Involvement of Toll-Like Receptor 4 Signaling in Interferon-γ Production and Antitumor Effect by Streptococcal Agent OK-432

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Background: **The streptococcal agent OK-432 has been used for immunotherapy of head and neck cancer, among other malignancies, but its mechanism of action is unknown. Because the Toll-like receptor 4 (TLR4)/MD-2 complex is important in enabling the mammalian immune system to recognize bacterial components, we investigated whether expression of the TLR4 and MD-2 genes is associated with OK-432-induced anticancer immunity.** ***Methods:*** **Peripheral blood mononuclear cells (PBMCs) from 28 patients with head and neck cancer were analyzed for TLR4 and MD-2 mRNA expression by reverse transcription–polymerase chain reaction (RT–PCR) analysis. PBMCs were treated *in vitro* with OK-432 or with OK-PSA (a lipoteichoic-acid-related molecule that is an active component of OK-432), and interferon-gamma (IFN-γ) mRNA expression, an immune response measure, was analyzed by RT–PCR. Patient sera collected 24 hours after OK-432 administration were examined for IFN-γ protein using an enzyme-linked immunosorbent assay. Lewis lung carcinoma-bearing wild-type C57BL/6 and TLR4-deficient mice (four mice per group) received intraperitoneal injections of OK-432, and tumor volumes and sera IFN-γ levels were measured over time. All statistical tests were two-sided.** ***Results:*** **Twenty patients expressed both TLR4 and MD-2. Expression of TLR4 and MD-2 genes was associated with the *in vivo* IFN-γ induction in 19 patients administered OK-432 (Fisher's exact test $P<.001$). Although both OK-432 and OK-PSA induced IFN-γ expression from PBMCs *in vitro*, expression of TLR4 and MD-2 was associated only with IFN-γ expression induced by OK-PSA ($P<.001$). *In vivo* intraperitoneal administration of OK-432 resulted in an increase of IFN-γ in sera from wild-type mice but not in sera from TLR4-deficient mice. Tumors in wild-type mice treated with OK-432 were statistically significantly smaller than those in mice treated with saline ($P = .007$). By contrast, in TLR4-deficient mice, there was no difference in tumor volume between the two treatment groups.** ***Conclusions:*** **TLR4 and MD-2 may mediate OK-432-induced anticancer immunity. [J Natl Cancer Inst 2003;95:316–26]**

OK-432, a penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus pyogenes* (group A) *(1),* has been successfully used as an immunotherapeutic agent in many types of malignancies, including head and neck cancer *(2–6).* We have also reported that OK-432-based immunotherapy has a marked therapeutic effect in patients with oral squamous cell carcinoma *(7,8).* OK-432 elicits antitumor effects by stimulating immunocompetent cells such as macrophages, T cells, and natural killer (NK) cells, and by inducing multiple cytokines including interleukin 1 (IL-1), IL-2, IL-6, tumor necrosis factor-α (TNF-α), and interferon-gamma (IFN-γ) *(9–11).* In addition, OK-432 induces IL-12 and polarizes the T-cell response to a helper T-cell 1 (Th1) dominant state *(12).*

Despite the diverse immune effects and encouraging clinical results *(2–12),* not all patients respond to OK-432 therapy. Two trials were undertaken to discriminate between those who respond to OK-432 and those who do not: one trial evaluated a skin test with a polysaccharide fraction of OK-432 *(13),* and the other evaluated the relationship between T-cell receptor expression and OK-432 responsiveness *(14).* However, no definitive methodology has been established to predict patient responsiveness. In part, this may be associated with limited progress made in elucidating the molecular mechanism of action of OK-432, i.e., the identification of effective component(s) of OK-432, and their molecular target(s), such as receptors or signal transducers in immunocompetent cells.

One component of OK-432 that appears to be important in the antitumor effect is a lipoteichoic acid (LTA)-related molecule, OK-PSA *(15,16).* OK-PSA was isolated from a butanol extract of OK-432 by using a cyanogen bromide (CNBr)-activated Sepharose 4B affinity column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) bound with the monoclonal antibody TS-2, which neutralizes the IFN-γ-inducing activity of OK-432 *(15,16).* OK-PSA is a more potent inducer of Th1 cytokines and killer cell activities from human peripheral blood mononuclear cells (PBMCs) than OK-432 and has a marked antitumor activity in tumor-bearing mice *(15,17–20).* Furthermore, we recently showed that, in mice, OK-PSA elicits cytokine-inducing and antitumor activities via Toll-like receptor 4 (TLR4) signaling

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See "Notes" following "References."

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(21), a finding which may help clarify the molecular mechanism of the OK-432-induced antitumor host responses.

TLRs are transmembrane proteins that belong to a newly recognized family of vertebrate pattern recognition receptors within the innate immune system *(22,23).* Subsequent to ligand binding, TLRs initiate signaling through the sequential recruitment of myeloid differentiation protein 88, IL-1R-associated kinase, and TNFR-associated factor 6, which in turn activate downstream mediators such as mitogen-activated protein kinases and nuclear factor-κB (NF-κB) *(24,25).* Among the 10 identified TLRs, TLR2 and TLR4 recognize gram-positive and gram-negative bacterial cell wall components, including lipopolysaccharide (LPS), LTA, lipoprotein, peptidoglycan (PGN), and lipoarabinomannan *(26–31).* TLR9 recognizes bacterial unmethylated CpG DNA and is the receptor that distinguishes bacterial DNA from self-DNA *(32).* It has also been reported that MD-2 *(33)* and CD14 *(34)* act as important cofactors in TLR signaling. In particular, MD-2 physically associates with TLR4 on the cell surface, and the TLR4/MD-2 complex confers responsiveness to bacterial components *(33).*

Because OK-432 is a bacterial preparation containing multiple components, including OK-PSA, PGN, and unmethylated CpG DNA, it may be possible that signaling via TLRs and their cofactors is involved in OK-432-induced anticancer immunity. In the current study, we examined the relationship between the expression of TLRs and response to OK-432, as indicated by changes in IFN-γ levels, in patients with head and neck cancer to identify target molecule(s) for OK-432-based immunotherapy.

Patients and Methods

Preparation of OK-PSA

OK-PSA was prepared from OK-432 (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) as described *(15).* The OK-PSA preparation was tested for LPS contamination by using the Endospecy ES-50M set (Seikagaku Kogyo, Tokyo, Japan), according to the manufacturer's recommended protocol. LPS concentrations in samples were calculated from a standard curve of known LPS concentrations (derived from a solution of *Escherichia coli* 0111:B4 endotoxin in distilled water) plotted against absorbance at 545 nm *(35).*

Patients

This study was carried out in accordance with the standards of our Institutional Committee for the Protection of Human Subjects. Written informed consent was obtained from all patients, and the collection of the samples was approved by the Institutional Review Board. From October 1996 through September 2001, 28 patients (19 male and nine female) with head and neck cancer were treated with OK-432 (Chugai Pharmaceutical Co., Ltd.) at the Second Department of Oral and Maxillofacial Surgery, The Hospital of Tokushima University School of Dentistry. The median patient age was 64 years (range = 36–77 years). On the basis of the Tumor–Node–Metastasis system for the classification of malignant diseases *(36),* there were 17 T2 cancers, seven T3 cancers, four T4 cancers, 15 N0 cancers, 12 N1 cancers, and one N3 cancer. All cancers were M0. There were 27 squamous cell carcinomas and one mucoepidermoid carcinoma. Primary sites of the tumors were tongue (n = 11), upper alveolus and gingiva (n = 7), lower alveolus and gingiva (n = 3), buccal mucosa (n = 2), floor of the mouth, hard palate, maxillary sinus, parotid gland, and oropharynx (each n = 1) (Table 1). All of the patients had primary disease and had not received any previous treatment. None of the patients were severely immunodeficient, as determined from clinical examinations.

Protocol for Clinical Study

Within 1 week of diagnosis, patients were enrolled in the study to receive OK-432 as part of a regimen for the treatment of head and neck cancer. The regimen included treatment with OK-432 administered peritumorally or intradermally at a dose of 0.5 Klinische Einheit (KE), i.e., 50 μg/week. The dose was increased to 5 KE/week. Patients were monitored for general symptoms related to OK-432 therapy, such as increased fever and/or fatigue. All patients received therapy with OK-432 and UFT, an oral fluoropyrimidine formulation combining tegafur and uracil in a 1:4 ratio (Taiho Pharmaceutical Co., Tokyo, Japan), in combination with radiation therapy.

Two blood samples were collected from each patient 5 hours before receiving the first injection of OK-432. From one sample, PBMCs were prepared by the standard Ficoll–Hypaque gradient density centrifugation method *(37).* From the other sample, serum was isolated and immediately frozen at −80 °C until assayed for IFN-γ levels. Twenty-four hours after patients received an injection of OK-432, an additional blood sample was collected and the serum was isolated and frozen until assayed for IFN-γ levels. Within 3 days of collection, all sera were assayed for IFN-γ levels with an enzyme-linked immunosorbent assay (ELISA) at the Otsuka Assay Laboratory (Tokushima, Japan). The ELISA performed has a lower limit of sensitivity of 7.8 pg/mL for detecting human IFN-γ.

In Vitro Culture and Treatment of PBMCs With OK-432 or OK-PSA

PBMCs (1×10^6/mL) were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD) in the presence or absence of OK-432 (0.1 KE, i.e., 10 μg/mL] or OK-PSA (10 μg/mL) for 24 hours. In some experiments, polymyxin B (25 μg/mL; Sigma Chemical Co., St. Louis, MO) was added to cultures treated with OK-PSA, and in other experiments, LPS (derived from *E. coli* 055:B5; Sigma Chemical Co.), with or without polymyxin B, was added to untreated cultures.

RNA Extraction and Semiquantitative Reverse Transcription–Polymerase Chain Reaction (RT–PCR)

After 24 hours, total RNA was extracted from the PBMC cultures by a modified acid–guanidinium–thiocyanate–phenol–chloroform method *(38)* using ISOGEN RNA extracting mixture (Nippon Gene, Toyama, Japan), according to the manufacturer's recommendations. Expression of mRNAs for IFN-γ, TLR2, TLR4, TLR9, MD-2, CD14, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene that was used as an internal control, was detected by the semiquantitative RT–PCR. First, 1 μg of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies Inc., Gaithersburg, MD) and random primers (Life Technologies Inc.) in a volume of 20 μL at 42 °C for 60 minutes. Next, 2 μL of the reverse-transcribed mixture was subjected to PCR (10 m*M* Tris–HCl [pH 8.3]; 50 m*M* KCl; 1.5 m*M* $MgCl_2$;

Table 1. Summary of the results obtained from head and neck cancer patients treated with OK-432

Case No.	Age, y	Sex	Primary site	TMN* classification	Histopathologic diagnosis	TLR2	TLR4	TLR9	MD-2	CD14	IFN-γ†	IFN-γ‡	IFN-γ§	Operation
1	68	M	Tongue	T2N0M0	SCC	+‖	+	+	−	+	−	+	ND	Yes
2	64	M	Tongue	T2N0M0	SCC	+	+	+	+	+	+	+	3420	Yes
3	77	F	Tongue	T3N1M0	SCC	+	+	+	+	+	+	+	153	No
4	59	M	Upper alveolus and gingiva	T2N3M0	SCC	+	+	+	+	+	−	+	ND	Yes
5	73	M	Tongue	T3N1M0	SCC	+	+	+	+	+	+	+	151	Yes
6	67	F	Tongue	T3N1M0	SCC	+	+	+	+	+	+	+	173	Yes
7	64	F	Tongue	T4N1M0	SCC	+	−	+	+	+	−	+	ND	Yes
8	69	M	Upper alveolus and gingiva	T3N1M0	SCC	+	+	+	+	+	+	+	244	Yes
9	58	M	Tongue	T3N0M0	SCC	+	+	+	+	+	+	+	467	No
10	77	M	Tongue	T2N1M0	SCC	+	+	+	+	+	+	+	120	Yes
11	69	M	Maxillary sinus	T3N0M0	SCC	+	+	+	+	+	+	+	90.6	No
12	76	M	Lower alveolus and gingiva	T2N1M0	SCC	+	+	+	+	+	+	+	99.7	No
13	58	M	Parotid gland	T4N1M0	SCC	±	+	+	+	+	+	+	49.9	Yes
14	73	F	Buccal mucosa	T2N1M0	SCC	+		−	+	+	+	+	1810	No
15	68	M	Floor of mouth	T2N0M0	SCC	+	+	+	+	+	+	+	255	No
16	59	F	Upper alveolus and gingiva	T2N0M0	SCC	+	+	+	+	+	+	+	388	No
17	36	F	Hard palate	T2N0M0	Mucoepidermoid carcinoma	+	+	+	−	+	−	+	ND	Yes
18	66	M	Buccal mucosa	T2N0M0	SCC	+	+	+	−	+	−	+	188	Yes
19	71	M	Oropharynx	T2N0M0	SCC	+	+	+	+	+	+	+	180	No
20	66	M	Tongue	T2N0M0	SCC	+	+	+	+	+	+	+	423	Yes
21	55	F	Lower alveolus and gingiva	T4N1M0	SCC	+	+	+	+	+	+	+	1512	No
22	55	F	Tongue	T3N0M0	SCC	+	+	+	+	+	+	+	95.4	No
23	52	M	Upper alveolus and gingiva	T2N0M0	SCC	+	−	+	+	+	−	+	ND	Yes
24	63	M	Tongue	T2N1M0	SCC	+	+	+	+	+	+	+	126	Yes
25	72	M	Upper alveolus and gingiva	T2N1M0	SCC	+	+	−	−	+	−	+	115	Yes
26	49	F	Upper alveolus and gingiva	T2N0M0	SCC	+	+	+	−	+	−	+	ND	Yes
27	64	M	Upper alveolus and gingiva	T2N0M0	SCC	+	+	+	+	+	+	+	134	Yes
28	64	M	Lower alveolus and gingiva	T4N0M0	SCC	+	+	+	−	+	−	+	ND	Yes

*Tumor–Node–Metastasis (TNM) classification according to 1997 International Union Against Cancer (UICC) CRITERIA *(36).* SCC = squamous cell carcinoma; TLR = Toll-like receptor; IFN-γ = interferon gamma; RT–PCR = reverse transcription–polymerase chain reaction; PBMC = peripheral blood mononuclear cells; ND = not detected (<7.8 pg/mL).

†Expression of IFN-γ messenger RNA (mRNA) from patient-derived PBMC treated with OK-PSA *in vitro.*

‡Expression of IFN-γ mRNA from patient-derived PBMC treated with OK-432 *in vitro*.

§Amounts of IFN-γ protein in the sera of patients 24 hours after receiving OK-432.

‖mRNA expression was assessed by RT–PCR. Each RT–PCR product was quantified by densitometry and expressed as a ratio to the relative density (RD) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). − = an RD ratio of <0.1; ± = an RD ratio of equal to or greater than 0.1 but less than 0.5; + = an RD ratio of greater than or equal to 0.5.

0.01% gelatin; dATP, dGTP, dTTP, and dCTP, each at 20 m*M*), 0.5 U *Taq* polymerase (Takara Bio Inc., Otsu, Japan), and 0.25 pmol of each primer in a total volume of 20 μL. This study used the primer sequences 5′-GCCAAAGTCTTGATTGATTGG-3′ as an upstream primer and 5′-TTGAAGTTCTCCAGCTCCTG-3′ as a downstream primer for TLR2, which yield a 394-base pair (bp) fragment *(39);* 5′-TGGATACGTTTCCTTATAAG-3′ as an upstream primer and 5′- GAAATGGAGGCACCCCTTC-3′ as a downstream primer for TLR4, which yield a 506-bp fragment *(39);* 5′-GTGCCCCACTTCTCC-3′ as an upstream primer and 5′-GGCACAGTCATGATGTTGTTG-3′ as a downstream primer for TLR9, which yield a 270-bp fragment *(40);* 5′-GAATTCATGTTACCATTTCTGTTT-3′ as an upstream primer and 5′-GAATTCTAATTTGAATTAGGTTGG-3′ as a downstream primer for MD-2, which yield a 493-bp fragment *(33);* 5′-GCAGCCTCCTCCTTGTCGCTACC-3′ as an upstream primer and 5′-TCAGGGCCTGCATCAGCTCATCAA-3′ as a downstream primer for CD14, which yield a 450-bp fragment *(41);* 5′-TGCAGGTCATTCAGATGTAG-3′ as an upstream primer and 5′-AGCCATCACTTGGATGAGTT-3′ as a downstream primer for IFN-γ, which yield a 306-bp fragment *(42);* and 5′-GAAATCCCAGCACCATCTCCAGG-3′ as an upstream primer and 5′-GTGGTGGACCTCATGGCCCAC-CATG-3′ as a downstream primer for GAPDH, which yield a 781-bp fragment *(43).* To amplify the fragments, 25, 28, or 30 PCR cycles were used, with each cycle consisting of 94 °C for 60 seconds, 55 °C for 90 seconds, and 72 °C for 150 seconds, with an initial denaturation step of 94 °C for 5 minutes and a final elongation step of 72 °C for 5 minutes. PCR was carried out in a DNA Thermal Cycler (Takara Bio Inc.). Amplified complementary DNA (cDNA) was electrophoresed through 1.5% agarose gels containing 100 ng/mL ethidium bromide. After elec-

trophoresis, gels were illuminated with ultraviolet light, viewed, and photographed (Polaroid type 667 film; Polaroid Corp., Cambridge, MA). Densitometric analysis for the RT–PCR band patterns was done by using NIH Image 1.59 software (National Institutes of Health, Bethesda, MD). The relative density (RD) of each specific RT–PCR band was expressed as a ratio to the density of GAPDH, with negative expression defined as RD less than 0.1, marginal expression defined as RD equal to or more than 0.1 but less than 0.5, positive expression defined as RD equal to or greater than 0.5. The identification of each amplified product was confirmed by automated DNA sequencing.

To determine the optimal condition for the semiquantitative RT–PCR described above, we reverse transcribed 0.1, 0.5, 1, and 5 μg of total RNA and, using identical PCR conditions, amplified the fragments with different numbers of PCR cycles (from 18 to 40). We decided to use 1 μg of total RNA as a template for RT and to use 25 cycles of PCR amplification for GAPDH, 28 cycles for TLR4, and 30 cycles for the other factors.

Established Cell Lines and Culture Media

The derivation of the IL-3-dependent mouse proB cell line Ba/F3 stably expressing human TLR2 (Ba/hTLR2), mouse TLR2 (Ba/mTLR2), human TLR4 (Ba/hTLR4), human TLR4 and human MD-2 (Ba/hTLR4/hMD-2), mouse TLR4 (Ba/mTLR4), and mouse TLR4 and mouse MD-2 (Ba/mTLR4/mMD-2) have been described elsewhere *(33,44,45).* All of the transfectants have also been transfected with p55IgκLuc, an NF-κB reporter construct *(33,44,45).* The construct p55IgκLuc contains the NF-κB p55 regulatory sequence from the immunoglobulin (Ig) kappa light chain promoter upstream of a *Photonius pyralis* luciferase gene. The construct is used as a measure of NF-κB activity. A control cell line that stably expressed only p55IgκLuc (Ba/κB) was also established. Parental Ba/F3 cells do not express TLR2, TLR4, or MD-2 *(33).*

The transfectants were grown in RPMI-1640 medium (Life Technologies Inc.) containing 10% heat-inactivated FBS and 100 U/mL of mouse IL-3. LL/2, a Lewis lung carcinoma cell line, was obtained from American Type Culture Collection (Manassas, VA) and grown in Eagle's minimum essential medium (Life Technologies Inc.) supplemented with 10% FBS. All cell lines were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Luciferase Assay

The stable transfectants expressing human or mouse TLRs and p55IgκLuc were seeded at 5×10^5 cells per well into each well of a 48-well dish (Corning Inc., Vernon, NY), and then 1 or 10 μg/mL of OK-PSA, OK-432, LPS (derived from *E. coli* 055:B5; Sigma Chemical Co.), or PGN (Fluka 77140; Sigma Chemical Co.) was added. After 5 hours at 37 °C, the cells were harvested and lysed in 100 μL of Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI). Luciferase activity was measured using 20 μL of lysate and 100 μL of Luciferase Assay Substrate (Promega). The luminescence was quantified in relative light units (RLU) by a luminometer (Bio-Orbit, Turku, Finland). Relative luciferase activity was obtained by dividing the RLU value for each treated sample with the RLU value of each untreated sample. The experiment was repeated three times. A 5-hour incubation period was chosen on the basis of our previous studies [Okamoto M, Sato M: unpublished observations and *(33, 44,45)*].

Animals and *In Vivo* Experiments

All mice were maintained according to the National Institutes of Health standards established in the Guidelines for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/guide/guide2.htm), and all experimental protocols were approved by the Animal Investigation Committee of Tokushima University, Tokushima, Japan. C57BL/6 mice deficient in TLR2 ($TLR2^{-/-}$) or in TLR4 ($TLR4^{-/-}$) were previously generated by gene targeting *(30,46).* C57BL/6 mice that express wild-type TLR2 and TLR4 were purchased from Japan SLC Inc. (Shizuoka, Japan). In total, 71 mice were used for the present study.

To examine the role of TLR4 signaling in *in vivo* IFN-γ induction by OK-432, wild-type C57BL/6, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were given a single intraperitoneal injection of 100 μg of OK-432. After 6 days, OK-432 (100 μg/mouse) or physiologic saline was administered intraperitoneally, and 6 hours later, sera were collected and immediately frozen at −80 °C until assayed for IFN-γ levels. ELISA was performed to determine the IFN-γ concentration in the sera within 3 days.

The antitumor effect of OK-432 was evaluated both in wild-type C57BL/6 and $TLR4^{-/-}$ mice. Syngeneic LL/2 cells (5×10^5/mouse) were injected subcutaneously into the footpads of the mice (one footpad per mouse). Approximately 7 days after the injection and when tumors were approximately 40 mm^3 in volume, the mice were randomly divided into two groups of eight, with almost equal mean tumor volumes. The mice were given 0.2 mL of physiologic saline or 100 μg of OK-432 peritumorally on days 8, 12, 15, 19, and 22. The mice were monitored twice a week, and the tumor volume was estimated by measuring tumor size and using the following formula: tumor volume $= 0.4 \times L \times W^2$, where L represents the largest diameter and W represents the smallest diameter.

Treatment of Mouse Spleen Cells With Immunomodulators

Spleen cells (2×10^6/mL) were isolated from wild-type C57BL/6, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice as described *(47)* and treated with OK-PSA (1 or 10 μg/mL), OK-432 (1 or 10 μg/mL), LPS (1 or 10 μg/mL), or PGN (1 or 10 μg/mL). After 48 hours, supernatants were collected and assayed for IFN-γ by ELISA (BioSource International, Inc., Camarillo, CA).

We also examined the effect of long-term OK-432 treatment of mouse spleen cells on IFN-γ induction. OK-432 (1 or 10 μg/mL) was added to the culture of spleen cells isolated from wild-type or $TLR4^{-/-}$ mice. Supernatants were collected at 0, 12, 24, 48, 72, 120, and 168 hours after adding OK-432 and immediately frozen at −80 °C until they were assayed for IFN-γ. All ELISAs were performed within 3 days of collecting the supernatants.

Statistical Analysis

The relationship between the TLR expression and IFN-γ induction by OK-432 or OK-PSA was statistically evaluated by a two-sided Fisher's exact test. In animal experiments, results were expressed as means with 95% confidence intervals (CIs). Comparisons between groups were made using Student's two-tailed *t* test. Values of $P<.05$ were considered statistically sig-

nificant. All statistical analyses were performed using StatView, version 5.0 (SAS Institute, Cary, NC).

RESULTS

Effect of Polymyxin B on OK-PSA-Induced IFN-γ Production

To determine whether the IFN-γ-inducing activity of OK-PSA was the result of endotoxin contaminating the OK-PSA preparation, we examined the effect of polymyxin B, an LPS inhibitor, on OK-PSA-induced IFN-γ production from PBMCs derived from healthy volunteers. IFN-γ production induced by OK-PSA was not inhibited by the addition of 25 μg/mL of polymyxin B, whereas IFN-γ production induced by LPS was almost completely neutralized by polymyxin B (data not shown). These results suggest that IFN-γ-inducing activity of OK-PSA was not the result of endotoxin contaminating the OK-PSA preparation.

Expression of mRNAs for TLRs and Their Cofactors in PBMCs Derived From Patients With Head and Neck Cancer

To identify the target molecule(s) for OK-432-based immunotherapy, we first examined the expression of TLR2, TLR4, TLR9, MD-2, and CD14 by RT–PCR analysis in PBMCs from 28 patients with head and neck cancer. We defined levels of expression on the basis of calculated relative signal intensities. Of 28 patients, 27 (96.4%) patients expressed TLR2 mRNA and one (3.6%) patient marginally expressed TLR2 mRNA; 26 (92.9%) patients expressed TLR4 mRNA and two (7.1%) patients did not; 26 (92.9%) patients expressed TLR9 mRNA and two (7.1%) patients did not; 22 (78.6%) patients expressed MD-2 mRNA and six (21.4%) patients did not. All patients expressed CD14 mRNA. Twenty (71.4%) patients expressed mRNA for TLR4 and MD-2 (Fig. 1, A, and Table 1).

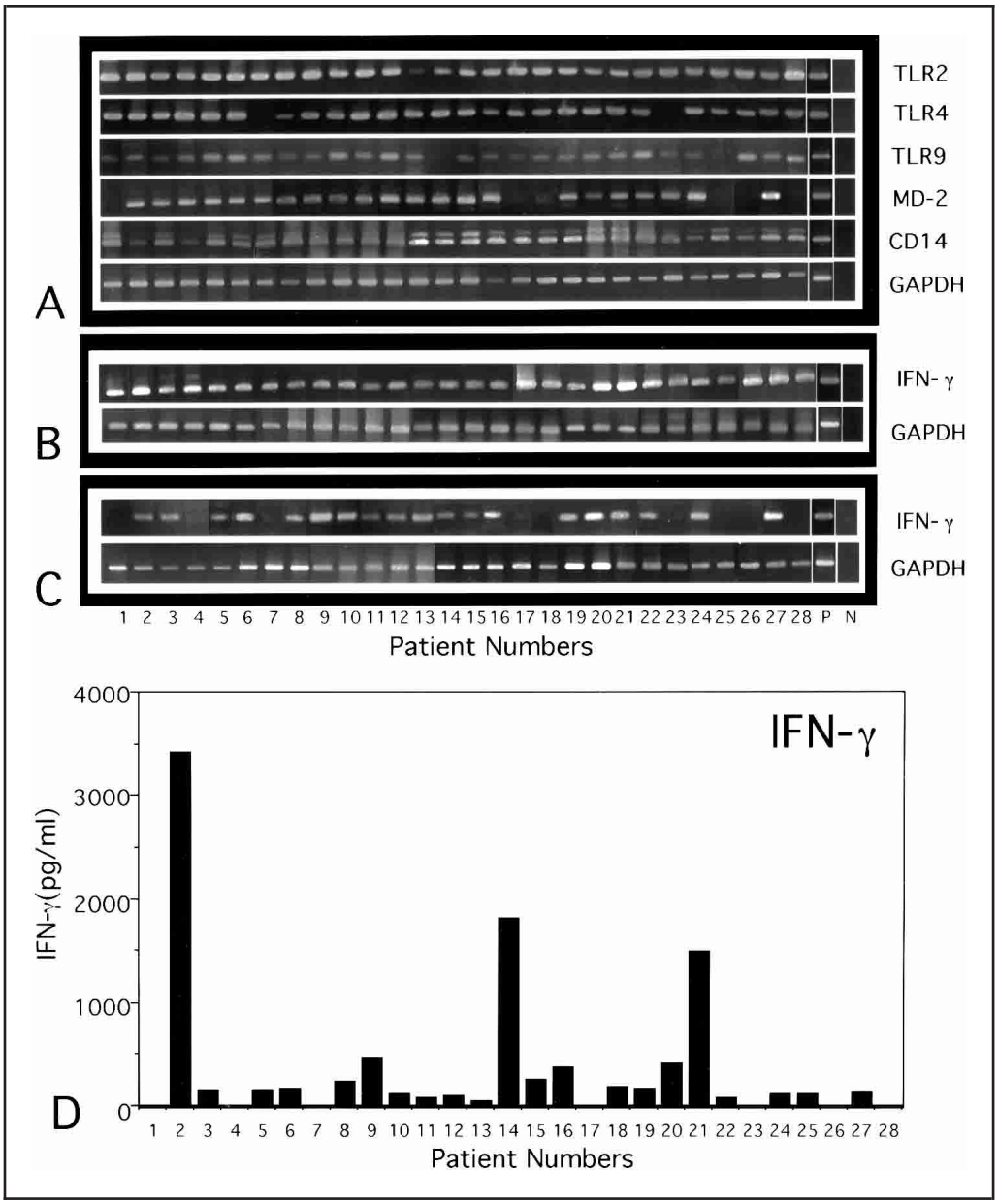

Fig. 1. Expression of Toll-like receptors (TLRs), MD-2, and CD14, and interferon-gamma (IFN-γ) in head and neck cancer patients. Total RNAs were extracted from the peripheral blood mononuclear cells (PBMCs) isolated from 28 patients with head and neck cancer, and expression of mRNAs for TLR2, TLR4, TLR9, MD-2, CD14, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed by reverse transcription–polymerase chain reaction (RT–PCR). RT–PCR products were separated by electrophoresis and visualized by staining with ethidium bromide (**A**). Patient-derived PBMCs (1×10^6/mL) were stimulated with 10 μg/mL of OK-432 (**B**) or OK-PSA (**C**) for 24 hours. Total RNAs were extracted, and expression of IFN-γ and GAPDH messenger RNAs (mRNAs) were analyzed by RT–PCR. Sera were collected from patients with head and neck cancer 24 hours after they received a peritumoral or intradermal injection of OK-432. IFN-γ levels in the sera were measured with a specific enzyme-linked immunoassay (ELISA) (**D**). In **A, B,** and **C,** positive controls (**P**) for the RT–PCR used total RNAs from Ba/F3 cells transfected with the expression plasmid containing each complementary DNA (cDNA) for human TLRs, MD-2, CD14, or IFN-γ. PCRs done without reverse transcription mixture were used as negative controls.

Effect of OK-432 and OK-PSA on IFN-γ Induction From Patient-Derived PBMCs

To determine the effect of OK-432 and OK-PSA on IFN-γ induction, patient-derived PBMCs were treated with OK-432 or OK-PSA for 24 hours, and the expression of IFN-γ mRNA was then analyzed by RT–PCR. Expression of IFN-γ mRNA was not detected or was only faintly detected (RD<0.1) in untreated PBMCs derived from all patients (data not shown). Expression of IFN-γ mRNA was observed in OK-432-treated PBMCs from all patients (Fig. 1, B, and Table 1). By contrast, expression of IFN-γ mRNA was detected in OK-PSA-treated PBMCs from 19 (67.9%) patients but not from nine (32.1%) patients (Fig. 1, C, and Table 1).

All 28 patients were administered OK-432 as a cancer immunotherapy. We measured IFN-γ levels in sera collected from the patients 5 hours before and 24 hours after OK-432 administration. Serum IFN-γ protein was not detectable (i.e., was <7.8 pg/mL) in any of the patients before OK-432 treatment (data not shown). Twenty-four hours after OK-432 injection, IFN-γ protein levels in the sera were detectable in 21 (75%) patients (Fig. 1, D, and Table 1).

Relationship Between Expression of TLR Genes and IFN-γ Induction by OK-PSA or OK-432

We next statistically analyzed the relationship between TLR expression and IFN-γ induction by OK-PSA or OK-432 with the two-sided Fisher's exact test. In *in vitro* experiments with patient-derived PBMCs, OK-PSA increased the expression of IFN-γ mRNA in 19 of 20 (95%) patients who expressed both TLR4 and MD-2 mRNA. Moreover, OK-PSA did not induce IFN-γ mRNA expression in all eight of eight (100%) patients who did not express TLR4 or MD-2 mRNA. There was a statistically significant relationship between TLR4 and MD-2 mRNA expression and IFN-γ induction by OK-PSA (P<.001) (Table 2), suggesting that IFN-γ induction by OK-PSA requires the TLR4/MD-2 complex. By contrast, IFN-γ mRNA expression induced by OK-432 was observed in all patients, which indicated that there was no statistically significant relationship between TLR4 and MD-2 expression and IFN-γ induction by OK-432. No statistically significant relationship was observed between TLR2, TLR9, or CD14 mRNA expression and OK-432- or OK-PSA-induced IFN-γ mRNA expression.

We detected a statistically significant relationship between increased IFN-γ protein levels in the sera of patients administered OK-432 and expression of TLR4 and MD-2 genes (P<.001, Table 3). An increase in serum IFN-γ protein in response to OK-432 was detected in 19 of 20 (95%) patients who expressed TLR4 and MD-2 mRNAs. Serum IFN-γ protein was not detected after OK-432 administration in six of eight (75%) patients who did not express TLR4 or MD-2 mRNAs. No relationship was observed between IFN-γ induction and TLR2 (P>.999), TLR9 (P>.999), or CD14 (P>.999) expression. Both TLR4 and MD-2 were apparently required for IFN-γ induction by OK-432 in patients with head and neck cancer, a result that was similar to the results from the *in vitro* study in which the PBMCs were stimulated to express IFN-γ with OK-PSA but not with OK-432.

Clinical Outcome of Patients Treated With OK-432 and UFT in Combination With Radiotherapy

All of the patients examined in the current study received OK-432 therapy with UFT in combination with radiotherapy. Ten of the 20 patients (50%) who expressed TLR4 mRNA and MD-2 mRNA became histopathologically tumor-free after therapy, without surgical resection. By contrast, all eight patients who did not express TLR4 mRNA or MD-2 mRNA became tumor-free only after having their tumors surgically resected after therapy (Table 1).

Relationship Between Expression of TLRs and IFN-γ Induction in PBMCs Derived From Healthy Volunteers

To examine the relationship between TLR mRNA expression and IFN-γ induction in healthy donors, we screened healthy donor-derived PBMCs for responsiveness to OK-432 or OK-PSA. *In vitro* experiments were done using PBMCs from eight healthy donors. Seven donors expressed TLR2 mRNA, and one donor marginally expressed TLR2 mRNA. Six donors expressed TLR9 mRNA, one donor marginally expressed TLR9 mRNA, and one donor did not express TLR9 mRNA. All donors expressed TLR4, MD-2, and CD14 mRNAs. After *in vitro* treat-

Table 2. Relationship between the expression of Toll-like receptor 4 (TLR4) and MD-2 genes and interferon-gamma (IFN-γ) from peripheral blood mononuclear cells (PMBCs) derived from patients with head and neck cancer and stimulated *in vitro* with OK-PSA*

Expression of IFN-γ mRNA†	No. of patients expressing TLR4 and MD-2 mRNAs		
	$TLR4^+$ and $MD\text{-}2^+$	$TLR4^-$ or $MD\text{-}2^-$	Total
Positive	19	0	19
Negative	1	8	9
Total	20	8	28

*Statistical relationship was detected by two-sided Fisher's exact test (P<.001).

†Expression of IFN-γ messenger RNA (mRNA) or TLR4 or MD-2 mRNA from patient-derived PBMCs treated with OK-PSA (10 μg/mL) *in vitro* for 24 hours. mRNA expression was determined by reverse transcription–polymerase chain reaction (RT–PCR). The relative density (RD) of each PCR product was compared with that of a control mRNA (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]), and a ratio of the RDs was used to quantify expression levels. Positive = an RD ratio of equal to or greater than 0.5; negative = an RD ratio of less than 0.1.

Table 3. Relationship between expression of Toll-like receptor 4 (TLR4) and MD-2 genes and serum interferon-gamma (IFN-γ) induction in patients with head and neck cancer administered OK-432*

Serum IFN-γ†	No. of patients expressing TLR4 and MD-2 mRNAs		
	$TLR4^+$ and $MD\text{-}2^+$‡	$TLR4^-$ or $MD\text{-}2^-$‡	Total
Positive	19‖	2	21
Negative	1	6	7
Total	20	8	28

*Statistical relationship was determined by two-sided Fisher's exact test (P<.001). mRNA = messenger RNA.

†IFN-γ protein detected by an enzyme-linked immunosorbent assay in patient sera 24 hours after receiving OK-432. + = IFN-γ protein levels higher than 7.8 pg/mL; – = IFN-γ protein levels lower than 7.8 pg/mL.

‡mRNA expression was determined by reverse transcription–polymerase chain reaction (RT–PCR). The relative density (RD) of each PCR product was compared to that of a control mRNA (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]), and a ratio of the RDs was used to quantify expression levels. Positive = an RD ratio of equal to or greater than 0.5; negative = an RD ratio of less than 0.1.

ment with OK-432 or OK-PSA, PBMCs from all donors expressed IFN-γ mRNA. Thus, the relationship between IFN-γ induction and expression of TLR4, MD-2, and CD14 genes could not be assessed from these donors. No clear relationship between the expression of TLR2 and TLR9 genes and IFN-γ expression was observed (P>.999) (data not shown).

Effect of OK-432 and OK-PSA on TLR4-Mediated NF-κB Activation

The data strongly suggested that TLR4/MD-2 signaling is closely associated with OK-432-mediated IFN-γ induction *in vivo,* but not *in vitro,* and OK-PSA-mediated IFN-γ induction *in vitro.* To test the hypothesis that TLR4/MD-2 signaling was directly associated with IFN-γ induction, we next examined TLR4/MD-2 downstream signaling events in response to treatment with OK-432 and OK-PSA.

TLR-mediated signaling activates the transcription factor NF-κB *(24,25).* We examined whether an NF-κB-dependent reporter construct could be activated in response to OK-432 and OK-PSA signals mediated through the TLR4/MD-2 complex. We examined the responsiveness of Ba/F3 transfectants that stably express human TLR2 (Ba/hTLR2), mouse TLR2 (Ba/mTLR2), human TLR4 (Ba/hTLR4), human TLR4 and human MD-2 (Ba/hTLR4/hMD-2), mouse TLR4 (Ba/mTLR4), or mouse TLR4 and mouse MD-2 (Ba/mTLR4/mMD-2) to OK-432 and OK-PSA.

Ba/F3 transfectants were cultured for 5 hours in the presence of OK-432, OK-PSA, LPS, or PGN (Fig. 2). Compared with control cells that did not express any TLR, OK-PSA dose-dependently induced NF-κB activation in Ba/hTLR4 (2.1-fold increase in NF-κB activity by 1 μg/mL of OK-PSA and 9.0-fold increase by 10 μg/mL of OK-PSA), Ba/mTLR4 (10.4-fold increase in NF-κB activity by 1 μg/mL of OK-PSA and 16.5-fold increase by 10 μg/mL of OK-PSA), Ba/hTLR4/hMD-2 (5.5-fold increase in NF-κB activity by 1 μg/mL of OK-PSA and 16.4-fold increase by 10 μg/mL of OK-PSA), and Ba/mTLR4/mMD-2 cells (20.9-fold increase in NF-κB activity by 1 μg/mL of OK-PSA and 27.4-fold increase by 10 μg/mL of OK-PSA). OK-432 did not induce NF-κB activation in any of the transfectants tested. Compared with control cells that did not express any TLR, LPS induced NF-κB activation in cells that expressed TLR4 (Ba/hTLR4, Ba/mTLR4, Ba/hTLR4/hMD-2, and Ba/mTLR4/mMD-2), and PGN induced NF-κB activation in cells that expressed TLR2 (Ba/hTLR2 and Ba/mTLR2) but not in cells that expressed TLR4. The data from the experiments using LPS and PGN were consistent with previous reports *(28,30).*

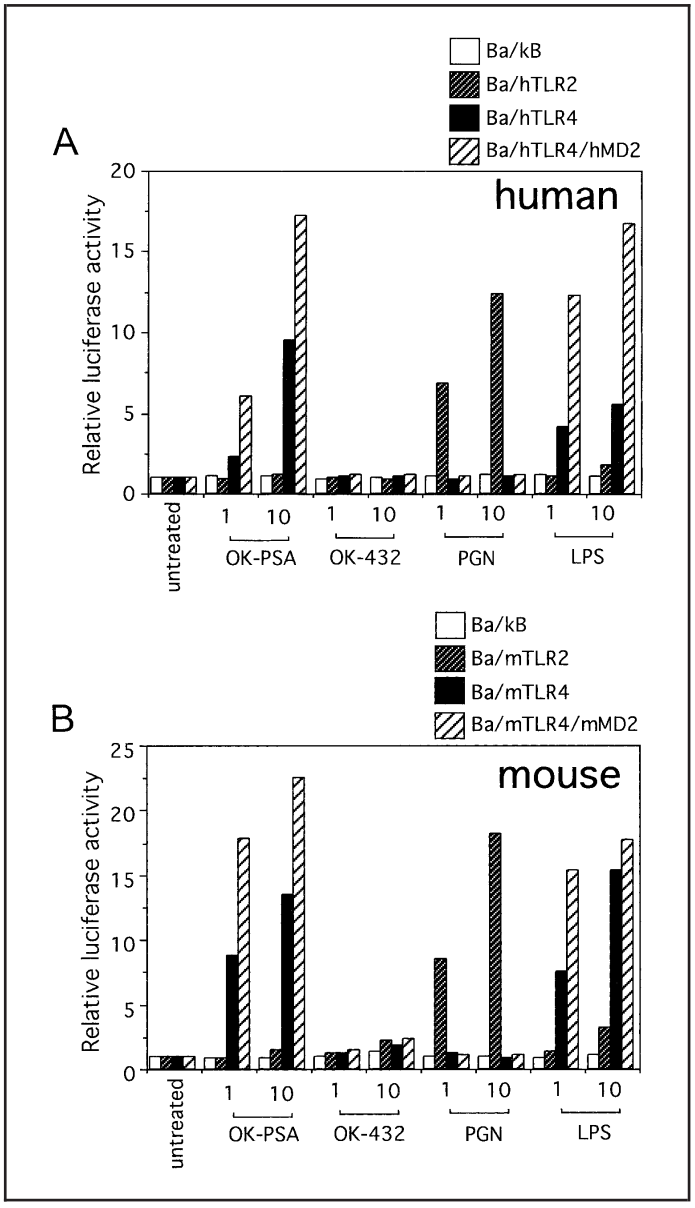

Fig. 2. Effect of OK-PSA and OK-432 on Toll-like receptor (TLR)-mediated nuclear factor-κB (NF-κB) activation. Stable Ba/F3 transfectants (5×10^5/well) expressing various human (**A**) or murine (**B**) TLRs (either TLR2 or TLR4) or MD-2 were transfected with an NF-κB-dependent luciferase reporter construct and stimulated with 1 or 10 μg/mL of OK-PSA, OK-432, lipopolysaccharide (LPS), or peptidoglycan (PGN) at 37 °C. After 5 hours, the cells were harvested and lysed, and the luciferase activity was measured according to the manufacturer's recommended protocol. The data are expressed as a ratio of relative luciferase activities to that in respective untreated controls. Each **bar** represents a single sample. The experiment was repeated three times with similar results, and representative data are shown.

Effect of OK-432 or OK-PSA on IFN-γ Induction *In Vitro*

To examine the role of TLR4 on IFN-γ induction by OK-432 or OK-PSA, mouse splenocytes from C57BL/6 mice that express wild-type TLR4 and TLR2 genes or from various TLR knockout mice were cultured in the presence of OK-432 or OK-PSA for 24 hours, and the supernatants were analyzed for IFN-γ protein. Untreated splenocytes derived from wild-type C57BL/6, TLR2$^{-/-}$, or TLR4$^{-/-}$ mice did not secrete IFN-γ. OK-432 induced IFN-γ from splenocytes derived from C57BL/6 mice that express the wild-type TLR4 gene and from TLR2$^{-/-}$ mice (Fig. 3, A). OK-432 also induced IFN-γ from splenocytes derived from TLR4$^{-/-}$ mice (Fig. 3, A). Although OK-PSA induced IFN-γ from splenocytes derived from C57BL/6 mice and TLR2$^{-/-}$ mice, it did not induce IFN-γ from splenocytes derived from TLR4$^{-/-}$ mice. LPS induced IFN-γ from splenocytes derived from C57BL/6 mice and TLR2$^{-/-}$ mice but not from TLR4$^{-/-}$ mice. PGN induced IFN-γ from splenocytes derived from C57BL/6 and TLR4$^{-/-}$ mice but not from TLR2$^{-/-}$ mice (Fig. 3, A).

We next examined the effect of long-term OK-432 treatment on changes in IFN-γ production from mouse splenocytes. Splenocytes derived from wild-type mice and TLR4$^{-/-}$ mice were stimulated with 0.1 μg/mL of OK-432 for 7 days, and IFN-γ levels in the supernatants were measured at regular intervals.

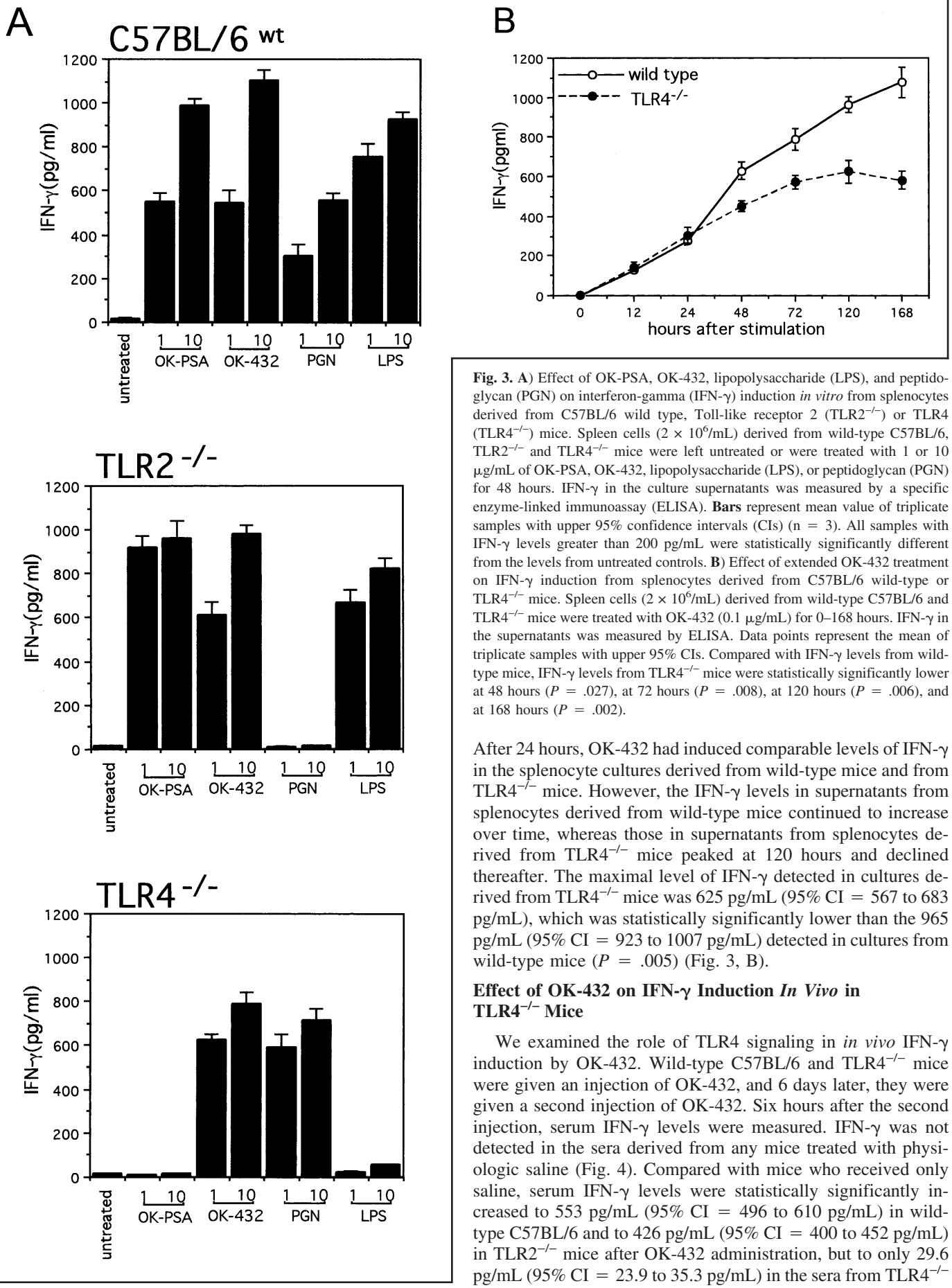

Fig. 3. A) Effect of OK-PSA, OK-432, lipopolysaccharide (LPS), and peptidoglycan (PGN) on interferon-gamma (IFN-γ) induction *in vitro* from splenocytes derived from C57BL/6 wild type, Toll-like receptor 2 (TLR2$^{-/-}$) or TLR4 (TLR4$^{-/-}$) mice. Spleen cells (2×10^6/mL) derived from wild-type C57BL/6, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were left untreated or were treated with 1 or 10 μg/mL of OK-PSA, OK-432, lipopolysaccharide (LPS), or peptidoglycan (PGN) for 48 hours. IFN-γ in the culture supernatants was measured by a specific enzyme-linked immunoassay (ELISA). **Bars** represent mean value of triplicate samples with upper 95% confidence intervals (CIs) (n = 3). All samples with IFN-γ levels greater than 200 pg/mL were statistically significantly different from the levels from untreated controls. **B**) Effect of extended OK-432 treatment on IFN-γ induction from splenocytes derived from C57BL/6 wild-type or TLR4$^{-/-}$ mice. Spleen cells (2×10^6/mL) derived from wild-type C57BL/6 and TLR4$^{-/-}$ mice were treated with OK-432 (0.1 μg/mL) for 0–168 hours. IFN-γ in the supernatants was measured by ELISA. Data points represent the mean of triplicate samples with upper 95% CIs. Compared with IFN-γ levels from wild-type mice, IFN-γ levels from TLR4$^{-/-}$ mice were statistically significantly lower at 48 hours ($P = .027$), at 72 hours ($P = .008$), at 120 hours ($P = .006$), and at 168 hours ($P = .002$).

After 24 hours, OK-432 had induced comparable levels of IFN-γ in the splenocyte cultures derived from wild-type mice and from TLR4$^{-/-}$ mice. However, the IFN-γ levels in supernatants from splenocytes derived from wild-type mice continued to increase over time, whereas those in supernatants from splenocytes derived from TLR4$^{-/-}$ mice peaked at 120 hours and declined thereafter. The maximal level of IFN-γ detected in cultures derived from TLR4$^{-/-}$ mice was 625 pg/mL (95% CI = 567 to 683 pg/mL), which was statistically significantly lower than the 965 pg/mL (95% CI = 923 to 1007 pg/mL) detected in cultures from wild-type mice ($P = .005$) (Fig. 3, B).

Effect of OK-432 on IFN-γ Induction *In Vivo* in TLR4$^{-/-}$ Mice

We examined the role of TLR4 signaling in *in vivo* IFN-γ induction by OK-432. Wild-type C57BL/6 and TLR4$^{-/-}$ mice were given an injection of OK-432, and 6 days later, they were given a second injection of OK-432. Six hours after the second injection, serum IFN-γ levels were measured. IFN-γ was not detected in the sera derived from any mice treated with physiologic saline (Fig. 4). Compared with mice who received only saline, serum IFN-γ levels were statistically significantly increased to 553 pg/mL (95% CI = 496 to 610 pg/mL) in wild-type C57BL/6 and to 426 pg/mL (95% CI = 400 to 452 pg/mL) in TLR2$^{-/-}$ mice after OK-432 administration, but to only 29.6 pg/mL (95% CI = 23.9 to 35.3 pg/mL) in the sera from TLR4$^{-/-}$

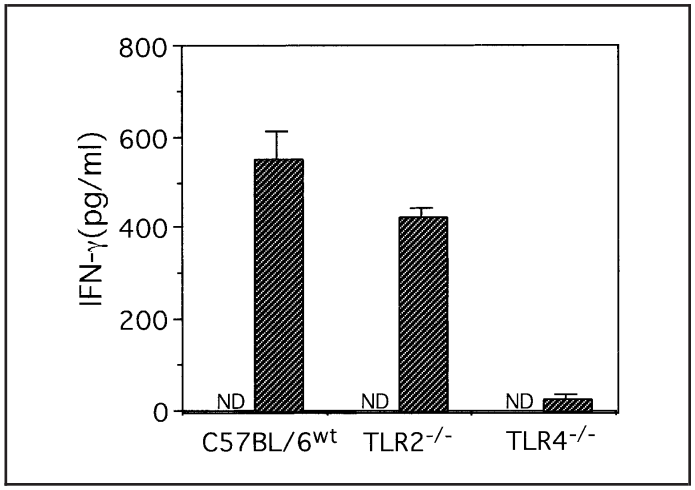

Fig. 4. Effect of OK-432 on interferon-gamma (IFN-γ) induction in C57BL/6 wild-type, Toll-like receptor 2 (TLR2) (TLR2$^{-/-}$), or TLR4 (TLR4$^{-/-}$) mice. All mice received an intraperitoneal injection of OK-432 (100 μg). After 6 days, all mice received a second intraperitoneal injection of OK-432 (100 μg) or physiologic saline. Six hours after the second treatment, IFN-γ levels in the sera were measured by enzyme-linked immunoassay (ELISA). **Bars** represent mean IFN-γ levels of four mice per group with upper 95% confidence intervals. Mean IFN-γ level in the sera derived from TLR4$^{-/-}$ mice given OK-432 was statistically significantly lower than that from C57BL/6 wild-type (P = .003) and from TLR2$^{-/-}$ mice (P = .019). ND = not detected (<7.8 pg/mL). **Shaded bars** represent OK-432.

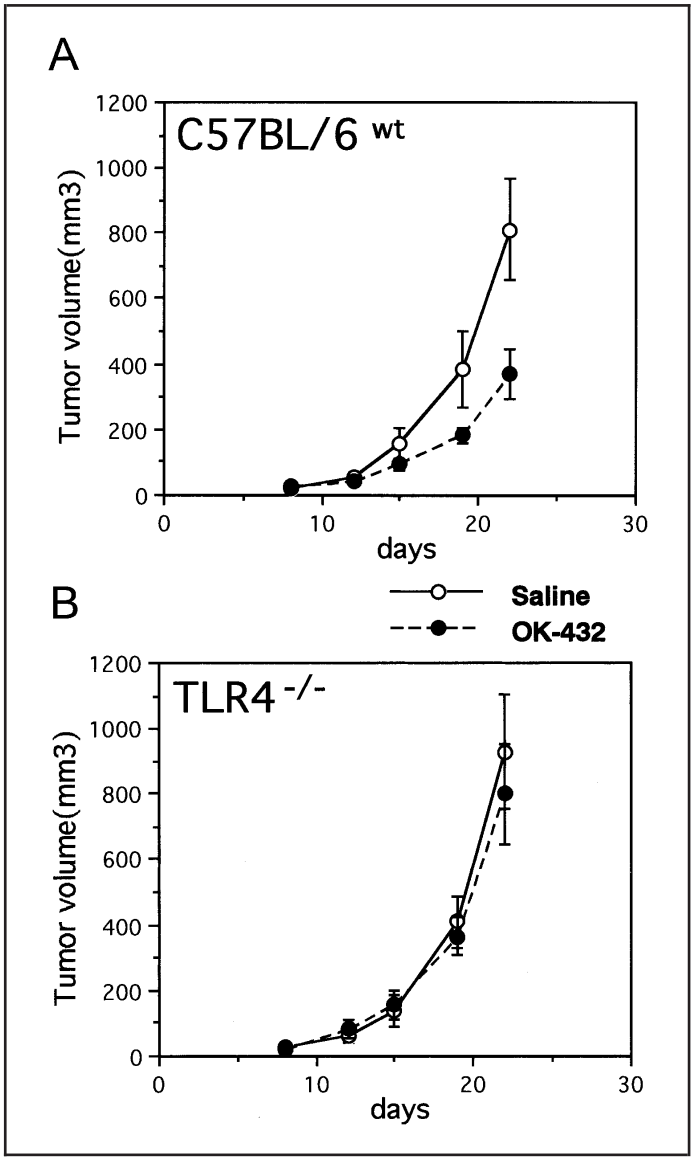

Fig. 5. Effect of OK-432 on tumor growth in C57BL/6 wild-type or TLR4 (TLR4$^{-/-}$) mice with Lewis lung (LL)/2 carcinomas. LL/2-bearing wild-type C57BL/6 (**A**) and TLR4$^{-/-}$ mice (**B**) received five peritumoral OK-432 injections over 5 days. The tumor volumes were measured twice a week. Data points represent mean tumor volumes of eight mice per group, with 95% confidence intervals. Compared with tumor volumes in control C57BL/6 mice who received saline, tumor volumes in C57BL/6 mice who received OK-432 were statistically significantly smaller at 19 days (P = .024) and at 22 days (P = .007).

mice after OK-432 administration (Fig. 4). The serum IFN-γ level in any nonprimed mouse did not increase after OK-432 administration (data not shown).

Effect of OK-432 in Tumor-Bearing TLR4$^{-/-}$ Mice

Finally, we compared the effect of OK-432 in wild-type C57BL/6 and TLR4$^{-/-}$ mice with syngeneic Lewis lung carcinomas. Twenty-two days after tumor inoculation, the mean tumor volume in wild-type mice that received peritumoral injections of OK-432 was statistically significantly smaller (369 mm^3, 95% CI = 293 to 445 mm^3) than the mean tumor volume in wild-type mice who received injections of saline (811 mm^3, 95% CI = 655 to 967 mm^3) (P = .007) (Fig. 5, A). By contrast, regardless of treatment, there was no statistically significant difference in mean tumor volumes in TLR4$^{-/-}$ mice (Fig. 5, B).

Discussion

IFN-γ, a representative Th1 cytokine, plays an important role in anticancer immunity. It has been reported that IFN-γ production is associated with a favorable clinical outcome in the patients with several types of malignancies, including colorectal carcinoma and cervical carcinoma *(48,49).* OK-432 augments anticancer host responses by increasing production of Th1 cytokines, especially IFN-γ *(10,50).* We therefore examined the relationship between the expression of TLRs and response to OK-432, indicated by changes in IFN-γ levels, in patients with head and neck cancer to identify target molecule(s) for OK-432-based immunotherapy.

We found that responsiveness of patients to OK-432 therapy, as represented by increased serum IFN-γ level, was statistically significantly related to the expression of TLR4 and MD-2. Because the TLR4/MD-2 complex confers responsiveness to bacterial components *(33),* it is suggested that both TLR4 and MD-2 may be required for signaling of OK-432 and for subsequent IFN-γ production *in vivo.* Thus, we conclude that expression of both TLR4 and MD-2 may be useful markers to discriminate between responders and nonresponders to OK-432.

It is interesting to note that the results obtained by using patient-derived PBMCs *in vitro* were different from those results obtained *in vivo.* Although responsiveness to OK-432 was closely associated with TLR4 and MD-2 expression *in vivo,* when PBMCs were treated *in vitro* with OK-432, increased expression of IFN-γ mRNA was irrespective of the expressed TLR4 and MD-2 phenotype. However, when PBMCs were treated with OK-PSA *in vitro,* increased expression of IFN-γ mRNA was dependent on expression of TLR4 and MD-2

mRNAs. Thus, in patients with head and neck cancer, the effect of *in vivo* OK-432 administration was similar to the effect of OK-PSA, but not with OK-432 stimulation *in vitro.* The discrepancy between OK-432-mediated IFN-γ induction *in vivo* and induction *in vitro* can be at least partially explained by the fact that, *in vitro,* IFN-γ induction by OK-432 in splenocytes derived from $TLR4^{-/-}$ mice was attenuated after 48 hours compared with that in splenocytes derived from wild-type mice. Furthermore, IFN-γ levels may decrease more rapidly *in vivo* than *in vitro.* Thus, IFN-γ induced by OK-432 not via TLR4 may be difficult to detect in sera, and it may also be hard to elicit an anticancer effect mediated by IFN-γ.

The results from our human studies were consistent with those from our $TLR4^{-/-}$ mice studies, with an impaired IFN-γ inducing an antitumor effect of OK-432 observed in $TLR4^{-/-}$ mice bearing syngeneic carcinomas. From our results we hypothesized that, in both *in vivo* and *in vitro* environments, because OK-432 consists of whole bacterial bodies, it is phagocytized by professional phagocytes such as macrophages, dendritic cells, and neutrophils. The phagocytes are then activated, and they release IFN-γ-inducing factors, including IL-12 and IL-18, which stimulate T cells and NK cells to produce IFN-γ. This suggests that OK-432 induces IFN-γ indirectly and not through a TLR4/MD-2 signal-transducing pathway. The results from the luciferase reporter assay using TLR4- and MD-2-expressing Ba/F3 cells support this hypothesis, suggesting that OK-432 could not directly induce IFN-γ without the presence of an additional cell type, most probably phagocytes. Further support for this hypothesis comes from the fact that OK-432 induces maturation of dendritic cells *in vitro,* not through TLR2 or TLR4 but through β2 integrins, which are important molecules used by dendritic cells and macrophages for phagocytizing antigens *(51).* In an *in vivo* environment, macrophages and dendritic cells should be activated by phagocytizing OK-432; however, this activation may be insufficient to increase IFN-γ levels in the sera of cancer patients and to elicit an antitumor effect in mice *in vivo,* because OK-432 was not effective in patients who did not express TLR4 or MD-2 mRNAs or in $TLR4^{-/-}$ mice *in vivo.* An association between an effective component(s) of OK-432 (e.g., OK-PSA) and TLR4/MD-2 complex may be required to elicit IFN-γ-inducing and antitumor effects. Furthermore, we hypothesized that the effective component(s) may be the molecule(s) that are released after the bacterial bodies are phagocytized and digested. In fact, we detected a soluble antigen recognized by the TS-2 monoclonal antibody (possibly OK-PSA) in culture supernatants derived from splenocytes treated with OK-432 (data not shown). This hypothesis is now under investigation in our laboratory.

In the current study, we observed one patient in whom IFN-γ was not induced by OK-432 *in vivo* or by OK-PSA *in vitro* despite expression of both TLR4 and MD-2. In this patient, we are now investigating possible mutations in the TLR4 and MD-2 genes. Several mutations in human TLR4 genes were observed in endotoxin-hyporesponsive hosts *(52),* but there has been no report, to our knowledge, of mutations in the human MD-2 gene. We noted that in two patients who did not express MD-2, IFN-γ was detected in the sera, although the patient-derived PBMCs showed no response to OK-PSA *in vitro.* It is possible that, in these patients, another component(s) in addition to OK-PSA may exhibit biologic activity via TLR2 or TLR9 but not via the TLR4/MD-2 complex or that immune cells might be activated more strongly by phagocytizing OK-432 in these patients than in other patients.

Our findings should help identify the molecular mechanism by which OK-432 enhances antitumor immunity, and they suggest that expression of both TLR4 and MD-2 mRNAs may be useful markers to discriminate between patients who may respond and those who will not respond to OK-432-based immunotherapy. In addition, introduction of TLR4 or MD-2 into patients predicted not to respond to recover TLR4/MD-2 signaling may be a potent strategy for improving the response to OK-432 therapy. This hypothesis is now under investigation in an animal study using mice deficient for the TLR4 or MD-2 gene.

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