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Nanogel-based pneumococcal surface protein A nasal vaccine induces microRNA-associated Th17 cell responses with neutralizing antibodies against *Streptococcus pneumoniae* in macaques

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We previously established a nanosized nasal vaccine delivery system by using a cationic cholesteryl group-bearing pullulan nanogel (cCHP nanogel), which is a universal protein-based antigen-delivery vehicle for adjuvant-free nasal vaccination. In the present study, we examined the central nervous system safety and efficacy of nasal vaccination with our developed cCHP nanogel containing pneumococcal surface protein A (PspA-nanogel) against pneumococcal infection in nonhuman primates. When [¹⁸F]-labeled PspA-nanogel was nasally administered to a rhesus macaque (*Macaca mulatta*), longer-term retention of PspA was noted in the nasal cavity when compared with administration of PspA alone. Of importance, no deposition of [¹⁸F]-PspA was seen in the olfactory bulbs or brain. Nasal PspA-nanogel vaccination effectively induced PspA-specific serum IgG with protective activity and mucosal secretory IgA (SIgA) Ab responses in cynomolgus macaques (*Macaca fascicularis*). Nasal PspA-nanogel-induced immune responses were mediated through T-helper (Th) 2 and Th17 cytokine responses concomitantly with marked increases in the levels of miR-181a and miR-326 in the serum and respiratory tract tissues, respectively, of the macaques. These results demonstrate that nasal PspA-nanogel vaccination is a safe and effective strategy for the development of a nasal vaccine for the prevention of pneumonia in humans.

INTRODUCTION

Streptococcus pneumoniae is a major cause of bacterial infections throughout the world and is involved in the induction of a wide variety of infectious diseases, including otitis media, pneumonia, bacteremia, and meningitis in children and adults. This organism is usually a commensal bacterium in the upper respiratory tract of humans. Currently, four pneumococcal vaccines, 7-, 10- and 13-valent polysaccharide conjugate vaccines (PCV7, 10, 13) for

children and a 23-valent pneumococcal polysaccharide vaccine (PPV23) for adults, have been developed for public use and are delivered by intramuscular injection.^{1–3} However, as the conjugate vaccine does not protect against other capsular types, it provides little or no protection against total colonization with pneumococci.^{4,5} The extensive carriage by other pneumococcal capsular types has led to strain replacement in disease with strains of non-conjugate vaccine capsular types.^{6,7}

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The development of effective protein-based vaccines, which have the potential to provide better coverage for all strains, and to protect against colonization with all strains requires a thorough understanding of the roles and relative contributions to pathogenesis of the various putative virulence proteins. The pneumococcal surface protein A (PspA) is a well-known highly immunogenic surface protein of *S. pneumoniae* and is considered to be a promising vaccine candidate.^{8,9} It is present on virtually all strains of pneumococci, and PspA-based vaccines against *S. pneumoniae* induce cross-reactive Abs in mice^{10,11} and humans.¹² Moreover, PspA-specific mucosal and serum Abs responses are induced, and these responses are mediated by both Th1- and Th2-type cytokine production by CD4⁺ T cells in infant mice via maternal immunization,¹³ as well as in aged mice.¹⁴ These findings indicate that PspA is a potent antigen for the development of effective pneumococcal vaccines not only in adults but also in children and the elderly.

S. pneumoniae commonly colonizes the nasal cavity, which can be protected by mucosal IgA.^{15–17} Nasal vaccination induces effective mucosal immune responses in the respiratory tract, where initial bacterial and viral infections commonly occur; it could therefore be an effective immunization strategy for delivering protection from pneumococcal infection. However, most subunit type vaccines are poor immunogens for the induction of antigen-specific immune response in both systemic and mucosal immune compartments when nasally administered. Thus, the co-administration of biologically active mucosal adjuvants (e.g., cholera toxin and heat-labile toxin) or a better delivery system is needed to overcome the disadvantages of nasal antigen exposure. However, there are currently no safe nasal adjuvants or delivery systems, as evaluated by safety pharmacology studies, such as absorption, distribution, metabolism, and excretion in preclinical studies.

To overcome these concerns, we recently developed an effective vaccine delivery system with a self-assembled nanosized hydrogel (nanogel), which is composed of a cationic type of cholesteryl group-bearing pullulan (cCHP).¹⁸ This cCHP nanogel efficiently delivers an antigen to epithelial cells in the nasal cavity, as well as to dendritic cells (DCs) under the basement membrane, and induces antigen-specific immune responses as an adjuvant-free vaccine.^{19,20} Furthermore, a radioisotope counting assay showed that nasally administered cCHP nanogel carrying the [¹¹¹In]-labeled non-toxic subunit of botulinum neurotoxin does not accumulate in parts of the central nervous system (CNS) in mice.¹⁹ In our separate study, we demonstrated that a nasally administered PspA-nanogel vaccine is safe and induces strong antigen-specific systemic and mucosal Ab immune responses, which can protect mice from invasive challenge with *S. pneumoniae*.²¹

MicroRNAs (miRNAs) have emerged as important regulators of many biological processes associated with the immune system, including the function of both innate and adaptive immune responses.^{22–24} Accumulating evidence indicates that miRNA has an essential role in eliciting immune responses. For example, mice with T lymphocytes in which the endoribonuclease dicer, which is critical for miRNA biogenesis,

has been conditionally knocked out show impaired thymic development and diminished Th-cell differentiation.^{25,26} Dicer deficiency in B cells also prevents B-cell development.²⁷ These findings indicate the critical functions of miRNAs in the biology of the cells that constitute the immune system, such as in the development and differentiation of lymphocytes. Therefore, it is important to identify the miRNA biomarkers that engage in both mucosal and systemic antibody responses induced by nasal immunization with PspA-nanogel. Together, better understanding of the precise engagement of miRNAs in mediating humoral and protective immunity will be beneficial for the development of effective mucosal vaccines.

Before pursuing a clinical trial of a PspA-nanogel-based vaccine, we designed experiments to assess its safety for the CNS and its immunological efficacy, including immunologically relevant miRNA expression, and to demonstrate its safety and efficacy in nonhuman primates.

RESULTS

[¹⁸F]-PspA-nanogel is retained for a long time in the nasal cavity but is not deposited in the olfactory bulbs or brain after nasal administration in macaques

We initially confirmed the physicochemical characterization of PspA-nanogel vaccine used in this study (**Supplementary Figure S1** online), and then investigated the retention of nasal PspA-nanogel in the nasal cavity and its accumulation in the olfactory bulbs and CNS in nonhuman primates by using three naive rhesus macaques. Because the results were nearly identical for the three macaques, we show the results for only one of the macaques in **Figure 1** (primate #1). The macaque's head was placed in the positron emission tomography (PET) scanner system and real-time imaging was performed for 6 h. To confirm the exact position of the cerebrum, we performed a magnetic resonance imaging (MRI) scan and then superimposed the PET images onto the MRI images. The real-time PET images clearly showed that nasally administered [¹⁸F]-PspA-nanogel was effectively delivered to the nasal mucosa and retained in the nasal tissues for up to 6 h (**Figure 1a,c**). In contrast, most of the free form of [¹⁸F]-PspA without a nanogel had disappeared from the nasal cavity by 3 h after nasal administration (**Figure 1a**). Furthermore, no deposition of [¹⁸F]-PspA was detected in the cerebrum or olfactory bulbs of macaques, even 6 h after nasal administration (**Figure 1b**). These results show that PspA-nanogel is a CNS-safe and effective nasal vaccine delivery system in nonhuman primates.

Nasal vaccination with PspA-nanogel induces mucosal and systemic Ab responses in macaques

We next examined whether the nasal PspA-nanogel vaccine induced PspA-specific immune responses in cynomolgus macaques (primates #2–#9). One week after the final immunization, PspA-specific serum IgG Ab responses were significantly increased in macaques nasally immunized with 25 µg of PspA-nanogel when compared with macaques immunized with PspA alone or PBS only (**Figure 2a**). Examination of the longevity of PspA-nanogel-induced serum antigen-specific IgG

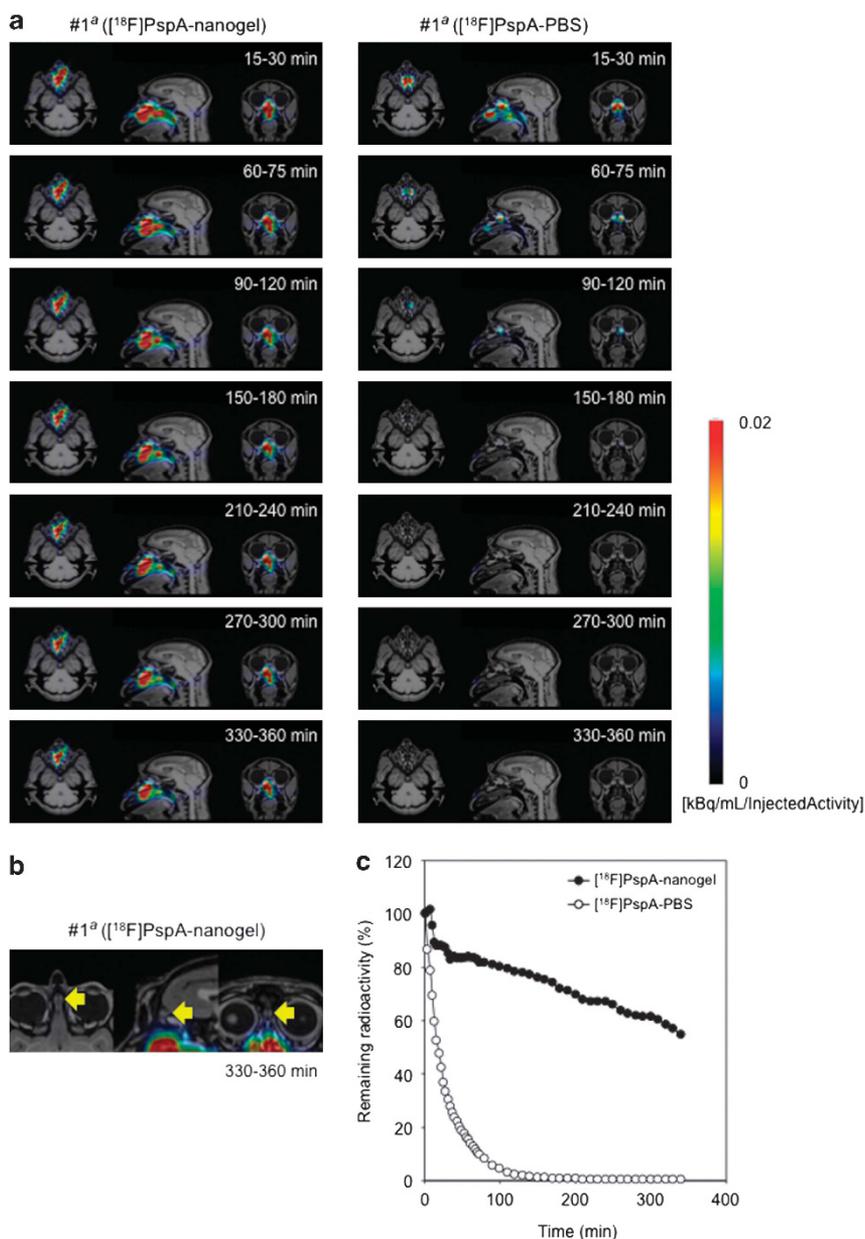


Figure 1 PET/MRI images (a,b) and TACs (c) for nasal administration of [¹⁸F]-PspA-nanogel or [¹⁸F]-PspA-PBS in a naive rhesus macaque. (a) After nasal administration of [¹⁸F]-PspA-nanogel or [¹⁸F]-PspA-PBS, the macaque's head was scanned for 6 h with a PET scanner. Real-time PET images overlaid on MRI images are shown for the indicated times post-administration. (b) To further check whether [¹⁸F]-PspA accumulated in the CNS or olfactory bulbs (indicated by arrowheads), PET images taken at 6 h post-administration of [¹⁸F]-PspA-nanogel were enlarged. (c) TACs for the nasal cavity for 6-h period after nasal administration of [¹⁸F]-PspA-nanogel or [¹⁸F]-PspA-PBS are presented. The data are expressed as percentages of the dose remaining after nasal administration. a: The same macaque was nasally administered of [¹⁸F]-PspA-nanogel or [¹⁸F]-PspA-PBS with a 1-week interval between administrations. CNS, central nervous system; MRI, magnetic resonance imaging; PET, positron emission tomography; TACs, time-activity curves.

Ab titers revealed that Ab levels gradually decreased over a period of 8 months in macaques nasally immunized with PspA-nanogel. Similarly, PspA-specific bronchoalveolar lavage fluid (BALF) IgG and nasal wash IgA Ab responses exhibited higher levels in macaques nasally immunized with PspA-nanogel when compared with macaques nasally immunized with PspA alone or PBS only (Figure 2b,c), and these Ab levels were also

gradually decreased. In addition, PspA-specific BALF IgA Ab responses were slightly increased in two of the immunized macaques (#3 and #5) (Figure 2c).

When these macaques were given a dose of nasal booster of PspA-nanogel 9 months after the final immunization, the levels of PspA-specific serum and BALF IgG and nasal wash IgA Ab responses immediately recovered to those observed at 9 weeks

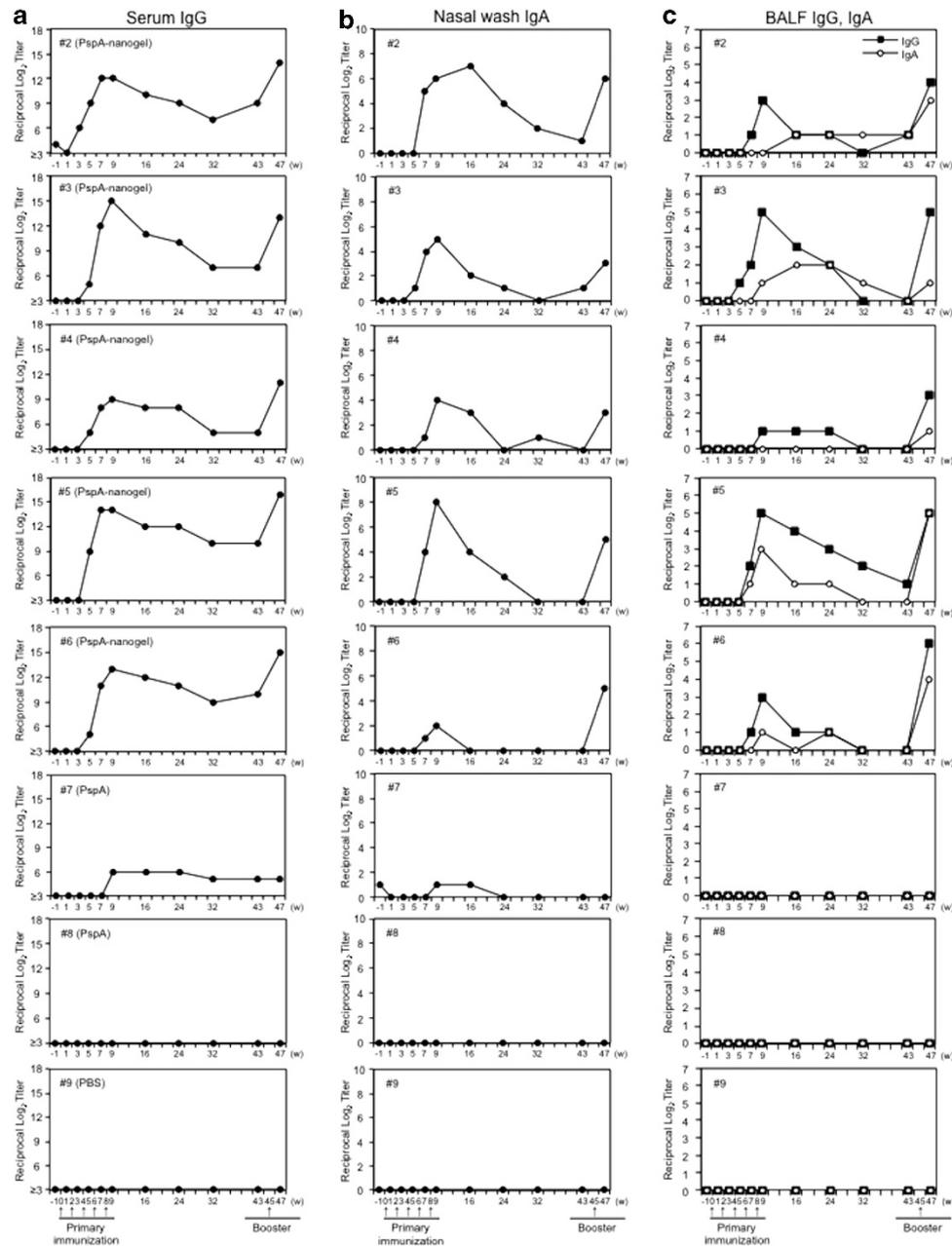


Figure 2 Nasal immunization with PspA-nanogel induced PspA-specific Ab responses in macaques. Each cynomolgus macaque was nasally immunized with PspA-nanogel (macaques #2-#6), PspA alone (#7 and #8), or PBS only (#9) at the times indicated with arrows. Serum, nasal wash, and BALF were collected, and the levels of PspA-specific serum IgG (a), nasal wash IgA (b), and BALF IgG and IgA (c) were determined by ELISA. BALF, bronchoalveolar lavage fluid.

after the initial PspA-nanogel immunization (Figure 2a-c). Of importance, a nasal booster induced higher levels of PspA-specific IgA Ab responses in BALF of two macaques (#5 and #6) than those observed after the primary immunization (Figure 2c).

These findings suggest that memory-type PspA-specific Ab responses are induced in nonhuman primates after nasal vaccination with PspA-nanogel. PspA-nanogel is therefore a promising nasal vaccine candidate that can induce long-lasting antigen-specific systemic and mucosal immunity and can elicit nasal booster activity in nonhuman primates.

Nasal immunization with PspA-nanogel induces neutralizing Abs against *S. pneumoniae* in macaques

To investigate whether the nasal PspA-nanogel vaccine induced neutralizing Abs, we examined whether PspA-specific serum Abs from macaques nasally immunized with PspA-nanogel would passively protect against pneumococcal infection. CBA/N mice were injected intraperitoneally with diluted pooled sera of macaques nasally immunized with PspA-nanogel, PspA alone, or PBS only. When all groups of mice were challenged with *S. pneumoniae* Xen10 or 3JYP2670 strain via the intravenous route, mice passively immunized with sera

from macaques nasally immunized with PspA-nanogel were fully protected for at least 2 weeks (Figure 3a,b). In contrast, mice that received sera from macaques given nasal PspA alone or PBS only died within 5 days post-challenge (Figure 3a,b). These results demonstrated that protective immunity with subtype cross-reactivity was induced by nasal PspA-nanogel vaccination.

Nasal immunization with PspA-nanogel induces Th2 and Th17 responses in macaques

As macaques nasally immunized with PspA-nanogel showed high IgG/IgA Ab responses, we next determined the levels of cytokine production in CD4⁺ T cells isolated from blood of the macaques. The macaques nasally immunized with PspA-nanogel showed increased levels of IL-4 and IL-17 production by CD4⁺ T cells when compared with macaques given PspA alone or PBS only (Figure 4b,c). However, essentially identical levels of IFN- γ were produced by CD4⁺ T cells isolated from macaques nasally immunized with PspA-nanogel, PspA alone, or PBS only (Figure 4a). Furthermore, we showed that nasal immunization with PspA-nanogel induced PspA-specific IgG1 Ab responses, which is the hallmark of the Th2-type immune response (Figure 4d). These results indicated that the nasal PspA-nanogel vaccine could induce Th2 and Th17 cytokine responses.

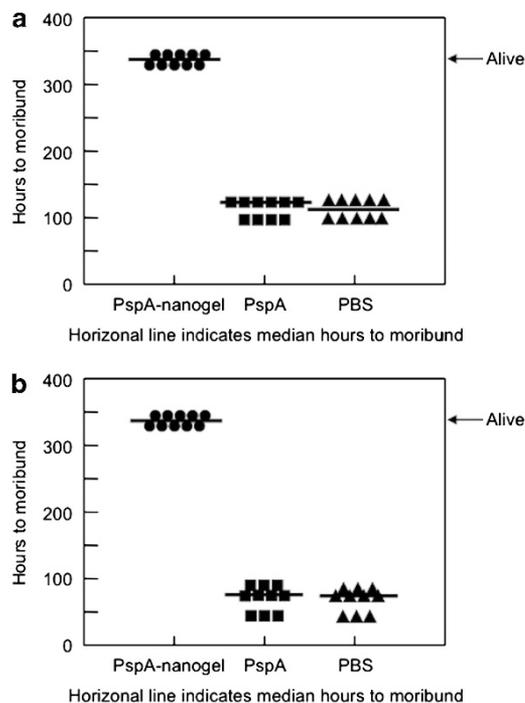


Figure 3 Neutralizing Abs induced by nasal immunization with PspA-nanogel. Serum from each of the macaques was collected 1 week after the final primary nasal immunization with PspA-nanogel, PspA alone, or PBS only. CBA/N mice (10 mice per group) were passively transferred with 100 μ l of diluted (1:20) pooled sera via i.p. route. Four hours later, mice were injected i.v. with 1.5×10^4 c.f.u. *S. pneumoniae* Xen 10 (a) or 1×10^3 c.f.u. *S. pneumoniae* 3JYP2670 strain (b). The mice were monitored daily for mortality. Each line represents the median survival time. c.f.u., colony-forming unit; i.p., intraperitoneal; i.v., intravenous.

Nasal immunization with PspA-nanogel increases the expression levels of miRNAs in serum and respiratory tract tissues in macaques

To examine the roles of miRNA in the induction of PspA-specific immunity, we performed miRNA microarray analysis to identify immunologically associated differences in serum miRNA profiles between pre-immunized and post-boosted macaques (data not shown). We selected some immunologically relevant miRNAs, namely miR-181a, miR-326, miR-155, miR-17, miR-18a, miR-20a, and miR-92a, the levels of which were upregulated in post-boost serum samples compared with pre-immunized serum samples. To further confirm whether these immunologically relevant miRNAs were upregulated or downregulated in post-boost serum samples compared with pre-immunized or pre-boost serum samples, we performed quantitative RT-PCR of them. Expression levels of miR-326, Th17-cell differentiation-related miRNA, and miR-181a, T-cell and B-cell differentiation-related miRNA, were significantly increased in the sera of macaques given a nasal booster dose of PspA-nanogel when compared with control macaques as pre-immunization (Figure 5a). The levels of the two miRNAs were also shown significantly higher in the respiratory tract tissues, including nasal tissues and lungs, of macaques given a booster dose of PspA-nanogel than the levels in the corresponding tissues of control macaques given PspA alone or PBS only that was set at 1 (Figure 5b,c). Furthermore, we analyzed the expression level of Ets-1, which is a known negative regulator of Th17 cells and is the functional target of miR-326. We detected a significant decrease in the expression level of Ets-1 mRNA in the lungs of macaques given a booster dose of PspA-nanogel compared with those of control macaques given PspA alone or PBS only (Figure 5c). These results suggest that these miRNAs have important roles in T-cell and B-cell differentiation and in Th17 cytokine responses after nasal immunization with PspA-nanogel in macaques.

DISCUSSION

By using a nonhuman primate system, we demonstrated that the nasal PspA-nanogel vaccine did not accumulate in the CNS and effectively induced both mucosal and systemic immunity associated with protection against pneumococcal infection. To our knowledge, this study is the first to report the safety and effectiveness of a nasal PspA vaccine in macaques; therefore, our results provide a concrete rationale for testing our nanogel-based PspA vaccine in humans.

The nanogel itself is non-immunogenic material, and a cancer-specific protein (e.g., Her 2) complexed with a neutral CHP nanogel produced by means of good manufacturing practices (GMP) has been used as an injectable cancer vaccine in clinical research.²⁸ In our previous study, nasal immunization with CHP-nanogel containing PspA induces effective antigen-specific immune responses in mice²¹ but not in macaques (data not shown); therefore, in the present study, we developed a cCHP nanogel containing 20 amino groups per 100 glucose units to improve antigen delivery to the nasal

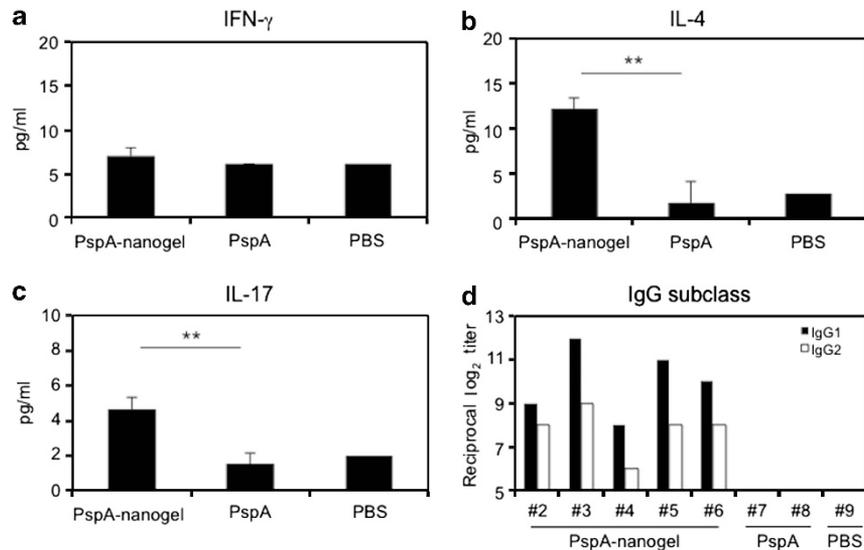


Figure 4 PspA-nanogel immunization produced CD4⁺ Th2- and Th17-type cytokine responses. CD4⁺ T cells were separated from the PBMCs 1 week after the booster. Purified CD4⁺ T cells were cultured with irradiated APCs and 5 $\mu\text{g ml}^{-1}$ of PspA with anti-CD28 and CD49d antibodies for 5 days. The levels of the cytokines, IFN- γ (a), IL-4 (b), and IL-17A (c) in the supernatants were measured. This experiment was repeated in triplicate. Values are shown as the means \pm s.d. in each experimental group. ** $P < 0.01$ compared between PspA-nanogel and PspA/PBS groups. (d) Serum from macaques was collected 1 week after the final primary nasal immunization with PspA-nanogel (#2-#6), PspA alone (#7, #8), or PBS only (#9). Expression levels of PspA-specific serum IgG subclass Abs were determined by using ELISA. APCs, antigen-presenting cells; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cells.

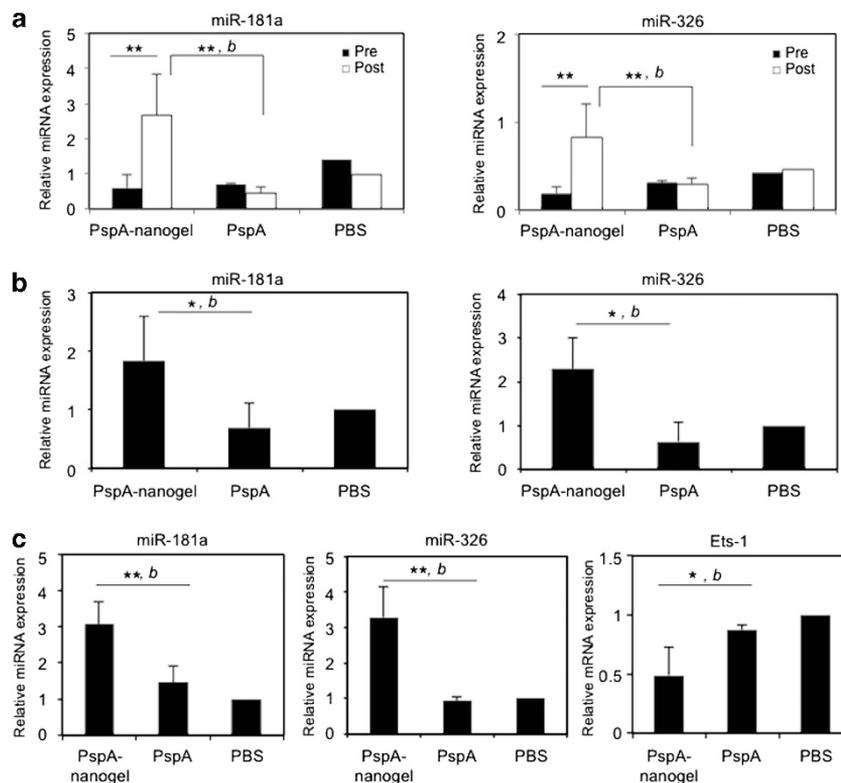


Figure 5 MiRNA expression levels in sera (a), nasal tissues (b), and lung tissues (c) of macaques nasally immunized with PspA-nanogel, PspA alone, or PBS only. Expression levels of the indicated miRNA and Ets-1 mRNA were analyzed by quantitative RT-PCR and normalized to the levels of miR-16 and β -actin, respectively. Values are shown as the means \pm s.d. in each experimental group. * $P < 0.05$, ** $P < 0.01$ when compared between pre-immunization and post-booster groups. *b*, Compared between PspA-nanogel and PspA/PBS groups in post-booster macaques. MiRNA, microRNA; Pre, pre-immunized serum; Post, post-booster serum.

epithelium layer of macaques. We confirmed the perfect complex formation and the size of PspA-nanogel complex using fluorescence resonance energy transfer (FRET) analysis and dynamic light scattering (DLS): the cCHP nanogel spontaneously formed nanoparticles after the incorporation of PspA (**Supplementary Figure S1a,b**).^{18,19} In addition, consistent with its positive zeta-potential (**Supplementary Figure S1b**), *in vivo* PET and MRI imaging in macaques clearly showed that nasally administered cCHP nanogel carrying [¹⁸F]-labeled PspA was more effectively delivered to and continuously retained at the nasal mucosa of macaques when compared with nasally delivered [¹⁸F]-PspA alone. These results indicated that the new cationic group-modified cCHP nanogel would be able to efficiently deliver the vaccine antigen to the anionic nasal epithelium following nasal administration in macaques. Indeed, our previous mouse model studies have shown that the nanogel-antigen complex is retained and taken up into the epithelium by endocytosis, where the antigen is released from the nanogel in the epithelium by strong chaperone-like activity. The antigen is then released from the nasal epithelium by exocytosis and subsequently taken up effectively by DCs.^{19,21}

Recent studies of nasal vaccines have raised concerns about the deposition and accumulation of candidate vaccine antigens or co-administered mucosal adjuvants in the CNS through direct transport from the nasal cavity to the cerebrum via the olfactory pathways.^{29,30} It has also been reported that many peptides and proteins bypass the blood-brain and blood-cerebrospinal fluid barriers to reach the CNS following nasal administration in humans.³¹ In this study, we showed that there was no deposition or accumulation of [¹⁸F]-PspA in the CNS over a period of up to 6 h after nasal administration of [¹⁸F]-PspA-nanogel in macaques. As we validated the detection limit of our PET system for [¹⁸F]-protein by direct tissue counting in our previous study,³² [¹⁸F] radioactivity in this study was <0.05 SUV in the cerebrum and olfactory bulbs of the macaques. Therefore, our current results demonstrated that the cCHP delivered nasal PspA vaccine did not reach the CNS of macaques, even though the olfactory epithelium in the nasal cavity is connected to the CNS,³¹ thereby confirming the safety of the vaccine in higher mammals.

The mucosal immune system consists of both inductive and effector sites and has a key role in the induction and regulation of dynamic immune responses, including the Th2-type-cell-dependent SIgA response, the mucosal cytotoxic T-cell response, and the Th17-cell-mediated immune regulatory response.³³ In general, IgA in mucosal tissue is thought to have an important role in protection against respiratory pathogens including *S. pneumoniae*.¹⁵⁻¹⁷ In this study, we showed that the PspA-nanogel vaccine also induced mucosal antigen-specific mucosal IgA and systemic IgG Ab responses in the macaques. Especially, serum and BALF IgG, the main isotype of antibody in the lower respiratory compartment, have key roles in survival against lethal challenge with *S. pneumoniae*.³⁴ Importantly, the macaque IgG antibodies to PspA, which are supported by

CD4⁺ Th2-type cytokine IL-4, possessed protective activity against *S. pneumoniae*. When mice were systemically challenged with *S. pneumoniae* Xen10 or 3JYP2670 after mice passively immunized with macaques' sera containing PspA-specific Abs, they showed complete protection. Our findings indicate that this protection is clearly due to antibody-mediated immunity to PspA. These results are consistent with those of a previous study in mice showing that nasal vaccination induces functional CD4⁺ Th2-type cytokine-mediated IgG Ab responses, which are sufficient to provide appropriate protection in the absence of Th1-type cytokine responses.¹⁶ In addition, induction of the BALF IgG responses is essential, as antigen-specific IgG is known to exert protection at the alveolar level following to promote phagocytosis and prevents local dissemination of the pneumococcus and its passage into the blood.³⁴ These results demonstrated that the nasal PspA-nanogel vaccine effectively induced PspA-specific serum IgG with protective activity in addition to SIgA Ab immune responses in nonhuman primates.

Recent studies have shown that specific miRNAs are involved in T-cell and B-cell development, differentiation, and regulatory functions.^{24,35} Especially, miR-181a is highly expressed in mature T cells and has an important effect on the positive and negative selection process by controlling the strength of TCR signaling during thymic development of T cells for subsequent Th1 and Th2 differentiation, indicating that miR-181a modulates T-cell development.³⁶ In this study, the expression levels of miR-181a in the serum and respiratory tract tissues, including nasal tissues and lungs, were significantly higher in macaques nasally immunized with PspA-nanogel than in those given PspA alone or PBS only, indicating that miRNAs are implicated in adaptive immunity by controlling the activation of T cells after nasal immunization with PspA-nanogel in nonhuman primates. Furthermore, we showed that the levels of miR-155, which is required for the production of high-affinity IgG1 Abs, were increased in PspA-nanogel-immunized macaques (**Supplementary Figure S2a-c**).³⁷ These results indicated that PspA-nanogel-induced Th2 cytokine response was mediated through the increased expression of miR-155.

MiR-181a is also highly expressed in B cells and within bone marrow cells and germinal center B cells, where it promotes the differentiation of hematopoietic stem cells into B cells.^{24,38} To explore the roles of other miRNAs that are also highly expressed in germinal center B cells and are essential for adult B-cell development, we examined the expression of the miR-17-92 cluster.^{39,40} The miR-17-92 cluster regulates follicular helper T cell (T_{fh} cell) differentiation by controlling the migration of CD4⁺ T cells into B-cell follicles,⁴¹ suggesting that these miRNAs have an important role in the production of antigen-specific SIgA Ab. We found here that not only miR-181a expression but also miR-17-92 cluster expression was markedly increased in the nasal tissues of nasally PspA-nanogel-immunized macaques (**Supplementary Figure S3**). Detection of these mucosal IgA-associated miRNAs in the nasal tissues of nasally PspA-nanogel-immunized macaques

indicates that they contribute substantially to the production of mucosal IgA.

It is well known that IL-17-mediated CD4⁺ T cells are important for the generation of resistance to mucosal colonization by respiratory pathogens including *S. pneumoniae* in humans and mice.^{42,43} Trzcinski *et al.*⁴⁴ demonstrated that antigen-specific CD4⁺ T-cell immunity is sufficient to protect against nasopharyngeal colonization by *S. pneumoniae* in mice. Studies in mice indicated that pulmonary Th17 responses are associated with migration of B cells into airways and with the promotion of polymeric Ig receptor (pIgR) expression by airway epithelial cells.⁴⁵ In addition, Th17 cells are a crucial subset of Th cells responsible for inducing the switch of germinal center B cells toward T-cell-dependent IgA production.⁴⁶ Furthermore, IL-17-secreting memory Th17 cells increased by human pneumococcal carriage have been reported to enhance innate cellular immunity against pneumococcal challenge.⁴⁷ Therefore, it is important to determine whether antigen-specific CD4⁺ Th17 responses are induced by nasal immunization with PspA-nanogel in nonhuman primates. Recent studies have shown that miR-326-mediated Th17 upregulation might provide the host with a potentiating effect to recruit functional immune cells to local effector sites in response to pathogen attack.⁴⁸ We found here that nasal immunization with PspA-nanogel in macaques prompted the generation of IL-17-producing cells in the peripheral blood CD4⁺ T cells. Furthermore, our miRNA analysis showed that expression levels of Th17-associated miR-326 in the serum, nasal tissues, and lungs were significantly increased and that the expression level of Ets-1 mRNA, a negative regulator of Th17 differentiation, was decreased in the lungs of the PspA-nanogel-vaccinated macaques. Therefore, our finding that miR-326-associated IL-17-secreting CD4⁺ T cells were generated after nasal vaccination with PspA-nanogel suggests that it would be useful for the development of safe and efficacious nasal vaccines against pneumonia and that serum miR-326 could be used as a biomarker to evaluate vaccine efficacy.

In summary, we demonstrated for the first time that a nasal PspA-nanogel vaccine induced both humoral and cellular immune responses in macaques. These results were supported by increased expression levels of miR-181a and miR-326, which are candidate miRNA biomarkers for induction of mucosal immunity. In addition, a [¹⁸F]-PspA PET study showed long-term retention of PspA in the nasal cavity and no deposition of PspA in the CNS of the macaques. Taken together, these findings demonstrate the efficacy and safety of nasal PspA-nanogel vaccine in nonhuman primates. We conclude that the nasal PspA-nanogel vaccine should now be studied in humans for its possible use as an adjuvant-free nasal vaccine.

METHODS

Animals. Eight female naive cynomolgus macaques (*Macaca fascicularis*, 5 years old, ~3 kg) were used for the immunization study and were maintained at the Tsukuba Primate Research Center for Medical Science at the National Institute of Biomedical Innovation (NIBIO, Ibaraki, Japan). In a separate experiment, one naive male rhesus

macaque (*M. mulatta*, 5–6 years old, ~5 kg) was used for the PET imaging study, which was conducted at PET Center of Hamamatsu Photonics K.K. To assay antibody protection against *S. pneumoniae*, female CBA/N mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). All experiments were performed in accordance with the Guidelines for Use and Care of Experimental Animals, and the protocol was approved by the Animal Committee of NIBIO, Hamamatsu Photonics K.K., and The University of Tokyo.

Recombinant PspA. Recombinant PspA of *S. pneumoniae* Rx1, which belongs to PspA family 1 and clade 2, was prepared as described previously, with slight modification.¹⁰ In brief, the plasmid encoding PspA/Rx1 (GenBank accession no. M74122; amino acids 1 through 302, pUAB055) was used to transform *E. coli* BL21 (DE3) cells. To construct pUAB055, a 909-bp fragment of PspA from a pneumococcal strain Rx1 was cloned into the pET20b vector (Novagen, Darmstadt, Germany) between the *Nco*I and *Xho*I sites. Recombinant PspA/Rx1 contains the first 302 amino acids of mature PspA plus six poly-histidines added through protein fusion at the C-terminal end. The sonicated cell supernatant was loaded onto a DEAE-Sephacrose column (BD Healthcare, Piscataway, NJ) and a nickel affinity column (Qiagen, Valencia, CA), followed by gel filtration on a Sephadex G-100 column (BD Healthcare).

Preparation of recombinant PspA–nanogel complex. The cCHP nanogel (~40 nm size) generated from cationic type of cholesteryl group-bearing pullulan was used for all experiments. This cCHP nanogel contained 20 amino groups per 100 glucose units. The PspA-cCHP nanogel complex for each immunization was prepared by mixing 25 µg of PspA with cCHP at a 1:5 molecular ratio (59.45 µl per macaque) and incubating for 1 h at 46 °C. FRET was determined with an FP-6500 fluorescence spectrometer (JASCO, Tokyo, Japan) with FITC-conjugated PspA and TRITC-conjugated cCHP nanogel.^{18,19} The hydrodynamic radius was assessed by means of DLS and the zeta-potential of cCHP carrying or not carrying, PspA was determined with a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK).^{18,19}

Nasal immunization and sample collection. Cynomolgus macaques were nasally immunized five times at 2-week intervals with PspA-nanogel under ketamine anesthesia. For the control group, macaques were nasally administered with 25 µg of PspA alone, or PBS only. Eight months after the final immunization, the macaques were nasally boosted with the same amount of PspA-nanogel, PspA alone or PBS only. Serum, nasal wash, and BALF were collected before primary immunization, 1 week after each immunization, 2, 4, 6, and 8 months after the final immunization, and 2 weeks after receipt of the booster.

PspA-specific ELISA. The antigen-specific Ab responses were analyzed by ELISA as described previously.²¹ In brief, 96-well plates were coated with 1 µg ml⁻¹ PspA in PBS overnight at 4 °C. After blocking with 1 % BSA in PBS-Tween, twofold serial dilutions of samples were added and incubated for 2 h at room temperature (RT). After washing of the samples, horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (Nordic Immunological Laboratory, Tilburg, The Netherlands) or HRP-conjugated goat anti-monkey IgA (Cortex Biochem, San Leandro, CA) diluted 1:1,000 was added and incubated for 2 h at room temperature. For subclass analysis, sheep anti-human IgG1 and IgG2 (Binding Site, Birmingham, UK) and HRP-conjugated donkey anti-sheep IgG (Rockland, Limerick, PA) were used for detection. The reaction was developed with the use of TMB Microwell Peroxidase Substrate System (XPL, Gaithersburg, MD). End-point titers were expressed as the reciprocal log₂ of the last dilution that gave an OD₄₅₀ of 0.1 greater than the negative control.

Passive protection of mice with macaques' serum samples. Pooled serum samples from macaques nasally immunized with PspA-

nanogel, PspA alone, or PBS only were diluted with PBS (1:20) and injected into CBA/N mice via the intraperitoneal route (100 μ l per mouse). Four hours later, all groups of mice were challenged with 1.5×10^4 CFU *S. pneumoniae* Xen 10 ($LD_{50} = 2 \times 10^2$ CFU for CBA/N mice) or 1×10^3 CFU *S. pneumoniae* 3JYP2670 strain ($LD_{50} = 7 \times 10^2$ CFU for CBA/N mice) via the intravenous route and observed daily for death for 2 weeks. Information about *S. pneumoniae* strains is available in **Supplementary Materials**.

PspA-specific CD4⁺ T-cell responses. One week after the macaques had received the booster, lymphocytes were isolated from the peripheral blood by using Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK). We could not separate the lymphocytes from two macaques (#3 and #6). After washing of the samples, CD4⁺ T cells were purified by using CD4 microbeads and magnetic cell sorting (AutoMACS; Miltenyi Biotec, Auburn, CA). The cells remaining after the removal of CD4⁺ and CD8⁺ T cells (by using CD8 microbeads) were used as antigen-presenting cells after irradiation at 3,000 rad. Purified CD4⁺ T cells (1×10^5 cells/well) and antigen-presenting cells (0.5×10^5 cells/well) were resuspended in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10 % FCS and penicillin-streptomycin (Gibco, Carlsbad, CA), and were cultured in 24-well plates for 5 days in the presence of $5 \mu\text{g ml}^{-1}$ PspA with anti-CD28 (clone CD28.2) and CD49d (clone 9F10) antibodies ($0.5 \mu\text{g ml}^{-1}$ each; eBioscience, San Diego, CA) at 37 °C in 5% CO₂. Supernatants were then collected. The concentrations of the cytokines, IFN- γ , IL-4, and IL-17 in the supernatants were measured with a Monkey Singleplex Bead Kit (Invitrogen, Carlsbad, CA) and Bio-Plex 200 (Bio-Rad, Hercules, CA).

Synthesis of [¹⁸F]-PspA. Purified PspA was radiolabeled by conjugation with *N*-succinimidyl-4-[¹⁸F]fluorobenzoate ([¹⁸F]SFB), which reacts with free amino groups, including the N-terminal and ϵ -Lys amino groups in the protein, as described previously.^{19,32} The product was purified by gel-permeation chromatography (Superose 12, PBS, 1 ml min⁻¹), and the radioactive peak that eluted at 12.7 min was collected. The 615 MBq [¹⁸F]-PspA was obtained at 150 min from the end of bombardment. The radiochemical purity and the decay-corrected radiochemical yield were 100 and 2.95%, respectively. The specific activity was 1,798 to 4,045 MBq mg⁻¹ protein.

PET/MRI imaging in rhesus macaques. Because the half-life of [¹⁸F] is only 110 min, we used the same naive macaque for nasal [¹⁸F]-PspA-nanogel or [¹⁸F]-PspA-PBS administration with a 1-week interval between administrations. After nasal administration of 50 MBq per 700 μ l of [¹⁸F]-PspA-nanogel or [¹⁸F]-PspA-PBS (350 μ l in each nostril), the macaque's head was tilted back for 10 min and then scanned in an upright position. PET scans were conducted for 345 min with frames of 25 \times 3 min, followed by 27 \times 10 min, with the use of a high-resolution animal PET scanner (SHR-7700; Hamamatsu Photonics, Shizuoka, Japan). MRI images were recorded with Signa Excite HDxt (3T; GE Healthcare) to identify the cerebrum regions.

Image data analysis. PET data were analyzed by means of the PMOD software package (PMOD Technologies, Zurich, Switzerland). Each PET image was superimposed on the corresponding MRI data to identify the volume of interest. Time-activity curves (TACs) of PET/MRI images were expressed as % remaining dose.

MiRNA expression levels in serum and respiratory tract tissues. Serum samples were collected before primary immunization and after booster with PspA-nanogel, PspA alone, or PBS only. The respiratory tract tissues, which included nasal epithelial and lung samples, were collected after booster immunization with PspA-nanogel, PspA alone, or PBS only. Total RNAs were isolated from serum by using TRIzol LS reagent, and from nasal tissue or lung by using TRIzol reagent (Invitrogen) following the manufacturer's protocol. All the miRNAs in the sample were polyadenylated by using poly(A) polymerase and ATP (Invitrogen). Following polyadenylation, SuperScript III RT and a specially designed Universal RT Primer (Invitrogen) were used to

synthesize cDNA from the tailed miRNA population. Each of the first-strand cDNAs was analyzed by quantitative RT-PCR with Fast SYBR Green Master Mix and Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The expression levels were normalized to miR-16, which is a commonly used internal control for miRNA expression.^{49,50}

Analysis of Ets-1 expression. After total RNAs were isolated from lung tissue, cDNA was synthesized by using PrimeScript RT Master Mix (Takara, Shiga, Japan) following the manufacturer's protocol. The cDNA was analyzed by quantitative RT-PCR with Fast SYBR Green Master Mix and Step One Plus Real-Time PCR System (Applied Biosystems). The PCR primers were used as follows: Ets-1: F, 5'-TGG AGTCAACCCAGCCTATC-3' and R, 5'-TCTGCAAGGTGTCTGTC TGG-3'; β -actin: F, 5'-TGACGTGGACATCCGCAAAG-3' and R, 5'-CTGGAAGGTGGACAGCGAGG-3'. The expression levels were normalized to that of β -actin.

Statistical analysis. The results are presented as means \pm s.d. Student's *t*-test was used for comparisons among groups. The *P* values < 0.05 or < 0.01 were considered to indicate statistical significance.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declare no conflict of interest.

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