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Polymer Nanomicelles for Efficient Mucus Delivery and Antigen-Specific High Mucosal Immunity**

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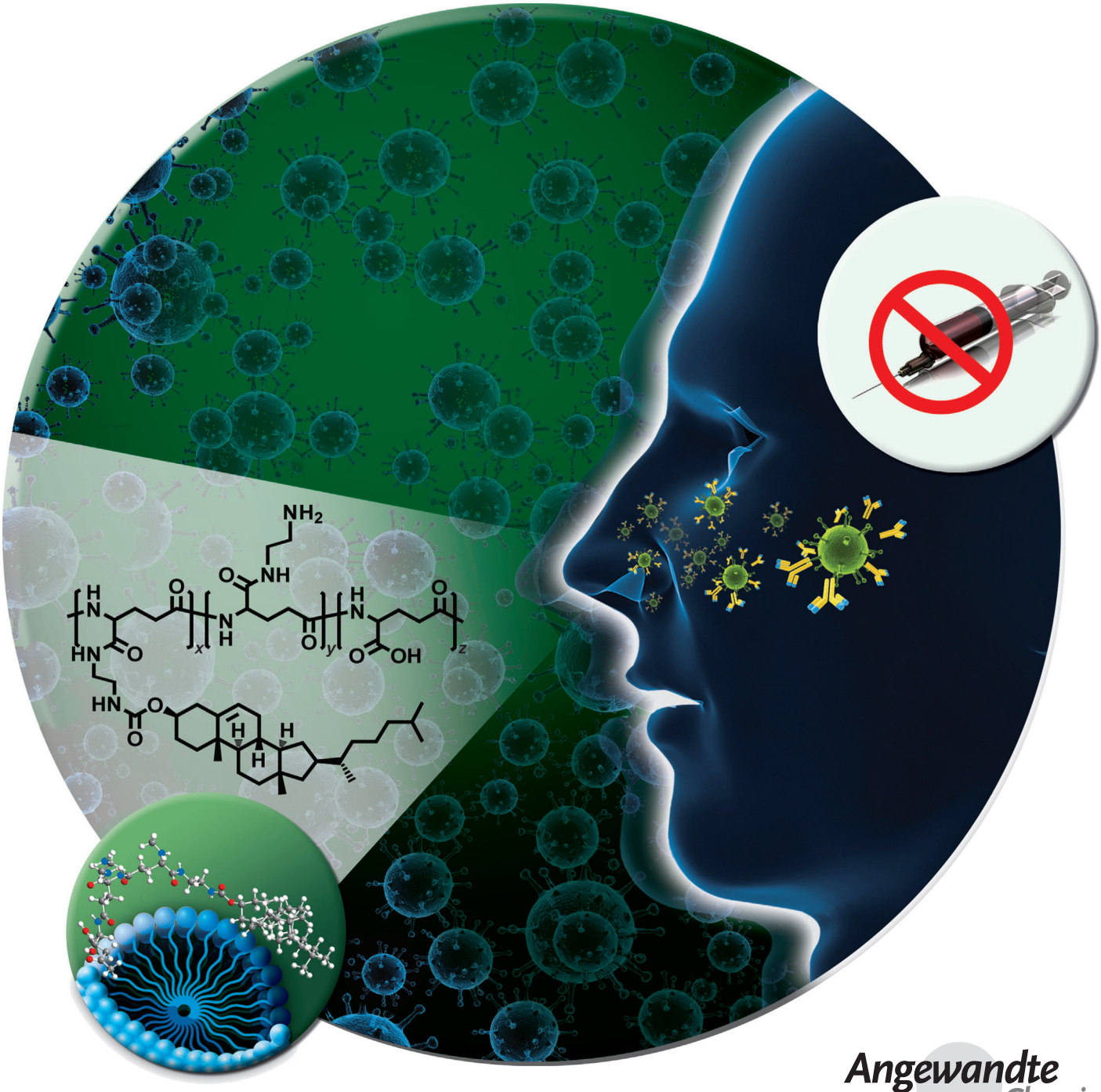

The design and synthesis of nanomaterials that can modulate the immune system are the hottest issues in the field of immunology-related nanomedicine because of the increasing interest in infectious diseases world-wide.[1] Because most pathogens access the body through mucosal membranes, effective vaccines that protect these sites are highly needed. There is general agreement that effective mucosal vaccines, including oral, nasal, sublingual, and genital tract vaccines, could dramatically contribute to the improvement of global health by stimulating a protective immune response not only against mucosal infections but also against HIV, Mycobacterium tuberculosis, and many other infections.[2] Mucosal vaccines are more advantageous than systemic vaccines from a production and regulatory perspective. Specifically, mucosal vaccines are practical for mass vaccination and do not involve the risk of blood-borne infections, which may occur due to contaminated injection needles. The ease of administration, improved compliance, and the possibility of delivery by personnel without medical training are also viewed as benefits of mucosal vaccine strategies, especially for preventing the pandemic spread of infections, including influenza virus infections.[2a–c] However, only a few commercially available mucosal vaccines currently exist because of challenges to effective mucosal vaccination, such as difficulties in generating effective mucosal immunity, as well as the lack of safe, effective mucosal adjuvants and delivery systems.[3–4] Vaccine adjuvants can act in different ways to increase both an innate and adaptive immune response and generate an effective immunological memory.[4] Adjuvants act by prolonging the exposure time of the antigen in the immune system, enhancing the delivery of the antigen to antigen-presenting cells, or providing immunostimulatory signals that potentiate the immune response. Although the mucus vaccine delivery strategy is regarded as a potent needle-free vaccine delivery, the limitations associated with the mucociliary clearance mechanism inhibit the delivery of antigens to the immune system.[5] Accordingly, new approaches for mucus vaccine delivery systems that can improve the antigen passage through biological barriers, such as the intestinal and nasal mucosa, are in high demand because they hold promise for the generation of efficient mucosal immunity using needle-free vaccines.[5,6]

Herein, we focus on the development of a nanodelivery system for the effective mucus delivery of a viral antigen that can induce strong mucosal immunity without additional immunostimulatory adjuvant materials, such as alum, emulsion, and cationic lipid.[7] To accomplish this goal, we designed and synthesized a mucosal vaccine delivery system based on biosynthetic mucoadhesive polymer nanomicelles (Figure 1). Figure 1a shows the chemical strategy for the synthesis of the poly (γ-glutamic acid) (γ-PGA)-based nanomicelle for mucus delivery of antigens. γ-PGA is a highly anionic polymer that is used in a variety of applications (e.g., food products, cosmetics, and medicine).[8] This polymer is synthesized naturally using microbial species, most prominently in various *bacilli*, and has been shown to have excellent biocompatibility and non-cytotoxicity.[8] In this study, γ-PGA was conjugated with hydrophobic cholesterol groups, which self-assembled into polymer nanomicelles and were then modified with amines (hereafter, γ-PGA nanomicelles; Figure 1a and Figure S1 in the Supporting Information). The presence of carboxy groups within γ-PGA is critical for the formation of a strong interaction with the mucus.[8b,9] These interactions are likely to be hydrogen bonding between the deprotonated carboxy group of γ-PGA and rich hydroxyl group in mucosal glycoproteins (Figure 1a). Further partial modification of the carboxy group of γ-PGA with amine groups induces a stronger interaction with the anionic epithelial cell layer, resulting in improved antigen delivery efficiency (Figure 1a). The size and distribution of synthesized γ-PGA nanomicelles were measured using dynamic light scattering and cryo-TEM. The results indicate that the nanomicelles exhibit a uniform size and morphology with a mean diameter of 20 nm (Figure 1b,c, Table S1). In addition, the zeta potential of γ-PGA nanomicelles changed from −65.35 to 36.43 mV following the amine modification, indicating that the negatively charged carboxyl groups were converted into positively charged amine moieties (Table S1).

To investigate the mucoadhesive properties of γ-PGA nanomicelles, OVA (ovalbumin), a model protein antigen, was added to the nanomicelles and the mixture was administered intranasally. To track the in vivo conditions of the administered dose, OVA was labeled with iodine (^{123}I), an isotope for SPECT (single positron emission computed tomography) imaging (Figure S2), and micro SPECT/CT imaging was performed.[10] By performing micro SPECT/CT whole-body imaging, we observed a strong SPECT signal from the administered OVA (^{123}I) in the γ-PGA nanomicelles (Figure 2). The signal remained strong in the nasal cavity even

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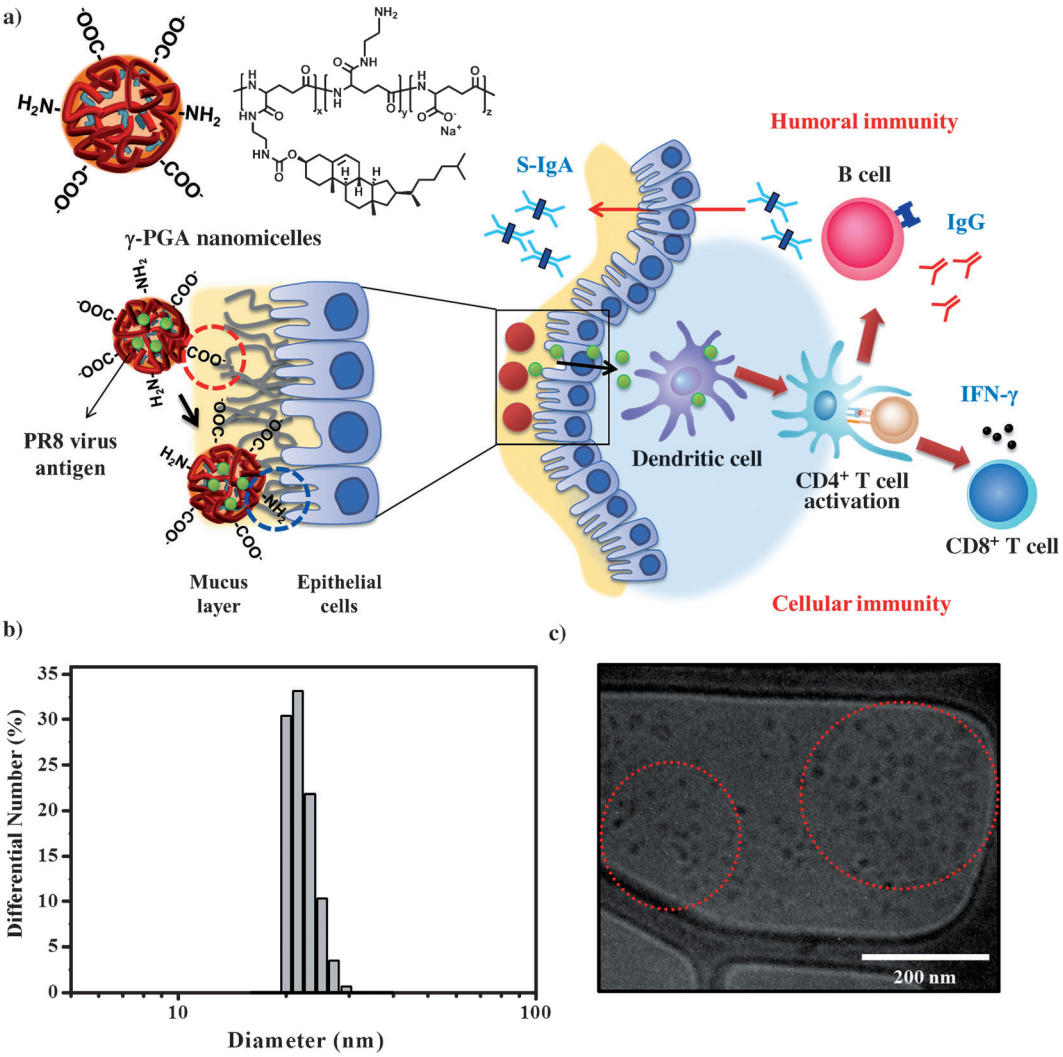

Figure 1. a) Polymer nanomicelle-based vaccination for anti-virus mucosal immunity. Poly(γ-glutamic acid) is conjugated with cholesterol and amine moieties (γ-PGA nanomicelles). The carboxy groups can act as a mucoadhesive in the presence of mucus layer glycoproteins (red dashed circle), whereas the amine moieties can interact with the anionic epithelial cell layer (blue dashed circle). b,c) Size and size distribution of γ-PGA polymer nanomicelles measured by b) dynamic light scattering (DLS) and c) cryo-TEM.

after 12 h (Figure S3). In contrast, the signal decreased drastically within 6 h when ^{123}I-OVA was administered by itself (Figure 2 a,b). The histological analysis of the nasal cavity also showed that intranasally administered FITC-labeled OVA (FITC-OVA) was effectively attached to the apical membrane of epithelial cells inside the nasal cavity and that FITC-OVA was still present in the nasal epithelial tissues even 6–12 h after the intranasal administration when it was co-delivered with the γ-PGA-based nanomicelles (Figure 2 b, Figure S3). In contrast, FITC-OVA in the absence of γ-PGA nanomicelles was easily removed from the nasal passages even 1 h following the intranasal administration *via* the mucociliary clearance mechanism. These experimental results suggest that the γ-PGA nanomicelles increased the residence time of co-delivered antigens in the mucus layer and acted as a controlled release reservoir for the nasal epithelial cells (Figure 2 c). We speculated that the γ-PGA nanomicelles could interact with the mucin molecules through physical chain entanglements and form hydrogen bonding with the sugar residues on the oligosaccharide chains.[9] Because the γ-PGA is in the expanded uncoiled state at the pH of the nasal secretions (pH 6.0–6.5) due to the electrostatic repulsion arising from the ionized functional groups, the γ-PGA is more susceptible to mechanical chain entanglement and secondary interactions with the mucous glycoprotein.[11] In addition to the mucoadhesive character of carboxylic groups in γ-PGA nanomicelles, amine moieties could also induce a stronger interaction with the anionic epithelial cell layer, resulting in improved antigen delivery efficiency. In fact, the potent capacity of chitosan polymer as a mucus delivery carrier is closely related to the cationic character induced by the amine moieties in the chitosan structure.[12]

We have also investigated the in vivo ability of γ-PGA nanomicelles to induce an antigen-specific systemic and mucosal immune response in a mouse model.[2b–d] To evaluate the OVA-specific humoral immune responses, we examined the level of anti-OVA IgG antibody titers in the serum using enzyme-linked immunosorbent assays (ELISA). Our results

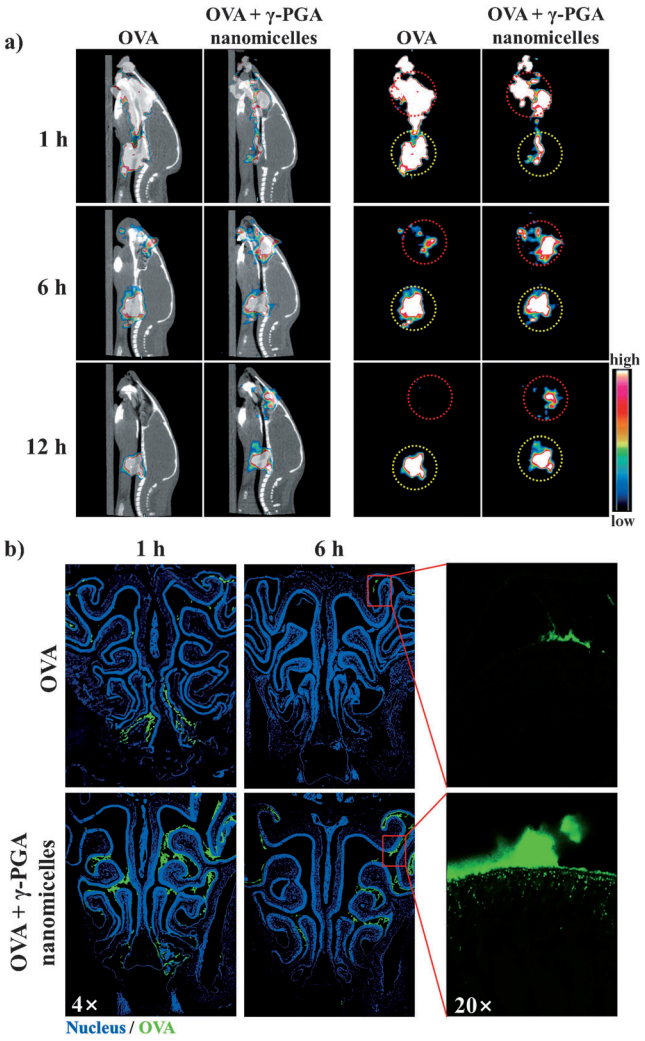

Figure 2. Nasal delivery of antigens using the γ-PGA nanomicelle system. a) The antigen residence time in the nasal cavity was determined using SPECT/CT. SPECT images were obtained at 1, 6, and 12 h following the nasal administration of [^{123}I]-labeled OVA (^{123}I-OVA) in the presence and absence of γ-PGA nanomicelles. Red dot circle: nasal cavity. Yellow dot circle: thyroid. b) Fluorescence images (4×) of the nasal cavity and nasal epithelium of mice administered FITC-labeled OVA (FITC-OVA) in the presence and absence of γ-PGA nanomicelles. c) Enlarged images (20×) of the region marked with a red square in (b). Blue: nucleus. Green: FITC-OVA.

demonstrate that intranasal immunization with OVA plus γ-PGA nanomicelles induced high levels (10.2-fold higher) of anti-OVA IgG antibody titers in serum than OVA alone (Figure 3a). We also examined the OVA-specific IgA antibody secretion in nasal washes from immunized mice. Nasal immunization with OVA plus γ-PGA nanomicelles also induced high levels (4.5-fold higher) of OVA-specific IgA antibody titers in the nasal tissue compared with OVA only, which indicates that a strong mucosal immune response was also induced (Figure 3b). As a comparison, we also investigated that effect of amine modification on the final immune response capability of γ-PGA nanomicelles. When we measured the OVA-specific immune response following the intranasal immunization with OVA in the presence of γ-PGA nanomicelles without amine groups (i.e., γ-PGA-cholesterol micelle), the OVA-specific antibody level was similar to that of OVA only (Figure S4). These experimental results suggest that the modification of γ-PGA nanomicelles with amines is critical in order to induce strong interactions with the anionic epithelial cell layer that leads to efficient antigen delivery, although the carboxylic groups of γ-PGA can act as mucoadhesives by interacting with the hydroxy groups of the glycoproteins of the mucus layer (Figure S5). To evaluate the OVA-specific cellular immune responses, we performed an ELISPOT assay targeting the H-2K^{b}-restricted $OVA_{257\text{-}264}$ epitope of IFN-γ-secreting T-cells from the spleen of mice. Interestingly, the immunization of mice with OVA plus γ-PGA nanomicelles resulted in a greater number of IFN-γ spots in the splenocytes restimulated with the OVA peptide (Figure 3c). The number of IFN-γ-secreting cells was 5.2-times greater for mice samples from mice receiving OVA plus γ-PGA nanomicelles, compared with those receiving OVA alone. In fact, IFN-γ-secreting cells were rarely observed in mice immunized with OVA alone (Figure 3c). The mice immunized with γ-PGA nanomicelles containing OVA exhibited the highest IFN-γ producing populations compared with OVA only when stimulated with the CTL (cytotoxic T lymphocytes) epitope of OVA, revealing a high antigen-specific CTL response. OVA plus γ-PGA nanomicelles strongly induced OVA-specific antibody production and IFN-γ-producing cells, suggesting that our nanomicelle system could function as an effective adjuvant that potently induces both humoral and cellular immune responses. The histological analysis indicated that the nanomicelles had no conspicuous toxicity and inflammatory responses in nasal tissue (Figure S6).

Inspired by our finding, we tested the adjuvant function of γ-PGA nanomicelles in the presence of an influenza A viral antigen, that is, the inactivated virus of influenza A/PuertoRico/8/34 (PR8;H1N1).[13] Intranasal immunization with PR8 in the presence of γ-PGA nanomicelles induced high levels of PR8-specific IgG titers in the sera of mice (Figure 4a; 28.6-fold higher comapred with PR8 only), as well as PR8-specific mucosal IgA antibody titers in their nasal washes (Figure 4b; 27.6-fold higher compared with PR8 only). In contrast, the levels of PR8-specific IgG and IgA were very low in mice immunized with PR8 antigen only (Figure 4a,b). The experimental results suggest that the co-delivery of influenza A/PR8 antigen with γ-PGA nanomicelles induced an abundant PR8-specific humoral immune response. The immunization of mice with PR8 antigen plus γ-PGA nanomicelles also resulted in a greater number of IFN-γ spots in splenocytes restimulated with the inactivated PR8 virus.

The number of IFN-γ producing cells in the PR8 antigen plus γ-PGA nanomicelles group that were re-stimulated with inactivated PR8 virus increased by 3.2 times ($p = 0.0004$), compared with the PR8 antigen only (Figure 4c). An enhanced response was also observed in the hemagglutination inhibition (HI) titers, which are based on blocking the ability of influenza HA to agglutinate red blood cells with specific antibodies.[14] Figure 4d shows that the HI titer of the PR8 plus γ-PGA nanomicelles was four times higher than that of the PR8 antigen alone ($p < 0.002$). These results demonstrate

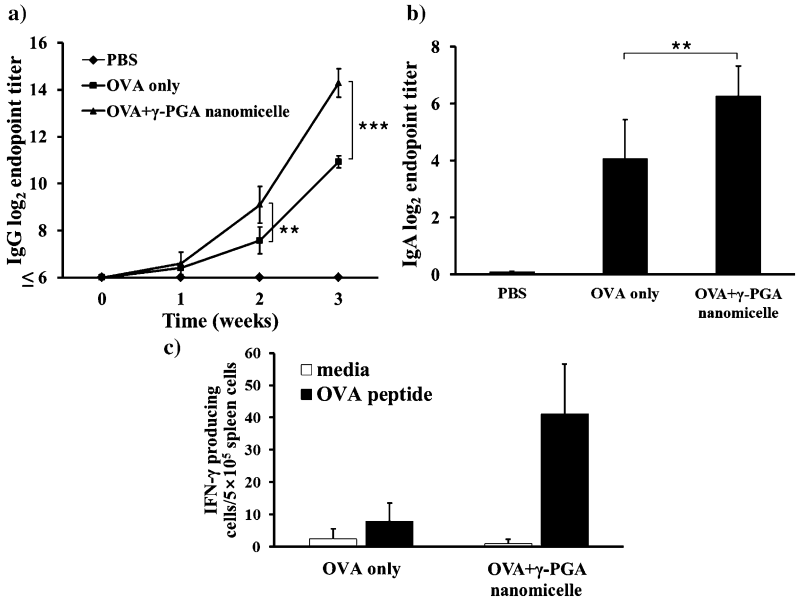

Figure 3. Induction of OVA-specific immune response by intranasal vaccination with γ-PGA nanomicelles. a,b) OVA-specific IgG (a) and IgA (b) antibody titers were observed using ELISA analysis in the serum and nasal washes collected following the intranasal vaccination. c) The IFN-γ cytokine production from OVA peptides (SIINFEKL, 10 μg mL^{-1})-stimulated splenocytes of vaccinated mice was determined using the ELISPOT assay. All data were obtained in triplicate and are presented as a mean value ± standard deviation (SD). Significant differences were observed only in comparison with OVA only. **, $p < 0.01$; ***, $p < 0.001$.

that PR8 plus γ-PGA nanomicelles were able to elicit high levels of functional antibodies and IFN-γ producing cells, suggesting that our nanomicelle system could function as an effective adjuvant for inducing both humoral and cellular immune responses. Because the immunized mice demonstrated a robust humoral and cellular immune response, we investigated the induction of protective immunity against a lethal dose of influenza virus infection. Following the intranasal dose of the mouse adapted influenza A/PuertoRico/8/34 (H1N1) virus with 10-times the 50% mouse lethal dose ($10 \times LD_{50}$),[2e] the mice immunized with PR8 plus γ-PGA nanomicelles exhibited 100% protective immunity to the lethal PR8 virus challenge, whereas the phosphate-buffered saline (PBS) inoculated mice exhibited 100% mortality six days after the challenge (Figure 4e,f). Mice immunized with only the PR8 virus exhibited a 50% survival rate on day 14. More importantly, these mice lost more than 10% of their body weight (Figure 4e,f). Although both humoral and cellular immunity levels were the highest in the case of 100 μg of γ-PGA nanomicelles, 10 μg of γ-PGA nanomicelles was enough to increase the survival rate from the lethal infection. The strong adjuvant-like function of the γ-PGA nanomicelles can play an important role in enhancing the antibody response in situations where the split virus preparation is not very immunogenic or if there is an imperative to provide "dose sparing" in the context of mass vaccinations with a virus to which the population is immunologically naïve.[15]

In this study, we designed and synthesized a mucosal vaccine system based on γ-PGA nanomicelle and viral antigens. The results described in this communication are significant for the following reasons. First, although various types of emulsions and particulate systems have been reported as adjuvant materials (and some are currently in use in human clinics),[7] most were found to induce only a good antibody (Th2) response, with little stimulation of the cellular immune (Th1) responses that are so important for protection against many pathogens, including viruses. In contrast, our γ-PGA nanomicelle have an adjuvant effect on both humoral and cellular immunity. Second, the γ-PGA nanomicelle system described in this study can be potent needle-free vaccine delivery tools. This is exactly the type of advanced technology that major global health entities, such as the World Health Organization and the

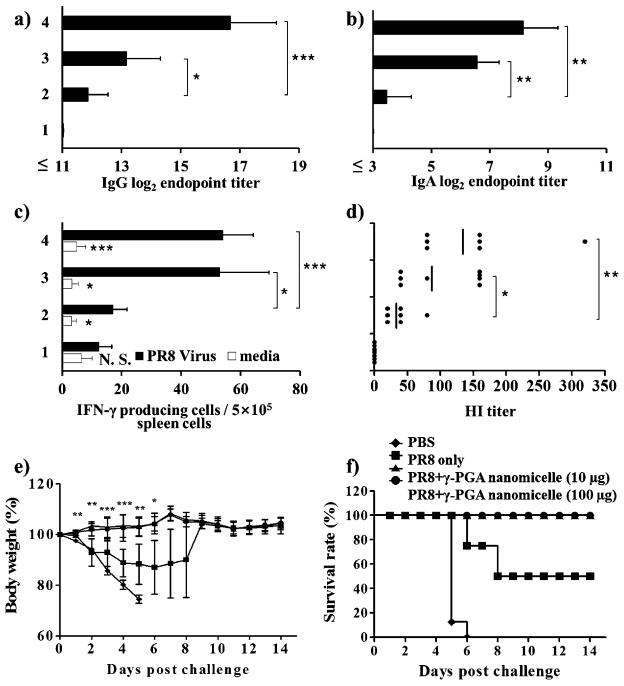

Figure 4. Influenza PR8 virus-specific immune response in vaccinated mice and their survival rates after a lethal challenge (1. PBS, 2. PR8 only, 3. PR8 plus γ-PGA nanomicelle (10 μg), 4. PR8 plus γ-PGA nanomicelle (100 μg). The PR8 virus-specific IgG (a) and IgA (b) antibody titers were observed in C57BL/6 mice ($n = 5$) that were immunized intranasally with 20 μg PR8 viral antigen with/without γ-PGA nanomicelles (10, 100 μg per mice). c) The cellular immune response was analyzed in the splenocytes by ELISPOT for IFN-γ production by re-stimulation with the PR8 viral protein. d) Hemagglutination inhibition (HI) titers ($n = 7$) were assessed against the PR8 virus. Bars indicate the geometric mean titer (GMT). e,f) Protection of mice from the lethal influenza virus challenge. The percentage of body weight (e) and survival rates (f) of immunized mice ($n = 8$) that were monitored for 14 days following an intranasal infection with a mouse-adapted PR8 virus. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *n.s.*, not significant.

Center for Disease Control and Prevention, have deemed essential. The accomplishment of these objectives should generate an improvement in the vaccination coverage of several infectious diseases, especially those in the developing countries. Third, the strong adjuvant function of the γ-PGA nanomicelle can be combined with recombinant protein antigens, which are usually not very immunogenic and require effective mucosal adjuvants. Finally, for convenient clinical usage, the γ-PGA nanomicelle would be useful for preparing adjuvant materials that can be mixed with the vaccine antigens prior to on-site medical administration, as is currently done in conventional emulsion-based adjuvant systems.

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