# Receptor-Mediated Targeting of Spray-Dried Lipid Particles Coformulated with Immunoglobulin and Loaded with a Prototype Vaccine

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*Received February 26, 2001; accepted April 4, 2001*

***Purpose.*** Spray-dried lipid-based microparticles (SDLM) serve as a platform for delivery of a wide variety of compounds including peptides, proteins, and vaccines to the respiratory mucosa. In the present study, we assessed the impact of IgG-mediated targeting to phagocytic cells of inactivated influenza virus formulated in SDLM, on subsequent immune responses.

***Methods.*** SDLM were produced containing inactivated influenza virus strain A/WSN/32/H1N1 (WSN), with or without IgG. Using phagocytic antigen presenting cells (APC) and a T cell hybridoma (TcH) line specific for a dominant influenza virus epitope, we compared the *in vitro* responses elicited by ligand-formulated (SDLM-IgG-WSN) and non-ligand particles (SDLM-WSN). The effect of including the IgG ligand in the formulation was further characterized by measuring the immune responses of rodents vaccinated with SDLM.

***Results.*** SDLM-IgG-WSN were internalized in an Fc receptor (FcR)-dependent manner by phagocytic APC that were then able to effectively present a dominant, class II-restricted epitope to specific T cells. While SDLM-WSN elicited a lower response than administration of plain inactivated virus in saline, the level of the T cell response was restored both *in vitro* and *in vivo* by incorporating the APC FcR ligand, IgG, in the SDLM.

***Conclusions.*** Incorporation of FcR ligand (IgG) in SDLM restored the limited ability of formulated virus to elicit T-cell immunity, by receptor-mediated targeting to phagocytes.

**KEY WORDS:** microparticles; ligand; cell targeting; vaccines; surfactant-lipid.

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**ABBREVIATIONS:** APC, antigen presenting cells; BAL, bronchoalveolar lavage; CTL, cytotoxic lymphocytes; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; FcR, Fc receptor; HA, hemagglutinin; HES, hydroxyethylstarch; IgG, immunoglobulin G; MHC, major histocompatibility complex; PFOB, perfluorocarbon; PLA, poly(lactide); SDLM, spray-dried lipid-based microparticles; SDS, sodium-dodecyl-sulfate; SFC, spot forming cells; TcH, T cell hybridoma; WSN, influenza virus strain A/WSN/32/H1N1.

## INTRODUCTION

Multiple strategies to encapsulate immune active compounds have been assessed for the many potential advantages this may offer for qualitative and quantitative modulation of the immune response. In the case of vaccines, the necessity to stabilize labile antigens as well as the use of various biocompatible excipients to control antigen release drove early advancements in the field (reviewed in 1).

Previous studies to improve on classical adjuvants such as Freund's oil-in-water based emulsion, focused on the use of liposomes both as a vehicle to encapsulate antigen as well as a means to be able to coformulate compounds with adjuvant activity together with the antigen (2,3). Subsequently, it was suggested both with liposomes as well as alternative particulate antigen formulations, that the enhanced response may be a function of whether the particle is actively internalized and the antigen targeted to endolysosomes (4,5).

Biopolymeric particles obtained by spray-drying or double-emulsion/solvent evaporation methods, offer similar versatility for coformulation of antigen with adjuvants, but have the advantage of being more stable than liposomes as well as being appropriate for dry powder applications that are compatible with delivery to the respiratory mucosa (1,6). More recently, it was demonstrated that biocompatible polymers might be used to generate micron-size, solid particles with intrinsic adjuvant activity resulting from phagocytic uptake by antigen presenting cells (APC) (7,8).

Despite the potential advantages that may be offered by vaccine encapsulation into microparticles (i.e., stability, slow-release, and adjuvant-like activity), only a few such vaccine formulations have yet reached the stage of clinical testing (1,9). The most important factors to be cited for the limited clinical development of encapsulated vaccines are: i) the lack of adequate methods for quality assessment (10); ii) safety concerns due to residual processing solvents (11); and iii) the need for sterility of the formulation because the majority of the prototype encapsulated vaccines have been designed for parenteral administration (1). Regarding the immunogenicity, the encapsulation of prototype vaccines into micron-size particles resulted in increased antibody and T helper (Th) responses, as compared to non-formulated vaccine in saline (7,8,12,13).

Recently, we developed and tested an alterantive strategy to encapsulate vaccines using a spray-dried lipid-based microparticle (SDLM) technology (14,15). The major excipients of these SDLM are biocompatible lipids present in normal lung surfactant, such as 1,2-dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) (14). Previous experiments showed that micron-size SDLM composed on DPPC/DSPC were poorly phagocytosed (15), consistent with data previously obtained by Evora *et al.* (16). This phosphatidylcholine-based composition allows mucosal vaccination via the respiratory tract (15) where the antigen may be liberated from particles in a process facilitated by lung surfactant-associated proteins (17,18) and internalized by mucosal-associated APC rather than cleared via the mucociliary escalator. This offers multiple potential advantages: i) priming of the lymphoid cells at the port of entry (local immunity) for microbes with specific mucosal tropism such as influenza

virus; ii) the need for complete sterility of formulated vaccine would be circumvented; iii) stability; iv) local bioavailability; and v) patient compliance may be all more favorable if the vaccine is delivered as dry powder to the respiratory tract.

We reasoned that encapsulation and mucosal vaccination would be beneficial for vaccines endowed with low intrinsic immunogenicity (19,20) that are currently administered parenterally, such as whole-inactivated influenza virus vaccine. In the present study, we describe a prototype SDLM vaccine loaded with inactivated influenza virus and present data on the immunogenicity of such a formulation. As a prototype vaccine antigen, we used the WSN influenza virus strain that was UV-inactivated prior to formulation. Here, we show that incorporating a ligand (IgG) into SDLM-WSN optimized the delivery of inactivated influenza virus to APC and modulated the ensuing Th response to the formulated antigen. Finally, we show that the delivery of formulated vaccine as dry powder via the respiratory tract resulted in induction of IgG responses, indicating the importance of both priming of Th immunity and the availability of soluble, released antigen to B cell receptors.

## MATERIALS AND METHODS

### SDLM Engineering

The microparticle shells were generated by a spray-drying process using a fluorocarbon-in-water emulsion stabilized with saturated phosphatidylcholine as feed material (14,15). Hydrophilic agents such as immune active compounds and hydroxyethylstarch (HES), were added in aqueous solution just before the spray-drying step (14,15). Besides the major lipid excipient (75–85% w/w phosphatidylcholine derivatives DPPC and DSPC; EPC3 Lipoid-Germany), the final SDLM preparation contained 10% w/w of HES, either with or without 10% w/w purified mouse $IgG_2b$ (Sigma, St Louis MO) and 5% w/w of UV-inactivated influenza virus strain A/WSN/32 H1N1. Typically, the size of the particles was between 1–5 μm and the density $\leq 0.2 g/cm^3$. SDLM loaded with 10% w/w of HA 110-120 peptide, NP 147-155 peptide, mouse $IgG_2b$, or human IgG served as control formulations.

### Virus Preparation and Inactivation

The WSN strain of influenza virus (A/WSN/32 H1N1) was grown on permissive Madin-Darby bovine kidney carcinoma (MDBK) cells. The virus was recovered and concentrated from the culture supernatant by sucrose-gradient centrifugation (21), resuspended in sterile phosphate buffered saline (PBS), and exposed to two rounds of inactivation using short-wave UV light. Inactivation was confirmed by standard titration on permissive Madin Darby canine kidney (MDCK) carcinoma cells, as described elsewhere (22). The amount of viral antigen was measured in a bioassay using M12 B lymphoma cells as APC and a T cell hybridoma (TcH) specific for HA 110-120 peptide. The B/Lee virus used as negative control was grown on 10-days old embryonated eggs in standard conditions.

### Immunization

SDLM administration to BALB/c mice was carried out by intraperitoneal injection or intranasal instillation. For injection or nasal instillation, the particles were suspended in perflubron (PFOB-*LiquiVent®,* Alliance Pharmaceutical Corp., San Diego, CA) at a concentration of 10 mg/ml. Blood samples and splenic tissue, were harvested at 7 days after immunization. For assessment of *in vivo* loading of APC, 4 × 40 μl of 10 mg/ml SDLM in PFOB were instilled directly into the airways of anesthetized BALB/c mice (total dose of 1.6 mg/mouse, corresponding to 80 μg of antigen) at 0, 3, 18, and 21 h. At 22 h the mice were euthanized and airway cells harvested by bronchoalveolar lavage (BAL).

Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were vaccinated with SDLM delivered as dry powder directly to the trachea by using an insufflator (Penn-Century Inc., Philadelphia, PA) loaded with 1mg of powder, which corresponds to 50 μg of antigen. At 7 days after immunization, bronchoalveolar fluid was harvested by lavage with heparinized (15 U/ml) phosphate buffered saline and blood was obtained by venipuncture.

### Particle Dissolution and Measurement of Antigen-Content and Release

SDLM were suspended in sterile PBS at a concentration of 1 mg/ml and incubated at 37°C under mild shaking conditions. One hundred-μl aliquots were sampled at set time intervals, centrifuged (5 min at 10,000 RPM) and the supernatants stored at –70°C. The lipid pellet was resuspended in the same volume of PBS containing 0.1% SDS (sodium-dodecylsulfate) and solubilized with gentle shaking for 30 min at room temperature. The samples were recentrifuged and the SDS removed from supernatants (using an SDS-Out kit, Pierce, Rockford-IL) according to the manufacturer's instructions. The amount of antigen was then determined using a bioassay based on activation of TcH specific for HA 110-120 peptide, when cocultured with M12 B lymphoma cells. Standard curves were generated using various concentrations of inactivated virus, with or without previous SDS-treatment, depending on the nature of the samples to be measured. Human IgG was quantitated using a sandwich enzyme linked immunosorbent assay (ELISA) assay based on mouse anti-human k chain monoclonal antibody (Sigma, cat. no. K-4377) and a mouse anti-human IgG monoclonal antibody coupled with alkaline phosphatase (Sigma, cat. no. A-2064), as previously reported (15).

### *In Vitro* T Cell Response

The specific response to formulated viral antigen was measured using BALB/c-derived APC (expressing the MHC class II allele $I\text{-}E^d$) and a T cell hybridoma specific for a dominant class II-restricted influenza epitope (HA 110-120 peptide with the sequence SFERFEIFPKE). This TcH clone is a stable transfectant carrying the β-galactosidase open reading frame under the control of the IL-2 promoter (23).

For antigen titration experiments, M12 B cell lymphoma cells were used as APC. Alternatively, splenic or BAL cells were used as APC, either with or without separation into adherent and non-adherent cells, by 1-h incubation on plastic Petri dishes at 37°C. To block endocytosis and antigen processing, the APC were fixed before antigen-exposure, by incubation in 1% paraformaldehyde—PBS for 20 min at 4°C. For experiments assessing the dependency of antigen

presentation on ligand-receptor interaction, APCs were pretreated for 1 h at 4°C with either excess soluble $IgG_2b$ (10-fold excess to the formulated IgG added subsequently) or anti-FcγRII/III monoclonal antibody (50 μg/ml; 2.4G2, PharMingen, San Diego-CA). Subsequently, the APC were washed and incubated with SDLM and TcH, respectively. Most often, the ratio of APC to TcH was 1:2 ($1 \times 10^4$ APC and $2 \times 10^4$ TcH) and the incubation time varied between 4 and 12 h.

To characterize the *in vivo* loading of airway APC with antigen, BAL and lung tissue were harvested from immunized mice. The BAL cells were obtained by centrifugation, washed and suspended in HL-1 cell culture medium (BioWittaker, Walkersville-MD). The APC associated with the lung interstitium were obtained by collagenase digestion of the tissue (24), followed by washing, tagging with magnetic beads coated with anti-mouse MHC-II (Ia) antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), and magnetic sorting according to the manufacturer's instructions.

The BAL, positively selected (MHC $II^+$) and negatively selected (MHC $II^-$) APC were incubated in various numbers with TcH overnight. As controls, we used similar numbers of BAL and interstitial APCs, that had been pulsed *in vitro* with supraoptimal amounts of antigen (20 μg/ml of inactivated WSN virus). In some experiments, the *ex vivo* prepared APC were resuspended at a concentration of $2 \times 10^6$ cells/40 μl of saline and adoptively transferred to BALB/c animals via the respiratory tract, using the nasal route.

### Measurement of Humoral and Cellular Response After Vaccination

For the measurement of virus-specific antibodies in BAL and serum samples, MaxiSorb 96 well microtiter plates (Nunc, Denmark) coated with sucrose gradient-purified WSN virus (8 μg/ml in carbonate buffer pH = 9, incubated overnight at 4°C) were used. After blocking with 30% SeaBlock (Pierce, Rockford-IL), serial dilutions of samples were incubated for 2–4 h at room temperature. Mouse antibodies were detected with goat-anti-mouse IgG polyclonal antibodies conjugated with alkaline phosphatase (cat. no. A7434; Sigma, St Louis MO) (15). Rat antibodies were detected with goat anti-rat IgG polyclonal antibody (cat. no. A8438, Sigma). The reaction was developed after 1 h of incubation at room temperature, by addition of soluble pNPP substrate (Sigma). The results were read using an automatic plate reader (ThermoMax, Molecular Devices; Menlo Park-CA) at an absorbance of 405nm.

For ELISPOT analysis, splenocytes ($4 \times 10^5$/well and serial dilutions) were stimulated *in vitro* with antigen (2 μg/ml sucrose-purified WSN virus) in 96 well nitrocellulose MAHA S4510 plates (Millipore S.A., Molsheim France) precoated with rat anti-mouse IFN-γ (cat. no. 18181D, PharMingen) or rat anti-mouse IL-4 antibodies (cat. no. 18031D, PharMingen, San Diego-CA). The reaction was developed after 72 h using 2 μg/ml biotinylated anti-cytokine antibodies (PharMingen), followed by streptavidin-coupled horseradish peroxidase (1:1000) and an insoluble substrate (AEC) (Sigma, St Louis MO). The data representing the frequency of spot-forming cells (SFC) were acquired using a camera (Navitar, Rochester NY) and analyzed with *Image-Pro Plus* software (Media Cybernetics, L.P.). In parallel with antigen-stimulation, splenocytes were incubated with medium alone to define the background level.

## RESULTS

### Effect of Incorporating IgG on the Immunogenicity of SDLM Loaded with Inactivated WSN Influenza Virus

To construct and further evaluate a prototype mucosal vaccine, we formulated inactivated WSN influenza virus in DPPC/DSPC-based SDLM (5% w/w antigen), using a previously described technology (14,15). The immunogenicity of antigen encapsulated into SDLM was tested *in vitro* using a sensitive and quantitative bioassay based on a transfected TcH that expresses a reporter gene (β-galactosidase) (23). Two different types of APC were used: BAL cells that contain a high percentage of phagocytes (25), or splenocytes that contain mostly non-phagocytic APC such as mature DC and B cells, as well as a small percentage of macrophages (26). APC were coincubated with SDLM-WSN or corresponding amounts of non-formulated virus for 4 h, in the presence of specific TcH. The activation of TcH was clearly higher in the case of non-formulated virus, irrespective of the type of APC used (Fig. 1A). A higher activation rate by low amounts of non-formulated virus was noted with splenocytes, which is expected given there were potent non-phagocytic APC such as mature DC present. The activation triggered by SDLM-WSN, while significantly above background, was much less than that obtained with non-formulated virus in this type of reporter gene assay. These data suggested that either the viral antigen was degraded during the spray-drying process and/or the antigen is not readily available to the APC and the class II-restricted pathway of presentation when formulated in SDLM. The latter may occur due to limited antigen release from SDLM into aqueous medium combined with the poor internalization of SDLM-WSN particles by phagocytic cells. To eliminate the possibility of antigen degradation during the spray-drying process, the amount of viral antigen released from SDLM following saline or saline plus detergent exposure was measured by bioassay. Detergent treatment resulted in 100% recovery of viral antigen, available to non-phagocytic M12 APC (Fig. 1B). In contrast, only 2% of the loaded antigen was available to M12 APC from saline-treated particles (Fig. 1B). BALB/c mice immunized intraperitoneally with SDLM-WSN or non-formulated virus (corresponding to 5 μg of antigen) mounted significant T cell responses as measured by ELISPOT analysis (Fig. 1C). However, the frequency of virus-specific T cells producing IFN-γ, as revealed by *in vitro* stimulation with live virus, was significantly decreased (approximately half) in the mice immunized with SDLM-WSN as compared to the frequency observed in mice immunized with non-formulated virus. In sharp contrast, the SFERFEIFPKE peptide (HA 110-120 epitope; M.wt. of 1.4kDa) was readily released from SDLM and the T cell response was high even in the presence of non-phagocytic APC (Fig. 2). HA epitope availability was not dependent on the status of APC, because fixed cells could present the peptide with the same efficacy as live APCs (Fig. 2A). As expected, this rules out internalization as a prerequisite for peptide presentation when the syn-

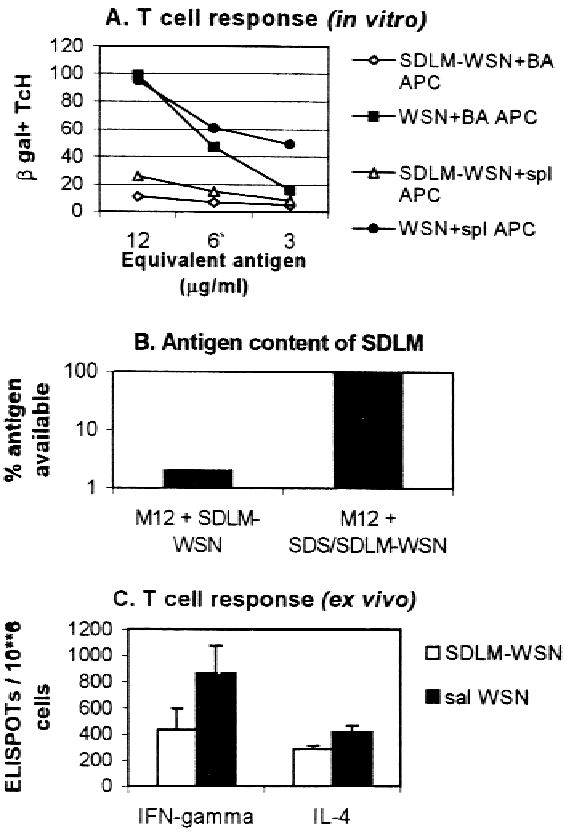


**Fig. 1.** The MHC class II-restricted T cell response to whole inactivated influenza virus formulated into SDLM. (A) Dose-dependent activation of HA 110-120 peptide-specific TcH by formulated virus (SDLM) in the presence of bronchoalveolar (BA) or spleen APC. As control, dose-matched non-formulated, inactivated virus was used (closed symbols). The activation of the reporter gene expressed by TcH was measured after 4-h incubation, as detailed in Materials and Methods. (B) The release of viral antigen was measured by coincubating SDLM non-treated or previously treated with SDS, with non-phagocytic B cell lymphoma APC (M12). After 1 h pulsing, the APC were washed and coincubated with TcH overnight. The number of TcH expressing β-galactosidase was quantified, and the amount of antigen released from SDLM was normalized to the amount of antigen formulated, after interpolation based on a standard curve obtained with non-formulated virus. (C) The T cell response of BALB/c mice immunized with formulated (SDLM-WSN) or non-formulated (sal-WSN) virus was measured at day 7 by *ex vivo* culture of splenocytes in the presence of antigen followed by ELISPOT analysis. The frequency of antigen-specific IFN-γ or IL-4 producing T cells, was expressed as number of spot forming cells (SFC)/$10^6$ responder cells (mean ± SEM, n = 3/group).

thetic peptide is formulated into SDLM. However, endocytosis was still required for the presentation of HA epitope by APC when it is presented to the system in the context of virus formulated into SDLM particles (Fig. 2B). Negative controls (NP 147-155 peptide loaded into SDLM or B/Lee virus) failed to activate TcH, ruling out the possibility that the SDLM excipients were exerting a non-specific effect on the T cell response.

Thus, formulation of inactivated influenza virus in SDLM does not lead to degradation of T cell epitopes within the influenza virion, but rather limits their availability to the class II-restricted presentation pathway.

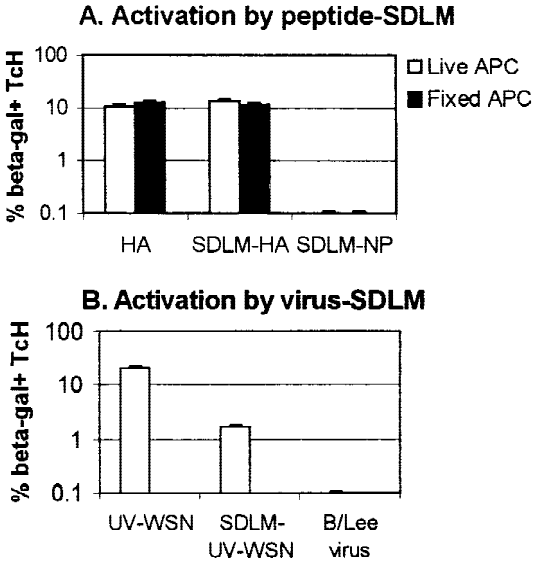


**Fig. 2.** Antigen-dependent, contrasting availability of HA 110-120 epitope presented in the context of SDLM. Particles loaded with HA peptide (A) or WSN influenza virus (B) were incubated with TcH and non-fixed or fixed M12 APC. The stimulation of TcH was quantified using β-galactosidase expression and the results were expressed as % activated TcH. As negative controls, we used dose-matched, non-formulated antigen and antigens devoid of HA 110-120 epitope (SDLM-NP peptide and B/Lee influenza virus).

### Receptor-Mediated Internalization of WSN-SDLM Formulated with FcR Ligand

The most likely explanation for the reduced immunogenicity of WSN virus formulated into SDLM is that the viral antigen is tightly bound to the lipid matrix of SDLM, potentially due to the size, complexity, and lipophilicity of influenza virus. When exposed to a saline environment, the SDLM undergo hydration and reorganization with release of hydrophillic constituents. To assess whether the reduced immunogenicity of WSN when formulated in SDLM was due to its limited solubility from the lipid shell, the kinetics of viral antigen release from SDLM-WSN as well as that of HA peptide or IgG from SDLM, were measured (Fig. 3). Most of the viral antigen segregated with the lipid matrix rather than entering the aqueous phase, irrespective of the incubation time (Fig. 3A). Approximately 2% of the viral antigen were released from SDLM-WSN in saline over a 24 h period. In contrast, smaller hydrophillic compounds such as HA peptide (1.4 kDa) or IgG (150 kDa) are readily released (Fig. 3B,C). The release of IgG however, was slower compared to that of HA peptide. Thus, 50% of IgG was still present within SDLM at one hour after the treatment with saline (Fig. 3C). This may be of significance for FcR-engagement by IgG formulated within the lipid matrix, resulting in the internalization of SDLM-IgG. Because the retention time of virus in SDLM is considerably higher than that of IgG, these results raise the possibility that the poor availability of SDLM-formulated viral antigen to APC could be corrected by using Fc receptor-mediated delivery to the APC endolysosomal compartment.

To test this hypothesis, SDLM containing $IgG_2b$ (10% w/w) together with inactivated influenza virus (5% w/w) were generated. We compared the ability of phagocytes to activate specific TcH when pulsed with SDLM-IgG-WSN or SDLM-

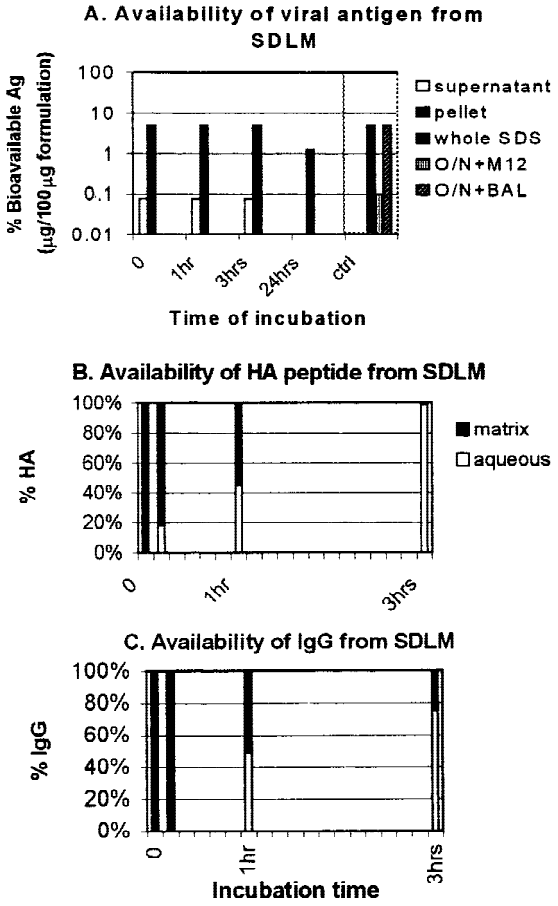


**Fig. 3.** Antigen-dependent kinetics of release from SDLM. Microparticles were exposed to normal saline, and the amount of released and matrix-associated antigen was subsequently quantified using TcH/M12 bioassay, at various intervals (Materials and Methods). In case of formulated virus (A) the results were normalized to the amount of formulation. Inset: SDLM were either dissoluted with SDS to completely extract the antigen, or incubated overnight with TcH in the presence of non-phagocytic M12 or bronchoalveolar (BA) APC. The results were similarly normalized to the amount of formulation. In case of formulated HA peptide (B), the data were normalized to the total amount of peptide formulated. The release of IgG (C) was quantified by ELISA using anti-IgG reagents, and the data were normalized to total formulated immunoglobulin.

WSN. As shown in Fig. 4A, the incorporation of IgG into SDLM as FcR ligand, improved the ability of pulsed phagocytes (plastic adherent cells) to activate TcH cells. As expected, the non-adherent cells (mostly non-phagocytic) pulsed with SDLM were poor activators of TcH (Fig. 4B). To test whether this improvement depended on ligand-receptor mediated internalization of SDLM-IgG-WSN into the endolysosomal compartment of APC, the effect of pretreating phagocytes with excess IgG, on subsequent responses to the SDLM-IgG-WSN or non-formulated virus, was studied. IgG-pretreatment of phagocytes pulsed with SDLM-IgG-WSN significantly inhibited their ability to activate TcH. In contrast, the activation of TcH by APC pulsed with non-formulated virus was not inhibited (Fig. 5A), demonstrating the importance of IgG-FcR engagement to the immunogenicity of SDLM-IgG-WSN. This was confirmed in an independent experiment by using anti-FcR monoclonal antibody to block the cellular IgG receptors (Fig. 5B).

Thus, APC targeting by SDLM loaded with whole-inactivated WSN influenza virus can be achieved by coformulating ligands for APC receptors, within SDLM, to mediate internalization into the endolysosomal compartment. This would overcome the limited availability of viral antigen to the class II-restricted pathway of antigen presentation, which was observed with SDLM-WSN.

### Immunogenicity of IgG-Containing SDLM Loaded with Inactivated Influenza Virus (SDLM-IgG-WSN)

To assess this hypothesis, the immune response of BALB/c mice to SDLM loaded with inactivated influenza virus, formulated with or without 10% w/w of IgG, was measured. Mice injected with SDLM-IgG-WSN corresponding to 50 μg of antigen, developed a greater T cell response compared to non-ligand containing SDLM-WSN, that was of similar magnitude to that obtained with non-formulated virus (Fig. 6A). The frequency of virus-specific T cells producing IFN-γ upon *in vitro* restimulation with live virus, was restored by coformulating $IgG_2b$ with WSN in SDLM. No significant difference regarding the frequency of IL-4-producing cells was noted among the different groups.

Although they mounted a substantial T cell response, the mice injected with SDLM-IgG-WSN failed to mount antibody

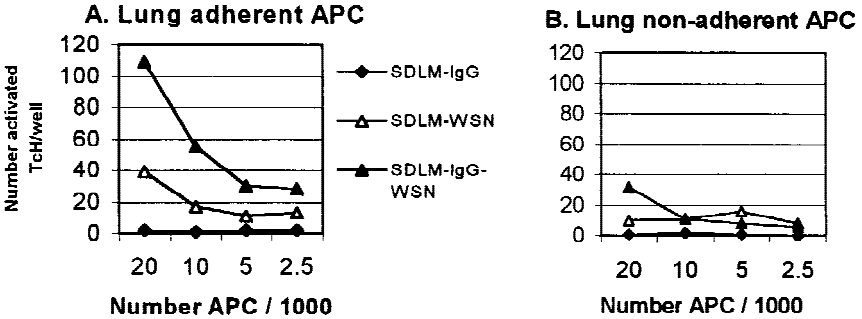


**Fig. 4.** Activation of class II-restricted TcH by ligand engineered and non-ligand SDLM loaded with inactivated virus. Plastic adherent (A) and non-adherent (B) APC were isolated from lung tissue of BALB/c mice and pulsed with SDLM-WSN or SDLM-IgG-WSN. Various numbers of pulsed APC were incubated with TcH, and the rate of activation was measured as % β-galactosidase-expressing cells. As negative control, we used SDLM-IgG devoid of viral antigen.

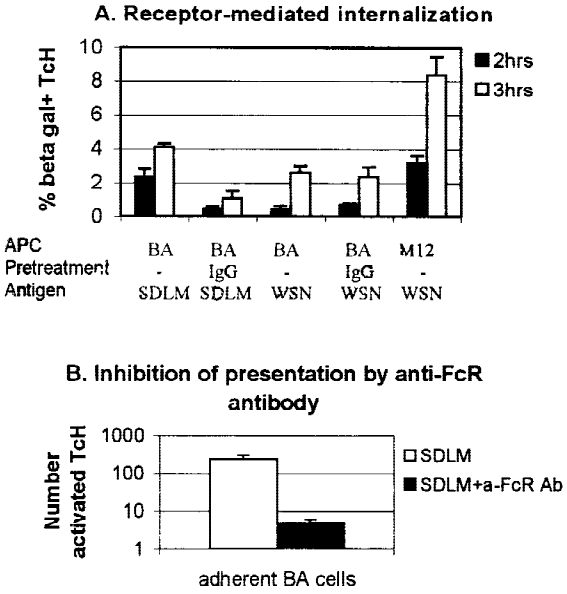


**Fig. 5.** Presentation of HA 110-120 epitope to specific TcH by phagocytic APC pulsed with IgG ligand-engineered SDLM depends on FcγR engagement. (A) APC obtained by bronchoalveolar (BA) lavage of BALB/c mice were pulsed with ligand-engineered virus-containing particles (SDLM) or dose-matched non-formulated virus, in the presence or without soluble IgG in excess. The pulsed APC were incubated with TcH and the activation was expressed as % β-galactosidase$^+$ cells (mean ± SE of triplicate wells). As control, we included non-phagocytic APC (M12 cells) pulsed with virus. (B) Plastic adherent APC harvested by bronchoalveolar (BA) lavage were pulsed with ligand-engineered virus-containing particles (SDLM) in the presence or absence of anti-FcγR monoclonal antibody. The washed APC were subsequently incubated with specific TcH, and the rate of activation was measured as number of β-galactosidase$^+$ cells / well (means ± SE of triplicate wells).

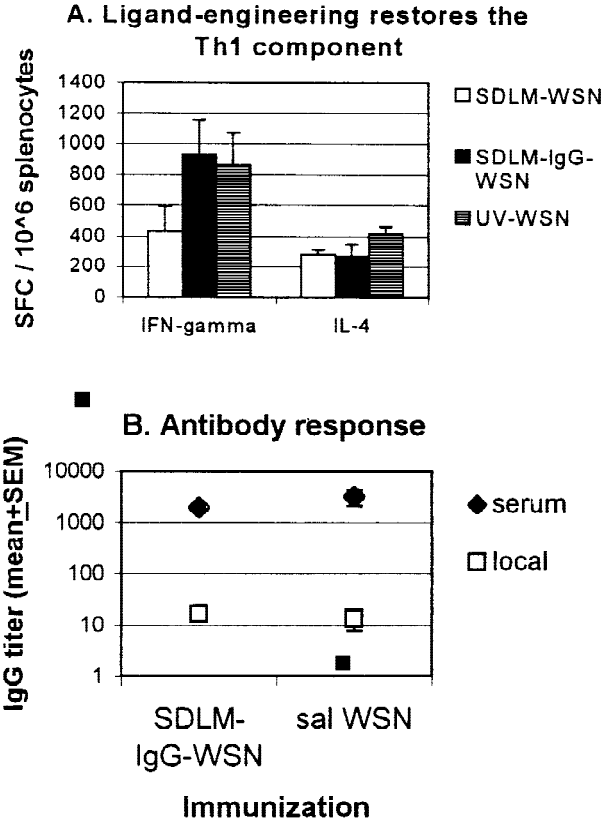


**Fig. 6.** Cellular and humoral response of BALB/c mice to inactivated influenza virus formulated into ligand-engineered SDLM. (A) The T cell response was measured at day 7 after parenteral inoculation of ligand-SDLM, non-ligand SDLM, or dose-matched non-formulated virus by ELISPOT analysis subsequent to in vitro antigen stimulation. The results were expressed as mean ± SEM of the frequency of spot forming cells (SFC) (n = 3 mice/group). (B) The systemic (serum) and local (bronchoalveolar lavage) humoral response was measured in Sprague-Dawley rats insufflated with dry powder containing ligand-SDLM loaded with virus. The results were expressed as geometric mean ± SEM of endpoint titers of virus-specific IgG, measured by ELISA (n = 3 rats/group). As controls, we employed rats treated by intratracheal instillation with non-formulated virus in saline (sal WSN).

responses at the same level as non-formulated WSN virus (data not shown). Because the antibody responses depend on interaction of viral antigens with B cell receptors, these results are consistent with the increased internalization of ligand-SDLM into phagocytes following coformulation with IgG and with the inherent limited solubilization of the antigen in aqueous environment.

While *in vitro* studies can provide considerable information on the release of antigen from SDLM, it is important to assess *in vivo* responses since the local millieu within the respiratory tract has some unique characteristics (i.e., surfactant-rich environment). To test this, SDLM-IgG-WSN were administered by dry powder insufflation: this resulted in induction of robust virus-specific IgG responses (Fig. 6B). The IgG antibody titers in blood and BAL were similar to those elicited by aqueous aerosols containing the same dose of killed virus (Fig. 6B). Evaluation of *in vivo* antigen loading of APC using TcH activation assay demonstrated responses that correlate with the *in vitro* data (Fig. 7). BAL APC from BALB/c mice that were treated via the respiratory tract with SDLM-IgG-WSN, activated TcH to a higher extent than BAL APC from mice immunized with non-ligand SDLM (Fig. 7A). The efficiency of BAL APC targeting by SDLM-IgG was near maximal, since their incubation with supraoptimal amounts of antigen shifted only slightly the activation profile (Fig. 7A). Whereas the intrinsic activation profile by interstitial MHC-II$^+$ APC was higher (Fig. 7B), the ability of APC from animals immunized with ligand-SDLM to activate TcH was limited and comparable with that of APC from mice immunized with non-ligand SDLM. Based on the *in vitro* titration curve (Fig. 7B) and the total number of MHC-II$^+$ APC isolated from lung tissue / mouse ($2.3x10^6$ cells), we inferred that approximately 2,000 interstitial APC (0.1%) were loaded with viral antigen by mucosal vaccination with SDLM, under the experimental conditions used in this study. In parallel, as expected, the MHC-II$^-$ interstitial cells failed to activate TcH (data not shown). As expected from these data, adoptive transfer to naïve animals of similar numbers of airway APC isolated within one day after *in vivo* administration of SDLM-IgG-WSN or SDLM-WSN, resulted in priming of T cell response of higher magnitude in the case of the former particle type (Fig. 7C, D). The bronchoalveolar APC were more potent in inducing a Th1-type immunity and the MHC-II$^+$ APC separated from lung parenchyma triggered a more balanced Th1/Th2 response.

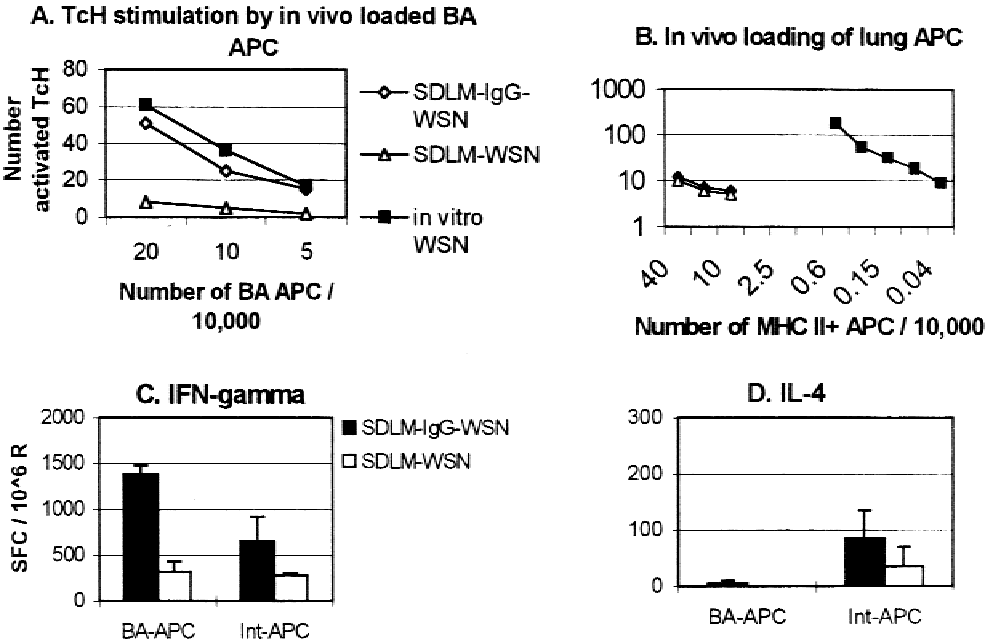


**Fig. 7.** *In vivo* loading of APC with viral antigen, subsequent to mucosal delivery of SDLM. The BALB/c mice were immunized and the BA and lung interstitial APC were harvested as described in Materials and Methods. (A) Various numbers of BA APC were incubated with TcH, and the results were expressed as number of activated TcH/well. As positive control ('in vitro WSN'), we added 20 μg/ml of viral antigen before incubation of BA APC harvested from SDLM-treated mice. (B) Similarly, various numbers of MHC-II$^+$ lung-interstitial APC were incubated with TcH. As positive control, we have used interstitial APC *in vitro* pulsed with supraoptimal amount of antigen (20 μg/ml of inactivated virus). (C, D) Induction of virus-specific IFN-γ (C) or IL-4 (D) producing T cells by adoptive transfer of APC loaded *in vivo* with ligand-engineered (SDLM-IgG-WSN) or non-engineered SDLM (SDLM-WSN). The quantification of specific T cells in the spleen was made by ELISPOT analysis, nine days after transfer of $2 \times 10^6$ APC (obtained by bronchoalveolar lavage - BA-APC; or by magnetic enrichment from lung - Int-APC) via the respiratory tract. The results are represented as mean ± SE of the number of spot-forming colonies (SFC) / $10^6$ splenocytes (triplicates).

## DISCUSSION

Producing dry powder formulations of complex, labile structures such as whole virus-vaccines offers two potential advantages over saline formulations: increased stability due to reduced exposure to aqueous environment and secondly, effective delivery to the mucosal surface of the respiratory tract, offering improved local bioavailability to the mucosal-associated lymphoid tissue. In the case of conventional influenza virus vaccine, whereas the elicited IgG antibodies directed against certain B cell epitopes on HA mediate protection from infection, the Th immunity is thought to exert a double role: to assist in the generation of protective antibodies (27) and secondly, to produce anti-viral effectors like cytokines (28).

We have generated and tested a spray-dried formulation of influenza virus, using biocompatible and immunologically inert excipients: DPPC/DSPC and HES. We previously demonstrated that such SDLM accommodate a broad array of antigens (small peptides, large proteins, immunoglobulins) without altering their immunologic properties, which generally depend on the primary and secondary structure and only to a limited extent on their tertiary structure. As a model system to evaluate the SDLM as platform for mucosal vaccines, we used UV-inactivated virus—i.e., devoid of replication ability, because the current commercially available killed influenza vaccines should have added benefit by changing the route of delivery from the parenteral to the mucosal route. We show that the viral antigens are preserved during the spray-drying procedure but are strongly bound by the lipid matrix of the SDLM (Figs. 1,2). Normally, peptides and hydrophillic proteins are rapidly released from SDLM upon contact with the aqueous environment (Fig. 3). However, most of the formulated whole killed influenza virus (WSN strain) remains attached to the lipid matrix, limiting its availability to the APC, precluding appropriate presentation to specific T cells (Fig. 3). Thus, the T cell response and T cell-regulated antibody response were decreased in animals immunized with killed influenza virus formulated in SDLM (Fig. 1). The most likely explanation is that the hydrophobic nature of the lipid excipient may have limited the antigen release as well as affected the surface exposure of sialoreceptor-engaging domains of the viral HA that mediate internalization of virus in target cells, which include APCs. This is concordant with previously reported data on the inhibitory effect of DPPC on phagocytosis of microparticles (16).

We sought to improve the intrinsic immunogenicity of SDLM-formulated influenza virus by reformulating the particles to facilitate antigen transfer to APC. Based on *in vitro* data (Figs. 1–3), we hypothesized that reduced antigen availability to APC was the main limiting factor associated with the reduced T cell immunogenicity observed with killed in-

fluenza virus formulated in SDLM. To overcome this limitation, we took advantage of our previous observation that formulated immunoglobulins facilitate receptor-mediated internalization of SDLM by phagocytic cells (15).

Internalization was dependent on IgG ligand - FcγR interaction, the amount of IgG ligand formulated in the SDLM, and the carbohydrate coexcipient used to stabilize the particle structure (15). Interestingly, although SDLM release IgG into saline at an exponential rate (Fig. 3C), this does not preclude receptor-mediated phagocytosis due to the rapid rate of this process (10–30 min). Since, as shown by the detergent-treatment experiments, inactivated influenza virus is largely trapped in the lipid matrix of SDLM (Fig. 3), we reasoned that ligand-engineering of lipid-particles may facilitate transfer of antigen to APC such as macrophages and immature DC, that express FcγR and are able to phagocytose micron-size particles. We generated IgG-SDLM loaded with inactivated virus and compared their immunogenicity to that of non-ligand containing WSN virus-SDLM. The following evidence support the conclusion that ligand-receptor mediated targeting aided APC uptake of whole inactivated virus formulated into IgG-SDLM:

i) the activation of epitope-specific TcH was increased when phagocytic APC were pulsed with ligand-engineered SDLM versus non-ligand SDLM (Fig. 4);

ii) competitive inhibition of ligand binding to FcγR on APC using soluble IgG in excess, or receptor blockage using anti-FcR antibodies, significantly reduced the activation of TcH by APC pulsed with ligand-SDLM (Fig. 5). This result strongly argues against other explanations for the enhanced availability of viral antigen, like a potential destabilizing effect of IgG on SDLM structure;

iii) immunization of BALB/c mice with ligand-SDLM loaded with inactivated virus restored the magnitude of the Th1 response obtained with inactivated virus in saline (Fig. 6);

iv) airway associated APC were more efficiently loaded, by mucosal immunization, with antigen formulated into ligand-containing as opposed to plain SDLM-WSN (Fig. 7).

Thus, incorporation of IgG into SDLM facilitated the transfer of whole-inactivated influenza virus from virus-formulated SDLM to phagocytic APC, in a manner that depended on FcγR-engagement. This resulted in the improvement of class II-restricted T cell response. However, in contrast to T cell epitope recognition, B cells recognize antigens in a non-processed form, without a prerequisite for endosomal processing (29). Nevertheless, the fact that only a small fraction of the antigen is readily released from SDLM into the aqueous environment (Fig. 3) may be a limiting factor in the induction of virus-specific IgG. In support of this hypothesis, we showed that despite the induction of a substantial Th response by parenteral administration of ligand-SDLM loaded with killed virus (Fig 6A), the IgG response was at the limit of detection (data not shown). In contrast to the response to parenteral administration, delivery of SDLM to the respiratory tract as dry powder resulted in a local and systemic IgG response similar to that observed with non-formulated virus in saline (Fig. 6B). This may be due to increased availability of viral antigen to mucosal-associated B cells, or enhanced dissolution of SDLM in the airways due to the presence of surfactant. Based on the data (Fig. 6B and 7A), the most likely mechanism involved is ligand-incorporation that facilitates antigen uptake by bronchoalveolar APC. The particles that fail to internalize within a certain time interval dissolute within the surfactant-rich environment of airways and release the antigen before clearance. This explains why a robust local and systemic T cell dependent antibody response is still generated by dry powder insufflation of ligand-SDLM loaded with virus (Fig. 6B). There is still an ongoing debate as to which type of airway APC phagocytoze the particulate antigens, migrate to the regional lymph nodes, and prime specific T cells. Some data suggest that both alveolar macrophages and dendritic cells have this ability (30), whereas others argue that only dendritic cells can transmigrate from airways to the interstitial tissue and regional lymph nodes (31). Our results generated following adoptive transfer of *in vivo* loaded airway APC (Fig. 7) support the hypothesis that rather than being a passive scavenging cell system, the airway APC may be connected into a complex monitoring network with the potential to directly impact the immune response (32).

In conclusion, ligand engineering of SDLM restores the Th1 component of the immune response to inactivated virus. Further, mucosal administration of IgG-SDLM leads to increased loading with antigen of airway APC. Finally, SDLM-mediated delivery of whole-inactivated influenza virus is compatible with antibody generation when the administration is carried out by dry powder delivery to the respiratory tract. Thus, dry powder-based vaccination of the respiratory tract constitutes an appealing strategy to raise both local and systemic immunity.

## ACKNOWLEDGMENTS

This study was supported in part by a grant from the N.I.H. to Alliance Pharmaceutical Corporation and A. B. (R21 AI47014-01).

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