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Brief Communication

Virus-like Particle (VLP) Mediated Antigen Delivery as a Sensitization Tool of Experimental Allergy Mouse Models

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
BCR, B cell receptor; DNP, 2,4-dinitrophenyl; HEL, hen egg lysozyme; IN, intranasally; IP, intraperitoneally; O/N, overnight; rHEL, recombinant hen egg lysozyme; RO, retro-orbital; RT, room temperature; SC,

ABSTRACT

Antigen delivery systems play critical roles in determining the quality and quantity of Ab responses *in vivo*. Induction of protective antibodies by B cells is essential in the development of vaccines against infectious pathogens, whereas production of IgE antibodies is prerequisite for investigation of allergic responses, or type 1 hypersensitivity reactions. Virus-like particles (VLPs) are efficient platforms for expression of proteins of interest in highly repetitive manners, which grants strong Ab responses to target antigens. Here, we report that delivery of hen egg lysozyme (HEL), a model allergen, through VLP could provoke strong HEL specific IgE Ab responses in mice. Moreover, acute allergic responses were robustly induced in the mice sensitized with VLPs that express HEL, when challenged with recombinant HEL protein. Our data show that antigen delivery in the context of VLPs could function as a platform for sensitization of mice and for subsequent examination of allergic reactions to molecules of interest.

Keywords: Antigen delivery; Virus-like particles (VLPs); Antigen specific antibody responses; IgE; Allergic reaction

INTRODUCTION

B cells produce antibodies, whose immunological functions are not only critical for host protection against infectious microbes but also associated with allergic responses (1,2). Differentiation of naïve B cells into Ab-secreting cells is regulated by cascade of activation signals, which are triggered downstream of various signaling receptors on the membrane of B cells, such as ones that recognize antigens (B cell receptors [BCRs]) or bind to co-stimulatory molecules and cytokines (3). Recognition of antigens leads to cross-linking of BCRs and convey activation signals inside B cells through phosphorylation of signaling molecules such as Lyn, Syk, and Btk (4,5). Activation of these signaling molecules results in transient increase in intracellular calcium level, which consecutively switches on various transcription factors for induction of target genes to support activation, proliferation and differentiation of B cells (6). Thus, initial interaction of B cells with antigens through BCRs is a critical step of B cell activation and is regarded as one of key factors that determine the magnitude of Ab responses to antigens.

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subcutaneously; TACA, tumor associated carbohydrate antigen; VLP, virus-like particle.

Author Contributions

Conceptualization: Choi YS; Data curation: Kim J, Oh J, Choi YS; Formal analysis: Kim J, Oh J, Choi YS; Funding acquisition: Choi YS; Investigation: Kim J, Oh J, Choi YS; Resources: Kang CS, Choi YS; Supervision: Choi YS; Writing - original draft: Kim J, Choi YS

Studies using synthetic antigens that exist at various conditions of valency demonstrated that activation and Ab production of B cells are greatly enhanced in response to polyvalent antigens than monomeric ones (7,8). While calcium influx was barely induced in B cells responding to monomeric 2,4-dinitrophenyl (DNP)-lysine, multivalent antigens, such as ones composed of 250 DNP molecules linked to lysine, led to calcium mobilization in B cells even at low concentration (10 nM) (7). Huang and colleagues reported that Ab responses to tumor associated carbohydrate antigens (TACAs) began to develop when mice were immunized with glycopolymers bearing more than 10 Thomsen-nouveau, the TACA found in breast and prostate cancers (8). Similarly, antigenic property of a soluble monomeric protein was greatly augmented when the monomeric protein is engineered to exist in polyvalent manners on virus-like particles (VLPs), as immunization with the latter gave rise to antigen specific IgG Ab responses over 1,000-fold higher than immunization with soluble protein antigen (9).

In 1960s, VLPs were first determined when nanoscale particles were observed in *in vitro* culture of virus-infected human and animal tissues (10,11). VLPs exhibit morphological similarities to the virus and do not contain genetic material (12), which enables strong activation and profound Ab production of B cells and curtails safety concerns of inoculation, respectively (13). These properties have led many researchers to investigate whether VLPs could function as an alternative vaccination platform (14). Several VLP-based vaccines are being administered to confer immune protection against human papillomavirus and poliovirus with high efficacy (15,16). Due to its potent B cell stimulating property, antigen delivery through VLPs is also being investigated to develop autoantibodies against pro-inflammatory cytokines by breaking self-tolerance of B cells for providing therapeutic strategies to cure autoimmune diseases (12).

IgE isotype of antibodies is required for host protection against infections with helminths or parasites (17). The production of IgE antibodies against harmless antigens, however, is strongly associated with the development of allergic reactions, or type 1 hypersensitive responses (18). Once IgE Ab responses develop against allergens, these IgE antibodies bind to the surface of mast cells or basophils through high affinity IgE receptor, or FcεRI (2). Subsequent exposures to the same allergen result in cross-link of IgE antibodies on the surface of mast cells or basophils, which leads to the activation of these cells for production of histamine and other mediators that trigger allergic reactions (19). Thus, activation and production of IgE antibodies of B cells in response to allergens is a prerequisite step for the development of IgE-mediated allergic reactions, and these processes are referred as sensitization (2). Using VLPs engineered to express hen egg lysozyme (HEL), here we established a novel method of sensitization to strongly elicit HEL specific IgE Ab responses in mice, with which acute allergic reactions could be examined in response to local and systemic challenge of HEL protein.

MATERIALS AND METHODS

Mice

Six-week-old female BALB/c mice and 7-week-old male C57BL/6J mice, purchased from OrientBio and DBL (Seongnam, Korea), were maintained under specific pathogen-free conditions in Institute for Experimental Animals at Seoul National University. All investigations were performed following guidelines of Seoul National University Institutional Animal Care and Use Committee (IACUC No. SNU170801-3-10).

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Recombinant hen egg lysozyme (rHEL)

rHEL (Sigma, St. Louis, MO, USA) stock was prepared at 5 mg/ml in PBS and kept in −20°C until use.

VLP production and quantification

VLPs were produced from HEK293 T cells after transfecting HEK293T cells with HEL-GTMCD and OTII-HgSyn plasmids (20-22). For this, both plasmids (20 μg per 15 cm dish) were incubated with polyethylenimine (PolyScience, Niles, IL, USA) in plain DMEM (Welgene, Gyeongsan, Korea), and then the mixtures were added to HEK293 T cells. Old media was replaced with pre-warmed new complete DMEM (plain DMEM, 1× Glutamax, 1% antibiotics, 10% FBS) 6 h after transfection. Supernatant was collected daily for 3 days, filtered, and then spun-down at 15,000 rpm for 1.5 h. After centrifugation, precipitated VLPs were resuspended, aliquoted with plain DMEM, and stored at −80°C until use.

For quantification of VLPs, 96 well plate (Nunc; Thermo Scientific, Waltham, MA, USA) was coated with concentrated VLP sample serially diluted in Na_2CO_3 coating buffer (21). Following overnight (O/N) incubation at 4°C, the plate was washed with wash buffer (PBS, 0.05% Tween20), blocked with blocking buffer (PBS, 0.05% Tween20, 5% fat free milk), and then incubated with anti-p24 Ab (Antibodies-online) diluted in the incubation buffer (PBS, 0.05% Tween20, 2% fat free milk) at room temperature (RT). After wash, the plate was incubated with anti-mouse IgG-HRP (GeneTex, Irvine, CA, USA) Ab diluted in the blocking buffer at RT. TMB chromogen solution (Invitrogen; ThermoFisher Scientific, Waltham, MA, USA) was added and the reaction was stopped with stop solution (Invitrogen). OD was measured by Sunrise Microplate Reader (Tecan, Männedorf, Switzerland) (measurement wavelength: 450 nm, reference wavelength: 570 nm). p24 concentration of VLP samples was calculated based on concentration of recombinant HIV1 p24 protein (Abcam, Cambridge, United Kingdom).

Immunization and sensitization

HEL expressing VLP, equivalent to 1 μg (or other amounts described in the manuscript) of p24, was mixed with PBS, which was then 1:1 mixed with addavax or with alum. Under anesthesia, mice were administered with 25 μL of VLP/addavax subcutaneously (SC) or 200 μl of VLP/alum intraperitoneally (IP). For sensitization of soluble rHEL, 10 μg of rHEL mixed with PBS and alum, was IP administered.

Challenge

On the day of challenge, rHEL protein was prepared in PBS, was then given intravenous retro-orbital (RO) (23,24), IP (25), or intranasally (IN) (26).

ELISA

The 96 well plate (Nunc) was incubated with 5 μg/ml of rHEL protein (Sigma) diluted in PBS O/N at 4°C. Next day, the plate was washed with wash buffer (PBS, 0.1% Tween20) and blocked with blocking buffer (PBS, 0.05% Tween20, 0.5% BSA). After block, the plate was incubated at RT with serum samples serially diluted in the blocking buffer. After wash, the plate was incubated with anti-mouse IgG-HRP (GeneTex) or with anti-mouse IgE-HRP (Abcam) diluted in the blocking buffer to measure HEL specific IgG and IgE, respectively. TMB chromogen solution (Invitrogen) was added. After stopping the reaction, the OD was measured by Sunrise Microplate Reader (Tecan) (measurement wavelength: 450 nm, reference wavelength: 570 nm).

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Enzyme immunoassay histamine assay

For quantification of serum histamine, histamine dihydrochloride, purchased from Sigma, was used as a standard (27). The stock (0.1 N HCl at 1 mg/ml and stored in −20°C) was serially diluted in PBS to prepare the standard samples. Serum samples were pre-diluted in PBS. The 200 μl of the standard and serum samples were mixed with 80 μl of 0.1 N HCl and 20 μl of 60% perchloric acid (Junsei, Tokyo, Japan). All samples, prepared in duplicate, were vortexed and centrifuged. After spin, 200 μl of the supernatant was transferred to fresh tubes and mixed with 100 μl of 5 N NaOH, 800 μl of n-butanol and 200 μl of 5 M NaCl. After vortexing and spin-down, 500 μl of the supernatant was transferred to fresh tubes, into which 200 μl of 0.1 N HCl and 500 μl of n-heptane were added. After vortexing and spin-down, 150 μl of bottom layer was transferred to 96-well fluorescent black bottom plate (Nunc). Five min after incubation with 40 μl of 1 N NaOH and 10 μl of 1% o-phthaldialdehyde at RT, the plates were added with 20 μl of 3 N HCl and were read by microplate reader (Tecan) (excitation: 360 nm, emission: 440 nm).

Behavior test

Open field test was performed in Seoul National University Animal Behavior Analytical Laboratory. The total horizontal moving distance of the mice was measured in the testing box, which was 40 cm wide×40 cm high. The mice were placed at the center of the box and movement was recorded for 40 min. The data was analyzed using Video Tracking System, Ethovision XT (Noldus, Wageningen, The Netherlands).

Single cell preparation

For flow cytometry analysis, popliteal lymph nodes, spleens, and lungs were processed into single cell suspension. Popliteal lymph nodes and spleens were minced on cell strainers (Falcon; BD Biosciences, San Jose, CA, USA). Lungs were chopped into small pieces using a razor blade, and were then subject to incubation with 1 mg/ml type IV collagenase (Worthington, Columbus, OH, USA) and 1 mg/ml of DNase (Sigma) diluted in complete RPMI 1640 media (RPMI 1640 [Welgene], Glutamax, 1% antibiotics, 10% FBS) for 1 h at 37°C shaking incubator. Lung samples were then minced on cell strainers. The samples were then treated with ACK lysis buffer (Gibco, Life Technologies, Grand Island, NY, USA) to remove red blood cells. Following spin-down, single cell suspension was obtained by resuspending the cell pellets in complete RPMI 1640 or in complete DMEM media.

Stimulation for intracellular staining of cytokines

Cells were stimulated for detection of intracellular cytokines. Briefly, cells were cultured with 4 μl of PMA (stock: 100 μg/ml in DMSO), 15 μl of ionomycin (stock: 1 mg/ml in DMSO) and 10 μl of GolgiPlug (stock: 2 mg/ml BFA in DMSO) for 2 h in 37°C CO_2 incubator.

Flow cytometry analysis

Freshly isolated mononuclear cells from lymph nodes, spleens and lung or cells after *in vitro* stimulation with PMA/ionomycin were incubated at 4°C with Ab cocktails including followings: CD4 (RM 4-5), CD19 (6D5), Streptavidin-APC, CD8α (53-6.7), IL-2 (JES6-5H4), IL-5 (TRFK5), and biotin-conjugated IL-10 (JES5) from Biolegend, San Diego, CA, USA; CD4 (RM 4-5), and IL-13 (eBio13A) from eBioscience, San Diego, CA, USA; CD4 (RM 4-5), Fas (Jo2), and IL-4 (11B11) from BD Biosciences; PNA from Vector. For intracellular staining of cytokines, Foxp3/transcription factor staining buffer (eBioscience) was used. The samples were read by BD LSRII (SORP), and data was analyzed using Flowjo (BD).

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Statistical analysis

Student's *t*-test, un-paired or paired, and Pearson's correlation were performed for statistical analysis using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The p-values less than 0.05 are considered to be significant.

RESULTS AND DISCUSSION

Strong antigen specific Ab responses elicited by delivery of antigen using VLP

VLP is regarded as one of the next generation vaccine platforms (14). While there is a little concern in inoculation due mostly to the lack of genetic material in VLPs, which is required for replication of virus in host cells, VLPs are considered highly immunogenic as target molecules could be expressed on the surface in highly repetitive manners (12). We thus tested whether antigen specific Ab responses could be elicited in mice by immunization with VLPs that were in-house generated to express antigen of interest. VLPs were engineered to express HEL, a model antigen, in association with transmembrane and cytoplasmic domain of VSV-g protein (20,21) (**Supplementary Fig. 1A**). Eight days after subcutaneous injection of HEL-VLPs (HEL-VLP, hereafter) with addavax, a squalene-based oil-in-water emulsion similar to MF59, a licensed adjuvant for human use in Europe (28), we found that HEL specific IgG was profoundly increased in blood (**Supplementary Fig. 1B and C**). The production of HEL specific IgG was associated with strong induction of Fas+PNA+ germinal center B cells in the draining popliteal lymph nodes, in comparison to those found in unimmunized mice (**Supplementary Fig. 1D**). Thus, our data demonstrate that antigen specific Ab responses could be strongly engendered by in-house engineered VLPs that express antigens or interest.

VLP mediated antigen delivery leads to the production of antigen specific IgE antibodies

Ab isotypes determine effector functions of antibodies, and the process leading to isotype switching of B cells occurs mostly in responding to cytokines produced by helper CD4 T cells (29). Following immunization with VLP emulsified in addavax (**Supplementary Fig. 1**), type 1 effector CD4 T (Th1) cells predominantly develop to produce IFN-γ (type 1 effector cytokine) in the draining lymph nodes, whereas CD4 T cells that produce type 2 effector cytokines (IL-4, IL-13) are rarely induced (data not shown). Given that type 1 and 2 effector cytokines favor isotype switching of B cells into IgG2a and IgE, respectively (30), we speculated that VLP mediated antigen delivery system could be modified to induce antigen specific IgE antibodies using aluminum hydroxide (alum), a potent adjuvant that enforces CD4 T cells to differentiate into Th2 cells (31).

To test this point, BALB/c mice were immunized SC or IP with HEL-VLP that were emulsified with either addavax or alum. IP route was incorporated as an additional experimental variable in the study, given that this route is normally utilized for sensitization with a model allergen (32). HEL-VLP, emulsified in addavax or alum, were SC or IP injected into BALB/c mice at days 0, 10, and 20, from which blood was taken at days –1 (pre-sensitization), and 7 and 17 (during sensitization), and 27 (post-sensitization) to measure the amount of HEL specific IgE antibodies (**Fig. 1A**). HEL specific IgE was strongly induced in mice, which were given IP with HEL-VLP/alum, whereas SC immunization of HEL-VLP/addavax resulted in production of the least amount of HLE specific IgE antibodies (**Fig. 1B**). In consistent with previous findings on the roles of Th2 cells in IgE isotype switching of B cells, we found that the strong induction of HEL specific IgE antibodies in mice after IP administration of HEL-VLP/alum was associated

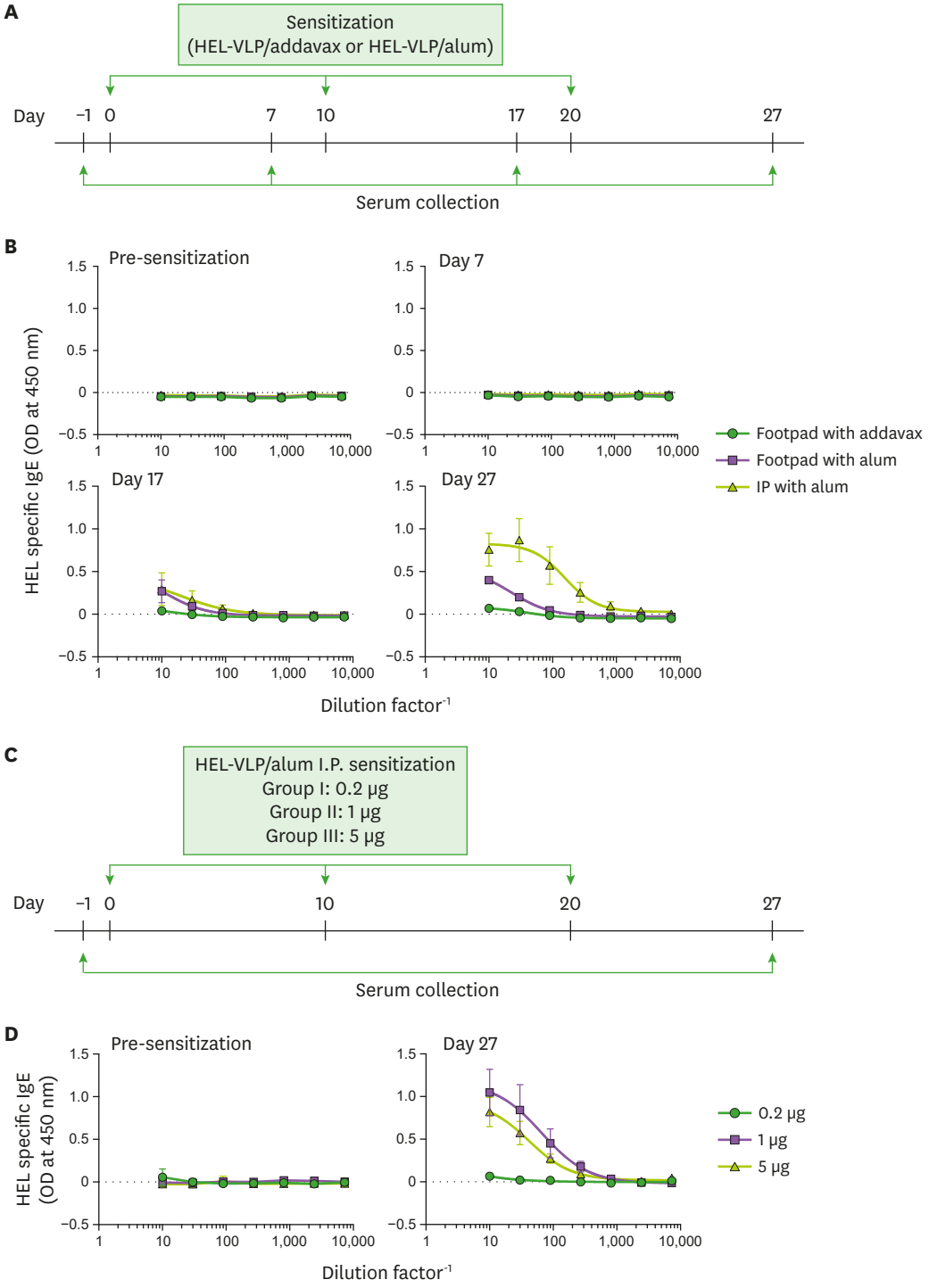

Figure 1. VLP mediated delivery of an antigen elicits antigen specific IgE responses. (A) HEL-VLPs emulsified in either addavax or alum were IP or SC injected into BALB/c mice at days 0, 10, and 20. Blood was taken at days −1, 7, 17, and 27. (B) Relative amounts (mean OD±SD) of HEL specific IgE present in the serum of the mice administrated with HEL-VLP/addavax SC (green circles), HEL-VLP/alum SC (purple squares) or IP (light green triangles). Three mice per group. Data are representative of 2 independent experiments. (C) The 0.2 μg, 1 μg, or 5 μg of HEL-VLPs (refer to Methods section for detailed information of VLP quantification), emulsified in alum, was given IP at days 0, 10, and 20. Blood was taken at days −1 and 27. (D) Relative amount (mean±SD) of HEL specific IgE present in mice before and twenty-seven days after IP administration of HEL-VLPs (0.2 μg [green circles], 1 μg [purple squares], or 5 μg [light green triangles]). Analyses performed with data compiled from 2 independent experiments. Six mice per group.

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with the differentiation of CD4 T cells into Th2 effectors, which produce IL-4, IL-10, and IL-13 in the spleen (**Supplementary Fig. 2**).

Sensitizing antigen doses also influence the magnitude of isotype switching of B cells to IgE (25). We thus explored to determine an optimal dose of HEL-VLP for strong induction of HEL specific IgE in the context of IP administration with alum. HEL-VLP doses could be determined based on the amount of p24 capsid protein, about 2,000 molecules of which are present in a virus particle (33). Previous experiments (**Supplementary Fig. 1**; **Fig. 1A and B**) were performed by administering HEL-VLP, which corresponds to 1 μg of p24 protein (refer to 1 ug of VLP hereafter), as 1 μg of VLPs that express influenza virus hemagglutinin (HA) induced strong HA specific IgG antibodies (data not shown). The 0.2, 1, or 5 μg of HEL-VLP emulsified in alum was IP injected at days 0, 10, and 20 into BALB/c mice, from which blood was obtained to measure pre- (day −1) and post- (day 27) sensitization HEL specific IgE (**Fig. 1C**). The 0.2 μg of HEL-VLP led to little if any induction of HEL specific IgE, whereas HEL specific IgE was strongly produced by sensitizing BALB/c mice with 1 μg and 5 μg of HEL-VLP (**Fig. 1D**). It is worth to note that the magnitude of antigen specific IgE response does not positively correlate with the amount of HEL-VLP used for sensitization. While it fails to reach statistical significance, 5 μg of HEL-VLP appears to induce lower HEL specific IgE responses than 1 μg of HEL-VLP (**Fig. 1D**). These results comply with a previous study that reported decreased IgE isotype switching of B cells induced in mice by escalating amounts of soluble recombinant protein antigens (25), which likely happens due to biased Th1 differentiation of CD4 T cells in responding to high doses of antigens (34).

Severe acute allergic reactions elicited in mice sensitized with HEL expressing VLP

Repetitive exposures to an allergen lead to the cross-link of IgE molecules that are bound on the surface of mast cells and basophils, which contribute to the local and systemic release of histamine and other mediators that are responsible for the onset of allergic responses (2). In comparison to a conventional sensitization method with recombinant protein antigen, we found antigen specific IgE responses engendered much more strongly in mice using antigen expressing VLP (**Supplementary Fig. 3**), which indicates remarkable potency of VLP mediated sensitization in triggering allergic reactions in mice. Thus, we addressed whether allergic response to rHEL could be induced in mice that had been sensitized with HEL-VLP/alum. Following a series of IP sensitization with 1 μg of HEL-VLP/alum, BALB/c mice were given (or challenged) intravenous RO (group I) and IP (group II) with rHEL; IP challenge was conducted with 10 mg of rHEL, as previously reported by other studies (35-37), whereas 10-fold less amount of the model allergen was chosen for the RO route due to much faster systemic dissemination of materials in mice than that of IP (38) (**Fig. 2A**). Forty min after challenge, blood was taken from both groups to measure the amount of plasma histamine. In comparison to ones obtained before sensitization, histamine levels were strongly increased by both RO and IP administration of rHEL (mean pre-sensitization: 63.38 ng/ml; mean RO challenge: 376.51 ng/ml; mean IP challenge: 282.89 ng/ml) (**Fig. 2B**). Paired comparisons of histamine levels of pre-sensitization and post challenge samples within each group revealed significant increases in the plasma histamine levels (RO challenge: $p<0.01$; IP challenge: $p<0.05$).

Stark increase of systemic histamine levels following challenges with rHEL implies that VLP mediated delivery of target allergens could function as an alternative sensitizing tool to study allergic reactions, including anaphylaxis, the most severe form of allergic reactions. We explored this point by performing open field tests to investigate locomotor behavior of

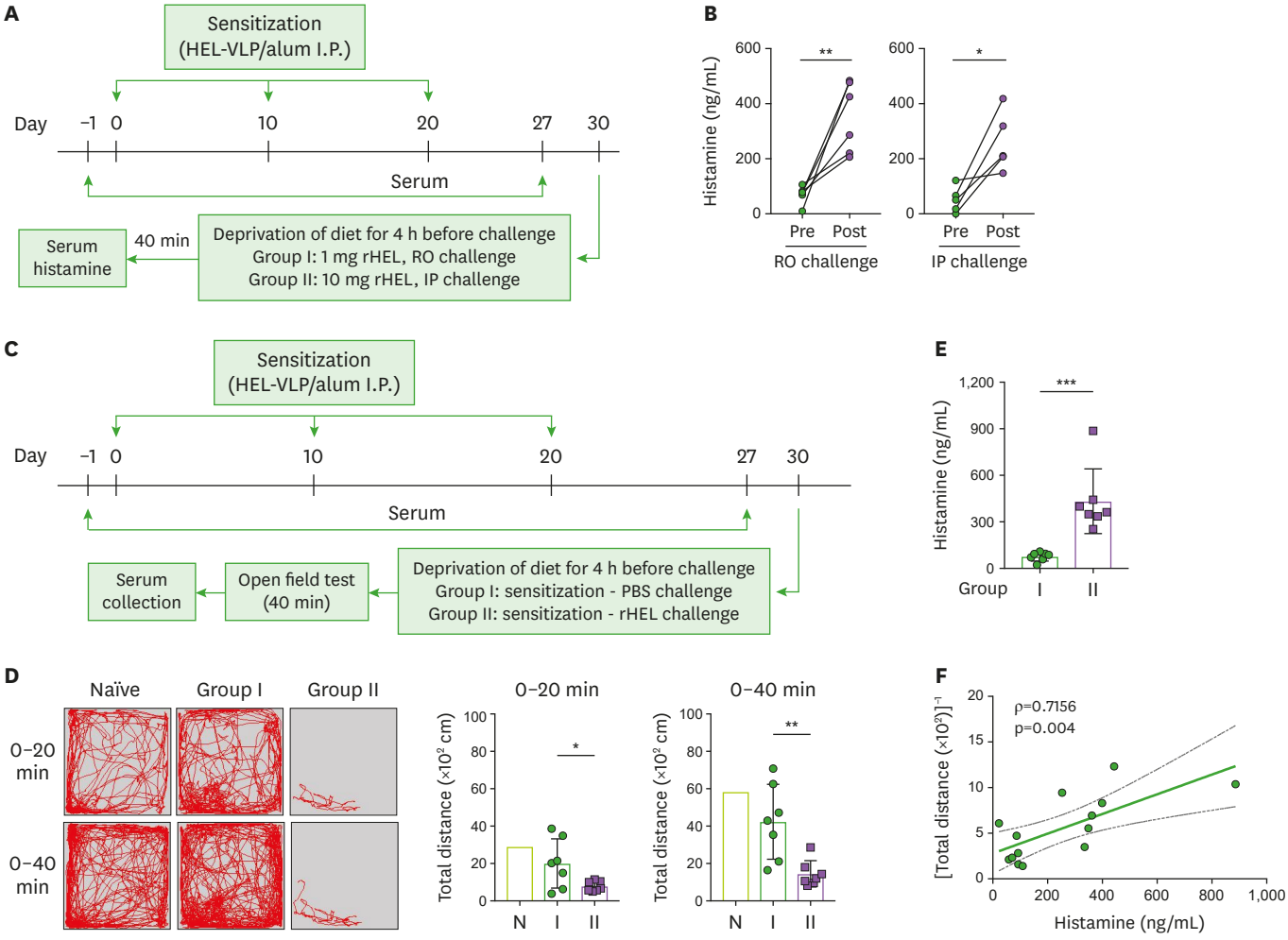

Figure 2. Systemic histamine release and acute anaphylaxis are induced in mice sensitized with HEL-VLP following systemic challenge of allergen. (A) BALB/c mice were given IP with HEL-VLP/alum at days 0, 10, and 20. Ten days after the last injection, the mice were challenged by intravenous RO (group I) or IP (group II) administration of rHEL. Forty min later, blood was taken to measure serum histamine level. (B) Paired comparisons of histamine concentrations present in blood of the mice before sensitization and post challenge with rHEL. Analyses performed with composite data (n=6 in group I, n=5 in group II) of 2 independent experiments. (C) HEL-VLP/alum was given IP in BALB/c at days 0, 10, and 20. Ten days after the last sensitization, mice were challenged intravenous RO with PBS (group I) or with rHEL (group II). The mice were subject to open field test to measure the locomotive movement of mice, after which serum was collected. (D) Representative heat image frames that demonstrate the movement of a mouse of each group in the 40×40 test box for 20 (upper panels) and 40 (lower panels) min (left) following challenge with PBS (group I) or rHEL (group II). Bar charts indicate mean±SD of the distance travelled by mice for 20 (middle) and 40 (right) min after challenge. (E) Plasma histamine concentration in group I and group II. (F) Correlation between serum histamine concentration (ng/ml) and inverted total distance (Total distance×10^2 cm)$^{-1}$ made by group I and group II. Composite data from 2 independent experiments including 7 mice per group and one naïve mouse. *p<0.05; **p<0.01; ***p<0.001.

mice, which was previously shown as an indicative of systemic anaphylaxis triggered in mice upon intravenous challenge with a model allergen (24). For this, BALB/c mice, following serial sensitization with HEL-VLP/alum, were given RO with PBS (group I) as a control or with rHEL (group II) (**Fig. 2C**). RO challenge with rHEL led to a profound reduction in locomotion of mice for the first 20 min (**Fig. 2D**; mean distance: group I, 20×10^2 cm; group II, <10×10^2 cm). Behavioral difference between 2 groups becomes more apparent for additional 20 min, and the total traveled distances were 40×10^2 cm in group I and up to 10×10^2 cm in group II during the entire period of the open field test (**Fig. 2D**). Interestingly, the amount of plasma histamine, which was considerably increased only after challenge with rHEL (**Fig. 2E**), exhibited a negative correlation with total traveled distance of the mice (**Fig. 2F**), indicating that the decline of mouse movement could result from acute increase of histamine that was

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induced during allergic reaction in mice sensitized with HEL-VLP/alum. Taken together, our data demonstrate that acute allergic reactions could be induced in mice, whose immune system is sensitized with allergens of interest expressed by VLPs.

Allergic reaction of mice sensitized with VLP to airway allergen challenge

VLP mediated delivery of an allergen functions as a novel tool to sensitize experimental mice, given the strong acute allergic responses including anaphylaxis provoked by subsequent exposures of the target allergen. To validate applicability of this system in the field, we tested whether allergic reactions could be triggered in the mice, sensitized with model allergen expressing VLP, by aerosol challenge with the allergen. BALB/c mice were IP sensitized with HEL-VLP/alum at days 0, 7, and 14, and were subsequently IN challenged with either PBS (group I) or with rHEL (group II) at days 21, 22, and 23 (**Fig. 3A**), following protocols used to study allergic responses to IN challenge with allergens (39). CD4 T cells in the lungs were examined for production of Th2 cytokines. The magnitude of IL-2 producing CD4 T cells was comparable between 2 groups (**Fig. 3B**), indicating that activation and recruitment of CD4 T cells in the lung were similarly induced in 2 groups of mice after sensitization. Aerosol challenge or rHEL, however, led to escalated production of Th2 cytokines (**Fig. 3C**); the frequency of IL-4 producing CD4 T cells seemed to be higher, while

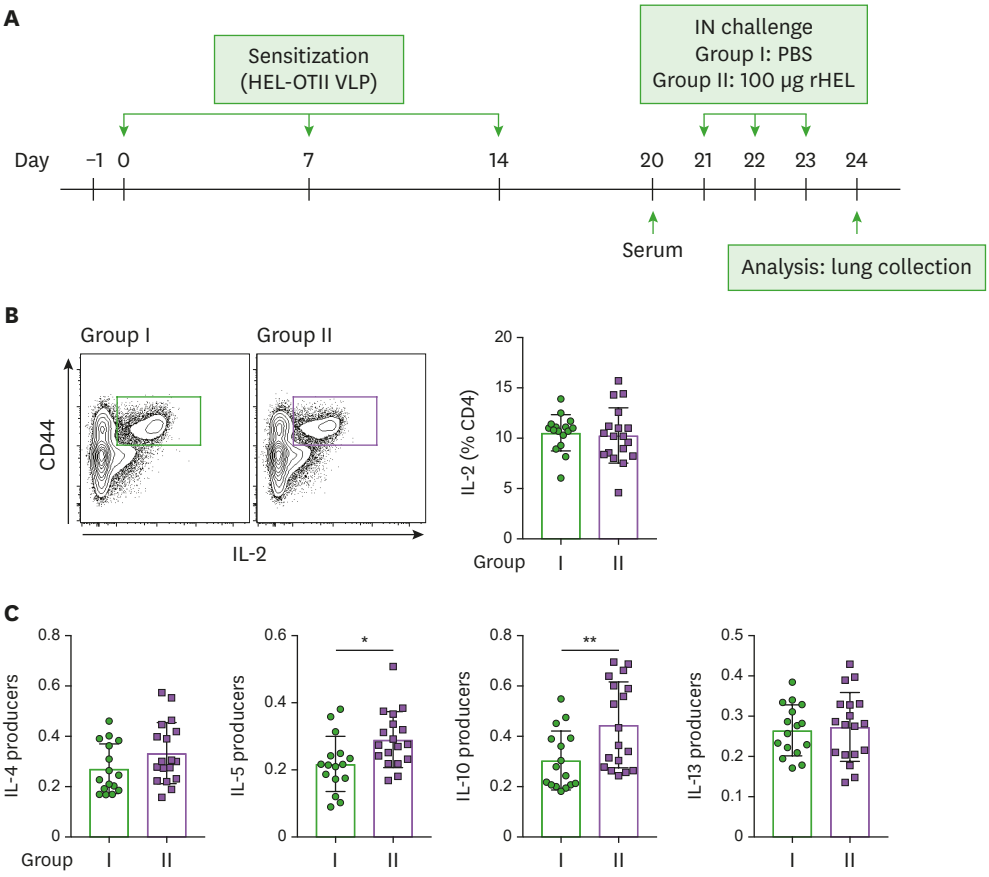

Figure 3. Intranasal challenge with rHEL leads to significant increases in Th2 cytokine production by CD4 T cells in the lungs of mice sensitized with HEL-VLP. (A) BALB/c mice were IP given with HEL-VLPs at days 0, 7, and 14. For 3 consecutive days (days 21 through 23), the mice were IN challenged with PBS (group I) or 100 μg of rHEL (group II). (B, C) Lungs were obtained at day 24 to measure cytokine producing CD4 T cells. (B) The 10% contour plots of CD4 T cells. Gates indicates IL-2 producing CD4 T cells in the lungs. (C) Mean±SD of IL-4, IL-5, and IL-10 producing CD4 T cell frequencies among IL-2 producers. Analyses performed with composite data from 4 independent experiments (n=16 in group I, n=18 in group II).
*p<0.05; **p<0.01.

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it fails to reach statistical significances, in group II than in group I (**Fig. 3C**), and both IL-5 ($p<0.05$) and IL-10 ($p<0.01$) producing CD4 T cell frequencies were strongly increased in the lung in group II. While Th2 cytokine secreting cells infiltrated in the lung following aerosol challenge of rHEL, histological analyses recognized no profound signs of inflammation in the lung (data not shown), indicating that VLP mediated sensitization system needs further optimizations prior to being used in studying local allergic reactions including ones developed by aerosol challenge.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Antigen delivery using VLPs elicits strong antibody response to the antigen in mice. (A) Schematic representation of HEL-VLPs. HEL (green) linked with TM and CTD (purple) of VSV-g protein. (B) Experiment schedule of immunization with HEL-VLP/addavax and analysis of HEL specific IgG level in serum and germinal center B cells in the popliteal lymph node of C57BL/6J mice. (C) Relative amount of HEL specific IgG in the serum of mice before (green circles) and 8 days after immunization with HEL-VLPs (purple squares). (D) Representative FACS plots of CD19 B cells in the spleen of naïve mice and in the popliteal lymph nodes 8 days after immunization. Gates indicate Fas+PNA+ germinal center B cells. The frequencies of germinal center B cells among total CD19 B cells (mean±SD) calculated. Shown are representative data of 2 independent experiments (n=3 per group). The popliteal LNs are too challenging to dissect from naïve mice.

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Supplementary Figure 2

Th2 cytokine producing CD4 T cells develop in the spleen following a series of sensitizations with HEL-VLPs. BALB/c mice were sensitized with either PBS (Neg Ctrl) or HEL-VLPs (Sensitized) at days 0, 10, and 20, as shown in **Figure 1**. Spleens were obtained at day 27 for intracellular staining of cytokines. Representative 10% contour plots of splenic CD4 T cells. Gates indicate IL-4 (A), IL-10 (B), and IL-13 (C) producing CD4 T cells. The frequencies (mean±SD) of IL-4 (A), IL-10 (B), and IL-13 (C) producing CD4 T cells among total CD4 T cells calculated. Data are representative of 2 independent experiments (n=4–5 mice per group).

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Supplementary Figure 3

HEL-specific IgE production in mice sensitized with rHEL or with HEL-VLP. (A) Two groups of BALB/c mice were sensitized with 10 μg of soluble rHEL or with 10 μg (p24) of HEL-VLP, after emulsified with alum, at days 0, 10, and 20. Blood was taken at day –1 and day 25 to measure HEL-specific IgE. (B) Average OD values obtained after developing HEL specific IgE

antibodies present in mice pre- (red) and post (blue)-sensitization with rHEL or with HEL-VLP. Data are representative of 2 independent experiments with n=3–5 mice per group.

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