lower CRP concentrations (12). We show that in our in vitro model system, lovastatin significantly inhibited LPS-stimulated TNF- α release.

The peroxisome proliferator-activated receptor- γ (PPAR- γ) agonists, as a class of drugs, improve insulin sensitivity and also have antiinflammatory properties (13, 14). This class of drugs has revolutionized the treatment of diabetes. They have been shown to up-regulate adiponectin. Numerous investigators have shown that PPAR- γ agonists decrease CRP (28, 29) and plasma MCP-1, TNF, and serum amyloid A (29) concentrations after therapy. In addition, it has been reported that PPAR- γ has pleiotropic functions, exhibiting immunomodulatory activity in obesity as well as type 2 diabetes (13). We therefore tested the effect of ciglitazone, a PPAR- γ agonist, in our model system. Ciglitazone significantly inhibited LPS-induced TNF- α release. The reduction in TNF- α in our model system could be a mechanism explaining the reduction in insulin resistance because TNF- α impairs insulin signaling (30).

Drugs that inhibit the renin-angiotensin system, such as angiotensin-converting enzyme inhibitors and ARBs, are also known to modulate the processes of inflammation and possibly the atherosclerotic process (15, 16, 31, 32). Importantly, candesartan has also been shown to exhibit pleiotropic effects by significantly lowering plasma TNF- α concentrations in hypertensive patients (31). In our in vitro model system, candesartan (an ARB) significantly inhibited LPS-stimulated TNF- α release, confirming these in vivo findings.

Thus, the findings in this study clearly demonstrate that our in vitro model system is suitable for testing antiinflammatory effects because the dietary supplements (21, 33, 34) and pharmacologic agents (13, 16, 17, 28, 31) tested in this study have been shown to display antiinflammatory properties after chronic intake in patients/volunteers. Importantly, TNF- α release has been implicated in cardiovascular-related diseases (30).

In conclusion, based on our results, we propose that the model system (LPS-stimulated monocytic release of

TNF- α) is a rapid in vitro screening assay suitable for direct testing of compounds for antiinflammatory effects. If a compound shows antiinflammatory effects in this in vitro cell-based assay, testing should be followed by studies in relevant animal models and in human volunteers, focusing on various aspects of the inflammatory cascade (soluble cell adhesion molecules, cytokines, and chemokines, as well as downstream proteins such as serum amyloid A and CRP).

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HOST RESPONSE TO INFECTION

Priming of human oral epithelial cells by interferon- γ to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans

A. UEHARA, S. SUGAWARA and H. TAKADA

Department of Microbiology and Immunology, Tohoku University School of Dentistry, Sendai 980-8575 Japan

An earlier study reported that human gingival epithelial cells in primary culture and oral epithelial cell lines KB and HSC-2 cells were devoid of membrane CD14 (mCD14) and did not show enhanced production of interleukin (IL)-8 or granulocyte macrophage-colony stimulating factor (GM-CSF) upon stimulation with bacterial cell-surface components such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN) and synthetic muramyl dipeptide (MDP) even in the presence of serum. The present study demonstrated that after treatment with interferon (IFN)- γ for 3 days, these cells secreted IL-8 and GM-CSF in response to the bacterial components. Treatment with IFN- γ enhanced Toll-like receptor (TLR) 2, TLR4, MD-2 and MyD88 mRNA expression as determined by reverse transcriptase PCR. Anti-TLR2 and anti-TLR4 monoclonal antibodies (MAbs) inhibited the IL-8 production induced by PGN and LTA as well as LPS, respectively, in IFN- γ -primed oral epithelial cells, whereas neither MAb inhibited IL-8 production induced by MDP. These findings suggested that IFN- γ primed oral epithelial cells to produce cytokines upon stimulation with various bacterial components by up-regulation of the TLR system.

Introduction

Oral epithelial cells are the first cells encountered by bacteria in the periodontal tissues. In addition to acting as a physical barrier against the invasion of pathogenic organisms, in inflamed sites oral (gingival) epithelial cells appear to produce several pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6, IL-8 and tumour necrosis factor (TNF) [1], indicating that the cells actively participate in the initiation and development of chronic oral inflammation. However, only a few studies [2, 3] have demonstrated cytokine production by human gingival epithelial cells and related cell lines derived from the oral cavity in response to several oral bacteria.

Recently, homologues of *Drosophila* Toll were found to participate in signalling of lipopolysaccharide (LPS) activity in man and mice, and were designated as Toll-

like receptors (TLRs). In the initial studies on human TLRs, TLR2, but not TLR4, was suggested to mediate LPS activity [4, 5]. In subsequent studies, the dominant role of TLR4 in LPS signalling was clearly demonstrated in mice by genetic studies on TLR4-knockout mice [6] as well as genetically LPS-non-responsive C3H/HeJ and C57BL/10ScCr mice [7–9]. More recent studies on human TLRs [10] supported the critical role of TLR4 in LPS signalling. In addition, MD-2 is necessary for LPS signalling of TLR4 in human and murine cells [11, 12]. In contrast, peptidoglycan (PGN)-induced cell activation is mediated by TLR2 [13–15]. Previous studies at this laboratory revealed the dominant role of TLR4 in lipoteichoic acid (LTA) signalling [14, 16], while other investigators suggested the involvement of TLR2 [13, 15, 17, 18]. MyD88 is an adaptor molecule for TLRs as well as IL-1 receptor (IL-1R) and IL-18R [19–21], and most signalling of bacterial components examined to date was mediated by MyD88, although recent studies showed that LPS also activated cells in a MyD88-independent manner [22–24].

Recently, this research group [25] reported that human

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Corresponding author: Professor H. Takada (e-mail: dent-ht@mail.cc.tohoku.ac.jp).

oral epithelial cells did not respond to bacterial cell-surface components even in the presence of soluble CD14 (sCD14), in contrast to human colonic epithelial cells. Previous studies [26–28] showed that human gamma-interferon (IFN- γ) primed human gingival fibroblasts (HGF) to enhance production of inflammatory cytokines upon stimulation with the LPS fraction from black-pigmented bacteria (BPB) such as *Porphyromonas gingivalis* and *Prevotella intermedia*. Furthermore, IFN- γ primed HGF which highly expressed membrane CD14 (mCD14) to increase expression of mCD14 and mRNA for CD14, TLR4 and MyD88, resulting in production of higher levels of IL-8 than non-primed cells upon stimulation with purified *Salmonella* LPS [29].

This study examined the possible priming effect of IFN- γ on oral epithelial cells in relation to the production of cytokines upon stimulation with LPS, LTA and PGN and the possible regulation of the TLR-MyD88 signalling pathway by IFN- γ treatment in these cells.

Materials and methods

Bacterial components and other reagents

Ultrapurified LPS from *Salmonella enterica* serovar Abortus-equi [30] was donated by C. Galanos (Max Plank Institut für Immunbiologie, Freiburg, Germany). LTA specimens prepared from *Staphylococcus aureus* and *Bacillus subtilis* were purchased from Sigma. These LTA specimens were slightly contaminated with extraneous LPS which was detected by a colorimetric *Limulus* test (Endospecy test; Seikagaku, Tokyo, Japan) as described previously [31]: 86.3 and 15.2 ng equivalent to the reference LPS of *Escherichia coli* O111:B4 (Difco) in 1 mg of *S. aureus* and *B. subtilis* LTA, respectively. Therefore, the repurified LTA prepared from the commercial *Streptococcus sanguis* specimen (Sigma) was also used as described previously [31]: *Limulus* activity of the LTA was <0.1 ng equivalent/mg. *S. aureus* PGN was purchased from Fluka Pharmaceutical (Bechs, Switzerland). A water-soluble PGN, SEPS, was prepared from the purified PGN of *S. epidermidis* ATCC 155 by cleaving pentapeptide cross-links between neighbouring stempeptide subunits with the PGN lytic endopeptidase SALE as described previously [32]. *S. aureus* PGN and SEPS were shown to be considerably contaminated with extraneous LPS on *Limulus* test: 369 and 120 ng equivalent to the reference LPS in 1 mg of *S. aureus* PGN and SEPS, respectively. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP) was supplied by Daiichi Pharmaceutical (Tokyo, Japan). *Limulus* activity of MDP was 0.3 ng equivalent to the reference LPS (in 1 mg of MDP). Anti-human TLR2 monoclonal antibody (MAb) TL2.1 (mouse immunoglobulin IgG2a; IgG2a) [33] was purchased from Cascade Bioscience (Winchester, MA, USA). Anti-human TLR4 MAb

HTA125 (mouse IgG2a) [11] was supplied by K. Miyake and S. Akashi (University of Tokyo, Tokyo, Japan). Control mouse IgG2a was purchased from Coulter (Miami, FL, USA) and dialysed against phosphate-buffered saline (PBS). Human natural IFN- γ (antiviral activity, 8.0×10^6 IU/mg of protein) was provided by Hayashibara Bioscience Institute (Okayama, Japan). All other reagents were obtained from Sigma unless otherwise indicated.

Cells and cell culture

Human gingival epithelial cells were prepared from the explants of normal human gingival tissues of a 7-year-old donor with her parents' informed consent. The explants were cut into pieces and cultured in tissue culture dishes (100 mm in diameter) (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA) in keratinocyte serum-free medium (Gibco BRL, Grand Island, NY, USA) containing bovine pituitary extract 0.05% v/w and recombinant human epidermal growth factor ($820 \mu\text{M}$) supplemented with kanamycin (Meiji Seiyaku, Tokyo, Japan) $200 \mu\text{g/ml}$ with a medium change every 4 days for 15–20 days until subconfluent cell monolayers were formed. Human gingival epithelial cells obtained in this manner were characterised as keratinocytes on the basis of immunostaining with anti-cytokeratin type I and II antibodies (AE1+AE3 mouse IgG1 MAb, Progen Biotechnik GmbH, Germany) as described previously [30]. These procedures were approved by the Ethical Review Board of Tohoku University School of Dentistry. The human oral epidermoid carcinoma cell line (KB cells) was provided by the Health Science Research Resources Bank (Osaka, Japan). Human oral epithelial HSC-2 cells were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). KB cells were cultured in 100-mm dishes in alpha-modified minimal essential medium (α -MEM; Flow Laboratories, McLean, VA, USA) supplemented with fetal calf serum (FCS; Flow Laboratories) 10% with a medium change every 3 days. HSC-2 cells were cultured in 100-mm dishes in PRMI 1640 medium supplemented with FCS 10% with a medium change every 3 days.

As noted above, some bacterial components used in this study were contaminated with endotoxin. To rule out the possibility that the bioactivities of test materials were attributable to the endotoxin in the materials, IFN- γ -primed HSC-2 cells were also stimulated with representative test materials in the presence of polymyxin B sulphate (ICN Pharmaceuticals, Irvine, CA, USA).

RT-PCR assay

Total cellular RNA was extracted from 2.5×10^6 cells (one plate 100 mm diameter) by Isogen (Nippon Gene,

Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of the RNA samples to cDNA was performed with AMV XL (Life Science, St Petersburg, FL, USA) and random primer (Takara, Otsu, Japan). To transcribe the total RNA into cDNA, 1.0 μ g of RNA, 50 pmol of random primer, 10 $\times$ PCR buffer (Takara), 0.2 mM deoxynucleoside triphosphates and 5 U of AMV reverse transcriptase XL were added to a total volume of 20 μ l. The reaction mixture was incubated first at 30°C for 10 min, next at 42°C for 30 min, then 99°C for 5 min to inactivate the reverse transcriptase, cooled on ice, and stored at -20°C. The primers used for PCR were as follows: CD14, forward 5'-CTCAACCTAGAGCCGTTTAT-3' and reverse 5'-CAGGATTGTCAGACAGGTCT-3'; human TLR2, forward 5'-GCCAAAGTCTTGATTGATTGG-3' and reverse 5'-TTGAAGTTCTCCAGCTCCTG-3'; TLR4, forward 5'-TGGATACGTTTCCTTATAAG-3' and reverse 5'-GAAATGGAGGCACCCCTTC-3'; MyD88, forward 5'-TAAGAAGGACCAGCAGAGCC-3' and reverse 5'-CATGTAGTCCAGCAACAGCC-3'; MD-2, forward 5'-GCACATTTTCTACATTCC-3' and reverse 5'-CCTTACATCTTTTACACG-3'; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-TGAAGGTCGGAGTCAACGGATTGGT-3' and reverse 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The primers for CD14, TLR2, TLR4, MD-2, MyD88 and GAPDH were constructed to generate fragments of 426, 368, 506, 157, 200 and 983 bp, respectively. The PCR mixtures contained 5 μ l of the cDNA mixture, 2 μ l of 10 $\times$ PCR buffer, 0.2 mM deoxynucleoside triphosphates, 50 pmol of each primer, and 0.1 μ l of Ex Taq DNA polymerase (Takara, Tokyo, Japan) in a total volume of 20 μ l. Amplification was performed in a model MP TP3000 PCR thermal cycler (Takara) as follows: CD14, 25 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min; TLR2 and TLR4, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 2 min; MD-2, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 90 s; MyD88, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 40 s and extension at 70°C for 2 min; GAPDH, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. Amplified samples were visualised on agarose 2.0% gels stained with ethidium bromide and photographed under UV light. The bands on the photographs were scanned and then analysed with an Image Master 1D (Pharmacia Biotech, Uppsala, Sweden). The results are expressed as relative mRNA accumulation corrected with reference to GAPDH mRNA as an internal standard.

Measurement of cytokines

Confluent epithelial cells were collected by trypsinisation and washed three times in PBS. The cells (5×10^4 cells/200 μ l) were seeded in culture medium

in the wells of 96-well plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA). After incubation for 1 day at 37°C in a CO₂ 5% incubator, cells were cultured with IFN- γ 1000 IU/ml for 3 days. After washing three times with PBS, the cells were stimulated with various test materials in 200 μ l of medium without serum for 24 h. Cultivation was performed in triplicate, and IL-8 and granulocyte macrophage-colony stimulating factor (GM-CSF) levels in the supernates were measured by enzyme-linked immunosorbent assay (ELISA) kits (PharMingen, San Diego, CA, and Biosource, Camarillo, CA, USA, respectively). The concentrations of the cytokines in the supernates were determined with the Softmax data analysis programme (Molecular Devices Corp., Menlo Park, CA, USA).

Data analysis

All experiments were performed at least three times to confirm the reproducibility of the results, and representative results are shown. In most experiments, values are presented as means and SD of triplicate assays. The statistical significance of differences between the two means was evaluated by one-way analysis of variance (ANOVA), by the Bonferroni or Dunn method, and p values <0.05 were considered significant.

Results

Effects of IFN- γ on production of IL-8 and GM-CSF by oral epithelial cells

First, the study examined whether oral epithelial cells produced IL-8 in response to *Salmonella* LPS, *S. aureus* and *B. subtilis* LTA, *S. aureus* PGN, a water-soluble PGN, SEPS, and MDP. In accordance with a previous report [25], primary gingival epithelial cells, oral epithelial HSC-2 and KB cells did not secrete IL-8 in response to the bacterial components even in the presence of FCS 10% (Fig. 1b). Under the same conditions, treatment with IFN- γ (1000 IU/ml) for 3 days made the oral epithelial cells responsive to stimulation with bacterial cell-surface components, resulting in the production of IL-8 (Fig. 1a). Clear dose-dependency of IL-8 secretion by IFN- γ -primed HSC-2 cells was observed upon stimulation with bacterial cell-surface components (Fig. 2). Then, the study examined the optimum concentration and duration of IFN- γ treatment required for the priming of HSC-2 cells. Marked IL-8 release in response to LPS was observed when the cells were treated with IFN- γ 1000 and 10 000 IU/ml for 3 days, whereas IFN- γ at 10 and 100 IU/ml showed only a marginal effect in this respect (Fig. 3a). As shown in Fig. 3b, treatment with IFN- γ for 1 and 2 days was not sufficient to prime oral epithelial cells, whereas after IFN- γ priming for 3 days, LPS significantly activated the cells to release IL-8.

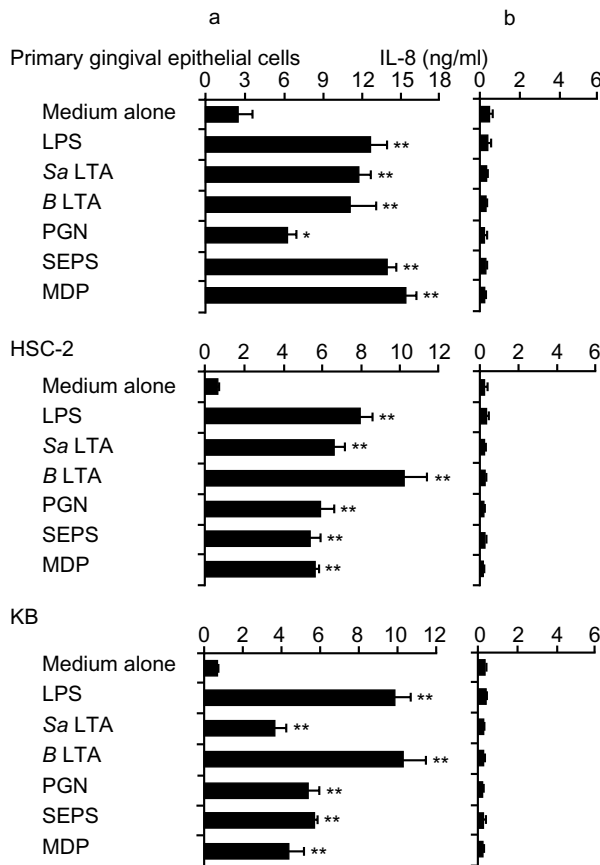


Fig. 1. IFN- γ priming of human oral epithelial cells to secrete IL-8 in response to bacterial cell-surface components. Human oral epithelial cells (primary gingival epithelial, HSC-2 and KB cells) were incubated with (a) or without (b) IFN- γ (1000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (0.1 μ g/ml), *S. aureus* LTA (Sa LTA; 100 μ g/ml), *B. subtilis* LTA (B LTA; 100 μ g/ml), *S. aureus* PGN (100 μ g/ml), SEPS (100 μ g/ml) or MDP (100 μ g/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. * $p < 0.05$, ** $p < 0.01$: significantly different from the respective control (medium alone). Results are representative of three different experiments.

Next, the study examined whether IFN- γ -primed oral epithelial cells also secreted another inflammatory cytokine, GM-CSF, in response to these bacterial cell-surface components. The IFN- γ -primed HSC-2 cells markedly secreted GM-CSF upon stimulation with bacterial cell-surface components, whereas non-treated cells did not show enhanced secretion of GM-CSF (Fig. 4).

Effect of polymyxin B on activities of LTA, PGN, SEPS and MDP

To examine the possible involvement of contaminating endotoxin in the bioactivities of the test materials, IL-8-inducing abilities of representative bacterial cell-surface components on IFN- γ -primed HSC-2 were also examined in the presence of polymyxin B. The activities of *S. aureus* LTA, PGN, SEPS and MDP were not inhibited but rather slightly increased by polymyxin B, whereas the activity of LPS was completely inhibited (Fig. 5). These findings clearly

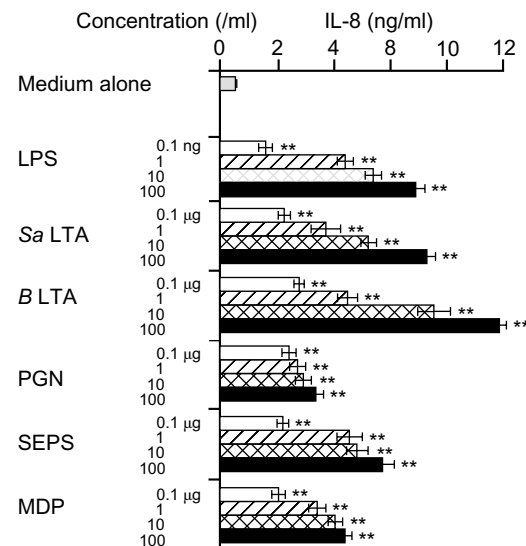


Fig. 2. IFN- γ primed human oral epithelial cells to secrete IL-8 upon stimulation with bacterial cell-surface components in a dose-dependent manner. HSC-2 cells were stimulated with IFN- γ (1000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (0.1–100 ng/ml), *S. aureus* LTA (0.1–100 μ g/ml), *B. subtilis* LTA (0.1–100 μ g/ml), *S. aureus* PGN (0.1–100 μ g/ml), SEPS (0.1–100 μ g/ml) or MDP (0.1–100 μ g/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. ** $p < 0.01$: significantly different from the respective control (medium alone). Results are representative of three different experiments.

indicated that bioactivities of test materials in this study are not attributable to the contaminating endotoxin.

Effects of IFN- γ on TLR2, TLR4, MD-2 and MyD88 mRNA expression in oral epithelial cells

HSC-2 cells were treated with IFN- γ (1000 IU/ml) for 3 days, then RT-PCR was performed to detect levels of CD14, TLR2, TLR4, MD-2 or MyD88 mRNA expression. As shown in Fig. 6, mRNA expression by the molecules of the TLR-MyD88 system were significantly up-regulated by IFN- γ . In contrast, the cells did not express CD14 mRNA even after treatment with IFN- γ .

Effects of anti-TLR2 and anti-TLR4 MAbs on IL-8 secretion by IFN- γ -primed HSC-2 cells in response to bacterial cell-surface components

To elucidate whether the responses of IFN- γ -primed oral epithelial cells to bacterial cell-surface components are mediated by the TLR-MyD88 signalling pathway, IFN- γ -primed HSC-2 cells were pre-incubated with anti-TLR2 MAb (10 μ g/ml), anti-TLR4 MAb (10 μ g/ml) or isotype-matched control antibody (IgG2a) (10 μ g/ml) for 30 min, then stimulated with various bacterial components. LPS-induced IL-8 secretion in IFN- γ -primed HSC-2 cells was inhibited by anti-TLR4 MAb to the control (medium alone) level, but not by anti-TLR2 MAb (Fig. 7). The activities of *S. aureus*

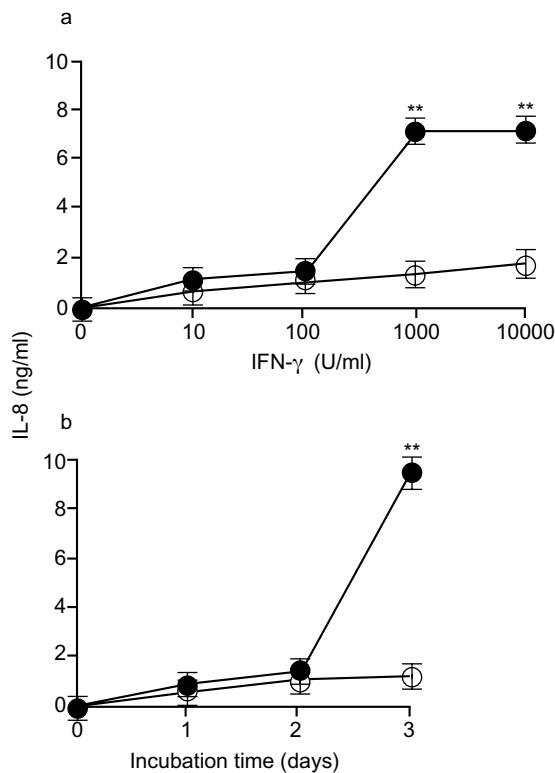


Fig. 3. Dose and time kinetics of IFN- γ -priming of human oral epithelial cells to secrete IL-8 in response to LPS. (a) HSC-2 cells were incubated with IFN- γ (0, 10, 100, 1000 and 10000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (100 ng/ml) for 24 h in triplicate. (b) HSC-2 cells were incubated with IFN- γ (1000 IU/ml) for 0, 1, 2 or 3 days, then stimulated with *Salmonella* LPS (100 ng/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. ** p < 0.01: significantly different from the respective control (medium alone). Results are representative of three different experiments.

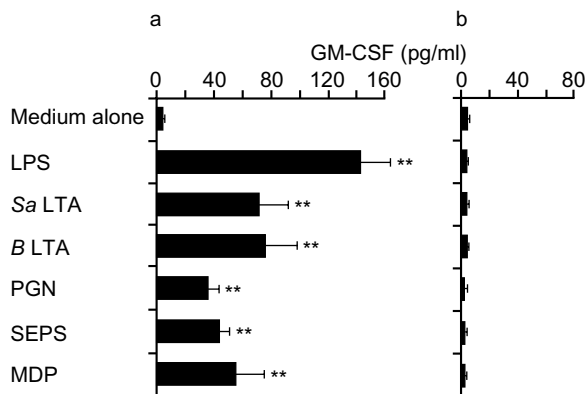


Fig. 4. IFN- γ primed human oral epithelial HSC-2 cells to secrete GM-CSF upon stimulation with various bacterial cell-surface components. HSC-2 cells were incubated with (a) or without (b) IFN- γ (1000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (100 ng/ml), *S. aureus* LTA (100 μ g/ml), *B. subtilis* LTA (100 μ g/ml), *S. aureus* PGN (100 μ g/ml), SEPS (100 μ g/ml) and MDP (100 μ g/ml) for 24 h in triplicate. Concentrations of GM-CSF in the culture supernates were determined by ELISA. Bars indicate SD. ** p < 0.01: significantly different from the respective control (medium alone). Results are representative of three different experiments.

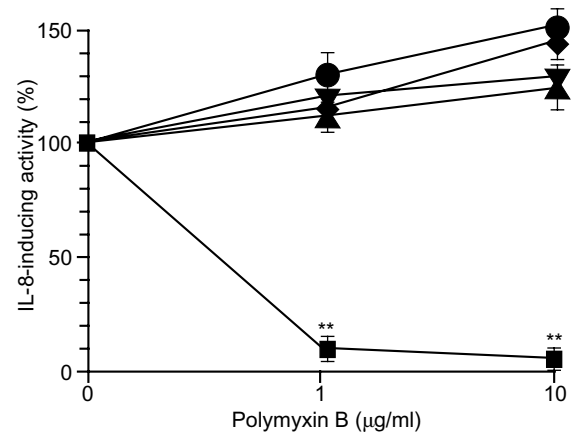


Fig. 5. Influences of polymyxin B (PB) on IL-8-inducing activity of LTA, PGN, SEPS and MDP and the reference LPS. Human oral epithelial HSC-2 cells were stimulated with IFN- γ (1000 IU/ml) for 3 days, then were incubated with *Salmonella* LPS (0.1 μ g/ml, ■), *S. aureus* LTA (100 μ g/ml, ●), *S. aureus* PGN (100 μ g/ml, ▲), SEPS (100 μ g/ml, ▼) and MDP (100 μ g/ml, ◆) in the presence or absence of 1 or 10 μ g/ml PB for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. The activities are expressed as percentages, where 100% and 0% indicate the IL-8 levels induced by the respective stimulant without PB and the negative control with medium alone or indicated concentrations of PB alone, respectively. ** p < 0.01: significantly different from respective positive control (stimulant alone). Bars indicate SD. Results are representative of three different experiments.

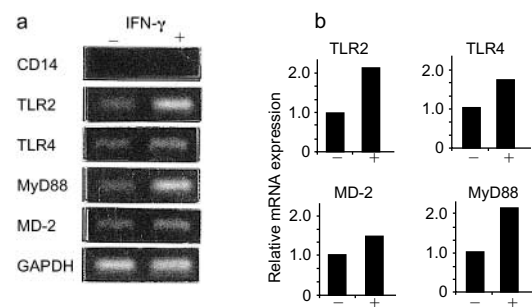


Fig. 6. IFN- γ up-regulated TLR2, TLR4, MD-2 and MyD88 mRNA expression by human oral epithelial cells. HSC-2 cells were cultured with or without IFN- γ (1000 IU/ml) for 3 days. Total cellular RNA was extracted from these cells and RT-PCR was performed to amplify specific signals of CD14, TLR2, TLR4, MD2 and MyD88 mRNA with the respective specific primer. The resulting PCR products were electrophoresed on agarose 2% gels and visualised by staining with ethidium bromide (a). Bands were further quantified by means of an imaging analyser. The results are expressed relative to GAPDH mRNA as an internal standard (b). Three additional experiments gave results similar to those shown here.

and *Str. sanguis* LTA were also inhibited by anti-TLR4 MAb, but not by anti-TLR2 MAb. In contrast, *B. subtilis* LTA-induced IL-8 secretion was inhibited by both anti-TLR2 and anti-TLR4 MAb. PGN- and SEPS-induced IL-8 secretion was significantly inhibited by anti-TLR2 MAb but not by anti-TLR4 MAb. However, MDP-induced IL-8 release was suppressed by neither anti-TLR2 nor anti-TLR4 MAb. These findings suggested that IFN- γ -primed HSC-2 cells produced IL-8 in

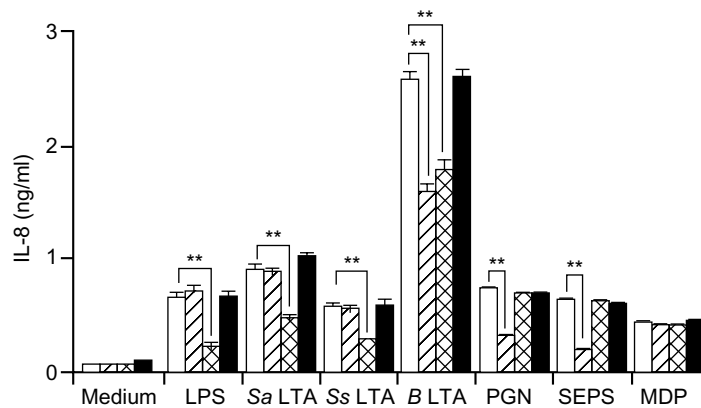


Fig. 7. Effects of anti-TLR2 and anti-TLR4 MABs on IL-8 secretion by IFN- γ -primed human oral epithelial cells in response to bacterial cell-surface components. HSC-2 cells were incubated with IFN- γ (1000 IU/ml) for 3 days, then pre-incubated with anti-TLR2 MAB TL2.1 (10 μ g/ml), anti-TLR4 MAB HTA125 (10 μ g/ml), or isotype-matched IgG2a Ab (10 μ g/ml) for 30 min, then stimulated with *Salmonella* LPS (0.1 ng/ml), *S. aureus* LTA (0.1 μ g/ml), *Str. sanguis* LTA (0.1 μ g/ml), *B. subtilis* LTA (0.1 μ g/ml), *S. aureus* PGN (0.1 μ g/ml), SEPS (0.1 μ g/ml) and MDP (0.1 μ g/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. ** $p < 0.01$: significantly different from the respective control (stimulant alone). Results are representative of three different experiments. □, Stimulant alone; ▨, + α -TLR2; ▩, + α -TLR4; ■, +IgG2a.

response to LTA as well as LPS via TLR4 and to PGN-related materials except MDP via TLR2. IL-8 production induced by these bacterial cell-surface components in IFN- γ -primed HSC-2 cell cultures was not inhibited by anti-CD14 MAB (data not shown).

Discussion

This study demonstrated that treatment with IFN- γ made human oral epithelium cells responsive to bacterial cell-surface components, whereas non-treated cells showed no response even in the presence of sCD14, as reported previously [25]. There is accumulating evidence that sCD14 in serum makes mCD14-defective cells responsive to LPS [34]. This is the case in some human colonic epithelial cell lines such as SW620 and HT29 cells [25, 35, 36] but not in others such as T84 and CaCo2 cells [36, 37]. The mechanism underlying the lack of responsiveness of these epithelial cells is not clear at present.

IFN- γ exhibits various regulatory effects in different cell types [38, 39]. This group has demonstrated the priming effect of IFN- γ on human gingival fibroblasts in culture to produce inflammatory cytokines, including IL-8, upon stimulation with LPS from BPB, but not from Enterobacteriaceae [26–28]. Stříž *et al.* [40] reported that IFN- γ enhanced secretion of IL-8 and expression of HLA-DR and ICAM-1 by human bronchial epithelial cells. The mechanism of the priming effect of IFN- γ on oral epithelial cells to respond to bacterial cell-surface components is not clear at present. One possible explanation might be an increased number or affinity of receptor(s) other than mCD14 on oral epithelial cells as suggested by Triantafyllou *et al.* [41]. In this context, Wan *et al.* [42] suggested that a potential mechanism of LPS

synergy with cytokines such as IFN- γ may be attributable to up-regulation of putative LPS receptors on cells by cytokines. With regard to the possible involvement of mCD14, Funda *et al.* [43] reported that human intestinal epithelial cells expressed mCD14 and released sCD14, in contrast to many previous reports. However, in the present study, human oral epithelial cells did not express CD14 mRNA as determined by RT-PCR (Fig. 5) and mCD14 determined by flow cytometric analysis, even after treatment with IFN- γ (data not shown). Furthermore, IL-8 induced by bacterial cell-surface components on IFN- γ -primed HSC-2 cells was not inhibited by anti-CD14 MAB even in the presence of serum (data not shown). Alternatively, the priming activity of IFN- γ may be due to augmentation of the TLR-MyD88 signalling pathway, which mediates signalling not only of gram-negative but also of gram-positive bacterial cell-surface components, irrespective of CD14 [44–46]. In this context, this group confirmed that IFN- γ up-regulated mCD14 expression and MyD88 mRNA expression by human gingival fibroblasts, and resulted in enhanced production of IL-8 by the cells in response to *Salmonella* LPS [29]. Faure *et al.* reported that IFN- γ induced TLR2 and TLR4 expression in human endothelial cells. In this study, oral epithelial cells expressed TLR2, TLR4, MD-2 and MyD88 mRNA, and IFN- γ up-regulated mRNA of all the molecules to a similar extent. However, cell surface expression of these molecules could not be detected by FACS (data not shown).

To elucidate the involvement of TLRs in the response of IFN- γ -primed oral epithelial cells to bacterial components, the study examined whether the responses of IFN- γ -primed HSC-2 cells to bacterial cell-surface components were inhibited by anti-TLR2 MAB or anti-TLR4 MAB. The dominant role of TLR4 in LPS

signalling was clearly demonstrated, in agreement with previous reports [7–10].

As described above, it has been suggested that both TLR2 and TLR4 are involved in LTA signalling. This group first reported that commercial LTA preparations were considerably contaminated with endotoxin and peptidoglycan, and prepared a purified LTA with bioactivity from the commercial *Str. sanguis* LTA by hydrophobic interaction chromatography, which was practically free from contamination with endotoxin and peptidoglycan [31]. On the other hand, Kusunoki *et al.* [48] prepared a non-hydrophobic bioactive fraction different from LTA from the commercial *S. aureus* LTA. With the purified LTA prepared from *S. aureus* and *Str. sanguis* by the same method, Takeuchi *et al.* [14] demonstrated the essential role of TLR4 in LTA signalling in macrophage cultures from TLR4-knockout mice, although several investigators [13, 15, 17, 18] reported that TLR2 was a signal transducer for LTA. In this study, IL-8 secretion induced by the purified LTA from *Str. sanguis* as well as the commercial LTA from *S. aureus* in IFN- γ -primed HSC-2 cells were significantly inhibited by anti-TLR4 MAb but not by anti-TLR2 MAb. However, *B. subtilis* LTA-induced IL-8 secretion was inhibited by both anti-TLR2 and anti-TLR4 MAbs. These findings suggested that some LTA preparations such as *B. subtilis* LTA used in this study exerted TLR2-mediated activity that might be attributable to other material(s) co-existing with LTA. Recently, Gao *et al.* [49] reported that major activities of commercial LTA from *S. aureus*, *Str. sanguis* and *B. subtilis* were attributable to contaminated endotoxin in the preparations. The possibility that the bioactivities of test LTA preparations shown in this study were also attributable to endotoxin contamination of the materials was clearly ruled out for the following reasons: (i) the activity of the purified LTA from *Str. sanguis*, which was practically free from endotoxin contamination, was dependent on TLR4 (Fig. 7); (ii) the activity of *S. aureus* LTA was not inhibited by polymyxin B sulphate (Fig. 5) [25].

S. aureus PGN- and soluble PGN, SEPS-induced IL-8 secretion was clearly inhibited by anti-TLR2 MAb, in accordance with previous reports [13–15, 50]. Although MDP is the minimal structure essential for the bioactivities of PGN, its signalling in relation to TLRs has not been elucidated. Recently, this group reported that MDP activated OCT (an analogue of the active form of vitamin D₃)-differentiated human monocytic U937 cells, which were devoid of TLR2 on their surface [16]. In accordance with these findings, the present study found that MDP activated human oral epithelial cells in a TLR2- and TLR4-independent manner (Fig. 6).

Three days were required for priming oral epithelial cells by IFN- γ – a 1–2-day period was not sufficient – perhaps suggesting that the priming effect of IFN- γ

was an indirect effect *via* other mediator(s) such as inflammatory cytokines and nitric oxide (NO). In fact, it was found that IL-1 β and TNF- α also primed oral epithelial cells to respond to bacterial components (Uehara *et al.*, unpublished observations). However, we confirmed that IL-1 β and TNF- α were not detected in the culture supernate of IFN- γ -primed cells by ELISA, and anti-NO antibody did not suppress the response of IFN- γ -primed cells to bacterial cell-surface components (Uehara *et al.*, unpublished observations). Therefore, treatment with IFN- γ might have directly modulated intracellular function(s) of oral epithelial cells, which in turn endowed the cells with responsiveness to bacterial components, although the intracellular events are not clear at present.

Oral epithelial cells might be exposed to various inflammatory mediators in the inflamed lesions. Excessive production of IFN- γ is characteristic of immune responses mediated predominantly by Th1 lymphocytes [51]. In fact, numerous Th1 cells infiltrate into the gingival epithelium and lamina propria in inflamed gingival tissues [52]. The intraepithelial $\gamma\delta$ T cells in chronically inflamed gingival tissues express IFN- γ [1]. Therefore, it is conceivable that in the inflamed gingival tissues, oral epithelial cells are primed with IFN- γ produced by these lymphoid cells and secrete various inflammatory cytokines in response to bacterial components such as LPS, LTA and PGN, as shown in this study, which in turn may augment periodontal inflammation.

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