Immunization with HSV-2 gB-CCL19 Fusion Constructs Protects Mice against Lethal Vaginal Challenge

Yan Yan, Kai Hu, Xu Deng, Xinneng Guan, Sukun Luo, Lina Tong, Tao Du, Ming Fu, Mudan Zhang, Yalan Liu and Qinxue Hu

*J Immunol* 2015; 195:329-338; Prepublished online 20 May 2015;
doi: 10.4049/jimmunol.1500198
http://www.jimmunol.org/content/195/1/329

Supplementary Material http://www.jimmunol.org/content/suppl/2015/05/20/jimmunol.1500198.DCSupplemental

References This article cites 61 articles, 26 of which you can access for free at: http://www.jimmunol.org/content/195/1/329.full#ref-list-1

Why *The JI*? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Immunization with HSV-2 gB-CCL19 Fusion Constructs Protects Mice against Lethal Vaginal Challenge

Yan Yan,*,† Kai Hu,*,† Xu Deng,*,† Xinneng Guan,*,† Sukun Luo,*,† Lina Tong,* Tao Du,* Ming Fu,*,† Mudan Zhang,*,† Yalan Liu,* and Qinxue Hu,*†

There is a lack of an HSV-2 vaccine, in part as the result of various factors that limit robust and long-term memory immune responses at the mucosal portals of viral entry. We previously demonstrated that chemokine CCL19 augmented mucosal and systemic immune responses to HIV-1 envelope glycoprotein. Whether such enhanced immunity can protect animals against viral infection remains to be addressed. We hypothesized that using CCL19 in a fusion form to direct an immunogen to responsive immunocytes might have an advantage over CCL19 being used in combination with an immunogen. We designed two fusion constructs, plasmid (pgBIZCCL19 and pCCL19IZgB), by fusing CCL19 to the C- or N-terminal end of the extracellular HSV-2 glycoprotein B (gB) with a linker containing two (Gly4Ser)2 repeats and a GCN4-based isoleucine zipper motif for self-oligomerization. Following immunization in mice, pgBIZCCL19 and pCCL19IZgB induced strong gB-specific IgG and IgA in sera and vaginal fluids. The enhanced systemic and mucosal Abs showed increased neutralizing activity against HSV-2 in vitro. Measurement of gB-specific cytokines demonstrated that gB-CCL19 fusion constructs induced balanced Th1 and Th2 cellular immune responses. Moreover, mice vaccinated with fusion constructs were well protected from intravaginal lethal challenge with HSV-2. Compared with pgB and pCCL19 coinmunization, fusion constructs increased mucosal surface IgA+ cells, as well as CCL19-responsive immunocytes in spleen and mesenteric lymph nodes. Our findings indicate that enhanced humoral and cellular immune responses can be achieved by immunization with an immunogen fused to a chemokine, providing information for the design of vaccines against mucosal infection by HSV-2 and other sexually transmitted viruses. The Journal of Immunology, 2015, 195: 329–338.

Herpes simplex virus-2, the major cause of genital herpes, is one of the most prevalent pathogens. HSV-2 is transmitted primarily through sexual contact and can cause ocular disease, long-term neonatal neurologic sequelae, or even mortality (1, 2), resulting in a significant financial burden on health systems worldwide. HSV-2 infection can also increase the risk for HIV-1 acquisition by 3- to 3-fold (3, 4). Despite intensive studies over the last two decades, an effective vaccine against HSV-2 has yet to be developed (5, 6).

HSV-2 glycoprotein B (gB) and glycoprotein D (gD) are the two commonly used immunogens in the development of HSV-2 subunit vaccines (5, 7–9). However, most findings suggested that one glycoprotein or several glycoproteins in combination are still not sufficient to induce effective immune responses against HSV-2 infection. One major limitation to subunit vaccine candidates is their poor immunogenicity compared with live-attenuated and inactivated viral vaccines (5, 10, 11). Adjuvantation is one of the simplest, but most effective, ways to improve the immunogenicity of vaccine candidates (5, 12). Components from the host immune system, such as cytokines, chemokines, and other costimulatory self-molecules, have received much attention as novel molecular adjuvants because of their essential roles in immunocyte differentiation, proliferation, expansion, maturation, and migration (13, 14). Among these components, chemokines have been widely investigated because they can bridge innate and adaptive immunity and/or direct the migration of immunocytes (14–17).

CCL19, a CCR7 ligand, is one of the key molecules in establishing functional microenvironments for the initiation of immune responses in secondary lymphoid tissues (17, 18). CCL19, through interactions with its cognate receptor CCR7, plays a pivotal role in recruiting immunocytes, including T cells (16), B cells (19, 20), and mature dendritic cells (DCs) (21–26), to secondary lymphoid organs, such as endothelial venules of lymph nodes and Peyer’s patches, as well as the T cell zones of spleen and lymph nodes (18, 19, 21, 26–30). The CCL19-CCR7 pathway is also important in mediating lymphocyte colocalization and Ag-specific T cell activation (26), as well as in directing the differentiation of DC-assisted B cells into Ab-secreting cells (ASCs) (16, 31). CCL19 as a molecular adjuvant was proved to be useful in several immunization models and is capable of promoting Ag-specific humoral and cellular immune responses in combination with vaccine candidates, including HSV-1 gB (14), HIV-1 Env (16), pseudorabies virus gB (32), and HCV core DNA (30).

Given that the main function of chemokines is to modulate the migration of responsive immunocytes, we hypothesized that fusion of an immunogen with a chemokine may represent an effective approach in enhancing Ag immunogenicity through directing the candidate immunogen to mucosae. It is generally accepted that...

---

The Journal of Immunology

*State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China; †University of Chinese Academy of Sciences, Beijing 100049, China; and Institute for Infection and Immunity, St. George’s University of London, London SW17 0RE, United Kingdom

Received for publication January 26, 2015. Accepted for publication April 20, 2015.

This work was supported by National Natural Science Foundation of China Grant 81273250, Ministry of Science and Technology of China Grants 2010CB530100, 2013ZX10001005-003-002, and 2012ZX10001006-002, and the Hotung Trust.

Address correspondence and reprint requests to Prof. Qinxue Hu, State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, 44 Xiaoxiaoshan Zhonggu, Wuhan 430071, China. E-mail address: qhx@whiov.cn.

The online version of this article contains supplemental material.

Abbreviations used in this article: ASC, Ab-secreting cell; CTX, cholera toxin; DC, dendritic cell; gB, glycoprotein B; gD, glycoprotein D; (G4S)2, (Gly4Ser)2; IZ, GCN4-based isoleucine zipper trimerization domain; i.vag., intravaginally; MLN, mesenteric lymph node; MLNL, MLN lymphocyte; p, plasmid.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500198

Copyright 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00
Ags fused to ligands of target cell surface molecules can facilitate Ag uptake and presentation and, consequently, induce the activation of responsive immune cells (33–37). Fusion of HIV-1 Env to several cytokines, including APRIL, BAFF, CD40L, IL-12, and GM-CSF, demonstrated improved immunogenicity of the DNA vaccines (33, 35, 36). However, whether such enhanced immunogenicity can protect immunized animals against virus infection remains to be fully addressed.

In this study, by using mice as a vaccination and challenge model, we designed and constructed two DNA constructs encoding GCN4-based isoleucine zipper trimerization domain (IZ)-linked CCL19 and HSV-2 gB and further investigated the ability of gB-CCL19 fusion constructs to induce protective immune responses against HSV-2 infection in mice.

Materials and Methods

Plasmid construction

pCDNA3.1 (+) (Invitrogen, Groningen, the Netherlands) was used as vector for p construction, unless otherwise indicated. Murine pCCL19 was described previously (16). Truncated gB (730R; pgB) was amplified from the HSV-2 genome (G strain; LGC Standards), as described previously (9) and subcloned into pCDNA3.1 (+) (16). Truncated gB also was subcloned into pET28a (Novagen; pET-gB) for prokaryotic expression. A linker composed of IZ and (Gly)8-12-(Gly)16 hydrophobic polypeptides [(Gly)8]-IZ-(Gly)16 was used in fusion constructs to promote gB trimerization (35), which was introduced between CCL19 and gB by splicing.

Forty-eight hours posttransfection, the protein-containing supernatants were harvested. Supernatants of sera and vaginal fluids were aliquoted and stored at −80°C until use.

Mice, HSV-2, and cell lines

Six- to eight-week-old female BALB/c mice were purchased from Beijing HFK Biotechnology (Beijing, China) and housed in specific pathogen–free conditions, with sterile food and water supplied. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee and performed according to the guidelines of the Hubei Laboratory Animal Science Association (Wuhan, China, Approval ID: WIVA1121301). HSV-2 (G strain) was propagated and titrated on Vero cells, as described previously (38). Virus stocks were aliquoted and stored at −80°C until use. Vero and 293T cells were purchased from the American Type Culture Collection and cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), penicillin (100 U/ml), and streptomycin (100 µg/ml).

Prokaryotic expression and purification of gB

pET-gB was expressed in an Escherichia coli system (Rosetta strain; Novagen) and purified as described previously with modifications (39). Briefly, bacteria bearing pET-gB were induced for 4 h with isopropyl-β-D-thiogalactoside, and gB proteins were harvested from the insoluble fraction of ultrasonically-treated bacteria by centrifugation (1200 × g, 30 min at 4°C). Insoluble gB proteins were subsequently denatured in denaturing buffer (10 mM Tris, 500 mM NaCl, 6 M GdCl4, 5 mM DTT [pH 8]) for 14 h and refolded in refolding buffer (20 mM Tris-Cl, 500 mM NaCl, 3 mM glutathione, 0.3 mM glutathione disulfide, 10% glycerol [pH 8]) for 24–48 h. After refolding, the refolded gB in supernatants was loaded onto a pre-equilibrated nickel-charged chelating Sepharose Fast Flow column (GE Healthcare). A six-His tag on the C-terminal end of gB facilitated the purification of the complex, followed by elution with imidazole buffer (20 mM Tris-Cl, 500 mM NaCl, 3 mM glutathione, 0.3 mM glutathione disulfide, 300 mM imidazole, 10% glycerol [pH 8]). Subsequently, purified gB was dialyzed against PBS and concentrated. Protein concentration was determined using a BCA Assay Kit (Pierce, Thermo Scientific). The quality of purified gB was determined by SDS-PAGE and Western blotting analysis. Purified products were aliquoted and stored at −80°C until use.

Expression analysis of fusion constructs

293T cells were transiently transfected with endotoxin-free pCDNA3.1, pgB, pgBIZCCL19, pgBIZCCL19, and pCCL19IgZgB using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Forty-eight hours posttransfection, the protein-containing supernatants were harvested for gB and CCL19 quantification. gB was detected by Western blotting, whereas CCL19 concentration was measured by ELISA using commercial mouse CCL19 DuoSet ELISA kits (R&D Systems), according to the manufacturer’s instructions.

SDS-PAGE, native PAGE, and Western blotting

293T cells were transfected with pgB, pgBIZCCL19, or pCCL19IgZgB for 48 h, and culture supernatants were harvested and resolved by 12% denaturing SDS-PAGE and 4–15% continuous gradient native PAGE, respectively. Subsequently, separated proteins were transferred onto a polyvinylidene difluoride membrane, and gB-specific bands were detected by Western blotting, according to a protocol described previously (9). gB (1–100 µg) (1:1000 dilution; Santa Cruz, sc-52425) and H126 mAb (1:1000 dilution; Santa Cruz, sc-69799) followed by HRP-labeled donkey anti-sheep IgG (1:5000 dilution; Santa Cruz) were used for gB detection in SDS-PAGE and native PAGE, respectively. The colorimetric reaction was developed as described previously (16, 39).

Immunizations and sampling

Mice (n = 10/group) were immunized using an “prime and boost” strategy twice in a 2-wk interval, according to the immunization procedures shown in Fig. 1D. For test groups, mice were immunized i.m. with 5 µg pgBIZCCL19 or pCCL19IgZgB. For control groups, mice were immunized with 5 µg pgB or pgB plus pCCL19 at a 1:1 ratio. For the negative control group, mice were immunized with 5 µg pcDNA3.1. Immunization was carried out as described previously (16, 40). In brief, pDNAs were dissolved and mixed well in sterile saline solution in a total volume of 10 µl/mouse and injected into the quadriceps muscles of one rear leg of each mouse, followed by electroporation. Fourteen days postboost, peripheral blood samples and vaginal fluids were collected. Forty-nine days postboost (i.e., the day before virus challenge), peripheral blood samples also were collected from fundus oculi. Sera were obtained from coagulated whole blood by centrifugation (800 × g, 15 min at room temperature). The vaginal fluids were collected by washing the vagina three times with sterile PBS plus protease inhibitors (Roche) in a total volume of 100 µl/mouse. Vaginal fluids were centrifuged (12,000 × g, 15 min at 4°C), and supernatants were harvested. Supernatants of sera and vaginal fluids were aliquoted and stored at −80°C until use.

Tissue harvesting

Mice were sacrificed by cervical dislocation at day 14 postboost. Spleens and mesenteric lymph nodes (MLNs) were excised under sterile conditions, and single-cell suspensions were obtained by passing tissue through a 70-µm Falcon cell strainer (BD Biosciences). Immune cells were purified using Mouse 1× Lymphocyte Separation Medium, according to the manufacturer’s instructions (Dakewe Biotech). RBCs were lysed using RBC lysis buffer (Sigma-Aldrich). Purified viable cells were suspended in complete RPMI 1640 medium and counted by a Bio-Rad Automated Cell Counter.

Ag-specific and isotype Ig ELISAs

gB-specific IgG and IgA end point titers in sera and vaginal fluids were measured by Ag-specific direct ELISA. Briefly, 96-well plates (Nunc MaxiSorp; Thermo Scientific) were coated with purified gB (2 µg/ml, 50 µl/well) overnight at 4°C, washed with wash buffer (PBS plus 0.05% Tween-20), and blocked with blocking buffer (PBS plus 1% BSA). Thereafter, plates were incubated with 5-fold serially diluted samples in assay buffer (PBS plus 1% BSA and 0.05% Tween-20) at 37°C for 1 h. For IgG detection, plates were incubated with HRP-conjugated goat anti-mouse IgG at a dilution of 1:5000 (Abcam) at 37°C for 1 h. For IgA detection, plates were incubated with biotin-conjugated goat anti-mouse IgA at a dilution of 1:5000 (Southern Biotechnology) and streptavidin-HRP (R&D System) at a dilution of 1:200 at 37°C for 1 and 0.5 h, respectively. After extensive washes, bound HRP conjugates were detected by TMB-based colorimetric reaction for 5 min at room temperature and stopped with 2 N H2SO4. The color intensity was read by an automatic ELISA reader (Tecan) at a test wavelength of 450 nm and a reference wavelength of 570 nm. End point titers were calculated using GraphPad Prism 6.0 software. Sample dilutions with an OD50 more than the OD450 of the negative control at the same dilution were considered positive.

The subclasses of gB-specific Abs in immunized murine sera were measured by HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, IgG3, and IgM (Southern Biotechnology; 1:500 dilution in PBS plus 0.05% Tween-20), according to the manufacturer’s instructions. Ab concentrations were calculated based on standard curves.
Virus-neutralization assay

The neutralizing activities of sera and vaginal fluids were tested using a plaque assay, as described previously with modifications (41). Briefly, heat-inactivated sera (56°C for 1 h) were prepared in 2-fold serial dilutions from 1:5 to 1:640 (post boost) or from 1:20 to 1:2560 (postchallenge) in DMEM (without FBS). Vaginal fluids were prepared in 2-fold serial dilutions from 1:5 to 1:40 (postbooster) in DMEM (without FBS). The diluted samples in triplicates were subsequently incubated with 100 PFU/ml HSV-2 for 1 h at 37°C. Following incubation, sample–virus mixture was applied to Vero cell monolayers grown in 48-well culture plate (preseeded at 1.2 × 10^4/well 1 d before the assay). Cells receiving HSV-2 alone served as positive controls, whereas cells in the absence of sample–virus mixture were used as background controls. Sera from pcDNA3.1-immunized mice at the same dilution were considered as controls of nonspecific backgrounds in inhibiting plaque formation. After incubation for 48 h, cells were stained with crystal violet, and the plaques were counted for HSV-2 titration. The Ab-mediated neutralization activity was determined based on the dilution titer that reduced the numbers of plaques by 50% compared with the positive control and is expressed as the log_{10} of the dilution titer.

Cytokine assay

Splenocytes (1 × 10^7/well) were added to duplicate wells in 24-well plates in a total volume of 1 ml complete RPMI 1640, with purified gB (1 μg/ml) as stimuli, and cultured at 37°C for 5 d. After incubation, cell culture supernatants were filtrated and used for the detection of Th1/Th2 cytokines (IL-2, IL-4, IL-5, IFN-γ, and TNF). Analysis was performed with a BD Cytometric Bead Array Mouse Th1/Th2 cytokine Kit (BD Biosciences), according to the manufacturer’s instructions. Data acquisition was conducted on a BD FACSAria III platform and analyzed by FCAP Array software 1.0.

Immunohistochemistry assay

Fourteen days postboost, murine colorectal samples were collected and analyzed for IgA+ cell number by immunohistochemistry, in accordance with the procedures described previously, with modifications (16). In brief, an ~2-cm segment from the anus end of each mouse was isolated, cut open longitudinally, and fixed in neutral buffered formalin (diluted 1:10 in PBS) for 24 h. After fixation, tissue was embedded and sectioned. Ag retrieval was performed with Ag Retrieval Reagent Basic (R&D Systems), according to the manufacturer’s instructions. For the detection of IgA+ cells, the slides were stained with goat anti-mouse IgA Ab (AbD Serotec) at a dilution of 1:400 overnight at 4°C, followed by incubation with the Polink-2 Plus Polymer HRP Detection System for Goat Primary Ab (GHI), according to the manufacturer’s instructions. Color reaction was developed with the addition of 3, 3'-diaminobenzidine free base, followed by counterstaining with hematoxylin.

Chemotaxis assay

A chemotaxis assay was performed using a microchamber Transwell system with 3-μm pores (Corning Costar). Purified splenocytes and MLN lymphocytes (MLNlbs; 2 × 10^6/well in 100 μl complete RPMI 1640 medium) were plated in triplicates in the upper chambers, whereas 600 μl complete RPMI 1640 medium, with or without (negative control) 30 ng/ml CCL19 (R&D Systems), was added to the lower chambers. Plates were incubated for 2 h in a 5% CO₂ incubator at 37°C, and the number of migrated cells was counted by a Bio-Rad Automated Cell Counter. Data were expressed as fold change in migration (migrated cell number in testing wells/migrated cell number in negative control wells).

Challenge, scoring, and virus DNA quantification

Five to seven days prior to challenge, vaccinated mice were injected s.c. in the neck ruff with Depo-Provera (medroxyprogesterone acetate, 2 mg/mouse). Forty-nine days postbooster, mice (n = 10/group) were anesthetized with pentobarbital sodium and challenged intravaginally (i. vag.) with 10 μl/mouse HSV-2 (G strain) at a concentration of 2.4 × 10^7 PFU/ml. The sera were collected on days 5, 9, and 11 postchallenge for virus-neutralization assays, and vaginal fluids were collected on days 1, 3, 5, 7, 9, 11, and 15 postchallenge for detection of virus shedding. Virus titers of vaginal fluids were determined by plaque assay on Vero cell monolayers, as described previously (42). Infected mice were monitored daily for weight loss, vaginal inflammation, and death and scored according to the 5-point scale reported by Toka et al. (14): 0, no apparent infection; 1, mild inflammation of the external genitals; 2, redness and moderate swelling of external genitals; 3, severe redness and inflammation; 4, genital ulceration and severe inflammation; and 5, hind limb paralysis and death. Thirty days...
postchallenge or on the day of animal death, the sacral ganglia of each mouse was collected for the detection of latent viral DNA load using a quantitative real-time PCR assay. DNA was isolated from nevy blood and tissue kits (QIAGEN) and stored at −20 °C until analysis. Copies of the HSV-2 genome were quantified by probe quantitative PCR with Premix Ex Taq (TaKaRa) on a Roche LightCycler 480, according to the manufacturer’s instructions. The following primers were used for the amplification reactions: 5′-gG-3′5′-GCCTGCCGTCAGCCCATCCTCT-3′, 3′-gG-5′-GCCTGCCGTCAGCCCATCCTCT-3′, and probe 5′- (FAM) CCTCGGCAGTATGGAGGGTGTCGC (TAMRA)-3′. A standard DNA was used to determine copy numbers, as described previously (41). All samples were tested in triplicates.

Statistical analysis

Data are presented as mean ± SEM. Comparisons between two groups were analyzed by the Student t test, whereas comparisons among more than three groups were analyzed by one-way ANOVA with Newman–Keuls multiple comparison analysis. The p values < 0.05 were considered statistically significant.

Results

Design and expression of gB-CCL19 fusion constructs

We constructed two fusion constructs, pgBIZCCL19 and pCCL19IZgB, by fusing CCL19 to the C- and N-terminal ends of HSV-2 gB (730t), respectively, with a flexible GCN4-based leucine zipper linker being used to facilitate protein folding and trimerization. Expression analysis of these two constructs was performed by Western blotting (gB) and ELISA (CCL19). Western blotting results showed that pgBIZCCL19 and pCCL19IZgB were successfully secreted in the culture medium to a similar level. SDS-PAGE analysis demonstrated that denatured gB and gB-CCL19 fusion proteins (expressed by pgBIZCCL19 and pCCL19IZgB) migrated at the expected apparent molecular masses ~94 and ~108 kDa, respectively (Fig. 1B). Native PAGE analysis demonstrated that gB-CCL19 fusion proteins appeared to be oligomeric, similar to gB protein (9) (Fig. 1B). ELISA results showed that CCL19 was successfully expressed by both fusion constructs, although the expression levels of the fusion constructs appeared to be slightly lower than those in cells transfected with pgB plus pCCL19 (Fig. 1C). The expression difference detected in CCL19 ELISA was likely due to the possibility that the CCL19 Ab is not able to access some of the epitopes in the gB-CCL19 fusion proteins. Nevertheless, altogether, our results indicate that the flexible linker between gB and CCL19 can enable the correct expression of both moieties.

gB-CCL19 fusion constructs enhance gB-specific systemic and mucosal Ab responses

To investigate whether CCL19 in gB-encoding fusion constructs could augment gB-specific humoral immune responses, mice were immunized with pgB, pgBIZCCL19, or pCCL19IZgB or coimmunized with pgB and pCCL19, using the “prime and boost” strategy, at 2-wk intervals. Sera and vaginal fluids were collected for gB-specific IgG and IgA titration. As shown in Fig. 2, coimmunization of pgB with pCCL19 enhanced the levels of gB-specific serum IgA and vaginal IgG and IgA, but not serum IgG, whereas immunization with gB-CCL19 fusion constructs pgBIZCCL19 and pCCL19IZgB further elevated gB-specific IgG and IgA levels in both sera and vaginal fluids. In general, pgBIZCCL19 and pCCL19IZgB showed a comparable ability to enhance gB-specific immune responses in serum and mucosal sites.

gB-CCL19 fusion constructs augment a Th1-biased gB-specific Ab response

It was described that DNA vaccines tend to induce a Th2-biased Ab response (43), with IgG1, but not IgG2a or IgG2b, predominant in HSV-2 gB-vaccinated mice (44). To test whether CCL19 in fusion constructs was functional in modulating the isotypes of Ab responses, four gB-specific IgG subtypes (IgG1, IgG2a, IgG2b, IgG3) and IgM subtype in sera collected at 2 wk postboost were examined. Among the four IgG subtypes in mice, IgG1 is associated with a Th2 profile, whereas the other subtypes are associated with a Th1 profile (45). As shown in Fig. 3, coimmunization of pgB with pCCL19 augmented IgG2a, whereas the levels of IgG1, IgG3, IgG2b, and IgM were similar to those in mice immunized with pgB alone. However, immunization with the two gB-CCL19 fusion constructs elicited robust Ab responses to gB compared with coimmunization of pgB with pCCL19, and it significantly enhanced the levels of gB-specific IgG2a, IgG2b, IgG3, and IgM but did not show an apparent impact on the levels of IgG1. Taken together, these results indicate that CCL19 is capable of regulating the gB-induced Ab response to a Th1-biased profile, and such regulation can be enhanced when CCL19 is fused to gB.

FIGURE 2. Induction of systemic and mucosal Ab responses against gB. Mice were immunized i.m. at days 0 and 14 with electroporation. At day 14 postboost, sera and vaginal fluids were collected, and end point titration of gB-specific IgG and IgA was determined by direct ELISA. Data are mean ± SEM (n = 10 mice/group) of three independent experiments, performed in duplicate for each condition. *p < 0.05, **p < 0.01, ***p < 0.001. NS, not statistically significant.
**gB-CCL19 fusion constructs promote an enhanced virus-neutralizing response**

It was well documented in animal models that an efficacious prophylactic HSV-2 vaccine will most likely induce a robust neutralizing-Ab response (11, 41, 46). Having demonstrated that fusion of CCL19 to gB at either end could significantly augment systemic and mucosal Ab responses to gB, we assessed the neutralizing activity of sera collected at days 14 and 49 postbooster and at days 5, 9, and 11 postchallenge, as well as vaginal fluids tested at day 14 postbooster. As shown in Fig. 4, the neutralizing activities of sera and vaginal fluids against HSV-2 were significantly enhanced at day 14 in all CCL19-immunized mice postbooster, with gB-CCL19 fusion constructs demonstrating a significantly stronger ability to induce anti–HSV-2–neutralizing activity in both sera and vaginal fluids compared with pgB plus pCCL19. The neutralizing activity of sera remained apparent until at least day 49 postbooster, suggesting that a sustained neutralizing-Ab response was elicited by vaccination with gB-CCL19 fusion constructs, with pCCL19IZgB exhibiting slightly stronger neutralizing activity than pgBIZCCL19. In addition, the neutralizing activity was rapidly recalled at day 5 postchallenge, peaked at day 9, and remained constant until at least day 11. In contrast, the neutralizing activity of sera from pgB- or pgB plus pCCL19-immunized mice was lower than that of mice immunized with fusion constructs and declined at day 11 postchallenge. Moreover, the neutralizing activity of sera postchallenge declined sharply in mice immunized with pgB plus pCCL19. Taken together, our results indicate that fusion of gB to CCL19 can enhance neutralizing systemic and mucosal Ab responses; such enhancement is sustainable and can be immediately recalled upon HSV-2 infection.

**gB-CCL19 fusion constructs induce balanced Th1 and Th2 cellular immune responses**

In vivo, CCL19 functions as a key regulator on the migration of T cells and DCs. In this regard, following CCL19 immunization, T cell–mediated immune responses might be upregulated more directly than humoral responses (16, 30). Given the central roles of cytokines secreted by activated immunocytes in defining the subsequent immune response, we evaluated gB-specific Th1- and Th2-like cellular immune responses by measuring the production of Th1-associated (IL-2, TNF, IFN-γ) and Th2-associated (IL-4, IL-5) cytokines. Splenocytes collected from immunized mice at day 14 postbooster were analyzed. As shown in Fig. 5, gB seemed to induce a Th1-dominant cellular response, with the levels of IFN-γ and TNF being significantly higher than those of the other three cytokines. Notably, pgB coimmunization with pCCL19 significantly elevated the production of IL-4, IL-5, IFN-γ, and TNF, and the production of these cytokines was further increased in pgBIZCCL19- or pCCL19IZgB-immunized mice. In contrast, samples from the control group (pcDNA3.1) demonstrated very low levels of cytokine background, with IL-2, IL-4, and IL-5 below the limits of detection. In general, these results indicate that CCL19 enhances balanced Th1- and Th2-like T cell responses, and the enhancement can be strengthened when CCL19 and gB are used in a fusion form.

**gB-CCL19 fusion constructs increase responsive immunocytes in secondary lymph nodes and mucosal sites**

The results above revealed that CCL19 could enhance gB-specific systemic and mucosal Ab responses, as well as balanced Th1- and Th2-like cellular responses. Furthermore, the enhanced immune response could be increased when CCL19 was fused to gB at either terminal. We further investigated the potential mechanisms underlying such immune enhancement. It is known that CCL19 can chemoattract CCR7-expressing immunocytes into certain effector sites or recruit IgA+ cells to colorectal mucosal sites (16, 31). Given that IgA constitutes the main Ab immune response against pathogens at mucosal sites and that a significant Ab increase was observed in our study when gB-CCL19 fusion constructs were used to immunize mice, we examined whether the CCL19-induced Ab elevation at mucosal sites correlated with the increased number of IgA+ cells. Immunohistochemical analysis of colorectal samples revealed that, compared with those in pgB-
immunized mice, IgA+ cells were significantly increased in mice coimmunized with pgB and pCCL19, whereas the cell number was further increased almost 1.8-fold in mice immunized with pgBIZCCL19 or pCCL19IZgB compared with pgB-immunized mice (Fig. 6A, 6B). Notably, these results were in agreement with the difference in gB-specific Ab levels at mucosal sites, with both gB-CCL19 fusion constructs showing a comparable ability to increase IgA+ cells in colorectal mucosal sites, reflecting an enhanced mucosal immune response.

The interaction between CCL19 and CCR7 is an essential process controlling the migration of CCR7+ immunocytes into lymph nodes where immune responses are initiated (24). We conducted assays to determine whether CCL19 fused to gB was functional in directing the migration of CCR7+ immunocytes to spleen and mucosa-related draining lymph nodes (MLNs). Freshly isolated splenocytes and MLNLs from immunized mice were examined to assess their ability to migrate to murine CCL19 protein. As shown in Fig. 6C, compared with pgB alone, immunization with pgB plus pCCL19, pgBIZCCL19, or pCCL19IZgB resulted in ∼1.5-, 2.1-, and 1.8-fold increases, respectively, in CCR7+ immunocytes in spleen, as well as ∼2.1-, 2.6-, and 3.1-fold increases, respectively, in MLNs. In general, coinmunization with pgB and pCCL19 consistently resulted in a significantly higher level of CCR7+ immunocyte influx into spleen compared with pgB alone but a significantly lower level of influx into spleen and MLNs compared with fusion constructs (Fig. 6C). These results were consistent with the findings that gB fused to CCL19 enhanced the numbers of IgA+ plasma cells in colorectal mucosal sites.

gB-CCL19 fusion constructs protect mice against lethal HSV-2 infection

Mucosal responses against HSV-2 are likely to be critical for protection because mucosal sites are the principal portals of HSV-2 sexual transmission. To test whether the gB-CCL19 fusion constructs can establish efficient immune defense against HSV-2 mucosal infection, immunized mice were challenged i.vag. with a lethal dose of HSV-2 at day 49 postboost. Thereafter, virus shedding, latent virus DNA load, development of clinical symptoms, and weight change were monitored.

**Virus shedding.** As shown in Fig. 7A, virus loads in vaginal fluids were detectable but dropped sharply on a daily basis in all groups. At the same tested time points, virus shedding was highest in the negative control group and moderate in the pgB group and the pgB plus pCCL19 group, whereas the lowest virus load was detected in the pgBIZCCL19 and pCCL19IZgB groups. Moreover, mice in the negative control group were all dead before day 13 postchallenge, whereas those from other groups survived, and virus loads in vaginal fluids dropped to levels under...
FIGURE 7. Protection of immunized mice against lethal vaginal HSV-2 challenge. Forty-nine days post-boost, mice were challenged i.vag. with a lethal dose of HSV-2. (A) Virus shedding at the indicated time points in vaginal fluids was tested by plaque assay. The dashed line represents the detection limit. (B) Latent virus DNA load was quantified in sacral ganglia by real-time PCR at day 30 after challenge or on the day of death. Number $\times$ ND indicates that the number of mice in which no virus was detected or was below the detection limit. Weight loss (C) and disease severity (D) were monitored on a daily basis for 15 d. Data are mean ± SEM ($n = 9–14$ mice/group) of results pooled from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS, not statistically significant.

### Table I. HSV-2 shedding incidences in HSV-2–challenged mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No. Animals</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 11</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1</td>
<td>14</td>
<td>14 (100)</td>
<td>14 (100)</td>
<td>14 (100)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pgB</td>
<td>12</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>10 (83.3)</td>
<td>2 (16.7)</td>
<td>ND</td>
<td>ND</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>pgB + pCCL19</td>
<td>11</td>
<td>11 (100)</td>
<td>11 (100)</td>
<td>3 (27.3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>pgBIZCCL19</td>
<td>12</td>
<td>12 (100)</td>
<td>11 (91.7)</td>
<td>4 (33.3)</td>
<td>3 (25.0)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pCCL19IZgB</td>
<td>12</td>
<td>12 (100)</td>
<td>11 (91.7)</td>
<td>6 (50.0)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

HSV-2 shedding incidences in vaginal fluids were detected by real-time PCR. Data shown are pooled from three independent experiments. HSV-2 shedding incidence = (no. vaginal fluids from which viral DNA was detected/total number of vaginal fluids analyzed) $\times$ 100. ND, no virus detected; –, severe disease or death.

the detection limit from days 7 to 9. Of note, virus reshedding was observed at day 15 in the pgB group and mice coimmunized with pgB and pCCL19 (Fig. 7A). HSV-2 shedding incidences in vaginal fluids are summarized in Table I, and similar tendencies were observed. From day 9 postchallenge, there was no detectable viral DNA in mice immunized with gB-CCL19 fusion constructs.

**Latent virus DNA load.** The copies of latent HSV-2 genomes in sacral ganglia of mice were quantified by real-time PCR at day 30 postchallenge or at the time of death. Latent HSV-2 genome copies from high to low were pcDNA3.1, pgB, pgB plus pCCL19, pgBIZCCL19 and pCCL19IZgB groups, indicating that CCL19-enhanced gB-specific responses could, to some extent, prevent HSV-2 from establishing latent infection and/or clear established infection (Fig. 7B).

**Weight change.** Upon HSV-2 challenge, mouse weight was monitored every day for a total period of 15 d. Mice immunized with pcDNA3.1 exhibited considerable weight loss after day 1 postchallenge, and an even sharper loss was observed from day 5 on. Mice immunized with pgB or pgB plus pCCL19 also demonstrated mild weight loss. In contrast, mice immunized with pgBIZCCL19 or pCCL19IZgB exhibited no apparent weight loss postchallenge. Rather, a slight weight gain was observed in mice immunized with pCCL19IZgB (Fig. 7C).

**Acute disease severity.** Upon viral challenge, the symptoms in mice were scored every day for a total period of 15 d. For all mice, acute symptoms began to appear on day 5. Mice with pcDNA3.1 immunization developed severe symptoms rapidly and were dead by day 13. All mice with pgB immunization also developed severe, but relatively milder, symptoms, whereas approximately half of the mice coimmunized with pgB and pCCL19 demonstrated mild disease symptoms. Of note, mice immunized with pgBIZCCL19 or pCCL19IZgB, unlike other groups, did not show any apparent symptoms postchallenge (Fig. 7D).

Overall, our study indicated that CCL19 as adjuvant promoted humoral and cellular responses against HSV-2 gB in a balanced Th1/Th2 cellular response fashion, as well as enhanced the clearance of latent virus load. Moreover, the adjuvant activity of CCL19 was enhanced significantly when fused to gB at either terminal. Our results further strengthen the notion that the adjuvant activity of CCL19 is likely due to its ability to target immunogen to immunocytes through the promotion of the migration of responsive immunocytes.

**Discussion**

Given that several HSV-2 glycoprotein-based subunit vaccines failed to show enough protection against viral infection in large-scale clinical trials (47, 48), there is a need to develop new immunization strategies. Among various means of improving vaccine efficacy, the adoption of adjuvants to promote Ag immunogenicity is one of the simple, yet most effective, ways (5, 12). CCL19 was shown to potentiate both cellular and humoral

(Table I. HSV-2 shedding incidences in HSV-2–challenged mice)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No. Animals</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 11</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1</td>
<td>14</td>
<td>14 (100)</td>
<td>14 (100)</td>
<td>14 (100)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pgB</td>
<td>12</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>10 (83.3)</td>
<td>2 (16.7)</td>
<td>ND</td>
<td>ND</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>pgB + pCCL19</td>
<td>11</td>
<td>11 (100)</td>
<td>11 (100)</td>
<td>3 (27.3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>pgBIZCCL19</td>
<td>12</td>
<td>12 (100)</td>
<td>11 (91.7)</td>
<td>4 (33.3)</td>
<td>3 (25.0)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pCCL19IZgB</td>
<td>12</td>
<td>12 (100)</td>
<td>11 (91.7)</td>
<td>6 (50.0)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

HSV-2 shedding incidences in vaginal fluids were detected by real-time PCR. Data shown are pooled from three independent experiments. HSV-2 shedding incidence = (no. vaginal fluids from which viral DNA was detected/total number of vaginal fluids analyzed) $\times$ 100. ND, no virus detected; –, severe disease or death.
It was reported that HSV-2 gD (1–23) peptide conjugated to cholera toxin (CTX) enhanced Ab responses and elicited protective antiviral immunity (50). In agreement, covalent linkage of an immunogen to CTX appeared to be more effective in enhancing Ag-specific immune responses than was a simple mixture of the immunogen and CTX (51). In addition, CCL19 fused to IgG2b possesses a long-lasting potent chemotactic activity, as a result of the extended half-life of the fusion protein (29). Together, these findings suggest that an immunogen fused to an adjuvant may offer better efficacy than an immunogen and an adjuvant being used in combination. When designing such fusion constructs, several factors should be taken into consideration. First of all, the function and structure of both moieties in the fusion protein should be maintained. To achieve this, a linker with proper length and structure is essential. An IZ linker has been commonly used for trimerization of fusion proteins, which has proven to be feasible in sustaining antigenic structure, thermal stability, and solubility (35, 36, 52, 53). Moreover, fusion proteins using an IZ linker do not bind to FcRs, which could avoid the generation of autoimmune Abs (54), whereas the G4S repeat is a linker with flexibility to allow both moieties to fold properly. In the current study, we found that some linkers favored the enhancement of immune responses, whereas others hindered such enhancement and sometimes even impaired Ag-induced immune responses. To this end, we tested gB-CCL19 constructs without a linker with a (G4S)3 repeat linker, or with a linker containing two G4S repeats and one IZ sequence [(G4S)3–IZ–(G4S)3]. Our results showed that only gB-CCL19 constructs with the (G4S)3–IZ–(G4S)3 linker were capable of enhancing immune responses (Fig. 2 and Y. Yan, K. Hu, and Q. Hu, unpublished observations). In addition to the linker, the construction order of the fusion proteins may affect protein activity (34, 54). We demonstrated that fusion constructs with CCL19 at the N- or C-terminal end had comparable abilities to enhance immune responses. Compared with the use of pG and pCCL19 in combination, fusion constructs (pgBZICCL19 and pCCL19IgZgB) enhanced gB-specific responses, likely via the following mechanisms. In contrast to gB and CCL19 being expressed in different vectors, construction of gB and CCL19 in the same vector guaranteed their simultaneous expression in terms of time and location. When responsive immunocytes were recruited to the sites where CCL19 was expressed, a more efficient Ag–immunocyte interaction might be achieved, consequently resulting in enhanced immune responses.

It is generally accepted that an ideal HSV-2 vaccine should be capable of protecting individuals against new infection, sterilizing circulating viruses in infected individuals, and eliminating latent viral reservoirs (11, 41, 46). We demonstrated that HSV-2 gB-CCL19 fusion constructs were highly effective in inducing systematic immune responses, in particular a Th1-based Ab response, which was different from the immune profile induced by HSV-2 gB alone (44). In addition, to function as a preventive vaccine against mucosally transmitted viruses like HSV-2, a strong and rapid mucosal response, especially an Ab response, is required, which can be achieved by a good memory B cell response (47, 55). Findings from the study of gD-specific immune response in guinea pigs demonstrated that IgG2a contributed to a potent neutralizing ability against HSV-2 (46). However, it was reported that only a small number of HSV-2 patients had IgG2 compared with IgG1 and IgG3 (56, 57). Therefore, a novel means is needed to enhance an IgG2 Ab response. In our study, we found that gB-CCL19 fusion constructs consistently induced an IgG2-biased immune profile better than did gB alone. In agreement, a humoral immune response with relatively potent neutralization activity was achieved by immunization with the fusion constructs. Such an immune response was sustained and rapidly recalled postchallenge, likely reflecting the increase in B cell differentiation. In contrast, we observed that the neutralizing activity of sera declined rapidly in mice coimmunized with pG and pCCL19 compared with that in mice immunized with gB-CCL19 fusion constructs. Likewise, gB-CCL19 fusion constructs demonstrated an advantage over pG plus pCCL19 in protecting mice from HSV-2 challenge.

Th1/Th2 cytokines, secreted by activated immunocytes postimmunization or challenge, play a pivotal role in controlling HSV-2 infection and disease progression in animal models (14, 46). In this study, we found that CCL19 as adjuvant promoted a balanced gB-specific Th1/Th2 response, including increased production of IL-4, IL-5, IFN-γ, and TNF. Moreover, gB-CCL19 fusion constructs demonstrated the ability to enhance the production of these cytokines. Nevertheless, in all immunized mice, the levels of IL-2 and IL-4 appeared to be significantly lower than those of other cytokines, whereas the levels of IFN-γ and TNF were high. In agreement with our findings, a previous study showed that the expression of IL-2 was lower than that of IFN-γ upon gB stimulation (58). As for IL-4, although the underlying mechanism remains to be further elucidated, it was reported that types I and II IFN can inhibit the expression of IL-4 (59). Previous studies by us (16) and other investigators (19–25) showed that the adjuvant effect of CCL19 is associated with its ability to recruit responsive T cells, DCs, and IgA ASCs to secondary lymph nodes and mucosal tissues. In the current study, we revealed that gB-pCCL19 fusion constructs, in comparison with coimmunization with pG and pCCL19, exhibited a stronger ability to increase CCR7+ immunocytes in secondary lymph nodes and IgA+ cells in mucosal sites and, consequently, resulted in enhanced immune responses. We speculated that, although CCL19 in combination with gB achieved an enhanced immune response over gB used alone, there was no guarantee that the two moieties could act simultaneously on immune cells. In contrast, fusion constructs have the advantage that both moieties act on the same immune cells. We found that the enhanced immune responses by gB-CCL19 fusion constructs were associated with an enhanced protection against lethal HSV-2 infection, as well as an improved elimination of latent viruses. Our findings highlight the application potential of gB-CCL19 fusion constructs over single agents being used in combination.

It is of interest that delivery of pCCL19 via the muscle surface can attract immunocytes into remote secondary lymphoid organs, such as spleen and MLNs. Previous studies by us (16) and other groups (60, 61) demonstrated that intranasally and i.m. administered pDNAs are distributed throughout the body, probably via the bloodstream. In the currently study, our data also showed that the DNA, DNA-transcribed RNA, and protein forms of the immunized fusion constructs were detected in blood, spleen, and MLNs, with CCL19 being elevated in mice vaccinated with gB-CCL19 fusion constructs (Supplemental Fig. 1), suggesting that the
elevated CCL19 may enhance immune responses by attracting CCL19-responsive cells into spleen and MLNs. Given that the level of CCR7 on B cells increases rapidly upon AgR engagement (62) and that CCL19–CCR7 interaction is involved in homing of B cells to mucosal draining lymph nodes, where B cells can differentiate into ASCs (31), we conclude that enhanced ASC trafficking is likely responsible for the elevated IgA* cells in the mucosal sites in gB-CCL19-immunized mice. However, we cannot rule out the possibility that gB-CCL19 may be capable of generating more IgA ASCs.

Despite the described favorable characteristics of gB-CCL19 fusion constructs, their optimal doses to trigger a maximum immune response warrant further investigation. It is well documented that an appropriate immunogen density is required to prime for a robust immune response, in particular a T cell response (61). Although a low density of immunogen is not sufficient to trigger an efficient response, an inappropriately high dose can inhibit the immune response (61). Given that a fixed amount of gB was administered in the currently studied model, an optimal dose remains to be determined in future studies. Moreover, we designed the constructs that express gB and CCL19 at a ratio of 1:1. If further investigation reveals that appropriate doses of gB and CCL19 are not equimolar, new constructs with a refined immunogen/adjuvant ratio, which may require more than one linker or even more types of linkers, need to be designed and tested. In addition, as a proof-of-concept study, we adopted a DNA-immunization strategy via i.m. delivery. Other delivery methods are worth testing for better efficacy. Given that the mouse model of HSV-2 has some limitations (e.g., no reactivation from the reservoir), future work is warranted to assess the fusion constructs in different animal models.

In conclusion, our findings in mice indicate that genetic gB-CCL19 fusion constructs have an attractive potential to promote systemic and mucosal responses against HSV-2 infection. To our knowledge, this is the first time that an immunogen–chemokine fusion construct was demonstrated to protect animals from virus challenge. Our study represents a promising approach when considering the development of an effective HSV-2 vaccine to induce protective systemic and mucosal immunity.

Acknowledgments
We thank Xiaofang An and Jimei Tao (Core Facility and Technical Support of Wuhan Institute of Virology) and Yan Wang (Institute of Hydrobiology) for assistance with animal studies and flow cytometry analysis.

Disclosure
The authors have no financial conflicts of interest.

References


