Published in final edited form as:

Carbohydr Res. 2015 March 20; 405: 87–92. doi:10.1016/j.carres.2014.11.007.

Sublingual injection of microparticles containing glycolipid ligands for NKT cells and subunit vaccines induces antibody responses in oral cavity

Elizabeth S DeLyria^{1,*}, Dapeng Zhou^{2,*†}, Jun Soo Lee¹, Shailbala Singh³, Wei Song^{1,4}, Fenge Li¹, Qing Sun¹, Hongzhou Lu⁴, Jinhui Wu⁵, Qian Qiao⁵, Yiqiao Hu⁵, Guodong Zhang⁶, Chun Li⁷, K. Jagannadha Sastry³, and Haifa Shen⁶

¹Department of Melanoma Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX

²Shanghai Pulmonary Hospital, Tongji University, Shanghai, China

³Department of Immunology, The University of Texas M.D. Anderson Cancer Center, Houston, TX

⁴Shanghai Public Health Clinical Center, Shanghai, China

⁵State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing, China

⁶Department of Nanomedicine, The Methodist Hospital Research Institute, Houston, TX

⁷Department of Experimental Diagnostic Imaging, The University of Texas M.D. Anderson Cancer Center, Houston, TX

Abstract

Natural Killer T (NKT) cells are a unique type of innate immune cells which exert paradoxical roles in animal models through producing either Th1 or Th2 cytokines and activating dendritic cells. Alpha-galactosylceramide (α GalCer), a synthetic antigen for NKT cells, was found to be safe and immune stimulatory in cancer and hepatitis patients. We recently developed microparticle-formulated α GalCer, which is selectively presented by dendritic cells and macrophages, but not B cells, and thus can avoid the anergy of NKT cells. In this study, we have examined the immunogenicity of microparticles containing α GalCer and protein vaccine components through sublingual injection in mice. The results showed that sublingual injection of microparticles containing α GalCer and ovalbumin triggered IgG responses in serum (titer $>1:100,000$), which persisted for more than 3 months. Microparticles containing ovalbumin alone also induced comparable level of IgG responses. However, immunoglobulin subclass analysis showed that sublingually injected microparticles containing α GalCer and ovalbumin induced 20 fold higher Th1 biased antibody (IgG2c) than microparticles containing OVA alone (1:20,000 as compared to 1:1000 titer). Sublingual injection of microparticles containing α GalCer and

[†]Correspondence: ; Email: dapengzhoulab@tongji.edu.cn.

*Equal contributors;

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ovalbumin induced secretion of both IgG (titer >1:1000) and IgA (titer =1:80) in saliva secretion, while microparticles containing ovalbumin alone only induced secretion of IgG in saliva. Our results suggest that sublingual injection of microparticles and their subsequent trafficking to draining lymph nodes may induce adaptive immune responses in mucosal compartments. Ongoing studies are focused on the mechanism of antigen presentation and lymphocyte biology in the oral cavity, as well as the toxicity and efficacy of these candidate microparticles for future applications.

Keywords

Microparticles; alpha-galactosylceramide; Natural Killer T cells; Vaccines; Mucosal Immunity; Antibody Class Switch

Introduction

Mucosal route of vaccination is a current focus of HIV vaccine research. Effective adaptive immune responses at mucosal sites are critical for the success of a prophylactic vaccine. Intranasal, intra-intestinal, intra-vaginal, and intra-rectal routes of immunization have been widely tested in multiple types of vaccines composed of proteins, peptides, DNAs, or adenoviral vector-based components. Evidence of successful induction of protective adaptive immune responses has been reported at both mucosal sites and systemic compartments (1–4).

The oral mucosal associated lymphoid tissues (MALT) are perhaps the least understood area of mucosal immunity. Oral MALT including tonsils and submandibular lymph nodes are known to have anatomical structures and cell types required for effective adaptive immune responses (5–7). Our recent study showed that the oral route of immunization by HIV env peptide vaccines in the presence of an adjuvant which activates Natural Killer T cells (NKT) can induce effective adaptive anti-viral CD8 responses (8).

Adjuvants bridge the innate and adoptive immune responses to prime strong and specific immunity (9–10). NKT cells are important members of the innate immunity that are activated in response to specific glycolipids such as α GalCer presented by dendritic cells (DC) in the context of the CD1d surface molecule (11). The potency of α GalCer delivery (both systemic and mucosal) to enhance immunity against tumors (12) and intracellular infections such as hepatitis (13) and malaria (14) has been well established. We have found orally delivered α GalCer enhanced humoral and cell-mediated immunity to the co-administered antigens (8).

While the mechanisms underlying the potential of α GalCer to serve as a vaccine adjuvant are not fully understood, it is generally believed that the DCs are the critical players in presenting α GalCer and vaccines to NKT cells and adaptive immune cells, respectively (15). It was found that targeting α GalCer and vaccines to the same DC population is critical for the adjuvant effect of α GalCer (16). There is a need for novel formulation of vaccines that can simultaneously deliver α GalCer and protein subunit vaccines to DCs.

Nanoparticle and microparticle formulations of vaccines have been shown to be superior to free form of vaccine (17–19), since these particles are selectively phagocytosed by professional antigen presenting cells, DCs. In this study, we tested the utility of microparticle-formulated α GalCer and subunit vaccines for sublingual injection.

Materials and Methods

Preparation of polylactic acid (PLA)-based microparticles and surface conjugation of streptavidin

The PLA-based microparticles (500 nm in size) were prepared according to our previously published methods (20). Four hundred milligrams of PLA was dissolved in 2 mL of dichloromethane in a glass tube, and 100 μ L of Milli-Q water was added to the polymer solution. The polymer solution was then sonicated for 15 s to create the primary emulsion. 4 mL of an aqueous 1% (w/v) solution of PEMA (poly[ethylene-alt-maleic acid]) was added to the tube, and the sonication step was repeated. After the second sonication, the emulsion was poured into 100 mL of 0.3% (w/v) aqueous solution of the same stabilizer used for the second emulsion, under rapid stirring with a magnetic stirrer. The resulting microparticles were stirred in the solution for 3 h to evaporate the organic solvent. The microparticles were then washed three times with Milli-Q water and re-suspended in 4 mL of Milli-Q water for use.

To conjugate streptavidin to microparticles, 12.5 mg microparticles were re-suspended in coupling buffer (50 mM MES, pH 5.2) at 170 μ L. 20 μ L of EDAC (N-[3-Dimethylaminopropyl]-N'-ethylcarbodiimide hydrochloride) solution (200 g/L) was added to the suspension and incubated at room temperature with continuous mixing for 15 min. Next, 200 μ g of streptavidin was added and mixed gently for 1 h at room temperature. The streptavidin-conjugated microparticles were stored in 10 mM Tris buffer, pH 0.5% with 0.05% bovine serum albumin. 2 different batches of microparticles were synthesized and studied in separate experiments.

Particle size was measured using dynamic light scattering at a 90° scatter angle on a ZetaPLUS particle electrophoresis system (Brookhaven Instruments Corp., Holtsville, NY). Correlation data were analyzed using a BI-9000AT Digital Autocorrelator (Brookhaven), and a nonnegativity-constrained least squares (CONTIN) approximation was used to calculate the particle size distribution (Figure 1). To measure the surface charges of particles, particles were suspended in 50 mM phosphate buffer (pH 7.2), and measured in Malvern DIP cell kit (Malvern Instruments, UK) by Malvern Zetasizer Nano ZS system (Table 1).

Preparation of microparticles containing α GalCer and ovalbumin

The synthesis of biotinylated α GalCer was previously published (21). Ovalbumin was from Sigma (Grade V A5503). Biotinylated ovalbumin was prepared by a EZ-Link Sulfo-NHS-Biotinylation Kit from Pierce Protein Biology products (Rockford, IL). For treatment of each mouse, 12.5 μ g of streptavidin-coated microparticles, 10 μ g of biotinylated ovalbumin and/or 2 μ g of biotinylated α GalCer were mixed and incubated for overnight at 4 °C under

constant shaking. Microparticles were washed by PBS for 3 times by centrifugation at 2000 × g.

Examining the rate of binding for biotinylated αGalCer and Ovalbumin

The biotinylated αGalCer, when present in solution, can be measured biologically in an NKT cell stimulation assay. We could not find detectable NKT stimulation activity of the post-binding supernatant after the biotinylated αGalCer was mixed overnight with streptavidin microparticles. Thus the biotinylated αGalCer was considered as 100% bound to microparticles. By ELISA detection with a rabbit polyclonal anti-ovalbumin antibody from Abcam (Cambridge, MA), we could not detect presence of ovalbumin in the post-binding supernatant after biotinylated ovalbumin was mixed overnight with streptavidin microparticles, which suggested 100% of biotinylated ovalbumin were bound to microparticles.

Sublingual and intravenous vaccination

Individual groups of 6 week old C57Bl/6 mice (n=3) were anesthetized by 80mg/kg of ketamine and 10mg/kg of xylazine, before being vaccinated with the soluble form of ovalbumin and αGalCer, the microparticles containing ovalbumin and αGalCer, and control PBS by intravenous or sublingual route of injection. Intravenous injection was via the tail vein in a total volume of 100 µl in PBS. Sublingual injection was underneath the mucous membrane in 20 µl of PBS. All injections were performed with 0.5 ml syringe and 28G1/2 needle (Becton Dickinson and Company, Franklin Lakes, NJ).

Immune monitoring of antibody responses

Saliva flow was induced by an intraperitoneal injection of 20µL of phosphate-buffered saline (PBS) containing isoproterenol (0.02 mg/ml) and pilocarpine (0.05 mg/ml). Saliva was then collected for 10 minutes from the mouse oral cavity using a micropipette. Ovalbumin antigen-specific IgA and IgG levels in serum and saliva were measured using the standard ELISA procedures and isotype-specific secondary antibodies. Goat anti-mouse total IgG, IgG1, IgG2c, IgG2b, IgG3, IgA, and IgE were from Southern Biotech (Birmingham, Alabama).

Statistic analysis of data

The raw ELISA measurements were transformed to the logarithmic scale prior to statistical analysis. Analysis of variance (ANOVA) method was used to estimate and compare the groups for each biomarker. Dunnett's adjustment was used to control overall type I error rate at 5% for pairwise comparisons against the control group. All tests were two-sided and p-values of 5% or less after adjustment were considered statistically significant. Statistical analysis was carried out using SAS version 9.3 (SAS Institute, Cary, NC).

Results

Sublingual immunization by microparticles triggers systemic antibody responses

Based on our observation that α GalCer conjugated to the surface of nanoparticles and microparticles could avoid the anergy of NKT cells, we have tested its application in delivering protein subunit vaccines by using ovalbumin as a model antigen. As shown in Figure 2, three doses of vaccination by sublingual route using microparticles containing OVA and α GalCer induced OVA-specific IgG titer of 1:200,000 one week after immunization, and this antibody titer lasted for at least 3 months post vaccination. Vaccination by intravenous route using microparticles containing OVA and α GalCer induced 20 to 50 fold weaker serum IgG titer as compared to sublingual route ($p<0.05$).

We have also compared the sublingual delivery of microparticle-formulated OVA and α GalCer to soluble OVA and α GalCer. The microparticle formulation caused a 10 fold of higher serum IgG titer through sublingual route as compared to mixed soluble OVA and α GalCer ($p<0.05$).

Interestingly, microparticles containing OVA alone also induced very strong serum IgG titer (1:100,000) when injected sublingually, but only a very weak serum IgG titer (1:4,000) was also observed when microparticles containing OVA were injected intravenously. This finding led us to focus our study on sublingual route of immunization and subsequently analyzed the subclasses of immunoglobulins with or without α GalCer adjuvant.

Subclasses of immunoglobulins induced by sublingually-injected microparticles

α GalCer is a well known NKT agonist which regulates the production of both Th1 and Th2 cytokines, which may influence the outcome of immunoglobulin class switch. In mice, IgG1, IgG2b, and IgE are enhanced by the Th2 cytokines such as IL4, while IgG2c is regulated by Th1 cytokines (e.g. IFN γ). We thus further studied the subclasses of immunoglobulins in mice vaccinated by microparticles.

Serum IgG1—Figure 3 shows that IgG1 was a main type of immunoglobulin induced by sublingual injection of microparticles containing OVA and α GalCer, with a titer of $>1:100,000$. Sublingual injection of microparticles containing only OVA also induced comparable titer of IgG1 (1:300,000). Sublingual injection of the soluble form of OVA and α GalCer induced much weaker IgG1 (1:5,000).

Serum IgG2c—The highest titer of IgG2c response was induced by sublingual delivery of microparticles containing OVA and α GalCer (1:40,000). In contrast, microparticles containing only OVA induced 20 fold weaker IgG2c response (1:2000, $p<0.05$).

Serum IgG2b—Sublingual injection of microparticles containing OVA and α GalCer induced IgG2b at a titer of 1:160,000. Sublingual injection of microparticles containing only OVA also induced comparable titer of IgG1 (1:40,000).

Serum IgG3—No IgG3 was detectable in any group of immunized mice.

Serum IgA—Sublingual injection of microparticles containing OVA and α GalCer induced a titer of IgA at 1:1000. IgA could also be detected in groups vaccinated by microparticles containing OVA alone. One way ANOVA analysis by Kruskal-Wallis test could not find significant difference in antibody titers between these 2 groups.

IgE—Sublingual injection of microparticles containing OVA and α GalCer induced a low titer of IgE (1:1000). IgE could also be detected in groups vaccinated by microparticles containing OVA alone by intravenously injected microparticles.

Sublingual immunization by microparticles triggers antibody responses in mucosal compartment

Immune responses in mucosal compartments are critical for protection against microbial infection and antitumor immunity. We have measured the secretion of IgG and IgA in saliva (Figure 4).

Mucosal IgG—Sublingually-injected microparticles containing OVA and α GalCer induced secretion of IgG in saliva (1:600). Similar level secretion of IgG in saliva was observed in mice which received sublingual injection of microparticles containing OVA alone. No secreted IgG was detected in saliva for the group sublingually vaccinated by soluble form of α GalCer and Ovalbumin.

Mucosal IgA—Sublingually-injected microparticles containing OVA and α GalCer induced IgA secretion in saliva. The average titer of IgA is 1:80. In contrast, no IgA was detectable in saliva of the group vaccinated by microparticles only containing OVA.

For the group which received sublingual injection of soluble OVA and α GalCer, IgA was detected at lower level in saliva ($p < 0.05$ as compared to microparticles containing α GalCer and ovalbumin).

Discussion

The immune response in mucosal compartments is the first line immune defense against microbes. It is also possibly involved in pathogenesis of mucosal cancer lesions such as oral, throat cancer, and cervical cancer (22). In this study, we examined the role of NKT cell function in jump starting adaptive immunity in the oral cavity. We found that the sublingual injection of vaccine formulation in microparticles induced antibody responses systemically, which suggested that the microparticles have effectively trafficked to draining lymph nodes and primed the naïve T cells and B cells. The antibody responses lasted for more than 3 months. IgG2c was induced, suggesting Th1-cytokine mediated antibody responses. However, weak IgE (titer lower than 1:1000) was also observed, indicating Th2 cytokines were also involved in NKT cell activation. Sublingually-injected microparticles containing OVA and α GalCer induced IgG and IgA secretion in mucosal compartments, which has previously been reported for intranasal immunization by virus antigens with α GalCer as adjuvant (23–25).

The cell types that are involved in inducing adaptive immune responses remain unclear. Microparticles are most likely phagocytosed and presented by macrophages and DCs according to our previous studies (20). It was reported by several groups that cognate help provided by NKT cells could induce short-term antibody responses (23–30) but failed to generate long lived memory pool of B cells (31). For the microparticles which we used as vaccine carrier, Bcl-6-expressing follicular CD4 T helper cells might be the key helper cells which provided help for the class switch and memory B cell differentiation (32–33).

CD8 T cells are essential for antiviral and antitumor immunity. We found sublingual delivery of microparticles triggered effective CD8 responses in the spleen, which could kill tumor cells (data to be published elsewhere). However, the T cells in the mucosal compartment remain to be studied, due to presence of few lymphocytes in the oral cavity. Tools like transgenic T cells (34) and B cells (35) which recognize antigens like ovalbumin and hen egg lysosome are being used to study the biology of these effector cells in oral cavity and other mucosal compartments.

When this work was in progress, Bershteyn et al. reported that strong class-switched, high avidity humoral immune responses can be elicited by subcutaneous vaccination with poly(lactide-co-glycolide) micro- or nano-particles enveloped by PEGylated phospholipid bilayers, with protein antigens covalently anchored to the lipid surface and lipophilic adjuvants inserted in the bilayer coating (36). In our study, we have focused on mucosal route of delivery by sublingual injection, with aim to induce antibody responses in mucosal compartments.

In this study, we have directly injected microparticles sublingually by 28G1/2 needle. This method may be feasible for treating head and neck cancer patients with mucosal lesions. However, it is clearly not applicable for prophylactic vaccines to prevent infectious diseases like HIV and HPV. It remains to be studied whether such microparticles can be delivered through micro injection patches and micro needle delivery systems.

In summary, this study suggests that NKT cells in the oral cavity serve as a critical arm in innate immunity, which exerts unique adjuvant effects in regulating immune memory, class switch of B cells, and mucosal secretion of antibodies.

Acknowledgments

We thank Wei (Auston) Wei for statistic analysis of data. ESD is supported by National Cancer Institute training Grant T32CA009598. DZ is supported by The University of Texas MD Anderson Cancer Center and National Institutes of Health grant AI079232. The University of Texas MD Anderson Cancer Center is supported in part by National Institutes of Health grant CA16672.

References

1. Lehner T, Anton PA. Mucosal immunity and vaccination against HIV. *AIDS*. 2002; 16(Suppl 4):S125–132. [PubMed: 12699009]
2. Simecka JW. Mucosal immunity of the gastrointestinal tract and oral tolerance. *Adv Drug Deliv Rev*. 1998; 34:235–259. [PubMed: 10837680]
3. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med*. 2005; 11:S45–S53. [PubMed: 15812489]

4. Wu H-Y, Abdu S, Stinson D, Russell MW. Generation of female genital tract antibody responses by local or central (common) mucosal immunization. *Infect Immun*. 2000; 68:5539–5545. [PubMed: 10992451]
5. van Kempen MJ, Rijkers GT, Van Cauwenberge PB. The immune response in adenoids and tonsils. *Int Arch Allergy Immunol*. 2000 May; 122(1):8–19. Review. [PubMed: 10859465]
6. Lü FX, Jacobson RS. Oral mucosal immunity and HIV/SIV infection. *J Dent Res*. 2007 Mar; 86(3): 216–26. Review. [PubMed: 17314252]
7. Boyaka PN, Wright PF, Marinaro M, Kiyono H, Johnson JE, Gonzales RA, Ikizler MR, Werkhaven JA, Jackson RJ, Fujihashi K, Di Fabio S, Staats HF, McGhee JR. Human nasopharyngeal-associated lymphoreticular tissues. Functional analysis of subepithelial and intraepithelial B and T cells from adenoids and tonsils. *Am J Pathol*. 2000 Dec; 157(6):2023–35. [PubMed: 11106575]
8. Courtney AN, Nehete PN, Nehete BP, Thapa P, Zhou D, Sastry KJ. Alpha-galactosylceramide is an effective mucosal adjuvant for repeated intranasal or oral delivery of HIV peptide antigens. *Vaccine*. 2009 May 26; 27(25–26):3335–41. [PubMed: 19200849]
9. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science*. 1996; 272:50–54. [PubMed: 8600536]
10. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell*. 1997; 91:295–298. [PubMed: 9363937]
11. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol*. 2003; 21:483–513. Epub 2001 Dec 19. Review. [PubMed: 12543936]
12. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, Yamaji K, Koezuka Y, Kobayashi E, Fukushima H. Structure-activity relationship of α -galactosylceramides against B16-bearing mice. *J Med Chem*. 1995; 38:2176–2187. [PubMed: 7783149]
13. Kakimi K, Guidotti LG, Koezuka Y, Chisari FV. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *J Exp Med*. 2000; 192:921–930. [PubMed: 11015434]
14. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, Wilson JM, Schmieg J, Kronenberg M, Nakayama T, Taniguchi M, Koezuka Y, Tsuji M. Natural killer T cell ligand α -galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med*. 2002; 195:617–624. [PubMed: 11877484]
15. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by α -galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to co-administered protein. *J Exp Med*. 2003; 198:267–279. [PubMed: 12874260]
16. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, Schmidt R, Harris AL, Old L, Cerundolo V. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol*. 2003 Nov 15; 171(10):5140–7. [PubMed: 14607913]
17. Zwiorek K, Bourquin C, Battiany J, Winter G, Endres S, Hartmann G, Coester C. Delivery by cationic gelatin nanoparticles strongly increases the immunostimulatory effects of CpG oligonucleotides. *Pharm Res*. 2008 Mar; 25(3):551–62. Epub 2007 Oct 3. [PubMed: 17912489]
18. Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, Lee LK, Swartz MA, Hubbell JA. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. *Nat Biotechnol*. 2007 Oct; 25(10):1159–64. Epub 2007 Sep 16. [PubMed: 17873867]
19. Uto T, Wang X, Sato K, Haraguchi M, Akagi T, Akashi M, Baba M. Targeting of antigen to dendritic cells with poly(γ -glutamic acid) nanoparticles induces antigen-specific humoral and cellular immunity. *J Immunol*. 2007 Mar 1; 178(5):2979–86. [PubMed: 17312143]
20. Thapa P, Zhang G, Xia C, Gelbard A, Overwijk W, Liu C, Hwu P, Chang DZ, Courtney A, Sastry JK, Wang PG, Li C, Zhou D. Nanoparticle formulated α -galactosylceramide activates NKT cells without inducing anergy. *Vaccine*. 2009 May 26; 27(25–26):3484–8. [PubMed: 19200815]
21. Xia C, Zhang W, Zhang Y, Woodward RL, Wang J, Wang PG. Facile Synthesis of Biotin Labelled α -Galactosylceramide as Antigen for Invariant Natural Killer T Cells. *Tetrahedron*. 2009; 65:6390–5.

22. Mitchell MF, Tortolero-Luna G, Wright T, Sarkar A, Richards-Kortum R, Hong WK, Schottenfeld D. Cervical human papillomavirus infection and intraepithelial neoplasia: a review. *J Natl Cancer Inst Monogr.* 1996; (21):17–25. Review. [PubMed: 9023824]
23. Kamijuku H, Nagata Y, Jiang X, Ichinohe T, Tashiro T, Mori K, Taniguchi M, Hase K, Ohno H, Shimaoka T, Yonehara S, Odagiri T, Tashiro M, Sata T, Hasegawa H, Seino KI. Mechanism of NKT cell activation by intranasal coadministration of alpha-galactosylceramide, which can induce cross-protection against influenza viruses. *Mucosal Immunol.* 2008 May; 1(3):208–18. [PubMed: 19079180]
24. Youn HJ, Ko SY, Lee KA, Ko HJ, Lee YS, Fujihashi K, Boyaka PN, Kim SH, Horimoto T, Kweon MN, Kang CY. A single intranasal immunization with inactivated influenza virus and alpha-galactosylceramide induces long-term protective immunity without redirecting antigen to the central nervous system. *Vaccine.* 2007 Jul 9; 25(28):5189–98. [PubMed: 17548137]
25. Ko SY, Ko HJ, Chang WS, Park SH, Kweon MN, Kang CY. alpha-Galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. *J Immunol.* 2005 Sep 1; 175(5):3309–17. [PubMed: 16116223]
26. Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, Besra GS, Brenner MB. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U S A.* 2008 Jun 17; 105(24):8339–44. [PubMed: 18550809]
27. Barral P, Eckl-Dorna J, Harwood NE, De Santo C, Salio M, Illarionov P, Besra GS, Cerundolo V, Batista FD. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. *Proc Natl Acad Sci U S A.* 2008 Jun 17; 105(24):8345–50. [PubMed: 18550831]
28. Noda K, Kodama S, Umemoto S, Abe N, Hirano T, Suzuki M. Nasal vaccination with P6 outer membrane protein and alpha-galactosylceramide induces nontypeable *Haemophilus influenzae*-specific protective immunity associated with NKT cell activation and dendritic cell expansion in nasopharynx. *Vaccine.* 2010 Jul 12; 28(31):5068–74. [PubMed: 20478344]
29. Devera TS, Aye LM, Lang GA, Joshi SK, Ballard JD, Lang ML. CD1d-dependent B-cell help by NK-like T cells leads to enhanced and sustained production of *Bacillus anthracis* lethal toxin-neutralizing antibodies. *Infect Immun.* 2010 Apr; 78(4):1610–7. [PubMed: 20123711]
30. Lindqvist M, Persson J, Thörn K, Harandi AM. The mucosal adjuvant effect of alpha-galactosylceramide for induction of protective immunity to sexually transmitted viral infection. *J Immunol.* 2009 May 15; 182(10):6435–43. [PubMed: 19414797]
31. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, Hogan JJ, Cerundolo V, Tangye SG, Bittman R, Nutt SL, Brink R, Godfrey DI, Batista FD, Vinuesa CG. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol.* 2011 Nov 27; 13(1):35–43. DOI: 10.1038/ni.2166 [PubMed: 22120117]
32. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang YH, Dong C. Bcl6 mediates the development of T follicular helper cells. *Science.* 2009 Aug 21; 325(5943):1001–5. [PubMed: 19628815]
33. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, Srivastava M, Linterman M, Zheng L, Simpson N, Ellyard JI, Parish IA, Ma CS, Li QJ, Parish CR, Mackay CR, Vinuesa CG. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity.* 2009 Sep 18; 31(3):457–68. [PubMed: 19631565]
34. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell.* 1994; 76(1):17–27. [PubMed: 8287475]
35. Phan TG, Amesbury M, Gardam S, Crosbie J, Hasbold J, Hodgkin PD, Basten A, Brink R. B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. *J Exp Med.* 2003 Apr 7; 197(7):845–60. [PubMed: 12668643]
36. Bershteyn A, Hanson MC, Crespo MP, Moon JJ, Li AV, Suh H, Irvine DJ. Robust IgG responses to nanograms of antigen using a biomimetic lipid-coated particle vaccine. *J Control Release.* 2012 Feb 10; 157(3):354–65. [PubMed: 21820024]

Highlights

- Alpha-galactosylceramide was used as adjuvant in microparticle vaccines
- Microparticle vaccines containing glycolipid were sublingually injected
- A 20 fold higher Th1-biased antibody response was induced
- Induction of IgG and IgA antibody secretion in oral cavity was accomplished

Average size: 606.4 ± 35.2 , Polydistribution: 0.068

Figure 1. Size analysis of microparticles

Microparticles were resuspended in 10 mM phosphate-buffered saline solution (pH 7.0) and analyzed by dynamic light scattering. Data were representative of 3 different batches of microparticles.

Figure 2. Sublingually-delivered microparticles induced sustained antibody responses in mice
 A. Groups of C57Bl/6 mice (n=3) were vaccinated by sublingual injection of microparticles containing OVA and αGalCer with control groups of different formulations and routes of vaccination; B. Serum IgG titer toward OVA antigen was measured by ELISA at day 21 and day 90 respectively. Data were representative of 3 independent experiments using 3 different batches of microparticles.

Figure 3. Sublingually-injected microparticles containing αGalCer and OVA induced 20 fold higher Th1 antibodies (IgG2c) than microparticles containing OVA alone
Data were representative of 3 independent experiments using 3 different batches of microparticles.

Figure 4. Sublingually-injected microparticles containing α GalCer and OVA induced secretion of both IgG and IgA antibodies in mucosal compartments

Data were representative of 3 independent experiments using 3 different batches of microparticles.

Table 1Surface charges of micro-particles after loading of glycolipid adjuvant α GalCer and Ovalbumin

Content of micro-particles	Surface charge (mV)
Unloaded particles	-21.2 $\pm$ 0.2
Particles loaded with Ovalbumin	-17.3 $\pm$ 1.9
Particles loaded with α GalCer and Ovalbumin	-19.9 $\pm$ 0.9

12.5 μ g of streptavidin-coated microparticles, 10 μ g of biotinylated ovalbumin and/or 2 μ g of biotinylated α GalCer were mixed and incubated for overnight at 4 °C under constant shaking. Microparticles were washed by PBS for 3 times by centrifugation at 2000 $\times$ g.

Particles were suspended in 50 mM phosphate buffer (pH7.2), and measured in Malvern DIP cell kit (Malvern Instruments, UK) by Malvern Zetasizer Nano ZS system. No significant difference on surface charges were found after the particles were loaded with ovalbumin, or with ovalbumin plus α GalCer. Data were representative of two independent experiments.