Original Article

A Novel System for Constructing a Recombinant Highly-Attenuated Vaccinia Virus Strain (LC16m8) Expressing Foreign Genes and Its Application for the Generation of LC16m8-Based Vaccines against Herpes Simplex Virus 2

Natsumi Omura^{1,2}, Tomoki Yoshikawa^{1*}, Hikaru Fujii¹, Miho Shibamura¹, Takuya Inagaki^{1,2}, Hirofumi Kato¹, Kazutaka Egawa¹, Shizuko Harada¹, Souichi Yamada¹, Haruko Takeyama², and Masayuki Saijo¹

¹Department of Virology 1, National Institute of Infectious Diseases, Tokyo 162-8640; and

²Department of Life Science and Medical Bioscience, Waseda University, Tokyo 162-8480, Japan

SUMMARY: A novel system was developed for generating highly attenuated vaccinia virus LC16m8 (m8, third-generation smallpox vaccine) that expresses foreign genes. The innovations in this system are its excisable selection marker, specificity of the integration site of a gene of interest, and easy identification of clones with a fluorescent signal. Using this system, recombinant m8s, which expressed herpes simplex virus 2 (HSV-2) glycoprotein B (gB)-, gD-, or both gB and gD (gB + gD), were generated, and their efficacy was evaluated. First, the induction of a specific IgG against these HSV-2 glycoproteins in mice infected with one of these recombinant m8s was confirmed by an immunofluorescent assay. Next, mice preinfected with one of the recombinant m8s were infected with HSV-2 at a lethal dose to examine the vaccine efficacy. The fatality rate among the mice preinfected with either the recombinant gB + gD- or gD-expressing m8 significantly decreased in comparison with the control. The survival rate in male and female mice preinfected with either the recombinant gB + gD- or gD-expressing m8 increased to 100% and 60%, respectively, while most of the control mice died. In summary, this new system may be applicable to creation of a novel m8-based vaccine.

INTRODUCTION

Vaccinia virus (VAC) has been used as a recombinant vaccine vector. There are many methodological reports involving natural homologous DNA recombination during replication for constructing a recombinant VAC with a desired property (1–5). Although the recombination occurs naturally, the selection process utilizing a selection marker gene with the desired phenotypic property is usually indispensable because of the low frequency of suitable recombinants (1,2). Nonetheless, especially for vaccine development, the recombinant VAC should be selection marker gene-free to avoid unexpected selection marker-related side effects.

Here, we established a novel system to generate a recombinant VAC based on a VAC strain LC16m8 (m8), a highly attenuated VAC LC16m8 (third-generation smallpox vaccine). Strain m8 is a smallpox vaccine that may possess protective efficacy and has a good safety profile (6). Approximately 100,000 people have been vaccinated with m8 so far without experiencing serious postvaccine complications, which occurred with the first- and second-generation smallpox vaccine strains. Furthermore, m8 is licensed for use in healthy people in Japan.

Herpes simplex virus 2 (HSV-2), an etiological patho-

gen of genital herpes (7), was chosen as the target to validate the capability of the novel system for construction of a recombinant m8 as a vaccine. To date, no HSV-2 vaccine has been approved for clinical use although several vaccine candidates have been developed. A subunit vaccine containing the HSV-2 envelope glycoproteins B (gB) and D (gD) with some adjuvants has been one of the most promising candidates. A large clinical study revealed that it is effective only in preventing HSV-1 genital disease, not in preventing HSV-2 disease, although the vaccine was targeted to HSV-2 infection (8). In the present study, recombinant m8s expressing HSV-2 glycoproteins (gB or/and gD) were generated, and their efficacy at protecting mice from a lethal HSV-2 infection was evaluated.

MATERIALS AND METHODS

Cells and viruses: 293FT cells (Thermo Fisher Scientific, Waltham, MA, USA), Vero cells, and RK13 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Wako, Osaka, Japan) supplemented with 5% of heat-inactivated fetal bovine serum (FBS) and antibiotics (DMEM-5FBS, Sigma-Aldrich Japan, Tokyo). Collagen-coated plates were specifically used for the cultured 293FT cells. VAC strain m8 (accession No. AY678275) was propagated in RK13 cells, and the infectious titer was determined by the standard plaque assay on RK13 cells (9). HSV-1 strain TAS (10) and HSV-2 strain G were propagated in Vero cells, and the infectious dose was determined by the standard plaque assay on Vero cells.

Plasmids: pCR-Amp-mCherry-XGPRT (accession No. LC325568) was constructed to harbor fluorescent genes: *mCherry* and *Escherichia coli* xanthine-guanine phosphoribosyltransferase (*XGPRT*). The genes were driven

Received October 16, 2017. Accepted January 25, 2018.

J-STAGE Advance Publication April 27, 2018.

DOI: 10.7883/yoken.JJID.2017.458

*Corresponding author: Mailing address: Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111, Fax: +81-3-5285-1180, E-mail: ytomoki@niid.go.jp

independently by a synthetic VAC early/late promoter (11). The genes *mCherry* and *XGPRT* were flanked by multiple cloning restriction enzyme sites (MCSs). Plasmid precB5R.1 (accession No. LC325569), a derivative of pCR-Amp-mCherry-XGPRT, harbored 2 upstream sites at sequence positions 170539–170982 and 170735–170979 around the m8 *B5R* gene that flanked the *XGPRT* and *mCherry* genes. A restriction site, *Bgl*II, for insertion of the gene of interest (GOI), which was located within the synthetic vaccinia early/late promoter, was flanked by shorter upstream and downstream portions (positions 171845–172308) around the m8 *B5R* gene. The open reading frame (ORF) of either gB or gD of HSV-2 strain G was amplified using the purified RNA from the HSV-2-infected cells by 2 step conventional RT-PCR. One of the ORFs (gB, gD, or fluorescent protein EGFP) was inserted into the *Bgl*II site of precB5R.1, and the vectors were named as precB5R.1-gB, -gD, and -EGFP. The ORF of gB with the vaccinia early/late promoter was inserted into a *Xho*I site of precB5R.1-gD to construct a plasmid named precB5R.1-gB + gD that contained both ORFs: gB and gD. A mammalian expression vector, pHEK293 Ultra Expression Vector II (Takara Bio Inc., Shiga, Japan), was constructed by insertion of the ORFs (gB and gD) at the *Bam*HI/*Hind*III sites, and was named pHEK293-gB or pHEK293-gD, respectively.

Generation of recombinant LC16m8s that express HSV-2 gB, gD, or both gB and gD: First, 2.5×10^5 293FT cells/ml were reverse transfected with 0.5 μ g of precB5R.1-gB, -gD, -gB + gD, or -EGFP with 1.5 μ l of FuGENE 6 (Promega, Fitchburg, WI, USA), and then the cells were infected with m8 at multiplicity of infection (MOI) of 0.05 per cell. The cells were cultured in DMEM-5FBS for 3 days. The culture medium along with the cells was then collected and was freeze-thawed 3 times to prepare the crude intermediate recombinant m8 stocks. RK13 cell monolayers were inoculated with one of the crude intermediate recombinant m8 stocks. After that, the inoculated stock was removed, and the cells were overlaid with Eagle's Minimum Essential Medium (EMEM, Wako, Osaka, Japan) containing 2% of FBS, 20 μ g/ml mycophenolic acid (MPA, Sigma-Aldrich, St Louis, MO, USA), 250 μ l/ml xanthine, 15 μ g/ml hypoxanthine, and 1% of agarose ME (Iwai Chemicals, Tokyo, Japan). After 3 days, the mCherry fluorescence-positive plaques were identified by either fluorescence microscopy or an LED transilluminator (GELmieu, WAKO) at the wavelength of 500 nm. The fluorescence-positive plaques in the agarose plug were mixed with DMEM-5FBS to prepare stocks of the intermediate recombinant m8 clones. RK13 cells in a monolayer in a 96-well plate were inoculated with the intermediate clones to confirm the expression capability of gB and/or gD. The inoculated cells were fixed with a 1:1 methanol:acetone mixture at 2 days postinfection. The cells were then reacted with either a mouse anti-HSV1 + HSV2 gB monoclonal antibody (clone 10B7; Abcam) or mouse anti-HSV1 + HSV2 gD monoclonal antibody (clone 2C10; Abcam), followed by a reaction with DyLight 488-conjugated goat anti-mouse IgG H + L antibody (Abcam, Cambridge, UK). The fluorescence-positive expression clones were then purified by plaque isolation at least 3 times. RK13 cells were infected with the purified intermediate clones without supplementation with MPA, xanthine, and hypoxanthine to prepare the

final crude recombinant m8 stocks. Monolayered RK13 cells were infected with one of the final crude recombinant m8 stocks, and the cells were overlaid with agarose containing MEM-2FBS without supplementation with MPA, xanthine, and hypoxanthine. After 3 days, the mCherry fluorescence-negative plaques were collected under the LED transilluminator. The gB and/or gD expression-positive clones were next purified by plaque isolation at least 3 times to establish the final recombinants.

Western blotting: The expression of gB and/or gD in the cells infected with one of recombinant m8s was confirmed by western blotting. Briefly, confluent RK13 cells were infected with one of the viruses—m8-gB, m8-gD, or m8gB + gD—at MOI of 0.01 per cell. The samples were fractionated by SDS polyacrylamide gel electrophoresis and subjected to western blotting. The membrane was reacted with either a mouse anti-HSV-1 + HSV-2 gB monoclonal antibody (clone T111; Thermo Fisher Scientific, Waltham, MA, USA) or with a mouse anti-HSV1 + HSV2 gD monoclonal antibody (clone 2C10), followed by reaction with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) antibody (KPL). The immunoreactive proteins were visualized by LAS-4000mini (Fujifilm, Tokyo, Japan) with the ECL western blotting detection reagents (GE Healthcare Bioscience, Tokyo).

Animal experiments: Male and female A/J and C57BL/6 mice (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan) aged 4 to 6 weeks at the time of primary infection were used in all the animal experiments. For preinfection, the mice were inoculated subcutaneously twice at a 2-week interval with 10^6 plaque-forming units (pfu) of one of the recombinant m8s in a volume of 100 μ l per mouse. Two weeks after the second preinfection, the mice were either euthanized for collection of peripheral blood or further challenged intraperitoneally with 10^5 pfu of HSV-2 strain G in a volume of 100 μ l per mouse. Although we could not determine the 50% lethal dose (LD_{50}) of strain G with the intraperitoneal inoculation into A/J and C57BL/6 mice as previously reported (12), all the mice infected with 10^5 pfu of strain G died. The mice were monitored daily regarding survival and body weight for 3 weeks. All the animal experiments were approved and performed under the strict regulations of the animal experimentation guidelines of the National Institute of Infectious Diseases in Japan.

An indirect fluorescent antibody (IFA) test: First, 293FT cells were transfected with either pHEK293-gB or -gD with an expression enhancer vector, pHEK293 (Takara Bio Inc.). The transfected cells were washed with PBS, spotted on glass slides (Matsunami glass IND., Ltd., Osaka, Japan), and fixed with a 1:1 methanol:acetone mixture. For measuring the gB- or gD- specific IgG titers in mice infected with recombinant m8, the serum samples were serially diluted twofold and were added onto the glass slides. The cells were then reacted with a DyLight 488-conjugated goat anti-mouse IgG H + L antibody (Abcam). The antibody titer was defined as the reciprocal of the highest dilution level at which a specific fluorescent signal was detected.

Statistical analysis: All statistical analyses were carried out in GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance with Tukey's

multiple-comparison test was performed to verify the equality of the means of specific IgG titers to either HSV-2 gB or gD in the serum samples from recombinant-m8-immunized mice. Survival curves were plotted according to Kaplan–Meier analysis, and the protective efficacy of recombinant m8 induced by infection of mice with m8 was evaluated by the logrank test. A significant difference was considered to be present for any p value < 0.05 .

RESULTS

Generation of plasmids for constructing recombinant LC16m8 (m8): A plasmid named pCR-Amp-mCherry-XGPRT and containing 2 selection marker genes, *mCherry* and *XGPRT*, was constructed (Fig. 1A), making it possible to insert a GOI at any intended site in the VAC genome via this plasmid. The *B5R* gene was chosen as the site of GOI insertion, because *B5R* is known to be indispensable (13). A derivative of pCR-Amp-mCherry-XGPRT, named precB5R.1, harbors the selection cassette that was flanked by upstream and downstream portions around m8 *B5R* (Fig. 1B). In addition, the markers were flanked by 2 upstream portions, one of which was shorter (i.e., F' in Fig. 1B). First, recombinant VAC was identified under MPA selection pressure with mCherry fluorescence under the LED transilluminator. Next, only the selection cassette, but not the GOI, was self-excised from intermediate recombinant m8 occurring

upstream and at F' in the absence of MPA because of intramolecular homologous recombination (Fig. 1C).

Generation of recombinant m8s expressing gB and/or gD: First, m8s expressing HSV-2 gB, gD, or both gB and gD in the infected cells were generated to validate the system for constructing recombinant m8s. Then, 293FT cells were transfected with one of the plasmids—precB5R.1-EGFP, -gB, -gD, or -gB + gD—and finally infected with m8. Intermediate recombinant m8s, which seemed to harbor both foreign genes and the selection marker genes, were purified and cloned by detection of both the mCherry fluorescent signal and XGPRT activity by addition of MPA. Intermediate recombinant m8s were confirmed to express the recombinant gB and/or gD glycoproteins in the infected cells by the IFA test with the antibody against either gB or gD. Final recombinant m8s, which express EGFP, gB, gD, or gB + gD (m8-EGFP, -gB, gD, -gB + gD) but not the selection marker genes, were cloned by selecting the fluorescent negative plaques (Fig. 1D). The selection marker genes were self-excised, resulting in their deletion. The cells infected with one of recombinant m8s were confirmed to express gB, gD, or both gB and gD by western blotting (Fig. 2).

Antibody responses induced in mice infected with the recombinant m8 viruses: Female C57BL/6 mice were infected with one of recombinant m8s twice subcutaneously at a 2-week interval with 10^6 pfu per mouse. Two weeks after the last infection, serum samples were

Fig. 1. Schematic of the system for making recombinant m8s. A plasmid, pCR-Amp-mCherry-XGPRT, which harbors the independently driven *mCherry* and *XGPRT* genes, was constructed (A). A plasmid, precB5R.1, enables integration of selection marker genes and GOI by homologous recombination, which occurs between the homologous sequences that flank the *B5R* gene in the m8 genome and those in the plasmid (B). The selection marker genes, *XGPRT* and *mCherry*, were self-excised from the intermediate recombinant viruses by intragenomic homologous recombination between 2 upstream sequences one of which was shorter (U') (C). The final recombinant m8 viruses, which express EGFP, gB, gD, and gB and gD, but not the selection marker genes, were generated (D). The dotted lines indicate the expected locations of the homologous recombination sites. A synthetic vaccinia early/late promoter is indicated as P in the arrow shaped box.

Fig. 2. Expression of gB and gD in the cells infected with recombinant m8 viruses. RK-13 cells were infected with authentic m8 and each of the recombinant m8 viruses (m8-gB, -gD, and -gB + gD) at an MOI of 0.01 per cell. Vero cells were also infected with either the HSV-1 strain TAS or HSV-2 strain G at an MOI of 1 or mock-infected. The cells were collected after 3 days post infection and were subjected to SDS-PAGE. The expression was confirmed by Western blotting using either anti-HSV gB or gD antibodies.

Fig. 3. Anti-HSV-2 gB or gD specific antibody titers induced in mice immunized with the EGFP-, gB-, and gD-expressing recombinant m8 viruses were determined with IFA. Each dot represents the antibody titer against either gB (A) or gD (B). Horizontal lines represent the mean value. The one-way analysis of variance with Tukey's multiple-comparison test was used to determine the level of statistical significance. The calculated p -values are shown above the groups that were compared. ns, not significant.

Fig. 4. Survival of mice immunized with each recombinant m8 after lethal challenge with HSV-2. Male (A) or female (B) A/J mice were inoculated subcutaneously twice at a two-week interval at the dose of 1×10^6 pfu of each recombinant m8 virus, m8-EGFP, m8-gB, m8-gD, and m8- gB + gD. Two weeks after the last immunization, mice were challenged intraperitoneally with a lethal dose (1×10^5 pfu) of the HSV-2 strain G. Survival was followed daily for 3 weeks. The log-rank test was used to determine the level of statistical significance. The calculated p -values are shown above the groups that were compared. ns, not significant.

collected, and a specific antibody titer was determined by the IFA test (Fig. 3). An antibody against either gB or gD was induced only in mice infected with either m8-gB and/or m8-gD, but not in mice infected with m8-EGFP. In contrast, a neutralizing antibody against HSV-2 strain G was not induced by infection of mice with m8-gB, -gD, or -gB + gD (data not shown).

Survival of the mice infected with wild-type HSV-2: Groups of either 5 male or female A/J mice were infected with 10^6 pfu of one of recombinant m8s twice subcutaneously at a 2-week interval. Two weeks after the last injection, the mice were infected intraperitoneally with 10^5 pfu of HSV-2 strain G. One of the 5 m8-EGFP-immunized male mice survived (Fig. 4A). In contrast, the survival rate was increased by preinfection of mice with either m8-gB or m8-gD, although the number of the mice that survived in these groups was not statistically significant in comparison with m8-EGFP-immunized mice. When the mice were preinfected with m8-gB + gD, all the mice survived. On the other hand, as compared with the male mice, the number of female mice that survived was not increased by m8-gB or m8-gB + gD preinfection in a statistically significant manner as compared with the mice preinfected with m8-EGFP (Fig. 4B).

DISCUSSION

VAC has served as a vaccine vector for many types of infectious diseases (14), and several candidates for HSV-1 and HSV-2 vaccines based on VAC have been reported (15–18). Nevertheless, the problem is that VAC is associated with severe side effects, such as encephalitis, encephalopathy, vaccinia conjunctivitis, and generalized fetal VAC infections (14). Hence, LC16m8, modified vaccinia Ankara (MVA) (19–21), and others, so-called third-generation smallpox vaccines, have been developed. These vaccines are highly attenuated but maintain immunogenicity as a smallpox vaccine (14). Therefore, m8 is a suitable vaccine vector.

The system for generating a recombinant m8, which expresses foreign genes, in this study, was developed because of the known advantages that have been reported independently, such as generation of selection marker-free VAC (3), specific insertion of a GOI at the intended site of the VAC genome (3,4), and cloning of the recombinant VAC with the cloning of fluorescent gene expression-positive VAC in the infected cells (5).

All the male m8-gB + gD-preinfected mice survived after the lethal HSV-2 challenge. In contrast, the female mice preinfected with m8-gD survived significantly better than controls after the lethal challenge, but the female mice preinfected with m8-gB + gD did not. Such a sex bias with HSV was previously reported, and the LD₅₀ of HSV-1 in male mice determined by intraperitoneal inoculation is 27-fold higher than that among female mice (22). Because the inoculation dose of HSV-2 was not changed by sex in this study, the female mice were probably under a more severe challenge of HSV-2 infection than the male mice were.

In summary, a novel method was developed here for constructing a recombinant VAC LC16m8 into which a foreign GOI can be inserted. By this method, recombinant m8s, which express gB, gD, or both gB and gD, were generated. Furthermore, it was confirmed that protective immunity is induced in both gB- and gD-expressing m8 virus-immunized male A/J mice and in gD-expressing m8 virus-immunized female A/J mice. The novel recombinant LC16m8 construction system may be a useful tool for the development of m8-based vaccines.

Acknowledgments We would like to thank Ms. Yoshiko Fukui and Ms. Mihoko Tsuda for their excellent technical assistance. This work was supported by grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan (H26-Sinkogyousei-Shitei-002, and H29-Sinkogyousei-Shitei-002) and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 15K09675 and No. 24591591).

Conflict of interest None to declare.

REFERENCES

1. Smith GL, Murphy BR, Moss B. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc Natl Acad Sci USA*. 1983;80:7155-9.
2. Lorenzo MM, Galindo I, Blasco R. Construction and isolation of recombinant vaccinia virus using genetic markers. *Methods Mol Biol* 2004;269:15-30.
3. Scheifflinger F, Dorner F, Falkner FG. Transient marker stabilisation: a general procedure to construct marker-free recombinant vaccinia virus. *Arch Virol*. 1998;143:467-74.
4. Cao JX, Upton C. gpt-gus fusion gene for selection and marker in recombinant poxviruses. *Biotechniques*. 1997;22:276-8.
5. Popov S, Mirshahidi S, Essono S, et al. Generation of recombinant vaccinia viruses via green fluorescent protein selection. *DNA Cell Biol*. 2009;28:103-8.
6. Kenner J, Cameron F, Empig C, et al. LC16m8: an attenuated smallpox vaccine. *Vaccine*. 2006;24:7009-22.
7. Felman YM, Nikitas JA. Sexually transmitted diseases and child sexual abuse. Part II. *N Y State J Med*. 1983;83:714-6.
8. Belshe RB, Leone PA, Bernstein DI, et al. Efficacy results of a trial of a herpes simplex vaccine. *N Engl J Med*. 2012;366:34-43.
9. Morikawa S, Sakiyama T, Hasegawa H, et al. An attenuated LC16m8 smallpox vaccine: analysis of full-genome sequence and induction of immune protection. *J Virol*. 2005;79:11873-91.
10. Saijo M, Suzutani T, De Clercq E, et al. Genotypic and phenotypic characterization of the thymidine kinase of ACV-resistant HSV-1 derived from an acyclovir-sensitive herpes simplex virus type 1 strain. *Antiviral Res*. 2002;56:253-62.
11. Chakrabarti S, Sisler JR, Moss B. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques*. 1997;23:1094-7.
12. Zawatzky R, Gresser I, DeMaeyer E, et al. The role of interferon in the resistance of C57BL/6 mice to various doses of herpes simplex virus type 1. *J Infect Dis*. 1982;146:405-10.
13. Kidokoro M, Tashiro M, Shida H. Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8. *Proc Natl Acad Sci USA*. 2005;102:4152-7.
14. Walsh SR, Dolin R. Vaccinia viruses: vaccines against smallpox and vectors against infectious diseases and tumors. *Expert Rev Vaccines*. 2011;10:1221-40.
15. Fleck M, Podlech J, Weise K, et al. A vaccinia virus-herpes simplex virus (HSV) glycoprotein B1 recombinant or an HSV vaccine overcome the HSV type 2 induced humoral immunosuppression and protect against vaginal challenge in BALB/c mice. *Med Microbiol Immunol*. 1994;183:87-94.
16. Cremer KJ, Mackett M, Wohlenberg C, et al. Vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D prevents latent herpes in mice. *Science*. 1985;228:737-40.
17. Rooney JF, Wohlenberg CR, Moss B, et al. Live vaccinia virus recombinants expressing herpes simplex virus genes. *Rev Infect Dis*. 1991;13 Suppl 11:S898-903.
18. Bernstein DI. Effect of route of vaccination with vaccinia virus expressing HSV-2 glycoprotein D on protection from genital HSV-2 infection. *Vaccine*. 2000;18:1351-8.
19. Belyakov IM, Earl P, Dzutsev A, et al. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. *Proc Natl Acad Sci USA*. 2003;100:9458-63.
20. Earl PL, Americo JL, Wyatt LS, et al. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. *Nature*. 2004;428:182-5.
21. Wyatt LS, Earl PL, Eller LA, et al. Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. *Proc Natl Acad Sci USA*. 2004;101:4590-5.
22. Geurs TL, Hill EB, Lippold DM, et al. Sex differences in murine susceptibility to systemic viral infections. *J Autoimmun*. 2012;38:J245-53.