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Th17 activation by dendritic cells stimulated with gamma-irradiated *Streptococcus pneumoniae*

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ABSTRACT

Dendritic cells (DCs) play an important role in antigen presentation, which is an essential step for the induction of antigen-specific adaptive immunity. Inactivated bacterial whole cell vaccines have been widely used to prevent many bacterial infections because they elicit good immunogenicity due to the presence of various antigens and are relatively inexpensive and easy to manufacture. Recently, gamma-irradiated whole cells of nonencapsulated *Streptococcus pneumoniae* were developed as a broad-spectrum and serotype-independent multivalent vaccine. In the present study, we generated gamma-irradiated *S. pneumoniae* (r-SP) and investigated its capacity to stimulate mouse bone marrow-derived DCs (BM-DCs) in comparison with heat-inactivated and formalin-inactivated *S. pneumoniae* (h-SP and f-SP, respectively). r-SP showed an attenuated binding and internalization level to BM-DCs when compared to h-SP or f-SP. r-SP weakly induced the expression of CD80, CD83, CD86, MHC class I, and PD-L2 compared with h-SP or f-SP. Furthermore, r-SP less potently induced IL-6, TNF-α, and IL-23 expression than h-SP or f-SP but more potently induced IL-1β expression than h-SP or f-SP in BM-DCs. Since Th17-mediated immune responses are known to be important for the protection against pneumococcal infections, r-SP-primed DCs were co-cultured with splenocytes or splenic $CD4^+$ T cells. Interestingly, r-SP-sensitized BM-DCs markedly induced IL-$17A^+$ $CD4^+$ T cells whereas h-SP- or f-SP-sensitized BM-DCs weakly induced them. Collectively, these results suggest that r-SP could be an effective pneumococcal vaccine candidate eliciting Th17-mediated immune responses by stimulation of DCs.

1. Introduction

Streptococcus pneumoniae, a Gram-positive facultative anaerobic bacterium, is a life-threatening pathogen that can infect the upper respiratory tract of humans. It is known to colonize the nasopharynx and the carriage rate is approximately 50% in adults (Lim et al., 2001). *S. pneumoniae* causes pneumonia, meningitis, otitis media, and pneumococcal septicemia (Bogaert et al., 2004). Particularly, pneumonia is the most common cause of death for children under 5 years old and *S. pneumoniae* is responsible for one million deaths of children per year worldwide (Black et al., 2010).

Capsular polysaccharides (C-PS) are one of the major virulence factors of *S. pneumoniae* and they interfere with the phagocytic capacity of neutrophils by inhibiting the complement pathway (Hyams et al., 2010). Currently-available pneumococcal vaccines, such as pneumococcal polysaccharide vaccine (PPSV) and pneumococcal conjugate vaccine (PCV), are based on C-PS (Daniels et al., 2016). The commercialized pneumococcal vaccine, 23-valent PPSV, protected adults and children over 2 years old from the disease caused by 23 different capsular serotypes of *S. pneumoniae*. However, it hardly induced protective immunity in infants and the elderly because it could only induce T cell-independent immune responses (Heilmann, 1990; Rubins et al., 1998). To overcome this limitation, PCV7 and PCV13 were developed and these elicited protective immune responses in immune compromised people through adaptive immune responses (Bhorat et al., 2015; Hoshina et al., 2016). However, PCV7 and PCV13 have limitations in that they only cover 7 and 13 serotypes, respectively, among 97 serotypes of *S. pneumoniae*, and are expensive to produce due to complicated conjugation processes. Furthermore, pneumococcal diseases by non-vaccine serotypes or nonencapsulated

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types have increased (Chapman et al., 2013). Therefore, the need for a serotype-independent, broad-spectrum, and cost-effective pneumococcal vaccine has increased.

Inactivated whole cell vaccines have been widely used against many bacterial pathogens. These vaccines are highly immunogenic because of a high number of various antigens and are safer than live-attenuated vaccines as they are completely inactivated using heat, chemicals, or radiation treatment (Kotloff et al., 2001; Seo, 2015). However, heat inactivation can cause antigen denaturation leading to poor adaptive immune responses (Magi et al., 2004). On the other hand, formalin-inactivated vaccines can cause side effects including tissue eosinophilia and allergic contact dermatitis (Kuritzky and Pratt, 2015; Moghaddam et al., 2006). Gamma-irradiation has been widely used for sterilization of medical devices (Bhatnagar et al., 2016) or foods (Anellis et al., 1972) due to negligible increase in temperature with no residual unwanted chemicals (Sadler et al., 2001). In principle, bacterial inactivation by gamma-irradiation mainly damages bacterial nucleic acids, preventing replication with minimal alterations of antigen structure, leading to higher immune responses and protective effects (Datta et al., 2006). In addition, whole cell vaccines inactivated by gamma-irradiation are relatively inexpensive and easy to prepare, and have multiple intact antigens compared with other inactivated vaccines (Seo, 2015). Recently, gamma-irradiated nonencapsulated *S. pneumoniae* was introduced as a novel intranasal vaccine candidate with appropriate immunogenicity (Babb et al., 2016).

Dendritic cells (DCs) are professional antigen-presenting cells that play an important role in linking innate and adaptive immunity. They are considered to be an essential target of vaccines because of their ability to uptake, process, and present antigen through major histocompatibility complex (MHC) molecules to T cells (Kranzer et al., 2005). When DCs recognize vaccines, they undergo maturation by changing their phenotypes and functions to evoke antigen-specific adaptive immune responses (Liu et al., 2016). During the maturation process, DCs migrate to draining lymph nodes and highly induce the expression of MHC molecules and co-stimulatory molecules to efficiently interact with T cells (Cohn and Delamarre, 2014). However, the role of DCs against pneumococcal whole cell vaccines is yet to be fully defined.

The use of PPSV or PCV leads to antibody responses against C-PS that mediate protective immunity against pneumococcal diseases (Chen et al., 2014; Dagan et al., 2015). However, many studies have suggested that not only antibody-dependent but also $CD4^+$ T cell-dependent immune responses are important for protection against pneumococcal infections (Lu et al., 2008; Malley et al., 2006). In pneumococcal infections, IL-17, but not IFN-γ, responses mediated by $CD4^+$ T cells are essential for the clearance of *S. pneumoniae* in the upper respiratory tract (Wright et al., 2013). Thus, in the present study, we investigated the maturation and activation of DCs stimulated with gamma-irradiated *S. pneumoniae* (r-SP) in comparison with those of DCs primed with heat-inactivated (h-SP) or formalin-inactivated *S. pneumoniae* (f-SP), and T cell activation mediated by these DCs.

2. Materials and methods

2.1. Reagents and chemicals

Tryptic soy broth (TSB), Todd-Hewitt broth (THB) and Bacto™ agar were purchased from BD Biosciences (San Diego, CA, USA). RPMI-1640 and penicillin-streptomycin solution were purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY, USA). Recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF) was obtained from CreaGene (Sungnam, Korea). Red Blood Cell Lysis Buffer and 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and from Molecular Probes (Eugene, OR, USA), respectively. Mouse CD4 T Lymphocyte Enrichment Set, PE-labeled anti-mouse CD69 antibody (clone: H1,2F3), and APC-labeled anti-mouse CD25 antibody (clone: 3C7) were purchased from BD Biosciences. FITC-labeled anti-mouse CD86 (clone: GL-1), PE-labeled anti-mouse CD80 (clone: 16-10A1), APC-labeled anti-mouse CD83 (clone: Michel-19), Alexa647-labeled anti-mouse MHC I (clone: 28-8-6), FITC-labeled anti-mouse MHC II (clone: 39-10-8), APC-labeled anti-mouse PD-L1 (clone: 10F.9G2), PE-labeled anti-mouse PD-L2 (clone: TY25), FITC-labeled anti-mouse CD3 (clone: 17A2), PerCP-labeled anti-mouse CD4 (clone: RM4-5), APC-labeled anti-mouse γδTCR (clone: GL3), and PE-labeled anti-mouse IL-17A (clone: TC11-18H10.1) antibodies were purchased from BioLegend (San Diego, CA, USA).

2.2. Generation of capsule-deficient S. pneumoniae

Capsule-deficient *S. pneumoniae* was generated by deleting cpsB (Wzh) and cpsC genes in TIGR4 strain as described previously (Dillard et al., 1995). A 669-bp fragment upstream of cps2B gene was amplified using primers Cps4BKO-UpF (5′-AAC TCG AGT GGA TAT CAA TTA CTA T-3′) and Cps2BKO-UpR (5′-TTA AGC TTT CAT CTA CCC TCC ATC-3′), and then digested with *Xho*I and *Hind*III. A 597-bp fragment downstream of cps2B gene was amplified with primers Cps4CKO-DnF (5′-AAG AAT TCT GGT AAA AGA CTA CCG TG-3′) and Cps2CKO-DnR (5′-TTG AAT TCT ATT TCA ACT TAC CCA AG-3′), and then digested with *Eco*RI. The upstream and downstream fragments were cloned sequentially into the multiple cloning sites of pE326 derived from pC326 (Seo et al., 2010). The resulting plasmid, pKO-CPS2B, was introduced into TIGR4 by natural transformation as previously described (Alloing et al., 1998). Briefly, TIGR4 culture was diluted 100-fold in fresh THB supplemented with 0.1% $CaCl_2$ and 0.5% bovine serum albumin, 200 ng/ml of competence-stimulating peptide-2 (CSP-2), and 10 μg/ml of pKO-CPS2B plasmid, followed by incubation at 37 °C for 2 h. The transformation mixture was centrifuged, resuspended with fresh THB, and then plated on blood agar containing 0.5 μg/ml erythromycin. Expression of C-PS in cpsBC mutant strain was confirmed by ELISA as described previously (Inzana and Champion, 2007).

2.3. Preparation of r-SP, h-SP, and f-SP

Three different inactivation methods (gamma-irradiation, heat treatment, and formalin treatment) were used in this study as described previously (Seo, 2015; Kim et al., 2017). Nonencapsulated TIGR4 strain was grown in TSB at 37 °C until optical density reached approximately 0.8 ~ 1.0. The cells were harvested by centrifugation (3,515 × *g*, 30 min), washed twice with phosphate-buffered saline (PBS), and lyophilized. For preparing r-SP, lyophilized cells were irradiated with 10 kGy of gamma-irradiation using cobalt-60 gamma-ray irradiator (point source AECL, IR-79, MDS Nordion, Ottawa, ON, Canada) followed by resuspension with PBS to reach optical density 1.0. For preparing h-SP, the lyophilized cells were resuspended with PBS to reach optical density 1.0. The cell suspension was incubated at 58 °C for 2 h. For preparing f-SP, lyophilized cells were resuspended with PBS with 0.2% formaldehyde (v/v). The cell suspension was stirred at 37 °C for 2 h, washed twice with PBS and the pellet was resuspended with PBS to reach optical density 1.0. All inactivated vaccines were inoculated in THB supplemented with 5% yeast extract and ascertained for sterility by culturing at 37 °C for 3 days.

2.4. Analysis of bacterial adherence and internalization

r-SP, h-SP, and f-SP were stained with CFDA-SE (10 μM) at 37 °C for 15 min and washed three times with the same volume of PBS. Immature DCs (5×10^4 cells) were incubated with CFSE-labeled r-SP, h-SP, or f-SP at 4 °C or 37 °C for 1 h for adherence or internalization assay, respectively. At the end of the incubation period, bacterial binding and internalization were measured by flow cytometry using FACSCalibur (BD Biosciences) as previously described (Hong et al., 2017).