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FEMS Immunology and Medical Microbiology 29 (2000) 101–106

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

Effect of *Alloiococcus otitidis* and three pathogens of otitis media in production of interleukin-12 by human monocyte cell line

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Received 17 January 2000; received in revised form 28 April 2000; accepted 11 May 2000

Abstract

Alloiococcus otitidis is detected in middle ear effusion of otitis media with effusion (OME). Only a limited number of studies are available concerning the immunological profile of *A. otitidis*. We have studied the ability of *A. otitidis* and three other representative pathogens of otitis media to stimulate the production of interleukin-12 (IL-12) from a monocytic cell line THP-1. Viable *A. otitidis* induced the production of IL-12 in THP-1 cells but IL-12 production was reduced if glutaraldehyde-fixed bacteria were used as stimulants. When viable bacteria were physically separated from THP-1 cells during the stimulation period, remarkable reductions of IL-12 secretion were shown after challenge with Gram-positive bacteria *A. otitidis* and *S. pneumoniae*. When stimulated with soluble extracts of *A. otitidis*, THP-1 secreted IL-12 in a dose-dependent manner. The subfraction with a molecular mass over 100 kDa showed a strong ability to induce IL-12 production. Our results show that *A. otitidis* has immunostimulatory capacity with regard to IL-12 production. We also show that soluble antigen(s) of *A. otitidis* can modulate the immune response in OME.

Keywords: *Alloiococcus otitidis*; Interleukin-12; Otitis media; Monocyte

1. Introduction

Interleukin-12 (IL-12) is a potent immunoregulatory cytokine that is crucially involved in the response to a wide range of infectious agents. In several experimental models of bacterial, parasitic, viral and fungal infection, endogenous IL-12 is required for the early control of infection and for the generation and perhaps maintenance of acquired protective immunity, under the direction of T helper type 1 (Th1) cells.

Otitis media with effusion (OME) is an important otological problem in childhood and is characterized by persistent inflammation with retention of mucinous fluid in the middle ear cavity. Although routine bacterial cultures of the middle ear effusions (MEE) showed bacterial growth in only 20–30% cases of childhood OME, the detection rate could be increased to up to 90% by using polymerase chain reaction (PCR) [1,2]. The representative pathogens of bacteria are considered to be *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*.

Alloiococcus otitidis is a Gram-positive bacterium which was first described in 1989 as a pathogen associated with OME and determined as a new species of bacteria in 1992 [3,4]. Only a limited number of studies are available concerning the immunological role of *A. otitidis* in OME. However, in a recent study using PCR for the detection of microbes, *A. otitidis* is found in over 40% of cases of children with OME and was more frequently detected than the other common pathogens of otitis media [1–2,5]. Therefore, the pathogenic role of this newly described bacterium in OME deserves a closer look.

IL-12 is believed to be an essential factor in the induction of protective immunity against intracellular pathogens. The intracellular location of *A. otitidis* was observed by studying infiltrating inflammatory cells of MEE [3].

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0928-8244/00/$20.00
PII: S0928-8244(00)00193-0

Therefore, in order to clarify the possible intracellular pathogenic role of *A. otitidis*, the ability of *A. otitidis* and another three representative pathogens of otitis media to stimulate the production of IL-12 from a monocytic cell line in vitro was studied. The ability of subcellular fragments of *A. otitidis* to induce IL-12 production was also investigated.

2. Materials and methods

2.1. Bacterial strains and preparation

The bacterial strains used in this study were *A. otitidis*, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*. The *A. otitidis* strain was NCFB118 and the other three bacterial strains were clinical isolates. The *A. otitidis*, *S. pneumoniae* and *M. catarrhalis* strains were grown in Todd–Hewitt broth (Difco Laboratories, Detroit, MI, USA) and *H. influenzae* was grown in chocolate II agar (Nippon Becton Dickinson Co., Tokyo, Japan) at 37°C for 2 days in 5% CO^2/95% air. The killed bacteria were produced by fixation with 0.25% (v/v) glutaraldehyde (GA) (Nakarai Chemicals, Kyoto, Japan) at room temperature for 1 h, or heat-killed at 56°C for 30 min. Killed bacteria were washed three times in phosphate-buffered saline (PBS) with antibiotics. The number of bacteria was determined by the McFarland method using the standard mixtures with 1% (v/v) barium chloride and 1% (v/v) sulfuric acid. The following reagents were purchased from commercial sources: lipopolysaccharide (LPS) (from *Escherichia coli*, serotype O127:B7; Sigma, St. Louis, MO, USA), lipoteichoic acid (LTA) (from *Streptococcus pyogenes*; Sigma), *Staphylococcus aureus* Cowan strain I (SAC) (Chemicon, Temecula, CA, USA), Staphylococcal enterotoxin B (SEB) (Toxin technology, Sarasota, FL, USA).

2.2. Cell culture and stimulation

The human monocytic cell line, THP-1 (ATCC CRL 1593), was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in suspension in 5% CO_2/95% air in RPMI 1640 medium (Life Tech., Grand Island, NY, USA) with 0.1% (v/v) 2-mercaptoethanol (Life Tech.), supplemented with 10% (v/v) fetal calf serum (FCS) (ICN Biomedicals, Aurora, OH, USA) containing 100 U of penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. THP-1 cells (1.0×10^{6} ml^{-1}) were non-treated, or pretreated for 24 h with 1.2% dimethyl sulfoxide (DMSO) (Katayama chemical, Osaka, Japan), incubated in the presence or absence of recombinant human γ interferon (IFN-γ 100 ng ml^{-1}) for 16 h. Monocytic cells were distributed to round-bottom tissue culture tubes (Becton Dickinson Co., Franklin Lakes, NJ, USA) at 1.0×10^{6} cells per tube in a volume of 1 ml medium.

Bacterial cells were added to the cultured cells at a bacterium/cell ratio of 100:1 in a 1-ml volume. An antibiotic-free medium was used in the experiment for viable bacteria. Other bacterial components, LPS (final concentration: 1 μg ml^{-1}), LTA (100 μg ml^{-1}), SAC (1:10 000 dilution) and SEB (10 μg ml^{-1}), were each added to the cultured cells. After incubation at 37°C in 5% CO_2/95% air, supernatants were collected at various time intervals.

2.3. A two-chamber system

We used a two-chamber system to test, in the same experiment, whether the four bacterial organisms can have an effect, per se or through the release of some materials, on the IL-12 production in the monocytic cell line. DMSO-treated IFN-γ plus THP-1 cells were seeded in 24-well tissue culture plates at 2×10^{5} cells per well in a volume of 1 ml per well. Prior to stimulation, the monocytic cell suspension was washed three times with fresh tissue culture medium without antibiotics, and then viable bacterial cells were added to the cultured cells at a bacterium/cell ratio of 100:1 in a 1-ml volume, in the presence or absence of cell culture inserts (Becton Dickinson Co., Franklin Lakes, NJ, USA). Supernatants were collected 16 h after stimulation.

2.4. Soluble extract of A. otitidis

Bacterial cells of *A. otitidis* (NCFB118) were grown in Todd–Hewitt broth for 3 days, and then antibiotics (1000 U ml^{-1} of penicillin G and 1000 μg ml^{-1} of streptomycin) were added to the culture. After treatment of the cells with antibiotics for 90 min, the bacterial cells were harvested by centrifugation ($2150\times g$ for 30 min) and washed three times with PBS. The resuspended cells in PBS were disrupted with a Branson cell homogenizer (Branson model 250 sonifer, Branson, CT, USA), and the unbroken cells were removed by centrifugation ($13\,000\times g$ for 10 min). Supernatants were collected as a soluble extract of bacterium. The soluble extract (protein concentration 200 μg ml^{-1}) was subdivided into four fractions categorized by molecular mass using a Microcon®-30, -50 and -100 (Amicon, Beverly, MA, USA) following the manufacturer's protocol. Each subfraction was diluted with PBS up to the same volume as the original extract and filtered with a 0.45-μm filter unit (Millex®-HV, Millipore, Bedford, MA, USA). The protein concentration was measured by Bio-rad protein assay (Bio-Rad Lab., Richmond, CA, USA).

2.5. Quantification of IL-12

THP-1 was incubated in the presence or absence of various bacterial cells, soluble extracts, LPS, LTA, SAC and SEB, and then the supernatants were collected 16 h after stimulation. The IL-12 in the resulting supernatants was assessed using an enzyme-linked immunosorbent as-

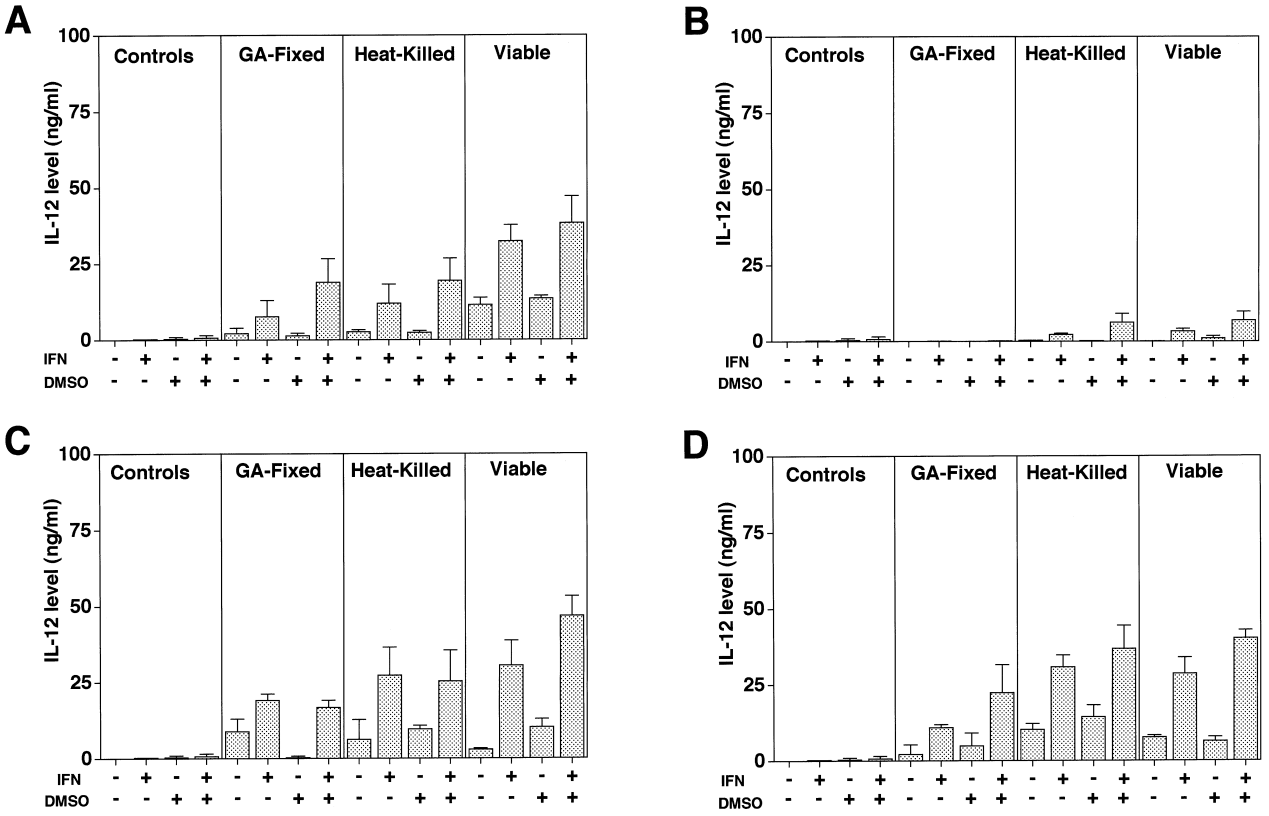

Fig. 1. Induction of IL-12 production in THP-1 cells after treatment with various bacteria. DMSO-treated (for 24 h) or non-treated THP-1 cells were incubated in medium alone, or preincubated with IFN-γ (100 ng ml^{-1}, 16 h) and stimulated with GA-treated, heat-killed and viable bacterial cells for 16 h (bacterium/cells ratio of 100:1). (A) *A. otitidis*; (B) *S. pneumoniae*; (C) *H. influenzae*; and (D) *M. catarrhalis*. Lane 1, control (no bacterial cells added); lane 2, GA-treated bacteria; lane 3, heat-killed bacteria; and lane 4, viable bacteria. Values represent the means and standard deviations of four experiments.

say kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturer's protocol.

3. Results

THP-1 cells were coincubated with four viable bacterial cells (*A. otitidis*, *M. catarrhalis*, *S. pneumoniae* and *H. influenzae*) and the level of IL-12 in the supernatants was measured. THP-1 cells secreted IL-12 in a time-dependent manner (data not shown) and significant levels of IL-12 protein were detected after a 16-h stimulation period. In Fig. 1 it is seen that all the tested bacteria were able to induce IL-12 synthesis in THP-1 cells. However, there were marked differences in the stimulatory capacity of the different bacteria, *S. pneumoniae* being the least effective.

We then tested whether killed bacteria were able to induce IL-12 production in THP-1 cells. As shown in Fig. 1, slight reductions in the capacity of *A. otitidis*, *H. influenzae* and *M. catarrhalis* to induce IL-12 production were observed after treatment with GA or heat. Only low levels of IL-12 production were observed after challenge of THP-1 cells with *S. pneumoniae* bacteria (either live or killed).

THP-1 cells pretreated with IFN-γ tended to produce higher levels of IL-12 protein than non-treated THP-1 cells after stimulation with all four bacteria. DMSO-treatment of THP-1 cells showed a minimal stimulatory effect on IL-12 production after bacterial challenge. Combined treatment with both IFN-γ and DMSO showed significant

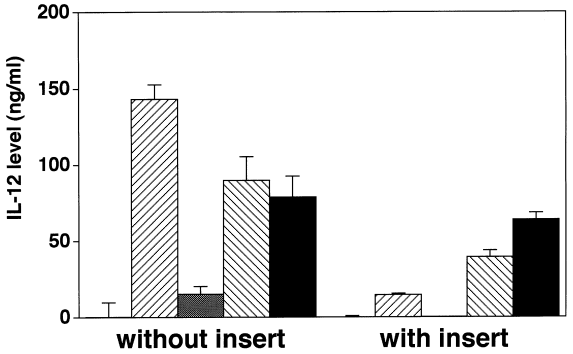

Fig. 2. Induction of IL-12 production after physical separation of bacterial cells from THP-1 cells with cell culture inserts. THP-1 cells were pretreated for 24 h with 1.2% DMSO, preincubated with IFN-γ (100 ng ml^{-1}, 16 h) and stimulated with viable bacterial cells without or with cell culture inserts (16 h, bacteria/THP-1 ratio 100:1) in 24-well tissue culture plates. *A. otitidis* (▨), *S. pneumoniae* (▩), *H. influenzae* (▧), *M. catarrhalis* (■), and tissue culture medium (□) were used. Lane 1, without insert; lane 2, with insert. Values represent the means and standard deviations of three experiments.

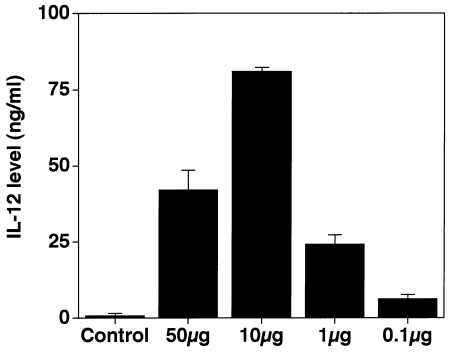

Fig. 3. Assessment of IL-12 stimulatory potentials of soluble extract prepared from *A. otitidis* strain on THP-1 cells (for 16 h). THP-1 cells were DMSO-treated (for 24 h) and then preincubated with IFN-γ (100 ng ml^{-1}, 16 h). Tissue culture medium as control, soluble extract of 50 μg ml^{-1}, 10 μg ml^{-1}, 1.0 μg ml^{-1} and 0.1 μg ml^{-1} were used. Values represent the means and standard deviations of two experiments.

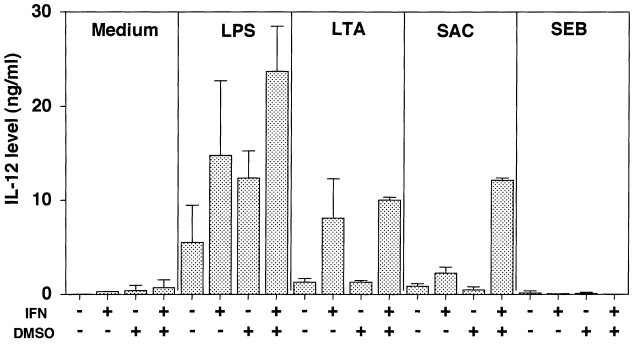

Fig. 5. Induction of IL-12 production after treatment of THP-1 cells with bacterial cell surface components and bacterial superantigen. DMSO-treated (24 h) or non-treated THP-1 cells were incubated in medium alone, preincubated with IFN-γ (100 ng ml^{-1}, 16 h), and were stimulated with various bacterial components; LPS (derived from *E. coli*; 1.0 μg ml^{-1}), LTA (derived from *S. pyogenes*; 100 μg ml^{-1}), SAC (1:10 000 dilution), SEB (10 μg ml^{-1}). Bar 1 and lane 1, control; bar 2 and lane 2, LPS; bar 3 and lane 3, LTA; bar 4 and lane 4, SAC; and bar 5 and lane 5, SEB. Values represent the means and standard deviations of two experiments.

stimulatory effects on the production of IL-12 (Fig. 1A–D).

To determine whether IL-12 production was triggered by a direct contact between cultured cells and viable bacteria, we performed experiments in a two-chamber system, in which bacteria were physically separated from the monocytic cells. In Fig. 2 it can be seen that IL-12 secretion was reduced when viable bacteria were physically separated from THP-1 cells. This effect was especially pronounced with *A. otitidis* and *S. pneumoniae*. Reductions in IL-12 production were less pronounced with Gram-negative bacteria, especially *M. catarrhalis* (Fig. 2)

We then investigated whether whole *A. otitidis* bacteria were needed for the induction of IL-12 secretion or whether subcellular fragments were also stimulatory. In Fig. 3 it can be seen that the monocytic cell line THP-1 (pretreated both with DMSO and with IFN-γ) secreted IL-12 in a dose-dependent manner following stimulation with soluble extracts of *A. otitidis* (Fig. 3). The subcellular fragments were then divided into four fractions by molecular mass and it was seen that although the subfraction with a molecular mass of over 100 kDa showed a strong ability to induce IL-12 production, the other three subfractions were only minimally inducive (Fig. 4).

We also tested the effect of different bacterial components on the capacity of THP-1 cells to produce IL-12. In Fig. 5 it is shown that THP-1 cells produced IL-12 protein when stimulated with LPS, LTA and SAC, but not with SEB. THP-1 treated with both IFN-γ and DMSO tended to produce higher levels of IL-12 protein than non-treated THP-1 cells (Fig. 5).

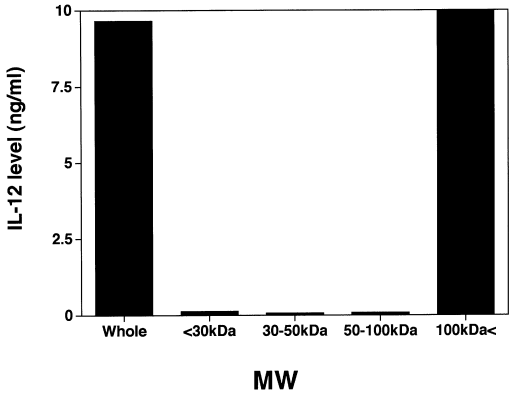

Fig. 4. Induction of IL-12 production in THP-1 cells after treatment with soluble extracts from *A. otitidis*. The soluble extract was subdivided into four fractions by molecular mass. THP-1 cells were DMSO-treated (for 24 h), were preincubated with IFN-γ (100 ng ml^{-1}, 16 h) and were incubated with various soluble fractions for 16 h. Values represent the means and standard deviations of two determinations.

4. Discussion

In a recent study, intact and metabolically active *H. influenzae* organisms were detected using PCR in culture-sterile MEE of OME [6]. Later the presence of *A. otitidis* was established and our previous study showed that the incidence of *A. otitidis* in culture-sterile MEE using PCR was higher than that of other representative pathogens used in this study [1].

A. otitidis is an intracellular pathogen and therefore the Th1-dependent cell-mediated immunity may play a role in the immune response against *A. otitidis*. The representative intracellular pathogens, *Listeria*, *Mycobacterium* and *Leishmania*, have been used extensively as models to study the role of Th1-dependent cell-mediated immunity in the control of infection by intracellular pathogens [7]. The experimental model systems using *L. monocytogenes* for evaluating the IL-12 profile can also be applied to a comparative study of *A. otitidis* as an intracellular pathogen. *L. monocytogenes* is able to live and replicate inside resting

macrophages but is rapidly destroyed as a result of macrophage activation. *L. monocytogenes*-infected macrophages produce IL-12 [8], which effectively activates natural killer cells [9]. The administration of recombinant IL-12 enhances, and its neutralization decreases, resistance to infection [10].

The present study showed that viable *A. otitidis* bacteria induced IL-12 production from THP-1 cells. However IL-12 production was slightly reduced if killed bacteria were used as stimulants. In accordance with our finding, Song et al. [11] showed that viable, but not killed, *L. monocytogenes* stimulated significant IL-12 production from macrophages. We also showed that priming of the THP-1 cells with IFN-facilitated IL-12 production.

In another study, priming with IFN-γ promoted IL-12 production after challenge of mononuclear cells with *A. otitidis* but priming with IFN-γ had no significant effect on IL-12 production if the cells were stimulated with *L. monocytogenes* [12].

The exposure of macrophages to group B *Streptococcus* and other extracellular bacterial agents of *Staphylococci* and *Enterococci* result in the production of bioactive IL-12 [13]. In our study, a reduction of IL-12 production was seen by pre-treatment of the bacteria with GA or by heat. Pretreatment of *A. otitidis* largely abolished the stimulatory capacity, whereas the effect on Gram-negative bacteria was minimal. Therefore, the molecules of *A. otitidis* responsible for the induction of IL-12 secretion from THP-1 cells were not resistant to treatment with GA or heat.

Augmentation of IL-12 production can be achieved by both Gram-negative and Gram-positive bacteria, but probably via different molecules. LPS of Gram-negative bacteria induces monocyte production of IL-12. Gram-positive bacteria also induce IL-12 production by monocytes and this effect is probably mediated by LTA [14]. However, our result showed that IL-12 production was still induced when the contact between *A. otitidis* and THP-1 cells was inhibited. The actions of soluble bacterial extracts on monocytes may be different from the actions of surface components of whole bacteria.

Soluble bacterial antigens also induce the production of IL-12 from macrophages, as observed with a soluble extract of *L. monocytogenes* as well as particulate immunogens [15]. As shown in the present study, microbial components that promoted IL-12 release include LPSs of Gram-negative bacteria [16] and LTAs of Gram-positive bacteria [14]. Other microbial components such as spirochetal lipoproteins [17], bacterial DNA containing CpG motif [18], bacterial superantigens and heat shock proteins [19] also have the ability to induce IL-12 production.

We ivestigated which components of *A. otitidis* were responsible for the immunostimulatory activity. We showed quite clearly that a subcellular fraction with a M_r over 100 kDa had an immunostimulatory activity. It can be speculated that *A. otitidis* utilizes not only bacterial surface components such as LTA but also soluble proteins to stimulate the IL-12 production by monocytes. In accordance with this line of thought, it was reported that soluble antigens of *Toxoplasma* induced IL-12 production [20], and soluble antigens of *L. monocytogenes* stimulated Th1 responses [21]. The precise characteristics of soluble extracts of *A. otitidis* presented here are still unknown.

In conclusion, we showed that *A. otitidis* has an immunostimulatory capacity on IL-12 production. Therefore *A. otitidis* may contribute to the pathogenesis of OME by modulating the immune response. Our results regarding *A. otitidis* provide the possibility of a new conceptualization of the etiology of OME.

Acknowledgements

We are grateful to Dr. Akikatsu Kataura for support and advice. This work was funded by the Japanese Ministry of Education (grant 07671872 to T.H.) and the Sapporo Medical University Foundation for the Promotion of Medical Science (Joint Research).

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