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Dynamics of dendritic cell migration and the subsequent induction of protective immunity in the lung after repeated airway challenges by nontypeable *Haemophilus influenzae* outer membrane protein

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Abstract

To determine the dynamics of dendritic cell (DCs) migration and their role in recurrent infections by nontypeable *Haemophilus influenzae* (NTHi), the migration of mature DC into pulmonary lymph nodes (LN) and the development of a P6-specific immune response and bacterial clearance in the lung were examined after repeated airway challenges with outer membrane protein (OMP) at 1-week intervals in mice. Although the migration of mature DC into the pulmonary LN is attenuated after repeated airway challenge with OMP, the in vitro P6-specific T cell proliferation in the cultured pulmonary LN cells was enhanced and was subsequently linked to the development of P6-specific IgA production and the development of protective immunity in the airway of mice.
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1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of acute otitis media in children, and is also frequently associated with the acute exacerbation of chronic pulmonary diseases (COPD) [1,2]. An increasing body of evidence is available that indicates a high rate of antibiotic resistance of NTHi in such diseases [3,4]. A vaccinating strategy against NTHi infections is, therefore, urgently required for these populations [5]. P6 is a 16 kDa peptidoglycan-associated lipoprotein that is commonly found in the outer membrane of all strains of NTHi and exhibits a high degree of sequence conservation among strains [6,7]. Since mucosal immunization with P6 enhances the bacterial clearance of NTHi in the airway and the middle ear, this protein is considered to be viable candidate for use in a mucosal vaccine [8–12].

Dendritic cells (DCs) are antigen presenting cells that have a unique ability to induce primary immune responses, thus allowing the establishment of immunological memories

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[13,14]. After capturing antigens, DCs mature and express high levels of MHC class II and costimulatory molecules such as CD86 on their surfaces [15,16]. A previous study reported that DC precursors rapidly emerged into the airway epithelium in advance of neutrophil influx after the inhalation of *Moraxella catarrhalis* [17]. These cells are thought to differentiate into mature DC in the airway epithelium and migrate via the lymphatic system to regional lymph nodes through CC chemokine receptors (CCR7)/CCL19 (Epstein-Barr virus-induced molecule-1 ligand chemokine: ELC) or CCL21 (secondary lymphoid tissue chemokine; SLC) interactions [18–20].

Although recurrent infections due to NTHi are typically observed in patients with COPD, the dynamics of DC migration into the regional lymph nodes (LNs) and the subsequent induction of specific adaptive immunity after a recurrent infection with NTHi have not been fully investigated. In this study, the relationship between DC–T cell interaction in regional LNs upon an P6 and protective immunity in the airway after repeated airway challenges with outer membrane protein (OMP) of NTHi was examined in a murine model.

2. Materials and methods

2.1. Mice

Specific pathogen-free, 6–9-week-old female BALB/c mice were purchased from Charles River Japan, Kanagawa, Japan. The mice were maintained in barrier-protected animal facilities under specific pathogen-free conditions using ventilated microisolator cages in the experimental animal facility of the Institute of Tropical Medicine, Nagasaki University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University and conformed to National Institute of Health guidelines.

2.2. OMP preparation

A clinical isolate of NTHi (H93-151) from a patient with a respiratory tract infection was obtained and stored at −80 °C. OMP was prepared by a previously described method, with minor modifications [6]. In a typical experiment, NTHi was cultured overnight on brain heart infusion (BHI) agar (BBL, Becton Dickinson Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) containing 5% Fildes enrichment (Becton Dickinson and Co.) and 1% isovital X (Becton Dickinson and Co.) at 37 °C in a CO_2 incubator. The bacteria were harvested, suspended in 10 mM HEPES (Sigma Chemical Co., St. Louis, MO) buffer (pH 7.4), and disrupted by sonication. Debris was removed by centrifugation at $1700 \times g$ for 20 min. The supernatants were pooled and centrifuged at $100{,}000 \times g$ for 1 h at 4 °C. The pellet was suspended in a solution containing equal volumes of 10 mM HEPES buffer (pH 7.4) and 2% aqueous sodium lauryl sarcosinate (Wako Pure Chemical Industries, Osaka, Japan). The suspension was then centrifuged at $100{,}000 \times g$ for 1 h min at 4 °C. The pellet was suspended in distilled water and lyophilized. The resulting powder was used as OMP, and stored until used.

2.3. Intratracheal challenge of OMP

Twenty micrograms of OMP from an NTHi strain (H93-151) in a volume of 25 μl was intratracheally administered to each mouse on days 0, 7 and 14. Day 0 was defined as the day of the initial intratracheal (IT) challenge. The procedures were performed under anesthesia with an intraperitoneal injection of 0.2 ml of a solution containing 3 mg of ketamine and 0.1 mg of xylazine. The day 7 was defined as immediately prior to the second IT challenge, and the day 14 was immediately before the third challenge, respectively. Heparinized blood was obtained, the plasma separated, and BAL was performed at the indicated times after the initial challenge, as previously described [21]. Plasma and BAL fluid were stored at −80 °C until used.

2.4. Cell preparation and flow cytometry

A single cell suspension was obtained from pulmonary LNs of mice that had received the IT challenges of OMP or untreated mice as described previously [16]. The cells were incubated in RPMI-1640 medium containing 1 mg/ml collagenase type I (Wako Pure Chemistry) and 40 μg/ml DNase I (Roche, Mannheim, Germany) for 30 min at 37 °C. Pulmonary LN cells were subjected to FACS analysis (FACSCalibur, BD Biosciences) and the data were analyzed using the Cell Quest software program. These cells were stained with PE-conjugated rat anti-mouse CD11c monoclonal antibody (mAb) (BD PharMingen, San Diego, CA) and FITC-conjugated hamster anti-mouse CD86 mAb (BD PharMingen). The cultured pulmonary LN cells were stained with FITC-conjugated anti-mouse CD3 (BD Phramingen) or PE-conjugated anti-mouse B220 mAb (BD Phramingen).

2.5. Cell proliferation assay

In vitro cell proliferation assays were carried out using the Prenix WST-1 cell proliferation assay system (TAKARA, Tokyo, Japan) according to the manufacturer's instructions. Mononuclear cells were prepared from the pulmonary LNs of mice that had received an IT challenge of OMP once, twice or three times at 1-week intervals (days 0, 7 and 14) as described previously [16]. LN cells at a concentration of 1×10^6 in 100 μl of RPMI medium containing 10% FCS were seeded in a 96-well chamber in triplicate and were stimulated at 37 °C for 72 h with or without antigens at concentrations of 5 or 10 μg/ml. Purified P6 protein, ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO) and lipooligosaccharide (LOS) from NTHi were used as antigens. The P6 protein was purified, as described previously [6]. The LOS from NTHi was also purified as described previously [22]. After incubation,

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