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# NK細胞活性型レセプターNKG2Dの生体内における機能

## 腫瘍，自己免疫疾患，骨髄移植

石﨑和沙，小笠原康悦

NK（natural killer）細胞は，腫瘍細胞や感染細胞など，生体にとって有害となった自己細胞を認識し排除することから，生体防御の最前線で活躍している細胞として知られている．NK細胞は機能的側面から追究されており，疾患への関与について多くの報告があるものの，明確に細胞集団を定義するレセプターが発見されておらず，謎も多い．近年種々のNKレセプターがクローニングされ，NK細胞の認識機構と生体内での役割が明らかになってきた．NK活性化レセプターの1つであるNKG2Dが，腫瘍免疫，自己免疫性糖尿病，骨髄移植拒絶の病態形成において，中心的な役割を果たしていることが判明した．

**キーワード●** NKG2D，腫瘍免疫，自己免疫性糖尿病，ハイブリッドレジスタンス

## はじめに

NK（natural killer）細胞は，生体内において感染免疫や腫瘍免疫，骨髄移植拒絶などにかかわっていることが知られている．NK細胞の機能は，基本的に，正常細胞と異常をきたした細胞を識別し，異常細胞を排除することにある．しかし，NK細胞は多くのレセプターを有しており，しかもレセプターの発現パターンが単一ではないため，制御メカニズムには不明な点も多い．

NK細胞は，名前の表すとおり，抗原による感作がなくとも標的細胞に対して細胞障害活性を示すことができる．NK細胞は抑制性レセプターと活性化レセプターを用いて，正常細胞と異常細胞を識別する．抑制性レセプターは自己MHC（major histocompatibility complex）クラスⅠを認識し抑制シグナルを伝える．そのためNK細胞は，T細胞のように選択を受けなくとも自己正常細胞に対して免疫寛容となる．また，活性化レセプターは細胞がストレスを受けると誘導される生体内リガンドを認識し，NK細胞を活性化する．NK細胞の細胞障害活性は，両者からのシグナルのバランスで制御されていると考えられている[1]．

NKG2Dは，NK細胞の機能に中心的な役割を果たすとして注目されている活性化レセプターである．われわれはNK細胞の生体内での役割を，NKG2Dを中心に解析してきた．その結果，自己免疫性糖尿病や骨髄移植拒絶において，NKG2Dが深く関与していることが明らかとなった．本稿では，腫瘍免疫，自己免疫疾患，骨髄移植拒絶におけるNK細胞の役割を，NKG2Dを中心に紹介する．

## 1 NK活性化レセプターNKG2D

### ❶ NKG2D

NKG2Dは，NK細胞の主たる機能を担うとして注目されている活性化レセプターである．すべてのNK細胞，一部のCD8$^+$T細胞，γδT細胞，NKT細胞など

Roles of NK activating receptor, NKG2D *in vivo*：tumor immunity, autoimmune disease, and bone marrow transplantation

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で発現している．構造的には，C型レクチン※1に属する．NKG2D自体は，シグナル伝達モチーフをもたないが，アダプター分子※2と会合し活性化シグナルを伝達する．マウス，ヒトNKG2Dと会合するDAP10は，YxxMモチーフをもち，PI3キナーゼを用いてシグナルを伝達する．また，ITAM (immunoreceptor tyrosine-based activation motife) をもつDAP12は，マウス活性化NK細胞のみ発現している．$CD8^+$T細胞におけるNKG2Dは，TCR（T cell receptor）刺激を増強する副刺激分子として働いている．一方，NK細胞においては，活性化レセプターとして機能している[2]．

### ❷ NKG2Dリガンド

NKG2Dリガンドは，MHCクラスⅠと構造的に類似している糖タンパク質である．ヒトではMIC（MHC class I related chain) A, MICB, ULBP (UL-16 binding protein）が，マウスではRAE-1（retinoic acid early inducible-1）ファミリー，H60，MULT1（murine ULBP-like transcript 1）が報告されている[2]．多くのNKG2Dリガンドは成体の正常細胞には発現していないが，病理的な状態になると発現が誘導される．つまり，NKG2Dリガンドは，細胞が何らかのストレスを受け異常をきたしたことを免疫系へと伝達する“danger signal”として働いていると考えられる．

## 2 腫瘍免疫におけるNKG2D

### ❶ 腫瘍免疫とNKG2D

NK細胞は，腫瘍細胞の免疫監視機構において第一線で働く細胞である．NK細胞の抗腫瘍作用において，NKG2Dが重要な役割を果たしていることが，近年の研究で明らかにされている．正常細胞では発現していないNKG2Dリガンドが多くの癌細胞株や腫瘍において発現していることから，腫瘍の進展に伴ってNKG2Dリガンドが誘導され，NK細胞の抗腫瘍活性が誘導されることが予想された[3)4)]．実際，マウスに発癌物質を投与するとNKG2Dリガンドが誘導される[5]ことや，抗NKG2D中和抗体の投与により，化学発癌に対する感受性が上昇することから，NKG2Dは生体内における腫瘍の免疫監視機構において，重要であることが示唆された[6]．

### ❷ NKG2Dリガンド発現制御メカニズム

それでは，腫瘍化に伴うNKG2Dリガンドの発現は，どのように制御されているのだろうか．その制御機構の1つとして，ATM (ataxia telangiectasia, mutated), ATR（ATM- and rad3 -related）の活性化があげられている[7]．癌細胞では，遺伝子に異常をきたしていることが多いことから，常にDNAダメージに対応する経路にあるATM，ATRが活性化され，NKG2Dリガンドの発現が上昇していると考えられる．

### ❸ NKG2Dを介した腫瘍細胞の免疫回避機構

一方，ヒトやマウスの多くの腫瘍においてNKG2Dリガンドが発現しているということは，これらの腫瘍細胞が免疫機構を回避しているということを意味している．腫瘍細胞は，NKG2D—NKG2Dリガンドによる抗腫瘍免疫機構を逆手にとって，巧妙に免疫監視を逃れている．ヒト癌患者の血清中に，高濃度の可溶性MICタンパク質が含まれており，$CD8^+$T細胞におけるNKG2Dがdown modulationされているという報告や[8]，RAE-1を過剰発現したトランスジェニックマウスではもともとNK細胞表面上のNKG2Dの発現レベルが低く，発癌物質に対する感受性が高くなっている[9]ことが報告されている．つまり，NKG2Dリガンドの恒常的な発現はNK細胞や$CD8^+$T細胞におけるNKG2Dのdown modulationを引き起こし，これらの細胞の細胞障害活性を抑制すると考えられる．NKG2Dのdown modulationはDAP10シグナルの下流であることが示唆されているものの[10]，詳しいメカニズムについては，まだ明らかになっていない．NKG2Dのdown modulationを抑制することで，腫瘍免疫の強化が期待されることから，さらなる解析が待たれる．

**※1　C型レクチン**
$Ca^{2+}$結合部位をもち，それに依存して糖鎖と結合するレクチンドメインと相同性をもつタンパク質の総称．

**※2　アダプター分子**
シグナル伝達物質との相互作用に必要なドメインを有する分子．それ自身は酵素活性をもたない．

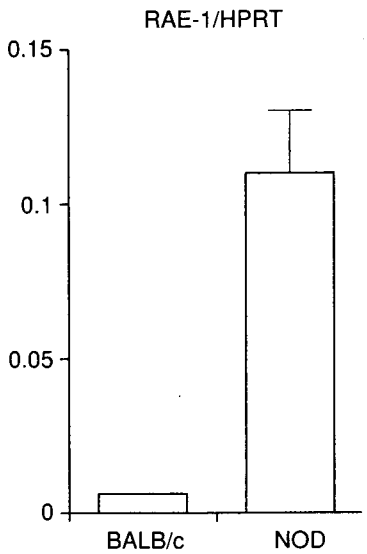



図1 BALB/cマウス，NODマウスの膵臓におけるRAE-1の発現
12～16週齢のNODマウスでは，標的臓器である膵臓におけるRAE-1の発現量が，同じ週齢のBALB/cマウスと比較して上昇している

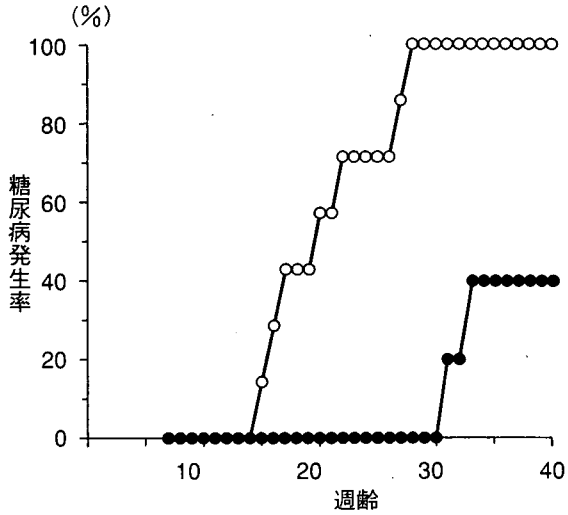



図2 抗NKG2D中和抗体による自己免疫性糖尿病発症の抑制
7週齢のNODマウスに，抗NKG2D中和抗体（●）またはPBS（○）を投与し，各週齢における糖尿病の発症率を比較した．抗NKG2D中和抗体投与群では，糖尿病の発症が著しく抑制された

## 3 自己免疫疾患におけるNKG2D

### ❶ 自己免疫疾患とNKG2D

NK細胞とCD8$^+$T細胞の役割として，ストレスを受けた自己細胞の除去があげられる．これまでに述べてきたように，正常細胞とストレスを受けた細胞の識別にNKG2Dリガンドが用いられている．しかし，NKG2Dリガンドの不適当な発現が，自己免疫疾患発症の引き金となり，病態の重篤化に寄与していることが示唆されている．実際，ヒトの関節リウマチ患者の滑膜細胞や，セリアック病患者の腸上皮細胞で，MICリガンドの発現が上昇していることが報告されている[11]．

### ❷ I型糖尿病とNKG2D

われわれは，I型糖尿病モデルマウス（NODマウス）を用いて，自己免疫性糖尿病におけるNKG2Dの重要性を明らかにした．I型糖尿病は，インスリン産生膵$\beta$細胞が自己反応性T細胞に破壊されることで病態が形成される．NODマウスでは，自己反応性CD8$^+$T細胞がインスリン産生細胞を攻撃することが知られていたが，発症メカニズムについて未だ不明な点も多い．NKG2Dは，この自己反応性CD8$^+$T細胞の増殖，活性化に寄与しており，病態形成に重要な役割を果たしていたのである[12]．

まずわれわれは，標的臓器である膵臓におけるNKG2Dリガンドの発現を検討した．NODマウスの膵臓ではNKG2DリガンドであるRAE-1の発現がBALB/cマウスと比較して著しく上昇しており（図1），12～16週齢になると他の臓器と比較して高い発現を示した．さらに，膵臓に浸潤するCD8$^+$T細胞においてNKG2Dが発現していることから，自己反応性CD8$^+$T細胞によるインスリン産生細胞の攻撃にNKG2Dが関与していることが示唆された．

次に抗NKG2D中和抗体をNODマウスに投与したところ，膵島内への自己反応性CD8$^+$T細胞の浸潤が抑制され完全に糖尿病の発症が抑制された（図2）．抗NKG2D中和抗体の投与によりCD8$^+$T細胞増殖・活性化が抑制されたことから，NKG2D$^+$CD8$^+$T細胞がI型糖尿病の病態形成に深く関与していることが明らかとなった．

## 4 骨髄移植におけるNK細胞

医療技術の発達により臓器移植が可能になったことで，多くの疾患を有効に治療できるようになった．し

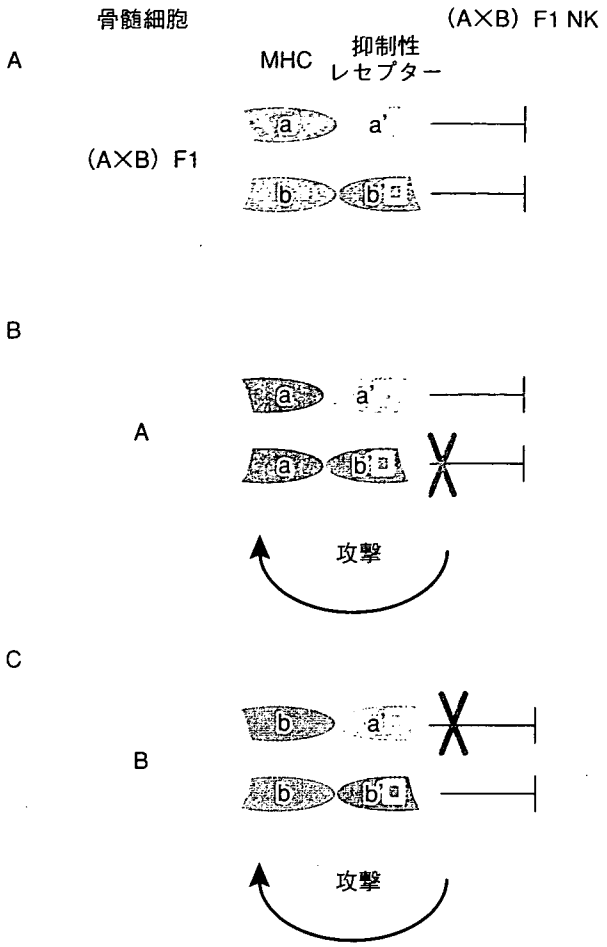



図3　ハイブリッドレジスタンスの原理
aというMHCをもつマウスAと，bというMHCをもつマウスの子供，(A×B) F1マウスは，a，bのMHCを有する．aを認識する抑制性レセプターをa'，bを認識する抑制性レセプターをb'とする．**A**) (A×B) F1マウスの骨髄からは，a'，b'両方から抑制シグナル(⊣)が入るため，拒絶しない．**B**) **C**) 親マウスからはa'またはb'からのシグナルしか入らないため，抑制が弱まり，拒絶する

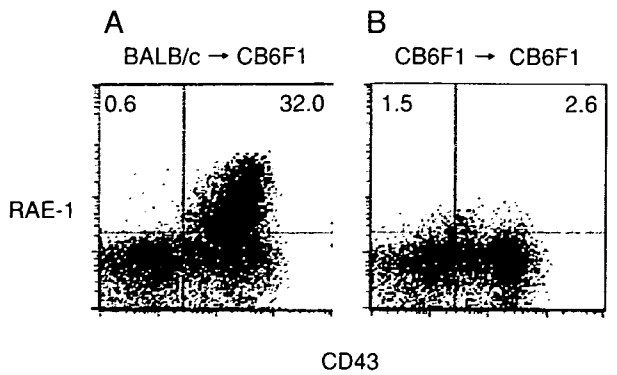



図4　移植骨髄細胞におけるRAE-1の発現
BALB/cマウスとC57BL/6マウスを掛け合わせたCB6F1に移植したBALB/cの骨髄細胞において，RAE-1が高発現している(**A**)．一方，拒絶の起こらない，CB6F1にCB6F1の骨髄細胞を移植した群では，RAE-1の高発現は認められない(**B**)

かし，移植には常に拒絶という問題がつきまとう．移植拒絶は，MHCの異なるドナー由来の細胞をレシピエントのT細胞が非自己と認識して攻撃することが主な原因で起こる．そのため，移植の成功には，ドナーとレシピエントのMHCを合わせること，レシピエントの免疫担当細胞を放射線照射によって排除しておくことが重要である．

### ❶ 骨髄移植とハイブリッドレジスタンス

移植骨髄の拒絶には，F1ハイブリッドレジスタンスという不思議な現象が知られている．aというMHCをホモでもつマウスAと，bというMHCをホモでもつマウスBを掛け合わせた(A×B) F1マウスは，a，b両方のMHCを発現する．そのため，(A×B) F1マウスでは，aおよびbに対して反応するT細胞が分化の過程で除去され，親のMHCに対して免疫寛容になっている．つまり，(A×B) F1にAまたはBの臓器を移植しても拒絶は起こらない．しかしT細胞が親のMHCに対して免疫寛容になっているにもかかわらず，親の骨髄細胞は拒絶されてしまう．これまでの研究から，ハイブリッドレジスタンスには放射線抵抗性のNK細胞が深く関与していることが明らかとなっている[13]．

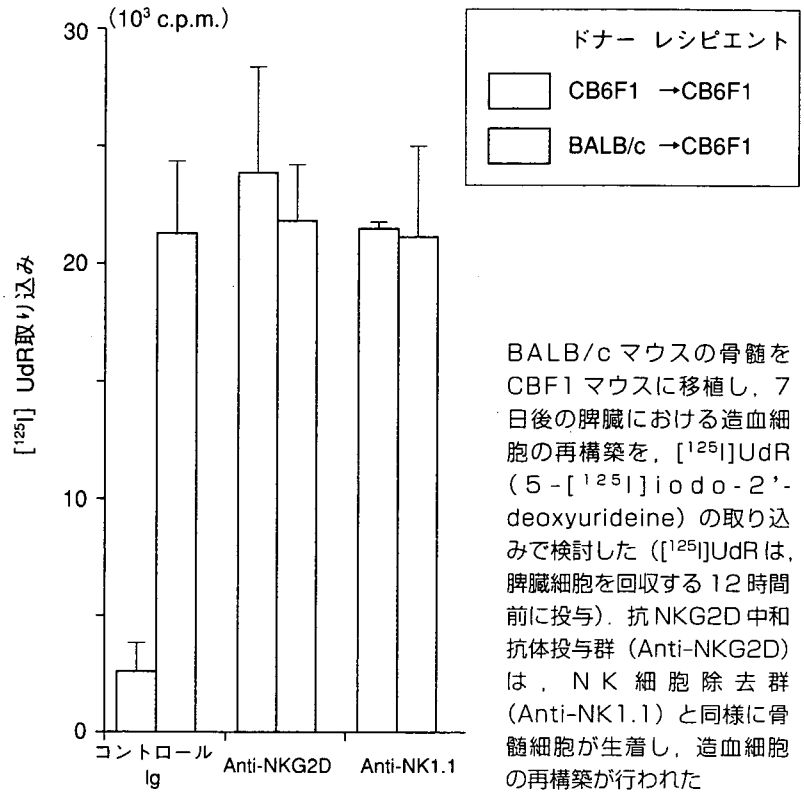
前に述べたように，NK細胞は，自己MHCを認識する抑制性レセプターからのシグナルで，自己細胞に対して免疫寛容になっていると考えられている．(A×B) F1のNK細胞は，通常，a，b両方から抑制性シグナルが入っている．しかし，親由来の骨髄細胞からは，aまたはbのシグナルしか入らないため，NK細胞の抑制が弱まり，拒絶が起こるとされている(図3)[13]．しかし，抑制性レセプターだけでは説明できない点も多く，ハイブリッドレジスタンスに活性化レセプターは関与しているのか否か，長年興味がもたれていた．

### ❷ NKG2Dとハイブリッドレジスタンス

われわれは，ハイブリッドレジスタンスに，活性化レセプターであるNKG2Dが関与していることを明ら

かにした[14]．BALB/cマウスとC57BL/6マウスを掛け合わせた（BALB/c×C57BL/6）F1マウスにBALB/cの骨髄を移植すると，BALB/c骨髄細胞におけるRAE-1の発現上昇が観察された（図4）．また，移植後に抗NKG2D中和抗体を投与すると，BALB/c骨髄細胞が生着した（図5）．しかしC57BL/6の骨髄を移植してもRAE-1の発現は上昇せず，抗NKG2D中和抗体の効果もみられなかったことから，マウスの系統によって拒絶のメカニズムが異なることも明らかとなった．

さらに，RAE-1トランスジェニックマウス（RAE-1 Tg）を作製し，骨髄移植拒絶がNKG2Dシグナルのみで起こりうるかを検討した．C57BL/6 RAE-1 Tgは野生型のC57BL/6と同じMHCを有しているが，骨髄細胞においてRAE-1が過剰発現している．C57BL/6 RAE-1 Tgの骨髄をC57BL/6に移植すると移植骨髄は拒絶され，抗NKG2D中和抗体の投与により生着した．つまり，ドナーのMHCが同一で，レシピエントのNK細胞に抑制シグナルが入ったとしても，NKG2Dシグナルの活性化のみで骨髄移植拒絶が起こることが明らかとなった．




BALB/cマウスの骨髄をCBF1マウスに移植し，7日後の脾臓における造血細胞の再構築を，[125I]UdR（5-[125I]iodo-2'-deoxyurideine）の取り込みで検討した（[125I]UdRは，脾臓細胞を回収する12時間前に投与）．抗NKG2D中和抗体投与群（Anti-NKG2D）は，NK細胞除去群（Anti-NK1.1）と同様に骨髄細胞が生着し，造血細胞の再構築が行われた

図5　抗NKG2D中和抗体投与によるハイブリッドレジスタンスの抑制

## おわりに

10年ほど前まではNK細胞にはNK活性化レセプターの存在すら知られていなかったが，NKG2Dとそのリガンドの発見によりNK細胞の多くの機能にNKG2Dが関与していることが明らかとなった．NKG2Dは，NK細胞やCD8⁺T細胞の機能に大きな役割を果たしており，多くの疾患の治療のターゲットとして期待される．今後の研究により，NKG2Dのdown modulationや，NKG2Dリガンドの発現メカニズムなどが追究され，腫瘍免疫や自己免疫疾患の発症機序などが解明されることを期待したい．

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**石﨑和沙**：2004年筑波大学大学院修士課程医科学研究科卒業．同年，武田薬品工業株式会社に入社．開拓研究所にて創薬研究に携わる．'06年から国立国際医療センター難治性疾患研究部臨床免疫室に所属．現在，協力研究員としてNKG2D，NKG2Dリガンドの研究に取り組んでいる．

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# Functional TLRs and NODs in Human Gingival Fibroblasts

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# Functional TLRs and NODs in Human Gingival Fibroblasts

## ABSTRACT

Since human gingival fibroblasts are the major cells in periodontal tissues, we hypothesized that gingival fibroblasts are endowed with receptors for bacterial components, which induce innate immune responses against invading bacteria. We found clear mRNA expression of Toll-like receptors (TLR)1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, MD-2, MyD88, NOD1, and NOD2 in gingival fibroblasts. Gingival fibroblasts constitutively expressed these molecules. Upon stimulation with chemically synthesized ligands mimicking microbial products for these receptors, the production of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1, was markedly up-regulated. Furthermore, the production of pro-inflammatory cytokines induced by TLR and NOD ligands was significantly inhibited by an RNA interference assay targeted to NF-κB. These findings indicate that these innate immunity-related molecules in gingival fibroblasts are functional receptors involved in inflammatory reactions in periodontal tissues, which might be responsible for periodontal pathogenesis.

**KEY WORDS:** TLR, NOD1, NOD2, fibroblasts, innate immunity.

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## INTRODUCTION

In the innate immune system, pattern recognition of micro-organisms should initiate host defense against invasive pathogens, where pathogen-associated molecular patterns are recognized by the pattern recognition molecules of hosts. Representative pathogen-associated molecular patterns are distributed on bacterial cell surfaces, such as peptidoglycans, lipoproteins, and lipopolysaccharides (LPS), and intracellularly, such as specific motifs of RNA and DNA derived from viruses and bacteria. Recent studies have demonstrated that, in mammals, these pathogen-associated molecular patterns are recognized specifically by respective Toll-like receptors (TLRs). Peptidoglycans and lipoproteins are mainly recognized by TLR2, double-stranded RNA is recognized by TLR3, LPS is recognized by TLR4, single-stranded RNA is recognized by TLR7/8, and bacterial CpG DNA is recognized by TLR9 (Akira *et al.*, 2006). More recently, it was reported that intracellular receptors for two active entities of peptidoglycans, desmuramylpeptides containing diaminopimelic acid and muramyldipeptide, were recognized by NOD1 and NOD2, respectively (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003ab; Inohara *et al.*, 2003).

Fibroblasts and their extracellular matrix products play pivotal roles in maintaining the structural integrity of connective tissues, in healing processes, and in pathological alterations (Buckley *et al.*, 2001). Fibroblasts are not a homogenous population among different anatomical regions, or even within a single tissue, and are considered actively to define the structure of microenvironments and modulate immune cell behavior by conditioning the local and cellular microenvironment (Buckley *et al.*, 2001). Human gingival fibroblasts are the major constituent of gingival connective tissue. In the initial studies on the innate immune responses of gingival fibroblasts, the cells were found to produce various inflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8, upon stimulation with lipopolysaccharide (LPS) from periodontopathic bacteria (Takada *et al.*, 1991; Tamura *et al.*, 1992). Subsequently, the heterogeneous expression of CD14 by gingival fibroblasts was reported (Sugawara *et al.*, 1998). Concerning TLR expression, gingival fibroblasts constitutively expressed TLR2 (Hatakeyama *et al.*, 2003; Wang *et al.*, 2003; Okusawa *et al.*, 2004), TLR4 (Tamai *et al.*, 2002; Hatakeyama *et al.*, 2003; Wang *et al.*, 2003; Okusawa *et al.*, 2004), TLR6 (Okusawa *et al.*, 2004), and MD-2 (Hatakeyama *et al.*, 2003), and produced various cytokines by interaction with their ligands (Sugawara *et al.*, 1998; Tamai *et al.*, 2002), indicating that gingival fibroblasts actively participate in inflammatory processes and immune responses. However, NOD molecules in gingival fibroblasts have not been reported so far.

To elucidate the possible expressions of TLR1, TLR3, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2, as well as TLR2 and TLR4, in gingival fibroblasts, we examined the mRNA and protein expressions of these molecules using RT-PCR, flow cytometry, and immunostaining *in vitro*. Additionally, we examined whether gingival fibroblasts secreted pro-inflammatory cytokines upon stimulation with

a

| | forward | reverse |
|---|---|---|
| TLR1 | CTTTGGACTTCTGACATCTTATC | GATATTAGATAGTTCCAGATTTG |
| TLR2 | GCCAAAGTCTTGATTGATTGG | TTGAAGTTCTCCAGCTCCTG |
| TLR3 | CCTCAGGAATCCCATGTATTTATTTGT | AACCATGCACTCTGCTTGCGAAGAG |
| TLR4 | GCACATTTTCTACATTCC | CCTTACATCTTTTACACG |
| MD-2 | TGGATACGTTTCCTTATAAG | GAAATGGAGGCACCCCTTC |
| TLR5 | AGGCCAGCAAATGTGTTCTGGTC | TGCTCCTTTGATGGCCGAATAG |
| TLR6 | TTGGACTCATATCAAGATGCTCTG | TCAGAATTTGTAGACTTTCTGTCTC |
| TLR7 | GCTCCCCTGAAATATGGGCAATCTC | CTGCTAAAATTAGAGGAATTAGACATC |
| TLR8 | AAAACTGTGGGCCAGCAAATGTGTTG | GAAGCACATCCCAAATGAAGCATTCC |
| TLR9 | TCCAGGGCCCGCAGCGACGTA | CAACTTCACCTTGGATCTGTCACG |
| MyD88 | TAAGAAGGACCAGCAGAGCC | CATGTAGTCCAGCAACAGCC |
| NOD1 | TAGTGCTGTTTCTGCCTCTC | AATTTGACCCCTGCGTCTAG |
| NOD2 | AGCCATTGTCAGGAGGCTC | CGTCTCTGCTCCATCATAGG |
| | TGAAGGTCGGAGTCAACGGATTTGGT | CATGTGGGCCATGAGGTCCACCAC |

b

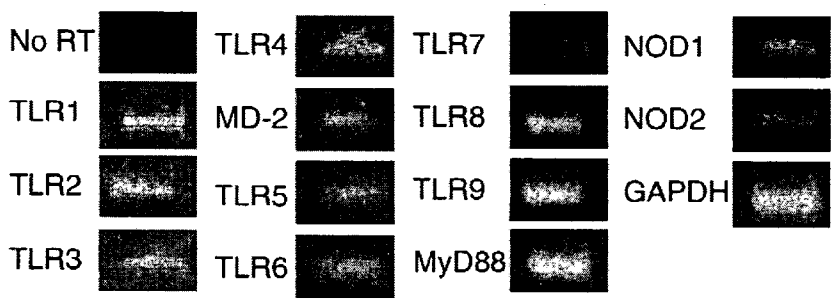


**Figure 1.** Expressions of TLR1, TLR2, TLR3, TLR4, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 mRNA in human gingival fibroblasts. Fibroblasts were cultured until confluent at 37°C. After incubation, the total RNA was extracted, and the mRNA expressions of TLR1, TLR2, TLR3, TLR4, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 were analyzed with PCR, with the primers shown in (**a**). The clear expressions of these mRNAs were detected (**b**). The results presented are representative of 3 different experiments demonstrating similar results.

respective TLR and NOD ligands, to determine whether these pattern recognition molecules are functional. We used only chemically synthesized components, because natural microbial preparations are inevitably contaminated with minor bioactive components that might confuse the results.

## MATERIALS & METHODS

### Reagents

Synthetic muramyldipeptide (MurNAc-L-Ala-D-isoGln) and an *Escherichia coli*-type lipid A (LA-15-PP) were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). Polyinosinic-poly(C) [poly(I:C)] was purchased from Sigma-Aldrich (St. Louis, MO, USA). Single-stranded (ss) PolyU was purchased from Invitrogen (San Diego, CA, USA). A conventional CpG DNA, CpG DNA 1826 (TCCATGACGTTCCTGACGTT [CpG motif is underlined]), was purchased from SIGMA Genosys (Tokyo, Japan). A synthetic *Mycoplasma*-type diacyl lipopeptide FSL-1 (S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-GDPKHPKSF) was purchased from EMC Microcollections (Tübingen, Germany). The synthetic desmuramylpeptides, a PGN fragment containing diaminopimelic acid (DAP), FK156 (D-lactoyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly), was supplied by Astellas Pharmaceutical Co. (Tokyo, Japan). Anti-TLR2 (TL2.1) (mouse IgG1), anti-TLR3 (mouse IgG1), anti-TLR4 (HTA125) (mouse IgG1), anti-MD-2 (rabbit IgG), anti-TLR5 (mouse IgG1), anti-TLR6 (mouse IgG1), anti-TLR7 (rabbit IgG), anti-TLR8 (mouse IgG1), anti-TLR9 (mouse IgG1), and anti-MyD88 (rabbit IgG) antibodies were purchased from eBioscience (San Diego, CA, USA). Anti-NOD1 (goat IgG) and anti-NOD2 (goat IgG) antibodies were obtained from Cayman Chemical (Ann Arbor, MI, USA). The isotype control mouse IgG1, rabbit IgG, and goat IgG were purchased from Sigma-Aldrich. Non-enzymatic cell dissociation solution (CDS) was obtained from Sigma-Aldrich. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

### Cells and Cell Culture

Human gingival fibroblasts were prepared from the explants of normal gingival tissues of six-year-old children, as described previously (Uehara *et al.*, 2005a), under informed consent given by the parents because of the age of the donors. The experimental procedure was approved by the ethical review board (Tohoku University Graduate School of Dentistry).

### Flow Cytometry

Flow cytometric analyses were performed with the use of a FACSCalibur cytometer (BD Biosciences, Mountain View, CA, USA). The cells were collected and washed in PBS. The cells were stained with anti-TLR2, anti-TLR4, and anti-MD-2 antibodies or control IgG at 4°C for 30 min, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA, USA) at 4°C for an additional 30 min. For TLR3, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 stainings, intracellular staining was performed. Briefly, the cells were washed with staining buffer, fixed, and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences) for 15 min at 4°C, and then the cells were incubated with primary antibodies or control IgG for 30 min, followed by FITC-conjugated secondary antibody at 4°C for anther 30 min.

### RNA Extraction, Reverse Transcription, and Quantitative Polymerase Chain-reaction (PCR)

Gingival fibroblasts were cultured in 10-cm-diameter dishes to subconfluence. Total RNA and cDNA were prepared according to a method described previously (Uehara *et al.*, 2005a). Using a programmed thermal cycler, we amplified cDNA in a solution containing 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.025 U/μL Taq DNA polymerase, and 0.2 μM of sense and

antisense primers specific for each mRNA (Fig. 1a) under optimal conditions for each primer set. Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide and photographed under UV light.

## Immunostaining

The cells were cultured on eight-chamber glass slides until confluent with or without test materials and washed with PBS. After fixation with 4% paraformaldehyde for 15 min, the cells were further treated with 0.5% Triton X-100 for 15 min for intracellular staining in the case of TLR3, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2. The cells were then incubated with anti-TLR1, TLR2, TLR3, TLR4, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 antibodies for 3 hrs at room temperature. The samples were then washed and incubated with Alexa Fluor 488 goat anti-mouse IgG1, Alexa Fluor 488 rabbit anti-goat IgG, and Alexa 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA), respectively. The nuclei were visualized by being stained with 4′,6-diamino-2-phenylindole (Molecular Probes). Samples were photographed with an AxioCamMR monochrome digital camera mounted on a Zeiss AxioImager Z1 microscope, equipped with Zeiss AxioVision 4 software (Zeiss, Nürnberg, Germany). As negative controls, mouse IgG1 and normal rabbit serum (DakoCytomation, Tokyo, Japan) and normal goat serum (ZYMED, San Francisco, CA, USA) were used.

## Cytokine Measurement

To investigate the production of inflammatory cytokines by gingival fibroblasts, we collected the supernatant from each culture. The production of cytokines (IL-6, IL-8, and MCP-1) was measured with the use of an OptEIA ELISA kits (PharMingen, San Diego, CA, USA). The concentrations of the cytokines in the supernatants were determined by means of the LS-PLATEmanager 2000 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

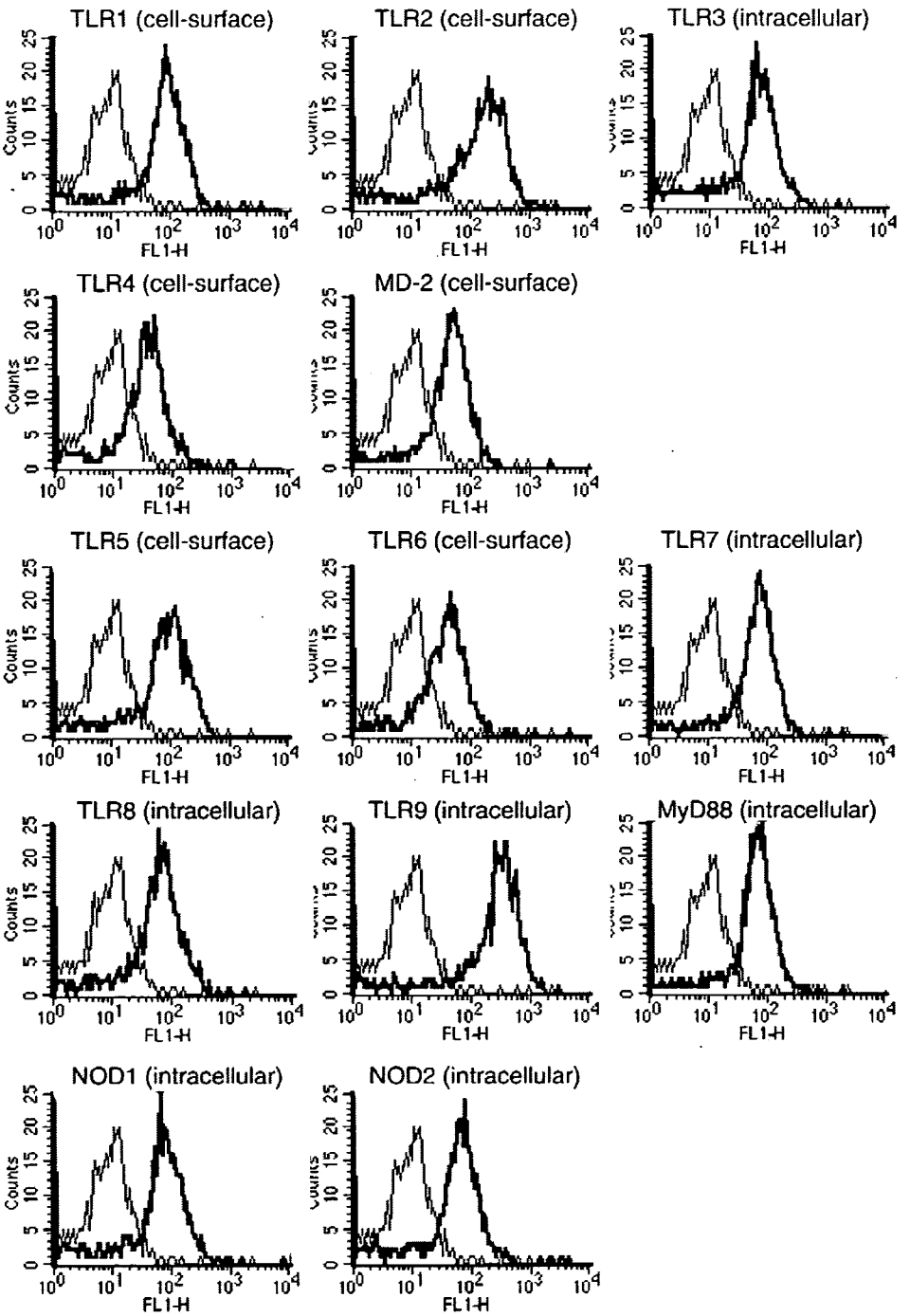


**Figure 2.** Expressions of TLR1, TLR2, TLR3, TLR4, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 in human gingival fibroblasts, detected by flow cytometry. Fibroblasts were cultured until confluent at 37°C. The cell-surface expressions of TLR1, TLR2, TLR4, MD-2, TLR5, TLR6, and intracellular TLR3, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 were assessed by flow cytometry. Thin lines represent the isotype Ab control. The results presented are representative of 4 different experiments demonstrating similar results.

## RNA Interference

Transfections for targeting endogenous NF-κB p65 were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and short-interfering (si) RNA (final concentration, 200 nM) for 24 hrs at 37°C, according to the manufacturer's instructions. The viability of the cells after transfection was more than 95%, as assessed by a 0.2% trypan blue exclusion test, and the morphological character was not changed after transfection. siRNA for NF-κB p65 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

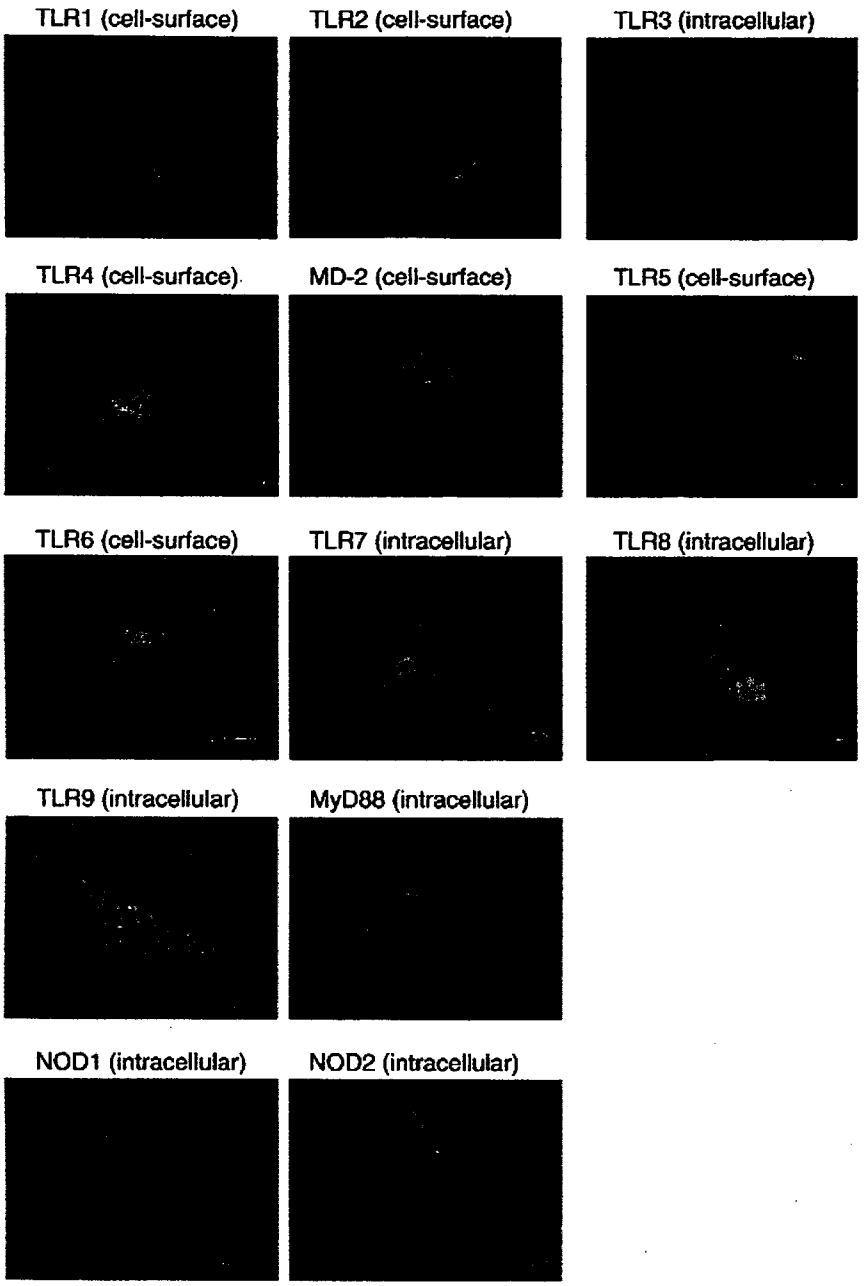


**Figure 3.** Expressions of TLR1, TLR2, TLR3, TLR4, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 in human gingival fibroblasts, detected by immunostaining. Fibroblasts were cultured until confluent at 37°C. After fixation, the cells were treated with anti-TLR2, anti-TLR3, anti-TLR4, anti-TLR5, anti-TLR6, anti-TLR7, anti-TLR8, anti-TLR9, anti-MyD88, anti-NOD1, and anti-NOD2 antibodies and then visualized with Alexa Fluor 488 (green). The nuclei were visualized by being stained with 4′,6-diamino-2-phenylindole (blue). Scale bars: 20 μm. The results are representative of 3 different experiments demonstrating similar results.

## RESULTS

### Gingival Fibroblasts Constitutively Expressed TLR1, TLR2, TLR3, TLR4, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2

First, we demonstrated, using RT-PCR, that gingival fibroblasts constitutively expressed the mRNAs for TLR1, TLR2, TLR3, TLR4, MD-2, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 molecules (Fig. 1). Then, using flow cytometry, we demonstrated the cell-surface expressions of TLR1, TLR2, TLR4, MD-2, TLR5, and TLR6, and the intracellular expressions of TLR3, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 in gingival fibroblasts (Fig. 2), consistent with the results of RT-PCR. With immunostaining, TLR1, TLR2, TLR4, MD-2, TLR5, and TLR6 were clearly expressed on the cell surface, and TLR3, TLR7, TLR8, TLR9, MyD88, NOD1, and NOD2 were constitutively expressed intracellularly (Fig. 3). In contrast, gingival fibroblasts were not stained with the negative controls mouse IgG, goat serum, or rabbit serum, followed by Alexa Fluor 488 (green) (data not shown).

### Induction of Pro-inflammatory Cytokines in Human Gingival Fibroblasts upon Stimulation with Chemically Synthesized TLR2/6, TLR3, TLR4, TLR7/8, TLR9, NOD1, and NOD2 Ligands

In this study, we examined whether TLRs and NODs expressed on human gingival fibroblasts actually functioned as receptors in terms of pro-inflammatory cytokine production upon stimulation with the respective ligands. It was found that FSL-1 (TLR2/6 agonist), Poly I:C (TLR3 agonist), lipid A (TLR4 agonist), ssPolyU (TLR7/8 agonist), CpG DNA (TLR9 agonist), FK156 (NOD1 agonist), and muramyl-dipeptide (NOD2 agonist) significantly induced the production of IL-6, IL-8, and MCP-1 (Fig. 4a).

### Suppression of IL-8 Production Induced by Synthetic TLR and NOD Ligands with siRNA Targeting NF-κB p65

To clarify the signaling pathway of cellular activation by these TLR and NOD ligands, we utilized RNA interference assays, targeting NF-κB p65 mRNA. Many studies have showed that NOD1 and NOD2 signaling, as well as TLR signaling, activated NF-κB (Ogura *et al.*, 2001; Akira *et al.*, 2006). Consistent with these reports, the up-regulated secretion of IL-8 induced by TLR and NOD ligands was significantly inhibited in NF-κB p65-silenced cells (Fig. 4b).

These results demonstrate that TLRs and NODs on human gingival fibroblasts function as pattern recognition receptors and signaling molecules.

## DISCUSSION

Since fibroblasts are the major constituent of gingival connective tissue and are capable of producing various inflammatory cytokines (Takada *et al.*, 1991; Tamura *et al.*, 1992), it has been speculated that gingival fibroblasts actively participate in the inflammatory processes associated with periodontal diseases. As described above, however, only a limited repertoire of TLRs in gingival fibroblasts has been reported so far (Sugawara *et al.*, 1998; Tamai *et al.*, 2002; Hatakeyama *et al.*, 2003; Wang *et al.*, 2003; Okusawa *et al.*, 2004). In the present study, we examined, in depth, the innate immune receptors in gingival fibroblasts, and demonstrated the clear expression of all TLRs (TLR1 to TLR9), MD-2, and MyD88 in gingival fibroblasts, although there were divergences in their expression (Figs. 1-3). This is the first report on the expression of TLR1, TLR3, TLR5, TLR6, TLR7, TLR8, and TLR9 in gingival fibroblasts. Furthermore, we also showed the expression of NOD1 and NOD2 in the cells. In addition, stimulation with respective specific ligands, chemically synthesized to mimic microbial components, markedly up-regulated the production of pro-inflammatory cytokines, IL-6, IL-8, and MCP-1, indicating that these molecules in gingival fibroblasts are functional innate immune receptors.

In the case of most of the TLRs, the signaling pathways are mainly mediated by the activation of NF-κB, although cell-surface TLR4 and intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) also activate the cells *via* IRF-3 and/or IRF-7 (Akira *et al.*, 2006). NOD1 and NOD2 signaling also activates NF-κB (Inohara *et al.*, 1999; Ogura *et al.*, 2001). In the present study, we clearly demonstrated that various synthetic TLR and NOD ligands induce IL-6, IL-8, and MCP-1 production by gingival fibroblasts (Fig. 4). Since IL-6, IL-8, and MCP-1 possess an NF-κB binding site in their promoter regions (Mizushima and Nagata, 1990; Yasumoto *et al.*, 1992), our findings led us to expect that the induction of IL-6, IL-8, and MCP-1 through respective TLR or NOD molecules in gingival fibroblasts may involve NF-κB activation. As we expected, the results with NF-κB p65-silenced cells demonstrated that various TLR and NOD ligands exerted cytokine-inducing activity, mainly *via* NF-κB (Fig. 4b).

It has been controversial whether oral epithelial cells express TLRs, especially TLR4 (Asai *et al.*, 2001; Kusumoto *et al.*, 2004), in relation to their apparent unresponsiveness to various microbial products in terms of pro-inflammatory cytokine products. Recently, we have demonstrated, by immunohistochemical analysis, the clear expression of TLR4 as well as TLR2, and the strong expression of NOD1 and NOD2, in normal oral epithelial tissues, and also showed, using PCR, flow cytometry, and immunostaining, that primary oral epithelial cells in culture expressed these molecules (Sugawara *et al.*, 2006). Furthermore, we found that oral epithelial cells expressed all TLRs (TLR1 to 9) (unpublished observations). It should be emphasized that the epithelial cells did not produce inflammatory cytokines upon stimulation with respective TLR or NOD ligands (Uehara *et al.*, 2001, 2005b). In contrast, the cells produced antimicrobial factors, such as peptidoglycan

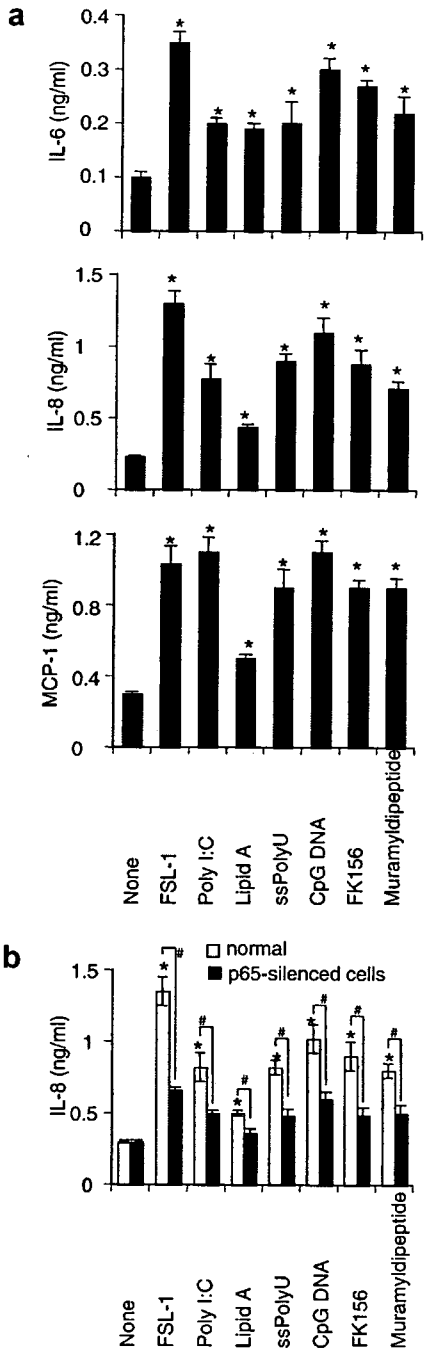


**Figure 4.** IL-6, IL-8, and MCP-1 production induced by TLR and NOD ligands in human gingival fibroblasts was mediated by NF-κB. **(a)** Fibroblasts were incubated for 24 hrs in the presence or absence of FSL-1 (1 nM), Poly I:C (10 μg/mL), lipid A (10 ng/mL), ssPolyU (10 μg/mL), CpG DNA (1 μM), FK156 (10 μg/mL), or MDP (10 μg/mL). IL-6, IL-8, and MCP-1 levels in the culture supernatants were determined with ELISA, and expressed as means ± SD. *Values marked differed significantly from those with medium alone (none). The results presented are representative of 3 different experiments demonstrating similar results. **(b)** Gingival fibroblasts transfected with siRNA targeting NF-κB p65 for 24 hrs were stimulated with FSL-1 (1 nM), Poly I:C (10 μg/mL), lipid A (10 ng/mL), ssPolyU (10 μg/mL), CpG DNA (1 μM), FK156 (10 μg/mL), or MDP (10 μg/mL). After 24 hrs of stimulation, the IL-8 levels in the culture supernatants were determined with ELISA, and expressed as means ± SD. *,#Values marked differed significantly from those with medium alone or respective cultures stimulated with the indicated ligands, respectively. The results presented are representative of 4 different experiments demonstrating similar results.

recognition proteins (PGRPs), especially PGRP-Iα and -Iβ, and β-defensin 2. It is reasonable for epithelial cells to produce antimicrobial factors without the accompanying inflammatory cytokines upon stimulation with microbial components, because oral epithelial cells interact constitutively with the normal flora, and inflammatory responses might result in tissue destruction. In contrast, gingival fibroblasts are physiologically isolated from the normal flora, and only in tissue injury situations do they interact with microbes, which should induce inflammatory reactions, such as the pro-inflammatory cytokine production shown in this study. In other words, the excessive inflammatory reaction might be involved in the tissue destruction typically observed in periodontal diseases. Although the cytokines should properly be host-defense factors, those produced by fibroblasts might be harmful. However, further studies, especially *in vivo* studies, are required to demonstrate the above putative periodontal pathogenesis.

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# Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines

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**Abstract**

Epithelial cells may form the first barrier of defense against bacteria in human tissues. We recently revealed that oral epithelial cells generated anti-bacterial factors, such as peptidoglycan recognition proteins (PGRPs) and β-defensin 2, but not proinflammatory cytokines, such as interleukin-8 (IL-8), upon stimulation with bacterial cell-surface components. In this study, we found clear expressions of Toll-like receptor (TLR)2, TLR3, TLR4, TLR7, NOD1 and NOD2 in oral, tongue, salivary gland, pharyngeal, esophageal, intestinal, cervical, breast, lung, and kidney epithelial cells. However, tongue, salivary gland, pharyngeal, esophageal, intestinal, cervical, breast, lung, and kidney epithelial cells, as well as oral epithelial cells, did not secrete IL-6, IL-8 or monocyte chemoattractant protein-1 in response to chemically synthesized TLR and NOD agonists mimicking microbial components: TLR2 agonistic lipopeptide (Pam3CSSNA), TLR3 agonistic Poly I:C, TLR4 agonistic lipid A (LA-15-PP), TLR7 agonistic single stranded RNA (ssPoly U), NOD1 agonistic iE-DAP (γ-D-glumtamyl-*meso*-diaminopimelic acid), and NOD2 agonistic muramyldipeptide (MDP). Although PGRPs on oral epithelial cells were significantly up-regulated upon stimulation with these synthetic components, PGRPs on pharyngeal epithelial cells were only slightly up-regulated, and PGRPs on esophageal, intestinal and cervical epithelial cells were not up-regulated upon stimulation with the components. In contrast, stimulation with synthetic TLRs and NODs ligands induced β-defensin 2 generation in all epithelial cells examined. These findings indicate that TLR and NOD in various epithelial cells are functional receptors that induce anti-bacterial responses in general without being accompanied by inflammatory responses.


*Keywords:* TLRs; NODs; Epithelial cells; β-Defensin 2

## 1. Introduction

The immune system provides protection against a wide variety of pathogens. The immune system can be divided into two major categories: innate immunity and adaptive immunity. The innate immunity is phylogenetically ancient and is found in plants as well as animals. Unlike adaptive immunity, innate immunity confers broad protection against pathogens without previous exposure, and most multicellular organisms depend on it to combat against microbial infections. The cellular components of the innate immunity include phagocytic cells, such as neutrophils, monocytes and macrophages, in tissues and circulating blood, microglia cells in the brain and Kupffer cells in the liver. These cells utilize pattern recognition molecules (PRMs) that recognize pathogen-associated molecular patterns (PAMPs). The well-known cell-surface receptors are Toll-like receptors (TLRs), which recognize a variety of bacterial and viral PAMPs; for example, bacterial lipopeptide, Poly I:C, lipopolysaccharide (LPS) and single-stranded RNA are recognized by TLR2, TLR3, TLR4 and TLR7, respectively (Akira et al., 2006). In addition, recent study demonstrated that NOD1

*Abbreviations:* PRMs, pattern recognition molecules; PAMPs, pathogen-associated molecular patterns; TLRs, Toll-like receptors; LPS, lipopolysaccharide; PGNs, peptidoglycans; DAP, diaminopimelic acid; IL, interleukin; PGRPs, peptidoglycan recognition proteins; MDP, muramyldipeptide; *meso*-DAP, *meso*-diaminopimelic acid; iE-DAP, γ-D-glutamyl-*meso*-DAP; TNF, tumor necrosis factor; CDS, cell dissociation solution; FCS, fetal calf serum; MCP-1, monocyte chemoattractant protein-1; RT-PCR, reverse transcriptional PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FITC, fluorescein isothiocyanate

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and NOD2 were intracellular PRMs that recognize the partial structure of bacterial peptidoglycan (PGN); NOD1 and NOD2 recognize diaminopimelic acid (DAP)-containing peptide moiety and muramyl-peptide moiety, respectively (Chamaillard et al., 2003; Girardin et al., 2003a,b; Inohara et al., 2003).

In various mucosal tissues, epithelial cells interact with bacteria in normal flora and have been thought to produce various proinflammatory cytokines in response to bacterial stimuli. We revealed, however, that oral epithelial cells did not show the enhanced production of proinflammatory cytokines, such as interleukin (IL)-8, in response to various bacterial PAMPs (Uehara et al., 2001). In this context, some investigators have shown that oral and colonic epithelial cells express low levels of TLR4 and are poorly responsive to bacterial LPS (Abreu et al., 2001; Asai et al., 2001; Naik et al., 2001; Suzuki et al., 2003). On the other hand, some colonic epithelial cell lines produced inflammatory cytokines upon stimulation with bacterial PAMPs (Pugin et al., 1993; Schuerer-Maly et al., 1994). The response to TLR2 ligands on intestinal epithelial cells is also muted (Melmed et al., 2003). Recently, we found the clear expression of TLR4 as well as TLR2, and the strong expression of NOD1 and NOD2 in normal oral epithelial tissues using immunohistochemical analysis, and also showed that primary oral epithelial cells in culture expressed these molecules, using PCR, flow cytometry and immunostaining (Sugawara et al., 2006), although the expression and function of these PRMs in various epithelial cells remains controversial.

On the other hand, we demonstrated that chemically synthesized bacterial PAMPs increase the expression of peptidoglycan recognition proteins (PGRPs) via TLR2, TLR4, NOD1 and NOD2 in human oral epithelial cells (Uehara et al., 2005). PGRPs are a novel family of PAMPs in innate immunity that have been conserved from insects to mammals (Dziarski and Gupta, 2006). Mammals have a family of four PGRPs, which were initially named as PGRP-S, PGRP-L, PGRP-Iα and PGRP-Iβ, but recently, the Human Genome Organization Gene Nomenclature Committee proposed that their names be peptidoglycan recognition proteins 1, 2, 3 and 4 (PGLYRP1, PGLYRP2, PGLYRP3 and PGLYRP4), respectively (Dziarski and Gupta, 2006). These findings suggest that epithelial cells interacting constitutionally with normal flora might respond to bacterial cell surface components with the enhanced production of anti-bacterial molecules, such as PGRPs, to prevent the bacterial invasion of host cells without producing proinflammatory cytokines to prevent possible tissue destruction through by excessive inflammatory responses. The secretory molecules, such as defensins and cathelicidins, directly destroy microorganisms (Froy and Gurevitz, 2003, 2004; Ganz, 2003). In mammals, defensins are the predominant anti-microbial polypeptides, as evidenced by the large number of expressed genes, the various forms, and the ubiquitous occurrence in inflamed or infected tissues (Ganz, 2003). β-Defensins are expressed in several organs, such as skeletal muscles, airways, esophagus, tongue, intestine and skin. To date, six β-defensins (hBD-1 to hBD-6) have been identified in humans (Bensch et al., 1995; García et al., 2001; Harder et al., 2000; Yamaguchi et al., 2002). hBD-1 is constitutively expressed, whereas hBD-2 and hBD-3 are inducible by bacterial and viral products and cytokines, such as IL-1β and tumor necrosis factor (TNF)-α. All defensins identified to date have the capability to kill and/or inactivate a large spectrum of bacteria, fungi, or some enveloped viruses in vitro (Yang et al., 2002). Thus, β-defensins play a crucial role in host defense against bacterial infection as constitutive or inducible components in the epithelial barrier.

In this study, we thoroughly examined the mRNA and protein expression of these PRMs on various human epithelial cells using RT-PCR, flow cytometry and immunostaining. Additionally, we examined whether various human epithelial cells secreted β-defensin 2 and PGRPs upon stimulation with TLR and NOD ligands to determine whether these PRMs are functional. We used solely chemically synthesized microbial PAMPs, because natural bacterial preparations are inevitably contaminated with minor bioactive components that might have confused the results. Namely, synthetic *Escherichia coli*-type triacyl lipopeptide Pam3CSSNA is an agonist for TLR2, Poly I:C is an agonist for TLR3, synthetic *E. coli*-type lipid A (LA-15-PP) is an agonist for TLR4, single-stranded RNA (ssPoly U) is an agonist for TLR7, DAP containing desmuramyl peptide iE-DAP (γ-D-glutamyl-*meso*-DAP) is an agonist for NOD1, and synthetic muramyldipeptide (MDP, *N*-acetylmuramyl-L-alanyl-D-*iso*glutamine) is an agonist for NOD2.

## 2. Materials and methods

### 2.1. Materials

The synthetic MDP and synthetic *E. coli*-type lipid A (LA-15-PP) were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). *E. coli*-type triacyl lipopeptide Pam3CSSNA and iE-DAP were synthesized as described previously (Chamaillard et al., 2003; Nakamura et al., 2002). ssPoly U and Poly I:C were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human natural gamma interferon (IFN-γ) was provided by the Hayashibara Bioscience Institute (Okayama, Japan). Recombinant human IL-1α and TNF-α were supplied by Dainippon Pharmaceutical Co. (Osaka, Japan). Anti-TLR2 monoclonal Ab TL2.1 (mouse IgG2a), anti-TLR3 monoclonal Ab (mouse IgG2a), anti-TLR4 monoclonal Ab HTA125 (mouse IgG2a), anti-TLR7 monoclonal Ab (mouse IgG2a) were purchased from eBioscience (San Diego, CA, USA). Goat anti-NOD1 polyclonal Ab L-17 was obtained from Cayman Chemical (Ann Arbor, MI, USA.). Rabbit anti-NOD2 polyclonal Ab and anti-β-defensin 2 polyclonal Ab C-17 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A non-enzymatic cell dissociation solution (CDS) was obtained from Sigma–Aldrich. All other reagents were obtained from Sigma–Aldrich, unless otherwise indicated.

### 2.2. Cells and cell culture

The human oral epithelial cell lines HSC-2, HSC-3, SAS, and HO-1-u-1, the human pharyngeal epithelial cell line HEp-2, the human esophageal epithelial cell line TE-1, human breast epithelial cell line MCF-7, human lung epithelial cell line A549,

and human kidney epithelial cell line Caki-1 were obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The human colon adenocarcinoma cell lines SW620 (CCL-227) and HT29 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human epitheloid carcinoma cell line HeLa (JCRB9904), derived from the cervix, was purchased from the Cancer Cell Repository, the Health Science Research Resources Bank (Osaka, Japan). Human salivary gland epithelial HSY was kindly provided by Dr. Sato (Tokushima University, Tokushima, Japan). These cells were cultured in 100-mm dishes in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS) with a medium change every 3 days. Primary human oral epithelial cells were prepared from explants of normal human gingival tissues with informed consent. In brief, the explants were cut into pieces and cultured in keratinocyte serum-free medium (Life Technologies, Grand Island, NY, USA) containing bovine pituitary extract (0.05%) and recombinant human epidermal growth factor (820 μM) supplemented with kanamycin (200 μg/ml) with a medium change every 3–5 days until confluent cell monolayers were formed. To avoid the possibility that trypsinization affects the amounts of PGRPs and other surface markers, we used Sigma's CDS. Because CDS contains no protein and allows the dislodging of cells without the use of enzymes, cellular proteins are preserved without enzymatic modification or the adsorption of foreign proteins.

### 2.3. Measurement of cytokines

Confluent epithelial cells were collected by CDS and washed three times with PBS. The cells ($10^4$ cells per 200 μl) were seeded in culture medium in 96-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA). After 1 day at 37 °C in a 5% $CO_2$ incubator, the cells were stimulated with test materials in 200 μl of the medium for a given period. Each culture was carried out in triplicate, and the level of IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1 in the supernatants were measured using OptEIA ELISA kits (Pharmingen, San Diego, CA, USA). The level of β-defensin 2 was measured using an ELISA Development Kit (PeproTech EC, London, UK). The concentrations of the cytokine in the supernatants were determined using the LS-PLATEmanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

### 2.4. Quantitative reverse transcriptional PCR (RT-PCR) assay

Total cellular RNA was prepared from human epithelial cells with Isogne (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Random hexamer-primed reverse transcription was performed using 2.5 μl of total RNA in a 50 μl reaction volume. Real-time PCR was performed with a LightCycler (Roche Diagnostics). The specificity of the PCR was confirmed by the molecular weight of the products and a melting curves analysis at each data point. The primers used for PCR had the following sequences: PGRP-L, 5′-ACTGAGGGC-TGCTGGGACCA-3′, 5′-GGCCTCAGTGAATTCCTTGG-3′; PGRP-Iα, 5′-GTTCCGTGTGTCCATGTGAA-3′, 5′-TTGGG-AAGCCAGAGAGACAC-3′; PGRP-Iβ, 5′-ATGTCTCCACC-ACGGTCTCT-3′, 5′-CACCCACTGTTGTTGTGGAC-3′; PG-RP-S, 5′-CCGTGGCTGGAACTTCACG-3′, 5′-GCACATCC-CGGTGTCCTTTG-3′; TLR2, 5′-GCCAAAGTCTTGATTG-ATTGG-3′, 5′-TTGAAGTTCTCCAGCTCCTG-3′; TLR3, 5′-CCTCAGGAATCCCATGTATTAATTTGT-3′, 5′-AACCA-TGCACTCTGCTTGCGAAGAG-3′; TLR4, 5′-TGGATACG-TTTCCTTATAAG-3′, GAAATGGAGGCACCCCTTC-3′; TLR7, 5′-AAAACTGTGGGCCAGCAAATGTGTTG-3′, 5′-GAAGCACATCCCAAATGAAGCATTCC-3′; NOD1, 5′-TA-GTGCTGTTTCTGCCTCTC-3′, 5′-AATTTGACCCCTGCGT-CTAG-3; NOD2, 5′-AGCCATTGTCAGGAGGCTC-3′, 5′-CGTCTCTGCTCCATCATAGG-3′; and human glyceraldeh-yde-3-phosphate dehydrogenase (GAPDH), 5′-CATCACC-ATCTTCCAGGAGC-3′, 5′-CATGAGTCCTTCCACGATACC-

Table 1
Expression of TLRs and NODs in various human epithelial cells

| Human cells | Positive cells (%) | | | | | |
|---|---|---|---|---|---|---|
| | TLR2 | TLR3 | TLR4 | TLR7 | NOD1 | NOD2 |
| Oral epithelial | | | | | | |
| HSC-2 | 56.2 | 32.1 | 36.4 | 40.7 | 90.1 | 77.4 |
| HO-1-u-1 | 48.9 | 38.3 | 33.0 | 41.6 | 85.4 | 70.8 |
| Pharyngeal epithelial | | | | | | |
| HEp-2 | 34.6 | 21.4 | 22.3 | 20.5 | 69.0 | 63.4 |
| Esophageal epithelial | | | | | | |
| TE-1 | 35.0 | 23.3 | 19.2 | 17.2 | 59.1 | 60.5 |
| Intestinal epithelial | | | | | | |
| SW620 | 29.0 | 21.4 | 19.6 | 15.7 | 85.3 | 70.8 |
| HT29 | 22.5 | 19.3 | 17.2 | 18.6 | 75.3 | 69.3 |
| T84 | 11.4 | 8.9 | 9.2 | 12.2 | 48.2 | 44.7 |
| Caco-2 | 9.6 | 8.1 | 7.7 | 9.4 | 39.8 | 33.8 |
| Cervical epithelial | | | | | | |
| HeLa | 35.6 | 14.8 | 24.7 | 11.2 | 40.7 | 51.1 |

3′. Cycling conditions were as follows: with 40 cycles at 95 °C for 15 s, 56 °C for 15 s (PGRP-L), 6 s (PGRP-Iα), 7 s (PGRP-Iβ), 12 s (PGRP-S), 8 s (TLR2), 10 s (TLR3), 7 s (TLR4), 11 s (TLR7), 12 s (NOD1), 11 s (NOD2), and 11 s (GAPDH), respectively, then 72 °C for 11 s. Results are expressed as the relative mRNA accumulation corrected using GAPDH mRNA as an internal standard.

### 2.5. Flow cytometry

Flow cytometric analyses were performed using FACSCalibur flow cytometer and CELLQuest software (BD Biosciences, San Diego, CA, USA). Human epithelial cells were collected by non-enzymatic CDS, and washed in PBS. Cells were stained with anti-TLR2 Ab, anti-TLR4 Ab, anti-PGRP-Iα, -Iβ, -S Ab (Imgenex, San Diego, CA, USA), or isotype matched control IgG (Beckman Coulter) at 4 °C for 30 min, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or FITC-conjugated rabbit goat IgG (Biosource International, Camarillo, CA, USA) at 4 °C for a further 30 min. For TLR3, TLR7, NOD1 and NOD2 staining, intracellular staining protocols were performed. Briefly, the cells were washed with staining buffer, fixed, and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences) for 15 min at 4 °C, and then incubated with anti-TLR3, anti-NOD1 or anti-NOD2 Ab, or control IgG for 30 min, followed by FITC-conjugated secondary antibody at 4 °C for another 20 min. The use of isotype-matched Ab excludes the possibility of the nonspecific binding of each Ab. To calculate the percentage of positive cells, the baseline cursor was set at a channel that yielded less than 2% of the events positive with a second Ab control. Fluorescence to the right was counted as specific binding.

### 2.6. Immunostaining

Human epithelial cells were cultured on eight-chamber glass slides (Falcon) until reaching confluence, treated with synthetic PAMPs for 24 h at 37 °C in a 5% $CO_2$ incubator, and washed three times with PBS. After fixation with 4% paraformaldehyde for 15 min at room temperature, the cells were treated with 0.5% Triton X-100 for 15 min for intracellular staining. Cells were then treated with mouse anti-TLR2 mAb, mouse anti-TLR3, mouse anti-TLR4 mAb, mouse anti-TLR7, goat anti-NOD1 Ab, goat anti-NOD2 Ab or goat anti-β-defensin 2 (1:100) mAb for 3 h at room temperature. As a negative control, mouse IgG1 or goat Ig (DakoCytomation, Tokyo, Japan) were used. Samples were then washed and incubated with Alexa Fluor488 rabbit

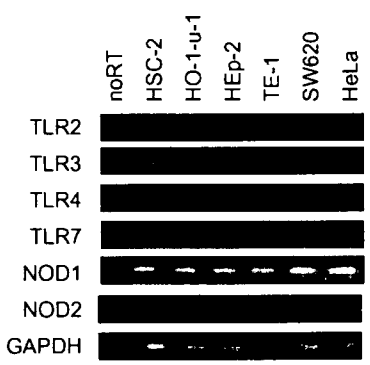


Fig. 1. Expression of mRNA of TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2 in various human epithelial cells. Human oral HSC-2, HO-1-u-1, pharyngeal HEp-2, esophageal TE-1, intestinal SW620 and cervical HeLa epithelial cells were cultured until reaching confluence at 37 °C. After incubation, the total RNA was extracted, and the mRNA expressions of TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2 were analyzed using PCR. The results presented are representative of three different experiments demonstrating similar results.

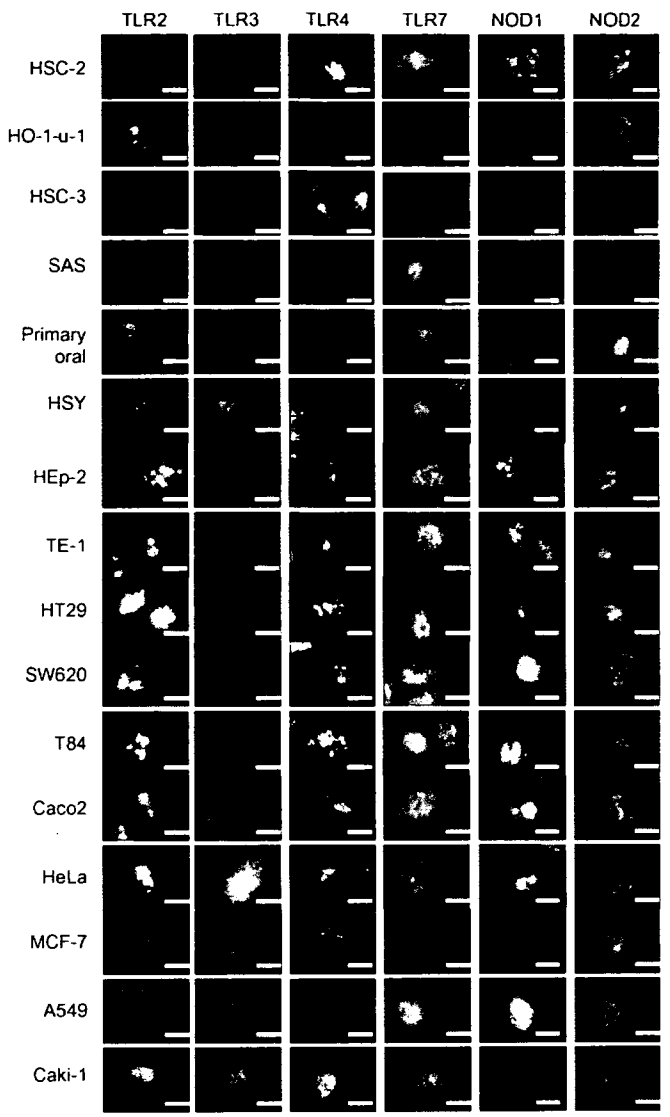


Fig. 2. Expression of TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2 molecules on human epithelial cells. Human oral HSC-2, HO-1-u-1, HSC-3, SAS, primary oral epithelial cells, salivary gland HSY, pharyngeal HEp-2, esophageal TE-1, intestinal HT29, SW620, T84, Caco-2, cervical HeLa, breast MCF-7, lung A549 and kidney Caki-1 epithelial cells were cultured until reaching confluence at 37 °C. After the fixation, cells were stained with anti-TLR2 mAb, anti-TLR3 mAb, anti-TLR4 mAb, anti-TLR7 mAb, anti-NOD Ab, anti-NOD2 Ab, mouse IgG1 or goat IgG, followed by incubation with Alexa Fluor 488 (green). Nuclei were visualized by staining with 4′,6-diamino-2-phenylindole (blue). The results presented are representative of four different experiments demonstrated similar results.

anti-goat IgG1 (1/500) or Alexa Fluor488 goat anti-mouse IgG1 (1/2000) (Molecular Probes, Eugene, OR, USA). Nuclei were visualized by staining with 4′,6-diamino-2-phenylindole in blue (Molecular Probes). Samples were photographed with an AxioCamMRm digital camera mounted on the Zeiss AxioImager Z1 microscope using the application Zeiss AxioVision 4 software (Carl Zeiss, Jena, Germany).

### 2.7. RNA interference

Transfections for targeting endogenous NF-κB p65 was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and siRNA (final concentration, 200 nM) for 24 h at 37 °C, according to the manufacturer's instructions. The cellular viability of the cells after transfection was more than 95%, as assessed

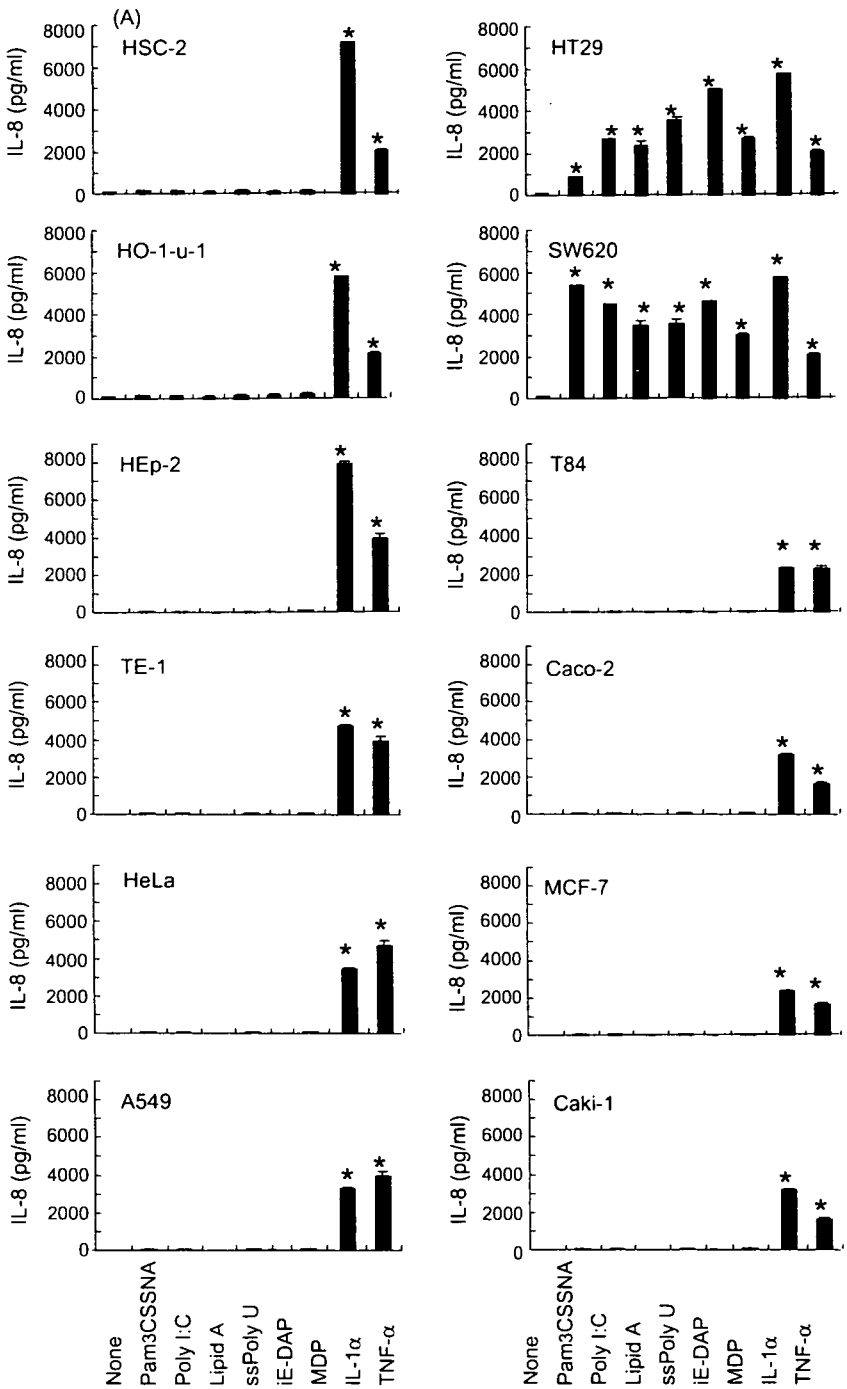


Fig. 3. Human oral, pharyngeal, esophageal, cervical, breast, lung and kidney epithelial cells did not secrete IL-6, IL-8 and MCP-1 in response to synthetic PAMPs. Human oral HSC-2, HO-1-u-1, pharyngeal HEp-2, esophageal TE-1, intestinal HT29, SW620, T84, Caco-2, cervical HeLa, breast MCF-7, lung A549 and kidney Caki-1 epithelial cells were incubated with Pam3CSSNA (100 ng/ml), Poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPoly U (1 μg/ml), MDP (100 μg/ml), iE-DAP (100 μg/ml), IL-1α (10 ng/ml) and TNF-α (10 ng/ml), respectively, for 24 h in triplicate. Human IL-1α and TNF-α were used as positive controls. The levels of IL-6 (A), IL-8 (B) or MCP-1 (C) in the culture supernatants were determined using ELISA. Data are expressed as mean values ± S.D., and significant differences are shown. $^{*}P < 0.01$ vs. medium alone. The results presented are representative of three different experiments demonstrating similar results.

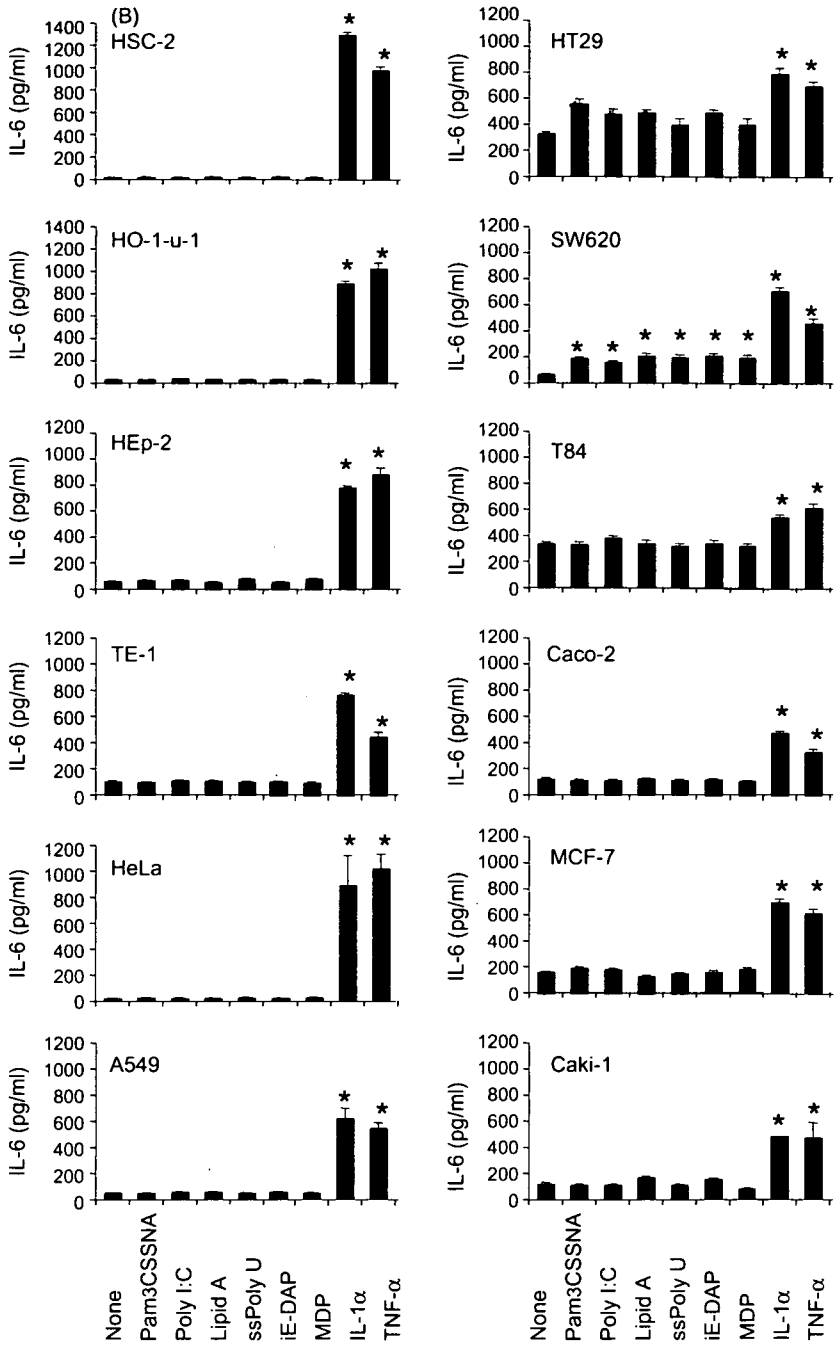


Fig. 3. (*continued*)

by a 0.2% trypan blue exclusion test, and morphological character was not changed after transfection. NF-κB p65 siRNA and anti-NF-κB p65 Ab (mouse IgG2a) were purchased from Santa Cruz Biotechnology.

### 2.8. Data analysis

All experiments were performed at least three times to confirm the reproducibility of the results. For most experiments, results are shown as the means ± S.D. of triplicate assays. The statistical significance of differences between two means was evaluated with a one-way analysis of variance, using the Bonferroni or Dunnett method, and *P*-values less than 0.05 were considered significant.

## 3. Results

### 3.1. Human oral, tongue, salivary gland, pharyngeal, esophageal, intestinal, cervical, breast, lung, and kidney epithelial cells in culture constitutively expressed TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2

Firstly, we examined whether human epithelial cells in culture expressed TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2

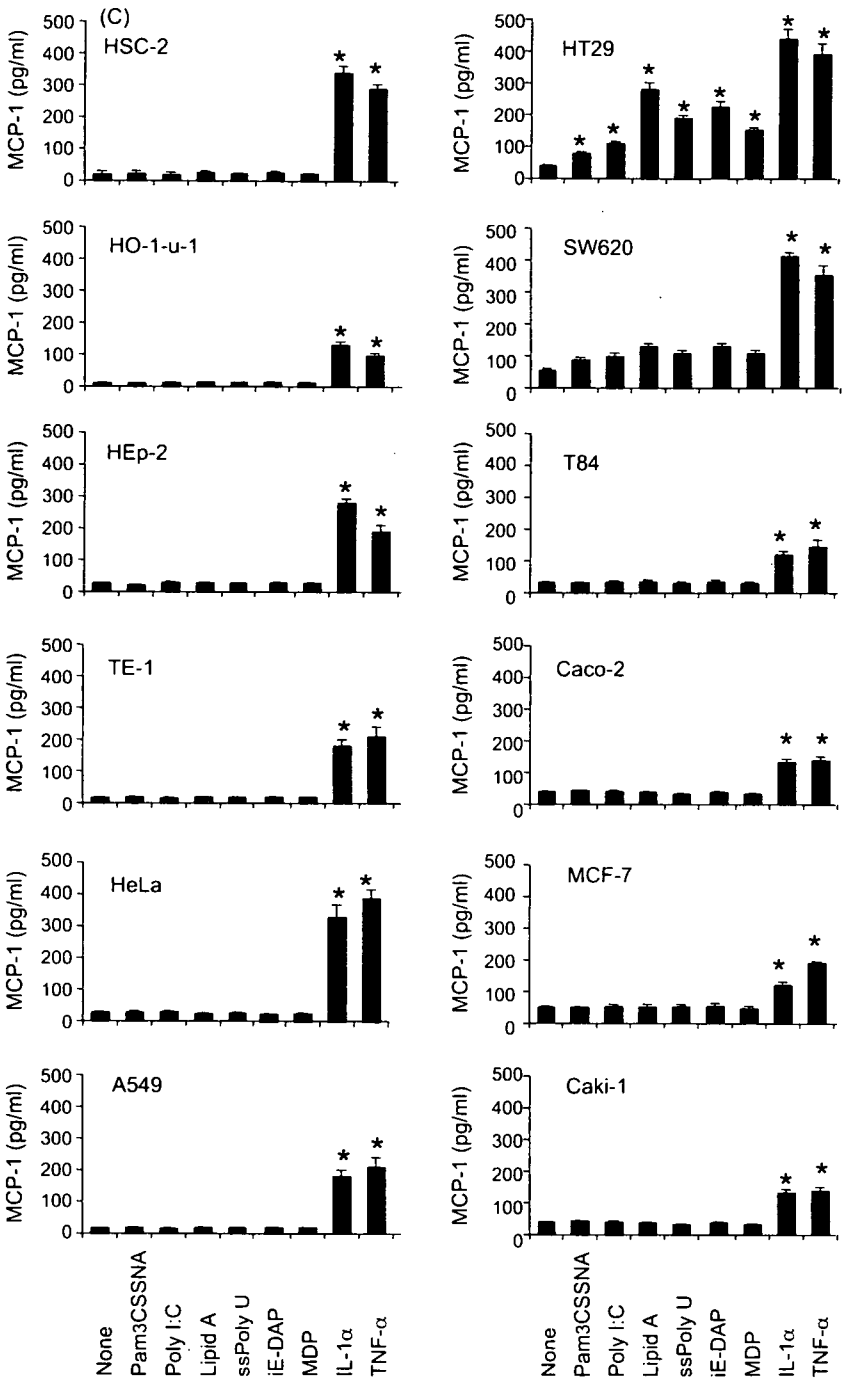


Fig. 3. (*continued*).

molecules by RT-PCR and immunostaining. It was found that human oral HSC-2, HO-1-u-1, pharyngeal HEp-2, esophageal TE-1, intestinal SW620 and cervical HeLa epithelial cells expressed the mRNA of these molecules (Fig. 1). Consistent with the results of RT-PCR, tongue, salivary gland, breast, lung, and kidney epithelial cells and primary oral epithelial cells in addition to these epithelial cells constitutively expressed TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2 molecules, as determined by immunostaining assay (Fig. 2). It must be noted that these cells were stained with goat IgG or mouse IgG1, followed by Alexa Fluor 488 (green) but did not show any positive fluorescence (data not shown). In flow cytometry analysis, we could clearly detect cell surface expressions of TLR2 and TLR4 and the intracellular expressions of TLR3, TLR7, NOD1 and NOD2, although the percentages of TLR-positive cells were lower than respective NOD-positive cells (Table 1).

### *3.2. Human oral, pharyngeal, esophageal and cervical, but not intestinal epithelial cells do not secrete proinflammatory cytokines upon stimulation with bacterial components*

We previously reported that the human primary oral epithelial cells and oral epithelial cell line HSC-2 and HO-1-u-1 did not