Oral Administration of the CCR5 Inhibitor, Maraviroc, Blocks HIV *Ex vivo* Infection of Langerhans Cells Within the Epithelium

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TO THE EDITOR

Preexposure prophylaxis (PrEP) with oral administration of an antiretroviral is a potential method for preventing acquisition of HIV. A controlled trial in men who have sex with men (the iPrEx trial) showed that daily oral use of tenofovir disoproxil fumarate-emtricitabine (TDF-FTC; Truvada) reduced transmission rates by 44% (Grant *et al.*, 2010). In addition, the HIV Prevention Trial Network (HPTN) 052 trial recently confirmed that antiretroviral treatment leads to 96% reduction in transmission among HIV-negative heterosexual partners of HIV-positive individuals (Cohen *et al.*, 2011). Similar trials, however, with TDF-FTC (the FEM-PrEP trial) or TDF alone (the VOICE trial) were stopped because of poor outcomes (van der Straten *et al.*, 2012). Different results among various trials, which used identical antiretroviral regimens, could be explained by varying compliance with drug use and/or varying drug concentration and activity within the exposed tissue (Patterson *et al.*, 2011).

Langerhans cells (LCs) are $CCR5^+$ dendritic cells located within genital skin and mucosal epithelium (Lederman *et al.*, 2006). In female rhesus macaques exposed intravaginally to simian immunodeficiency virus, up to 90% of initially infected target cells were LCs (Hu *et al.*, 2000). *Ex vivo* experiments with human foreskin explants show that epidermal LCs are target cells for HIV, providing a likely explanation for why circumcision greatly reduces the probability of acquiring HIV (Ganor *et al.*, 2010). LCs also express CD4 and CCR5, but not CXCR4, within the tissue and demonstrate the distinctive characteristics of emigrating from tissue to draining lymph nodes in order to interact with T cells after contact with pathogens (Kawamura *et al.*, 2000). Indeed, epidermal LCs are readily infected *ex vivo* with R5 HIV, but not with X4 HIV, and promote high levels of infection upon interaction with cocultured $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). Thus, LCs probably have an important role in disseminating HIV soon after exposure to virus.

Epidemiologic observations have found that the majority of HIV strains isolated from patients soon after initial infection are R5 HIV strains (i.e., they utilize CCR5; Lederman *et al.*, 2006). Not surprisingly, individuals with homozygous defects in *CCR5* are largely protected from sexually acquiring HIV (Lederman *et al.*, 2006). In addition, three different CCR5-binding topically applied compounds protected female macaques from sexually acquiring SHIV: the N-terminally modified chemokine analog PSC-RANTES, the small-molecule inhibitor CMPD167, and maraviroc (MVC) (Lederman *et al.*, 2006; Veazey *et al.*, 2010). In addition to topical application to vaginal mucosa, oral delivery of CMPD167 protected macaques from vaginal SHIV challenge (Veazey *et al.*, 2005). Given these data, orally administered MVC may prove to be particularly important in PrEP regimens, although its ability to prevent HIV acquisition is unknown.

In the current study, 20 healthy volunteers were randomly divided into four equal groups; they received 300 mg of MVC orally twice daily for 1, 2, 3, or 14 days. To obtain epidermal tissues, all subjects underwent suction blistering of the skin before and 2 hours after the last MVC dose. All subjects had plasma and semen collected 2 hours after their last dose. MVC concentrations in serum, semen, and epidermal tissues were determined by using the liquid chromatography– mass spectometry method, as described previously (Takahashi *et al.*, 2010). Mean concentration ± SD in the epidermis was 21.91 ± 13.80, 23.36 ± 13.28, and 31.54 ± 20.61 nM for individuals taking drug for 1, 2, or 3 days ($n = 5$ for each), respectively. MVC concentrations tended to be higher with a longer dosing period. Consistent with recent data showing high levels of MVC in genital tissue (Dumond *et al.*, 2009), these results indicate that MVC rapidly distributes into the skin at high concentrations. In addition, MVC was detected in semen of all subjects (Supplementary Figure S1 online).

To understand how HIV traverses skin and genital mucosa, an *ex vivo* model was developed whereby resident LCs within epithelial tissue explants are exposed to HIV and then allowed to emigrate from tissue, thus mimicking conditions that occur after mucosal exposure to HIV (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). In this model, although relatively few productively infected LCs are identified, these cells induce high levels of HIV infection when cocultured with resting autologous $CD4^+$ T cells (Kawamura *et al.*, 2000). In preliminary experiments, HIV infection of LCs, as well as subsequent virus transmission from emigrated LCs to cocultured $CD4^+$ T cells, was decreased in a dose-dependent manner when skin explants were pretreated with

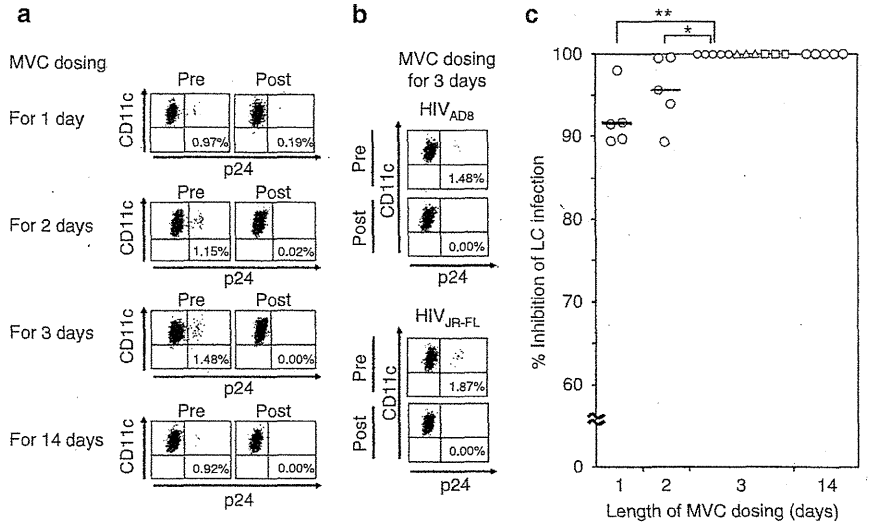

Figure 1. Oral administration of maraviroc (MVC) protects epidermal Langerhans cells (LCs) from *ex vivo* R5 HIV infection. Skin explants were isolated from healthy individuals who had received oral MVC (300 mg twice daily) for the indicated periods of time. These tissues were exposed to HIV_{Ba-L} (**a**, **c**), HIV_{AD8}, or HIV_{JR-FL} (**b**, **c**) and then cultured for 3 days. Emigrated LCs were collected and assessed for HIV infection by flow cytometry. Representative FACS analyses of CD11c and p24 mAb double-stained cells are shown (**a**, **b**). Percent MVC inhibition of LC infection with HIV_{Ba-L} (○), HIV_{AD8} (△), or HIV_{JR-FL} (□) was calculated as described in the text (**c**). $^*P<0.05$; $^{**}P<0.01$. Mean values obtained from different donors are shown as horizontal marks.

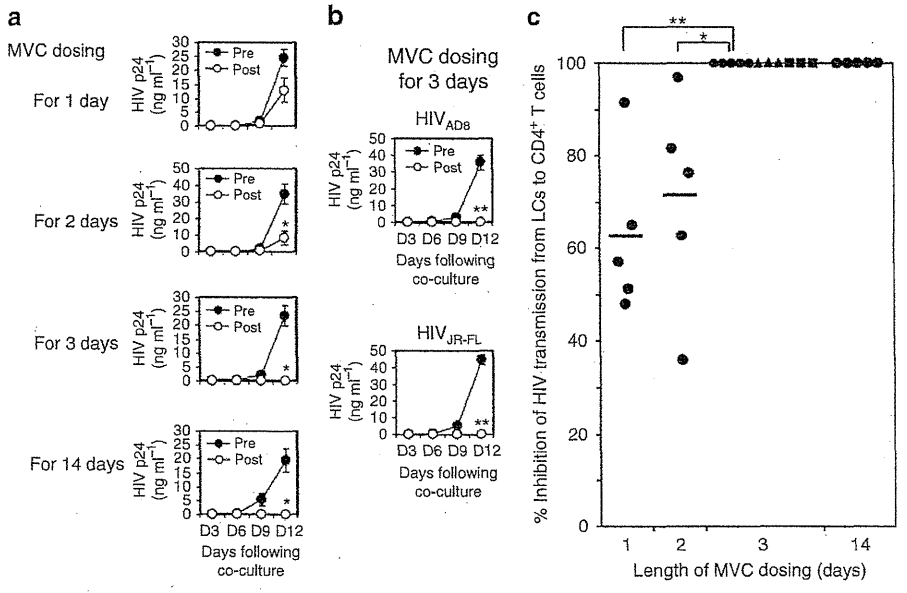

Figure 2. Oral administration of maraviroc (MVC) blocks viral transmission from HIV-exposed Langerhans cells (LCs) to cocultured $CD4^+$ T cells. Skin explants isolated from healthy individuals who had received oral MVC (300 mg twice daily) for the indicated periods of time were exposed to HIV_{Ba-L} (**a**, **c**), HIV_{AD8}, or HIV_{JR-FL} (**b**, **c**), as described in Figure 1. Emigrated LCs were cocultured with autologous $CD4^+$ T cells, and culture supernatants were assessed for p24 content by ELISA. Representative ELISA results are shown (**a**, **b**). Percent MVC inhibition of HIV_{Ba-L} (●), HIV_{AD8} (▲), or HIV_{JR-FL} (■) transmission to cocultured $CD4^+$ T cells was calculated as described in the text (**c**). $^*P<0.05$; $^{**}P<0.01$. Mean values obtained from different donors are shown as horizontal marks.

various concentrations of MVC before HIV exposure (Supplementary Figure S2 online), similar to experiments reported earlier with PSC-RANTES (Kawamura *et al.*, 2004).

Next, the epithelial tissue explants were collected from study subjects after oral treatment with MVC (Supplementary Materials and Methods online). Importantly, oral MVC pretreatment for either 1 or 2 days partially inhibited subsequent *ex vivo* HIV_{Ba-L} infection of LCs within epithelial tissue, whereas MVC administration for either 3 or 14 days completely blocked LCs from *ex vivo* HIV_{Ba-L} infection (Figure 1). These data demonstrate the importance of the length of MVC dosing period before HIV exposure. MVC treatment also consistently prevented HIV_{Ba-L} transmission from LCs to cocultured $CD4^+$ T cells (Figure 2). Furthermore, MVC administration for 3 days blocked *ex vivo* virus infection of LCs as well as

subsequent virus transmission when different R5 HIV strains, HIV_{AD8} and HIV_{JR-FL}, were utilized for an additional six subjects ($n=3$ for each strain, Figures 1 and 2). These data demonstrate that oral administration of MVC for at least 3 days is capable of fully protecting HIV infection of LCs within epithelial tissue.

These experiments provide perhaps the best proof-of-concept test for MVC as a potential PrEP drug, as it would be unethical to expose MVC-treated volunteers to HIV *in vivo*. As proven here, orally delivered MVC rapidly distributes to skin and functionally acts to block infection of relevant target cells, LCs, supporting randomized controlled trials of MVC as a PrEP therapy for individuals at high risk of becoming infected with HIV through sexual exposure.

CONFLICT OF INTEREST

The authors state no conflict of interests.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Effects of immunization of pregnant guinea pigs with guinea pig cytomegalovirus glycoprotein B on viral spread in the placenta

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ABSTRACT

Background: Cytomegalovirus (CMV) is the most common cause of congenital virus infection. Infection of guinea pigs with guinea pig CMV (GPCMV) can provide a useful model for the analysis of its pathogenesis as well as for the evaluation of vaccines. Although glycoprotein B (gB) vaccines have been reported to reduce the incidence and mortality of congenital infection in human clinical trials and guinea pig animal models, the mechanisms of protection remain unclear.
Methods: To understand the gB vaccine protection mechanisms, we analyzed the spread of challenged viruses in the placentas and fetuses of guinea pig dams immunized with recombinant adenoviruses expressing GPCMV gB and β-galactosidase, rAd-gB and rAd-LacZ, respectively.
Results: Mean body weight of the fetuses in the dams immunized with rAd-LacZ followed by GPCMV challenge 3 weeks after immunization was 78% of that observed for dams immunized with rAd-gB. Under conditions in which congenital infection occurred in 75% of fetuses in rAd-LacZ-immunized dams, only 13% of fetuses in rAd-gB-immunized dams were congenitally infected. The placentas were infected less frequently in the gB-immunized animals. In the placentas of the rAd-LacZ- and rAd-gB-immunized animals, CMV early antigens were detected mainly in the spongiotrophoblast layer. Focal localization of viral antigens in the spongiotrophoblast layer suggests cell-to-cell viral spread in the placenta. In spite of a similar level of antibodies against gB and avidity indices among fetuses in each gB-immunized dam, congenital infection was sometimes observed in a littermate fetus. In such infected fetuses, CMV spread to most organs.
Conclusions: Our results suggest that antibodies against gB protected against infection mainly at the interface of the placenta rather than from the placenta to the fetus. The development of strategies to block cell-to-cell viral spread in the placenta is, therefore, required for effective protection against congenital CMV infection.

1. Introduction

Human cytomegalovirus (HCMV) is the most common cause of congenital virus infection. Congenital infection occurs in 0.2–1% of all births, and causes birth defects and developmental abnormalities, including sensorineural hearing loss (SNHL) and developmental delay [1–3]. Since one of the major routes of transmission to pregnant mothers is suggested to be *via* the excretions of their own children [4,5], development of a vaccine is the only effective way for protection against primary HCMV infection. Indeed, a review panel from the Institute of Medicine indicated that the development of a vaccine against HCMV, particularly with the aim of preventing primary infection in pregnant women, was of the highest priority among those for infectious diseases other than HIV [6]. As one of the promising approaches, purified glycoprotein B (gB) protein in combination with the MF59 adjuvant was used for a phase 2 clinical trial on CMV-seronegative women who had recently delivered a child and had intention of having another, and this subunit gB vaccine protocol demonstrated 50% efficacy against primary infection [7]. Although such results are encouraging, further studies are required to improve the efficacy and rapid waning of protection.

Animal models are generally valuable in gaining a better understanding of pathogenesis as well as in developing therapeutics for

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infectious diseases. In contrast to murine and rat CMVs, guinea pig CMV (GPCMV) crosses the placenta and causes infection *in utero*. Importantly, congenital GPCMV infection causes diseases similar to congenital HCMV diseases, such as IUGR and labyrinthitis [8–11]. Previous studies using a guinea pig model demonstrated that congenital infection and mortality in pups were reduced by the administration of anti-gB antibodies [12] or by immunization with gB in the form of a DNA or purified subunit vaccine [13,14]. In the placenta, GPCMV-induced histopathological lesions with viral antigens were localized at the transitional zone between the capillarized labyrinth and the noncapillarized interlobium [15]. However, the mechanism by which gB immunization inhibits such viral spread in the placenta and fetus remains unclear. In this study, to better understand the mechanism, we analyzed the spread of viruses in the placentas and fetuses of gB-immunized dams after virus challenge.

2. Materials and methods

2.1. Cells and viruses

Guinea pig lung fibroblasts (GPL, ATCC) were initially cultured in F-12 medium supplemented with 10% fetal bovine serum (FBS) and subsequently, after infection with GPCMV, in F-12 medium supplemented with 2% FBS. GPL cells were infected with a GPCMV (strain 22122) stock purchased from ATCC. Salivary glands (SGs) of a guinea pig (Hartley strain) infected with the original GPCMV stock were recovered, minced, sonicated briefly, and then centrifuged to remove debris. The supernatant (SG-P0) was used for the infection of GPL cells, and viral stocks were prepared after propagation of the cell-free virus 5 times in GPL cells (SG-P5). Virus stocks were concentrated by ultracentrifugation (82,000 × *g* for 2 h) in a 20% sucrose step gradient. Infectious units (IUs) of the stocks were determined by immunostaining of GPL cells infected with the diluted stocks in 12- or 24-well plates and cultured for 2–3 days as described previously [16].

A recombinant GPCMV expressing red fluorescent protein (RFP) was prepared as follows: The sequence region from position 4244 to 8013 (positions are based on Ref. [17]) of GPCMV (SG-P5) was replaced with a 1.8-kb DNA fragment covering the TurboRFP gene under the control of the CMV IE promoter (Evorgen JSC, Russia) by homologous recombination in GPL cells. The RFP-expressing GPCMV candidates were then plaque-purified several times in GPL cells. One of the candidates, GPCMV-RFP(4A), was used for neutralization assay.

2.2. Recombinant adenoviral vectors

The gene encoding the extracellular domain (amino acids 1–674) of GPCMV gB (rAd-gB) and the LacZ gene encoding β-galactosidase were cloned into a pENTR-3C vector and then into pAd/CMV/V5/DEST by using the LR recombinase system (Invitrogen), resulting in pAd-gB and pAd-LacZ, respectively. Recombinant adenoviruses, rAd-gB and rAd-LacZ, were recovered by transfection of 293A cells with pAd-gB and with pAd-LacZ, respectively, amplified, and purified by centrifugation through two CsCl step gradients as described previously [18].

2.3. Animal studies

Female guinea pigs at the indicated weeks after birth (Hartley, Japan SLC, Inc.) were inoculated intraperitonealy (i.p.) with 10^6 IUs of GPCMV and euthanized 3-weeks later. Blood specimens were drawn directly from the heart, and organ specimens, including liver, spleen, kidney, lung, and salivary gland, were harvested. Dams at 1-week of gestation (Japan SLC, Inc.) were inoculated i.p. with 10^{10} transducing units (TUs) of rAd viruses, infected subcutaneously with 10^6 IUs of GPCMV (SG-P5) 3-weeks after the inoculation, and euthanized 3-weeks later. Blood specimens were drawn from the dams and their fetuses. Salivary glands were also obtained from the dams. The placentas and fetuses were weighed and organs were harvested from the fetuses. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), and were conducted according to the 'Guidelines for Animal Experiments Performed at the NIID'.

2.4. Immunological assays

Transfection was performed by using a commercial reagent (Fugene6, Roche). GPCMV-infected and -transfected cells were fixed with acetone for 5 min and expression of antigens were examined by an immunofluorescence assay as described previously [19].

Anti-gB antibody levels in the dams and fetuses were measured by ELISA using the cytoplasmic fraction (0.8 μg of protein/well) of 293T cells transfected with a gB construct. Absorbance values obtained using sera diluted at 1:200 had a good correlation with titers determined as a maximum dilution (in a range of 1:200–3200) that gives the threshold absorbance (data not shown). Avidity indices of anti-gB IgG were determined by a 10-min treatment with 4 M urea.

Neutralizing activities in sera were measured as follows. A total of 1×10^4 IUs of GPCMV-RFP(4A) in 50 μl of medium was mixed with 50 μl of serially diluted serum specimens, and incubated at 37 °C for 1 h. The reaction mixtures were then diluted and inoculated into GPL cell cultures. RFP-positive foci were counted 3 days after infection.

2.5. Immunohistochemistry

All organs obtained from sacrificed animals were fixed in 10% buffered formalin. Formalin-fixed specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE), as described previously [8]. Immunohistochemical analysis was performed using the monoclonal antibody g-1, which detects a GPCMV early antigen, or the rabbit polyclonal antibody against immediate-early proteins 1 and 2, which was generated by the immunization of rabbits with a GST-IE1/2 fusion protein, as primary antibodies. For the second- and third-phase immunostaining reagents, a biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (DAKO) or of goat anti-rabbit immunoglobulin (DAKO) and peroxidase-conjugated streptavidin (DAKO) were used. DAB was used as a chromogen and the slides were counterstained with hematoxylin.

2.6. Quantification of viral DNA

DNA samples were prepared from the placentas and fetal organs, and viral DNAs in the samples were detected by real-time PCR assays for GPCMV GP83 and β-actin genes as described previously [16].

2.7. Statistical analysis

Mann–Whitney *U* test was used to analyze statistical differences in the weight of animals, fetuses, and placentas, and in the number of viral foci in placentas. Chi-square test was also used to analyze differences in the rates of CMV-positive placentas and fetal organs.

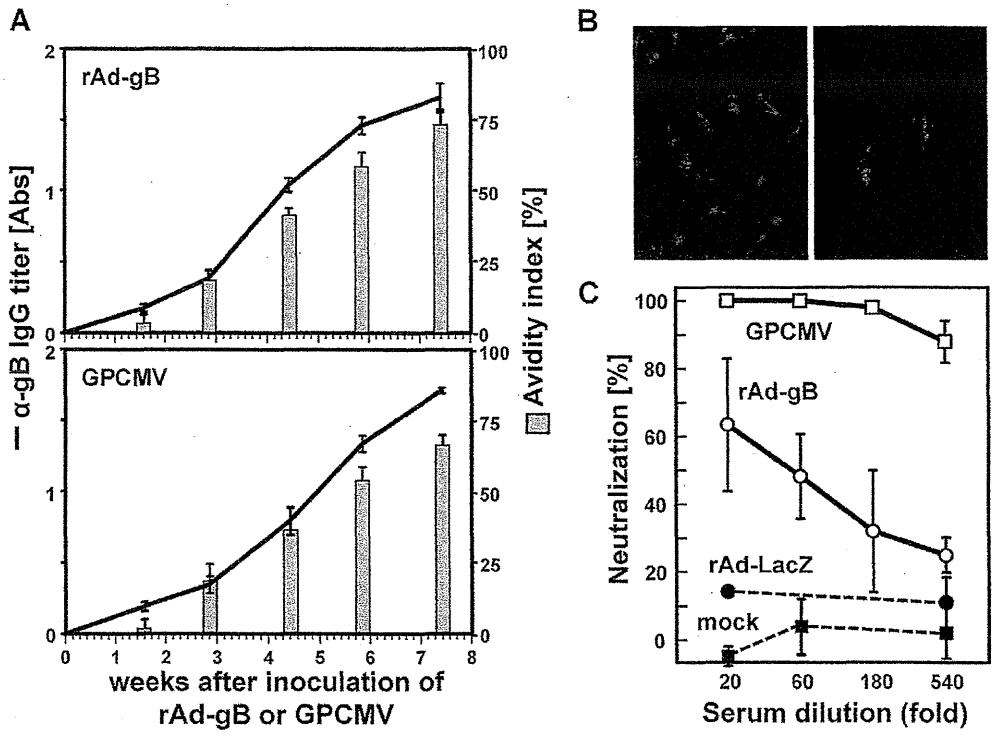

Fig. 1. Induction of anti-gB antibodies by immunization with rAd-gB. (A) Three 4-week old guinea pigs were inoculated with 10^{10} TUs of rAd-gB and GPCMV, respectively. Blood specimens were drawn from the great saphenous vein at the indicated days after inoculation, and their anti-gB IgG titers and avidity indices were measured. (B) RFP-expressing GPCMV, GPCMV-RFP(4A), was used for the detection of neutralizing antibodies. Examples of RFP foci detected in GPL cells after treatment of the virus with sera obtained from the mock-infected (left) and GPCMV-infected (right) animals are shown. (C) Neutralization assay was performed at indicated serum dilutions in triplicate. Serum specimens obtained from 3 guinea pigs immunized with rAd-gB and rAd-LacZ 7-weeks after immunization were analyzed and the averages and SDs of neutralization (%) by the respective 3 specimens are plotted. No reduction and complete inhibition of RFP-positive foci are indicated as 0% and 100% neutralization, respectively. Sera obtained from mock- and GPCMV-infected guinea pigs were used as negative and positive controls, respectively, for the detection of neutralizing antibodies.

3. Results

3.1. gB immunization of young animals

Administration of 10^{10} TUs of rAd-gB to young guinea pigs induced anti-gB antibodies, and the avidity index for the anti-gB antibodies increased gradually (Fig. 1A). Neutralizing activities against GPCMV in sera were measured by using RFP-expressing GPCMV (Fig. 1B and C). Although the anti-gB IgG titers and avidity indices of sera obtained from the animals immunized with rAd-gB were at a level similar to those of sera obtained from the animals infected with GPCMV (SG-P5), the neutralizing activities of the former sera were weaker than those of the latter sera, suggesting the presence of neutralizing antibodies other than those against gB.

Next, young animals were inoculated with rAd-gB or rAd-LacZ, and challenged with GPCMV at 2- to 4-weeks after inoculation. Although the GPCMV challenge resulted in a short-term weight loss of 6–17% in the rAd-LacZ-inoculated control animals, gB immunization suppressed weight loss (Fig. 2). It seems that younger animals are more prone to body weight loss after virus challenge. In addition, the amount of viral DNA in the salivary glands in the rAd-gB-inoculated animals was less than 1% that in the rAd-LacZ-inoculated animals (data not shown), indicating that gB immunization reduced viral dissemination to the salivary glands.

3.2. Protection of the placentas and fetuses against infection in gB-immunized dams

We observed that viral antigens and DNA were rarely detected in the placenta 1-week after infection at 4-weeks of gestation, but

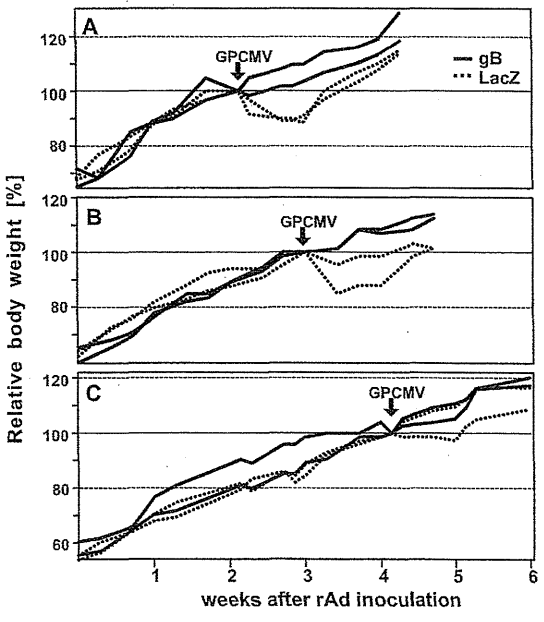

Fig. 2. Relative body weights of animals immunized with rAd-gB (continuous lines) and with rAd-LacZ (dashed lines) followed by GPCMV challenge (arrows) at 2-weeks (A), 3-weeks (B) and 4-weeks (C) after immunization are shown by using the weight of each animal at the time of the challenge as a 100% control.

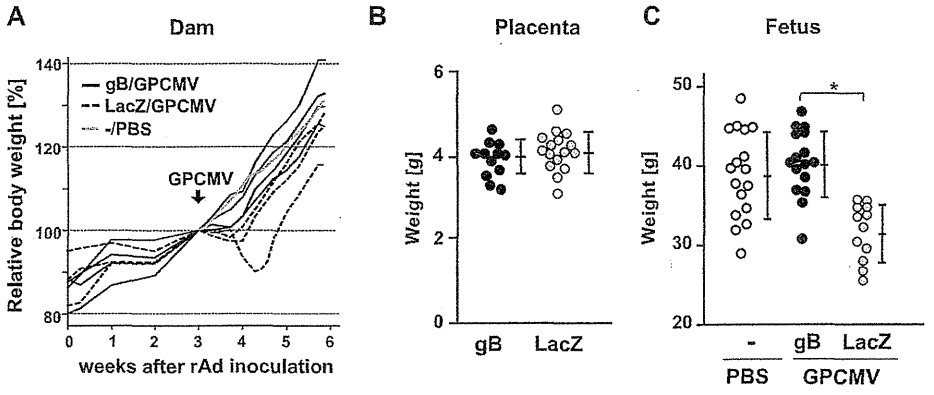

Fig. 3. (A) Relative body weights of dams immunized with rAd-gB (continuous lines), with rAd-LacZ (dashed lines), and with PBS (gray) followed by GPCMV challenge (arrow) are shown by using the body weight of each animal at the time of the GPCMV challenge (arrows) as a 100% control. Comparison of weights of the placentas (B) and fetuses (C) from dams without any treatment (open circles) and from dams immunized with rAd-gB (closed circles) or rAd-LacZ (gray circles). Each circle indicates one placenta or fetus. Means and SDs are shown. An asterisk means $p < 0.01$.

became detectable by 3-weeks after infection irrespective of the gestational age of the dams (data not shown). Based on this observation, we designed the experimental schedule as follows: guinea pigs were inoculated with 10^{10} TUs of Ad-LacZ or Ad-gB at 1-week of gestation, infected subcutaneously with 10^6 IUs of GPCMV at 4-weeks of gestation, and then sacrificed at 7-weeks of gestation. Although there was no difference in weight between the placentas from the gB- and LacZ-immunized dams, there was a significant difference in weight between the fetuses from the gB-immunized dams and those from the LacZ-immunized dams (Fig. 3). Taking the mean and standard deviation (SD) of the weight of the fetuses from untreated dams as the standard, we found that 42% of the fetuses from the LacZ-immunized dams exhibited IUGR, which was defined as a fetal weight less than the mean–1.5SD, whereas none of those from the gB-immunized dams did. Viral DNAs were detected by PCR in 75% and 13% of the fetuses from the LacZ- and gB-immunized dams, respectively (Fig. 4A). Two out of 16 fetuses from the gB-immunized dams (#A-1 and #B-5) were GPCMV positive. GPCMV DNAs were detected in most of their organs, and the viral loads in each organ were comparable to those from the LacZ-immunized dams (Fig. 4B).

3.3. Serological observations after challenge

Anti-gB antibody titers in the fetuses from the gB-immunized dams were similar to those of the dams (Fig. 5A). This was also true for the avidity indices against anti-gB IgG (Fig. 5B). As compared with the dams inoculated with rAd-LacZ, the anti-gB antibody titers and the avidity indices were significantly higher in the dams inoculated with rAd-gB, suggesting the presence of efficient pre-existing immunity in the dams immunized with rAd-gB.

No differences in weight in the placentas or fetuses, in the anti-gB IgG levels, or in the avidity indices (data not shown) were observed among the littermates of the gB-immunized dam A (#1–#4) as well as among those of dam B (#5–#8), suggesting

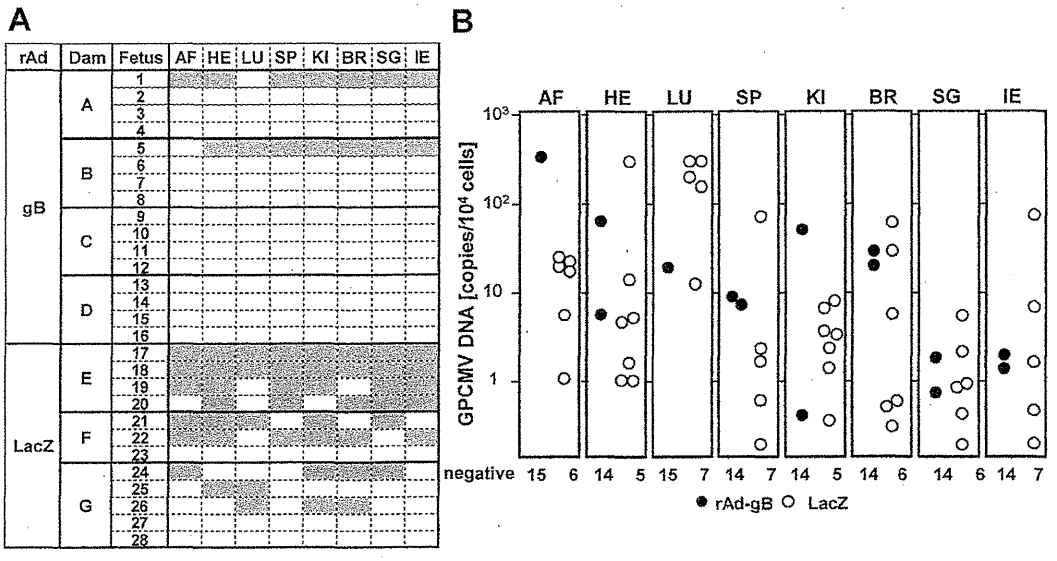

Fig. 4. GPCMV DNA detected in the organs of fetuses from dams immunized rAd-gB or -LacZ. (A) Shaded boxes indicate the presence of GPCMV DNA. (B) Viral loads (GPCMV DNA copies per 10^4 cells) of the organs from the fetuses are plotted. AF: amnion fluid, HE: heart, LU: lung, SP: spleen, KI: kidney, BR: brain, SG: salivary gland, and IE: inner ear.

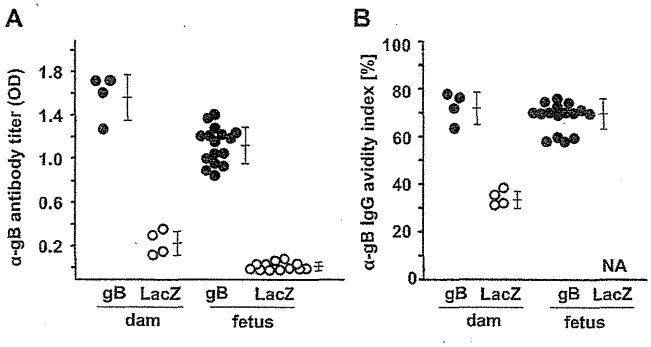

Fig. 5. Comparison of the anti-gB IgG titers and avidity indices against gB in sera obtained from dams immunized with rAd-gB (closed circles) or rAd-LacZ (open circles) and in sera from the fetuses. Each circle indicates one dam or fetus. Means and SDs are shown.

that gB-immunization protects the fetuses from IUGR irrespective of congenital infection and that the presence of anti-gB antibodies in the fetuses is not a sufficient determinant for protection from congenital infection.

3.4. Localization of GPCMV antigens in the placenta from gB-immunized dams

Immunohistochemical analysis using the anti-GPCMV monoclonal antibody g-1, which recognizes a 50-kDa viral protein expressed during the early phase of infection, showed that viral antigens were present focally in the placentas. In experiment A, in which the placenta specimens indicated in Figs. 3–5 were analyzed, 5 out of the 16 placentas from the gB-immunized dams were CMV positive, while 8 out of the 12 placentas from the LacZ-immunized dams were positive (Fig. 6B). The use of polyclonal antibodies against the IE2 protein gave similar results (data not shown). Taking into account the number of foci per slice, we found that more viral antigens were detected in the placentas of the LacZ-immunized dams ($p < 0.05$). However, as the difference was small, an additional set of dams were analyzed in the same way (experiment B), with similar results obtained in those animals. A comparison of CMV-positive rates in the placentas from gB- and LacZ-immunized dams in the combined results from experiments A and B revealed a statistical significance ($p < 0.05$), indicating the gB-immunization reduced CMV infection in the placentas. In the placentas from the dams immunized with rAd-LacZ, viral antigens

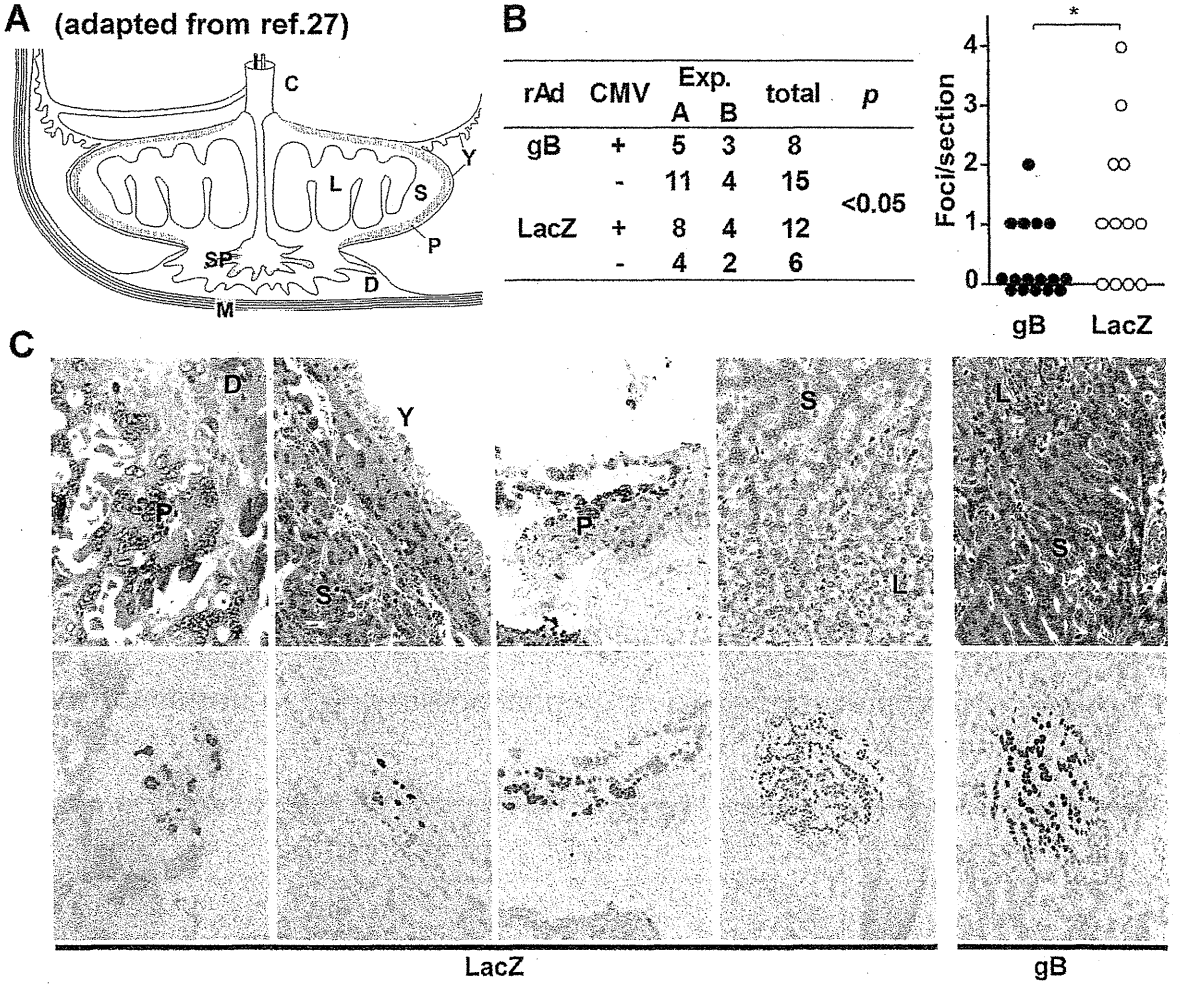

Fig. 6. Detection of viral antigens in the placentas. (A) Schematic presentation of the placental structure of guinea pigs (adapted from Ref. [27]). C: cord, M: myometrium, D: decidua, SP: subplacenta, Y: visceral yolk sac, P: parietal yolk sac, S: spongiotrophoblast layer, and L: labyrinth. (B) Numbers of CMV-positive foci in the placentas from rAd-gB- and rAd-LacZ-immunized dams. Two experiments, A and B, were performed. In the experiments (Exp.) A and B, the placenta specimens indicated in Figs. 3–5 and those obtained from an additional set of immunized dams were analyzed. A comparison of the number of foci per section in the placentas from dams immunized with rAd-gB (closed circles) or rAd-LacZ (open circles) in Exp. A is shown. An asterisk represents $p < 0.05$. (C) Localization of viral antigens in the placenta. HE staining (upper panels) and immunohistochemistry with anti-GPCMV antibody g-1 (lower panels) are shown.

were mainly detected in the spongiotrophoblast layer, occasionally in trophoblast giant cells in the parietal yolk sac, and only sporadically in the visceral yolk sac (Fig. 6C). No destruction of the spongy structure or inflammation was observed in the spongiotrophoblast layer. In contrast, some structural destruction and inclusion bodies were occasionally observed in the yolk sac. In the gB-immunized dams, viral antigens were detected only in the spongiotrophoblast layer. However, there was no apparent difference in the size of the CMV-positive foci in the spongiotrophoblast layer between the rAd-gB- and rAd-LacZ-immunized groups.

4. Discussion

Guinea pig models have been used for many years in an attempt to clarify the pathogenesis of congenital CMV infection. However, most prior studies using the guinea pig model for vaccine studies have focused on infection in pups. Since guinea pigs consume the placentas after delivery, there is little information on how CMV spreads in the placentas and then into the fetuses and on how gB vaccination prevents viral spread to and in the placenta. In addition, most prior studies used sera obtained from GPCMV-infected animals; in other words, sera in which active infection cannot be distinguished from abortive infection. Thus, our study is unique in undertaking the precise analysis of viral spread in placentas using a well-characterized monoclonal antibody. The most important findings in this study are as follows: (i) immunization of dams with gB reduced the level of viral antigens in the placentas slightly, but significantly, (ii) this slight reduction in viral load resulted in marked differences in the rates of IUGR (42% *vs.* 0%) and congenital infection (75% *vs.* 13%), (iii) gB immunization did not inhibit focal virus growth in the placentas, and (iv) the presence of anti-gB antibodies in the fetuses did not protect against viral spread in the fetuses.

Schleiss et al. demonstrated that preconceptional immunization with purified gB in combination with Freund's adjuvant reduced both pup mortality to 14% and CMV-infection in liveborn pups to 22% *vs.* 76% and 50%, respectively, in the unvaccinated group [13], and that a DNA vaccine expressing gB did not improve pup mortality but reduced the rate of viral transmission by half [14]. Similarly, immunization with GPCMV glycoproteins reduced pup mortality to 14% *vs.* 56% in the unvaccinated group [20]. The absence of any difference in the birth weight of pups born from dams with and without immunization is thought to be due to their comparison of the weights of only liveborn pups. The rate of congenital infection and the efficacy of gB immunization in our study were generally consistent with the results observed in those studies.

GPCMV infection in the placenta occurred focally in the spongiotrophoblast layer at a relatively low frequency, suggesting cell-to-cell spread of the virus in the placenta. This is consistent with previous studies that reported localization of viral antigens at the transitional zone between the capillarized labyrinth and the noncapillarized interlobium, presumably the spongiotrophoblast layer, with GPCMV-related lesions [15]. In humans, HCMV proteins have also been detected in focal areas in the term placenta [21]. The fact that gB immunization did not change the focal spread of GPCMV in the spongiotrophoblast layer, but reduced the number of foci, suggests that anti-gB antibodies are not effective against this form of viral spread in the placenta and that anti-gB antibodies mainly protect against infection at the interface of the placenta rather than inside the placenta or from the placenta to the fetus.

Recent studies on HCMV have demonstrated that human sera obtained from individuals naturally infected, but not from those immunized with the gB subunit vaccine or with the attenuated strain Towne, contain strong neutralizing activities against infection to endothelial and epithelial cells [22]. HCMV entry into endothelial cells can be inhibited with antibodies against UL128, UL130 and UL131A [23]. Since the major cell types comprising the placenta are cytotrophoblasts and syncytotrophoblasts, both of which possess similarities to epithelial cells, and vascular endothelial cells, it is plausible that neutralizing antibodies against gB cannot efficiently inhibit viral spread in the placenta. The neonatal Fc receptor mediated-transcytosis of CMV virions has been proposed as one possible mechanism for viral dissemination in the placenta, thus highlighting the importance of the quality and quantity of CMV-specific neutralizing antibodies to the prevention of viral transmission to the fetus [24]. It would be interesting to see whether immunization with the gH/gL/UL128/UL130/UL131A pentamer complex could protect the placenta against CMV infection. Although we tried to induce antibodies against GPCMV GP131, the HCMV UL130 homolog, by using the same rAd system, we could not induce a high level of anti-GP131 antibodies (data not shown).

The significant rate of IUGR observed in spite of the limited viral activity in the placenta suggests that infection induces secondary effects on placental functions. It would be important to identify which of the cellular genes are affected by CMV infection in the placenta. A recent study on amnion fluid specimens from pregnant women with congenital HCMV infection demonstrated that HCMV infection during pregnancy was associated with a shift in cytokine expression toward a proinflammatory state [25]. Pathological analyses on the placentas from women with congenital infection indicated that vascular endothelial growth factor (VEGF) and its receptor, fms-like tyrosine kinase 1 (Flt1), were up-regulated, and the amniotic fluid contained elevated levels of soluble Flt1 (sFlt1), an antiangiogenic protein, relative to placental growth factor. Hyperimmune globulin treatment reduced both VEGF and Flt1 expression as well as the sFlt1 levels [26]. Microarray analyses of cellular gene expression in the placentas of GPCMV-infected guinea pigs are currently under way.

In conclusion, this study demonstrated that a slight reduction in placental infection by immunization with gB can result in a significant prevention of IUGR and congenital infection, and that gB immunization may not be effective in reducing viral spread in the placenta.

Acknowledgements

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ORIGINAL ARTICLE

Mast Cells Play a Key Role in Host Defense against Herpes Simplex Virus Infection through TNF-α and IL-6 Production

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The essential contribution of mast cells (MCs) to bacterial host defense has been well established; however, little is known about their role in viral infections *in vivo*. Here, we found that intradermal injection with herpes simplex virus 2 (HSV-2) into MC-deficient $Kit^{W/W-v}$ mice led to increased clinical severity and mortality with elevated virus titers in HSV-infected skins. *Ex vivo* HSV-specific tetramer staining assay demonstrated that MC deficiency did not affect the frequency of HSV-specific cytotoxic T lymphocytes (CTLs) in draining lymph nodes. Moreover, the high mortality in $Kit^{W/W-v}$ mice was completely reversed by intradermal reconstitution with bone marrow–derived MCs (BMMCs) from wild-type, but not $TNF^{-/-}$ or $IL\text{-}6^{-/-}$ mice, indicating that MCs or, more specifically, MC-derived tumor necrosis factor (TNF) and IL-6 can protect mice from HSV-induced mortality. However, HSV did not directly induce TNF-α or IL-6 production by BMMCs; supernatants from HSV-infected keratinocytes induced the production of these cytokines by BMMCs without degranulation. Furthermore, IL-33 expression was induced in HSV-infected keratinocytes, and blocking the IL-33 receptor T1/ST2 on BMMCs significantly reduced TNF-α and IL-6 production by BMMCs. These results indicate the involvement of MCs in host defense at HSV-infected sites through TNF-α and IL-6 production, which is induced by keratinocyte-derived IL-33.

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INTRODUCTION

Mast cells (MCs) are not only effector cells in allergic responses but are also initiator and regulator cells in both innate and adaptive immune responses (Galli *et al.*, 2005). They are widely distributed throughout the body, in particular at host/environmental interfaces such as the skin and airways, where they preferentially localize around nerves and blood vessels (Galli *et al.*, 2005). They can therefore act as important sentinels for the immune system and control effective innate responses against invading pathogens by releasing various mediators, including a diverse array of cytokines, chemokines, and lipid mediators (Metz and Maurer, 2007; Abraham and St John, 2010).

The development of MC-deficient mouse models allows testing of MC contribution to biological responses of interest, both through the analysis of experimental outcomes in MC-deficient mice and those in mice in which MC deficiency has been selectively repaired by local engraftment (Galli *et al.*, 2005; Metz and Maurer, 2007). Using this so-called "MC knock-in mouse" model, several studies have demonstrated that MCs are critical effector cells in eliciting protective immune responses against bacteria, and that MC-derived tumor necrosis factor-α (TNF-α) is largely responsible for bacterial clearance by inducing neutrophil recruitment into sites of infection (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996). For the many MCs located proximal to blood vessels, the release of factors such as other cytokines, histamine, proteases, and chemokines also contributes to increased local vascular permeability and recruitment of other participants in the inflammatory response at the site of infection (Abraham and St John, 2010).

In contrast to the well-established contributions of MCs to host defense against bacteria, the function of MCs in antiviral immunity has not been well defined. We and others have previously reported that Toll-like receptor 3 (TLR3)-, TLR7-, and TLR9-mediated activation of MCs can induce selective production of cytokines and chemokines, suggesting that MCs

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Abbreviations: BMMC, bone marrow–derived mast cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DLN, draining lymph node; HSV-2, herpes simplex virus-2; MC, mast cell; MOI, multiplicity of infection; NK, natural killer; TNF-α, tumor necrosis factor-α; TLR, Toll-like receptor; WT, wild type

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are capable of secreting these mediators in response to virus-derived pathogen-associated molecular patterns (Kulka *et al.*, 2004; Matsushima *et al.*, 2004). Indeed, several viruses, including dengue virus and adenovirus, have been shown to activate MCs *in vitro* through TLR3, TLR7, and possibly other mechanisms (Dawicki and Marshall, 2007). Nevertheless, the *in vivo* contribution of MCs to host defense in viral infections is less clear, mainly because suitable mouse models of viral infection have not been tested in the "MC knock-in mouse" model until recently (Metz *et al.*, 2008; Abraham and St John, 2010). However, two very recent studies have revealed the *in vivo* role of MCs in protective immune responses against viral infection using "MC knock-in mouse" model, and demonstrated that natural killer (NK) and NK T-cell recruitment promoted by MCs or MC-derived antimicrobial peptides has a pivotal role in viral clearance during dengue virus or vaccina virus infection, respectively (St John *et al.*, 2011; Wang *et al.*, 2012).

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects more than 500 million people worldwide and causes most cases of genital herpes (Looker *et al.*, 2008). In cutaneous herpes simplex lesions of humans and in murine models, keratinocytes, dendritic cells (DCs), and infiltrating lymphocytes, especially HSV-specific CD8 T lymphocytes, are known to have a central role in controlling primary and recurrent HSV infections (Simmons and Tscharke, 1992; Chew *et al.*, 2009). Moreover, the important role of other innate immune effectors such as NK cells, NK T cells, plasmacytoid DCs, macrophages, and γδ T lymphocytes has been recently re-emphasized, either in direct immune control or via modulation of adaptive immune responses in HSV infection (Cheng *et al.*, 2000; Chew *et al.*, 2009; Melchjorsen *et al.*, 2009). However, very little *in vitro* and *in vivo* data exist regarding the role of MCs in HSV infections.

Here, we examined the *in vivo* contribution of MCs to the immune responses against HSV using the "MC knock-in mouse" model and demonstrated that MCs were critically involved in host defense at HSV-infected sites through TNF-α and IL-6 production. Our study also suggests an important role for IL-33 derived from HSV-infected keratinocytes as a trigger for the production of these inflammatory cytokines by MCs.

RESULTS

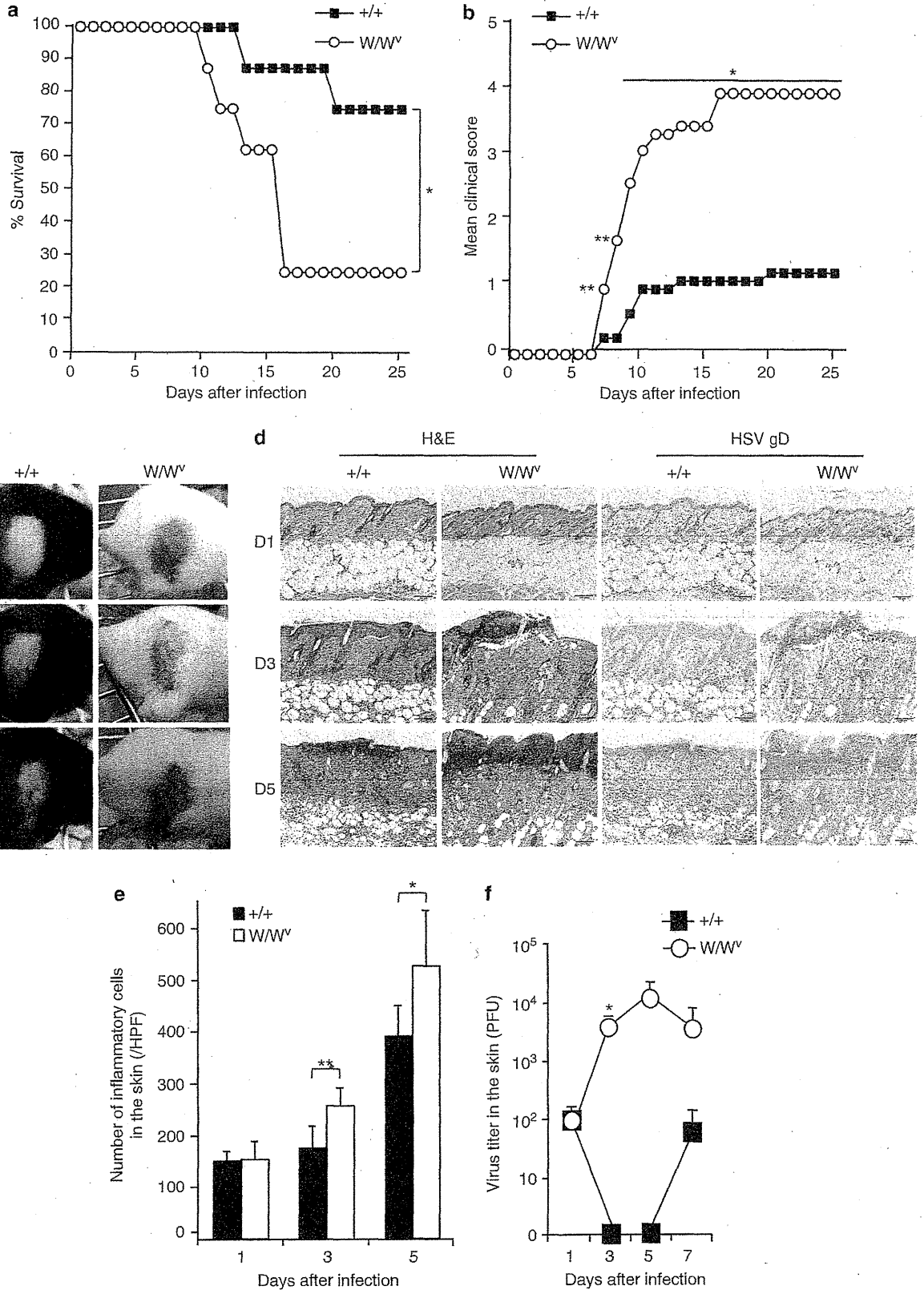
MC-deficient mice exhibit high mortality and local inflammation following cutaneous HSV infection

We first assessed the role of MCs *in vivo* by using a murine model of lethal HSV encephalitis (Corey and Spear, 1986), whereby peripheral infection with HSV involves local replication in the skin, followed by rapid dissemination of the virus via sensory axons, causing zosteriform lesions to spread from the primary inoculation site along the affected dermatomes, and leading to paralysis and death. Using this model, MC-deficient $Kit^{W/W\text{-}v}$ mice and $Kit^{+/+}$ wild-type (WT) mice were injected intradermally with HSV-2 (7.5×10^4 PFU) and were monitored for survival and scored for paralysis and skin lesions. Intriguingly, $Kit^{W/W\text{-}v}$ mice exhibited markedly decreased percent survival when compared with $Kit^{+/+}$ mice (Figure 1a). We also compared clinical severity in these mice by using the experimental autoimmune encephalomyelitis score, which reflects paralysis. The clinical score in $Kit^{W/W\text{-}v}$ mice was significantly higher than that in $Kit^{+/+}$ mice (Figure 1b). We obtained similar findings when we repeated the experiments with MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice injected intradermally with HSV-2 (Supplementary Figure S1a and b online). As shown in Figure 1c, the zosteriform eruption in the skin lesions of $Kit^{W/W\text{-}v}$ mice appeared at an earlier time point, and exacerbated more rapidly and severely than that of $Kit^{+/+}$ mice. Consistent with these findings, histological examination of the zosteriform lesion in $Kit^{W/W\text{-}v}$ mice revealed increased inflammation and an extended infection area, as demonstrated by HSV glycoprotein D antigen expression in subepithelial tissue, when compared with that in $Kit^{+/+}$ mice, at 3 and 5 days post infection (Figure 1d and e). On the basis of this histopathological observation, we assessed whether MCs contribute to HSV clearance by measuring virus titers in the HSV-infected skins of $Kit^{+/+}$ and $Kit^{W/W\text{-}v}$ mice. The virus titers in $Kit^{W/W\text{-}v}$ mice rapidly increased, reaching maximal levels at day 5, and dropped afterward (Figure 1f). In contrast, the skin lesions of $Kit^{+/+}$ mice contained lower virus titers, which were sustained until day 7. These results suggested that the severe inflammation and mortality observed in $Kit^{W/W\text{-}v}$ mice may be attributed to impaired virus clearance during these early stages of HSV infection, particularly during the first 3 days.

MCs produce TNF-α and IL-6, but not IFN-α, in response to soluble factors released from HSV-infected keratinocytes

The impaired clearance of HSV-2 in the skin lesion of $Kit^{W/W\text{-}v}$ mice was observed during the first 72 hours after infection (Figure 1f), suggesting that MCs are involved in innate immunity, rather than acquired immunity, against HSV-2. In early immune responses in HSV infection, several studies highlight the importance of TNF-α for protection against lethal HSV encephalitis (Rossol-Voth *et al.*, 1991; Lundberg *et al.*, 2007). In addition, TNF-α has been shown to control HSV replication, independent of T and B cells (Feduchi *et al.*, 1989; Heise and Virgin, 1995). Other studies indicated that IL-6 and IFN-α decrease the susceptibility to HSV infection (Murphy *et al.*, 2008; Melchjorsen *et al.*, 2009). Consistent with these findings, we could detect the production of TNF-α, IL-6, and IFN-α at HSV-2 injection sites 72 hours after infection in WT mice (Supplementary Figure S3a online). Therefore, we next assessed whether HSV-2 directly induced cytokine production by BMMCs. However, MCs are resistant to HSV-2 infection (Supplementary Figure S4a online), and HSV-2 exposure did not induce TNF-α and IL-6 production or degranulation by BMMCs (Supplementary Figure S4b–d online).

Because the major cellular targets for HSV-2 were found to be epidermal keratinocytes in the skin lesion of $Kit^{+/+}$ and $Kit^{W/W\text{-}v}$ mice at 3 days after infection Figure 1d), we hypothesized that soluble factors released from HSV-infected keratinocytes may induce the cytokine production by MCs. Strikingly, as shown in Figure 2a and b, the supernatants of HSV-treated keratinocytes, Pam-212, induced significant TNF-α and IL-6 production by BMMCs in a multiplicity of infection (MOI)–dependent manner, whereas they failed to induce


a
+/+
W/W^v
% Survival
Days after infection
b
Mean clinical score
Days after infection
c
+/+
W/W^v
D2
D5
D7
d
H&E
HSV gD
D1
D3
D5
e
Number of inflammatory cells in the skin (/HPF)
Days after infection
f
Virus titer in the skin (PFU)
Days after infection

degranulation in, and IFN-α production by, BMMCs (Figure 2c and d). Notably, *in vitro* HSV infection of keratinocytes did not directly induce the production of these cytokines (Supplementary Figure S3b online). These results imply that MCs may be capable of producing TNF-α and IL-6 in response to cutaneous HSV-2 infection *in vivo* by indirect stimulation via soluble factors derived from HSV-infected keratinocytes. Interestingly, as shown in Supplementary Figure S2a and c online, HSV-2 did not significantly increase MC degranulation in the skin *in vivo*.

MCs, particularly MC-derived TNF-α and IL-6, protect mice from HSV-induced severe mortality

To confirm whether the different responses against cutaneous HSV-2 infection in $Kit^{+/+}$ and $Kit^{W/W\text{-}v}$ mice observed in Figure 1 reflect the lack of MCs in $Kit^{W/W\text{-}v}$ mice rather than other c-Kit-related differences (Galli *et al.*, 2005), we examined $Kit^{W/W\text{-}v}$ mice locally reconstituted with BMMCs derived from $Kit^{+/+}$ mice (WT BMMC→$Kit^{W/W\text{-}v}$). As expected, the decreased survival rate and the severe clinical and lesion score in HSV-infected $Kit^{W/W\text{-}v}$ mice were significantly improved by local reconstitution with MCs, comparable to those in $Kit^{+/+}$ mice (Figure 3a–c). Nevertheless, local reconstitution of $Kit^{W/W\text{-}v}$ mice with BMMCs derived from TNF-deficient mice ($TNF^{-/-}$ BMMC→$Kit^{W/W\text{-}v}$) or IL-6-deficient mice ($IL\text{-}6^{-/-}$ BMMC→$Kit^{W/W\text{-}v}$) did not significantly improve percent survival, clinical score, or lesion score (Figure 3a–c). These results clearly indicate that both MC-produced TNF and IL-6 critically contribute to protective antiviral responses to HSV-2 *in vivo*.

IL-33 derived from HSV-2-treated keratinocytes can induce cytokine production by MCs

Next, we explored which soluble factors released by HSV-2-treated keratinocytes induce TNF-α and IL-6 production by MCs. Recent studies have highlighted the important roles of IL-33 as an "alarmin" in innate immune responses, as IL-33, as a key product of epithelial barrier tissues such as the skin, can be released into the extracellular space after epithelial cell damage during infection or trauma, and it functions as an alarmin to alert the immune system (Moussion *et al.*, 2008; Luthi *et al.*, 2009). It is notable that IL-33 has recently been shown to be upregulated in the lung during viral infection with influenza virus and spleen during infection with lymphocytic choriomeningitis virus (Chang *et al.*, 2011; Bonilla *et al.*, 2012). In line with these findings, we found that increased IL-33 expression was selectively detected on damaged or degenerating Pam-212 cells after *in vitro* HSV-2 exposure, and the frequency of IL-33-expressing cells was increased in an MOI-dependent manner (Figure 4a). In addition, we found a significant increase in IL-33 production by HSV-infected Pam-212 cells (Figure 4a). Importantly, we found that IL-6 and TNF-α production by BMMCs in response to the supernatants from HSV-2-treated Pam-212 cells was significantly and most effectively reduced by blockade of the IL-33 receptor using the T1/ST2 antibody (Figure 4b). Conversely, IL-6 production was not affected by neutralization of other cytokines including IL-1α, IFN-β, and thymic stromal lymphopoietin (Supplementary Figure S5 online). Furthermore, we found that IL-33 stimulates the production of TNF-α and IL-6 by BMMCs in a dose-dependent manner (Figure 4c), without inducing degranulation (data not shown). On the basis of these results,

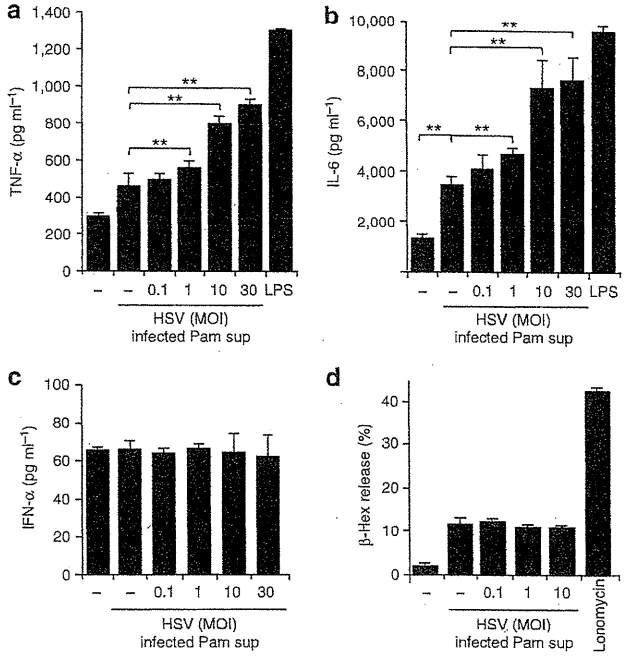


Figure 2. Supernatants of herpes simplex virus 2 (HSV)–infected keratinocytes induce tumor necrosis factor-α (TNF-α) and IL-6, but not IFN-α, production by bone marrow–derived mast cells (BMMCs). BMMCs were stimulated with culture supernatants (sup) from Pam-212 cells treated with or without HSV at multiplicities of infection (MOIs) of 0.1, 1, 10, or 30, or with lipopolysaccharide (LPS; 10 ng ml^{-1}) as a positive control for 24 hours, and assessed for (**a**) TNF-α, (**b**) IL-6, and (**c**) IFN-α production by ELISA. (**d**) β-Hexosaminidase (β-Hex) assay of BMMCs at 1 hour after stimulation with the culture supernatants from Pam-212 cells treated with or without HSV at MOIs of 0.1, 1, or 10 for 24 hours, or with ionomycin (1 μM) for 10 minutes as a positive control. $^{**}P<0.01$ versus results for untreated Pam-212 cells. Data are representative of three independent experiments, showing the means ($n=3$) ± SD.

Figure 1. Mast cell (MC)-deficient mice exhibit increased clinical severity and mortality following cutaneous herpes simplex virus 2 (HSV-2) infection. MC-deficient C57BL/6-$Kit^{W/W\text{-}v}$ mice (white circles) and the corresponding wild-type (WT) C57BL/6-$Kit^{+/+}$ mice (black squares) ($n=8$) were injected intradermally with HSV-2 186 strain (7.5×10^4 PFU) on the right back. (**a**) Survival rates, (**b**) mean clinical scores, (**c**, **d**) representative clinical photos and cross-sections stained with anti-HSV antibody (Ab) or by hematoxylin and eosin (H&E) staining of HSV-infected skins (original magnification ×200, scale bar = 100 μm), (**e**) quantification of the cell infiltrate in **d** throughout 10 high-power fields (HPFs) of view, and (**f**) HSV titers in the skin ($n=3$) measured in plaque assays at the indicated time points after HSV-2 inoculation are shown. gD, glycoprotein D. The results of $Kit^{+/+}$ mice at days 3 and 5 were below the limit of detection. $^{*}P<0.05$ and $^{**}P<0.01$ versus the corresponding WT mice. Data are representative of at least three independent experiments with similar results, showing the means ($n=3$) ± SD.

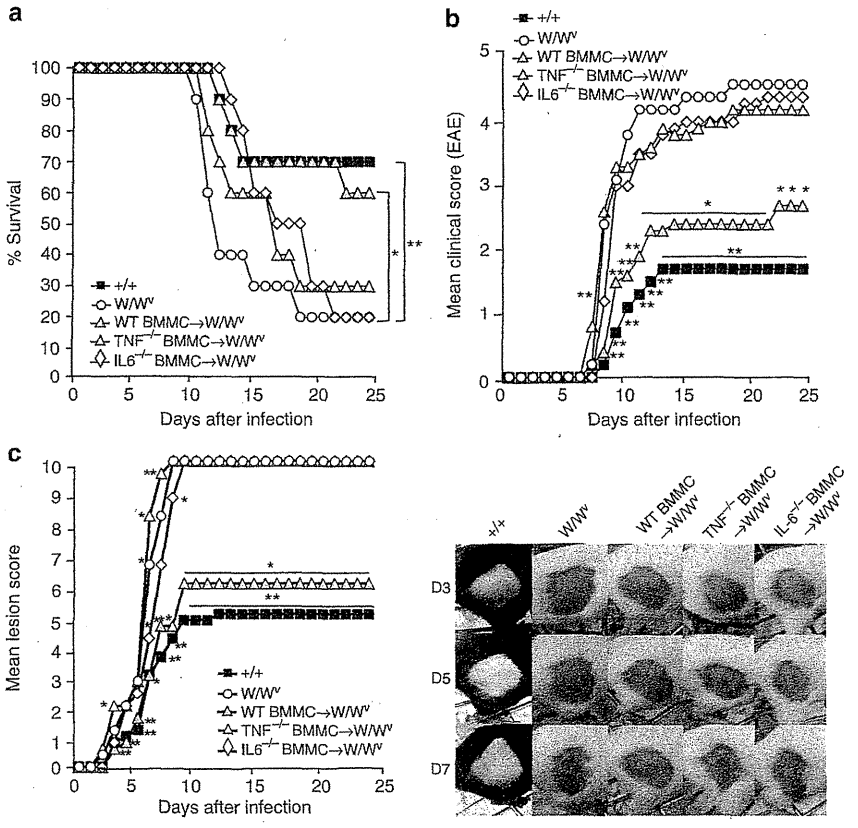


Figure 3. Reconstitution with bone marrow–derived mast cells (BMMCs) derived from wild-type (WT) but not $TNF^{-/-}$ or IL-$6^{-/-}$ mice prevents mortality in $Kit^{W/W\text{-}v}$ mice. (**a**) Survival rates, (**b**) clinical (experimental autoimmune encephalomyelitis (EAE)) scores, (**c**) lesion scores, and representative clinical photographs of skin lesions of $Kit^{W/W\text{-}v}$ mice (white circles), WT $Kit^{+/+}$ mice (black squares), WT BMMC-reconstituted $Kit^{W/W\text{-}v}$ mice (gray circles), $TNF^{-/-}$ BMMC-reconstituted $Kit^{W/W\text{-}v}$ mice (white triangles), and IL$6^{-/-}$ BMMC-reconstituted $Kit^{W/W\text{-}v}$ mice (white lozenges) at the indicated time points after intradermal injection with herpes simplex virus 2 (HSV-2; 7.5×10^4 PFU) are shown. TNF, tumor necrosis factor. $^*P<0.05$ and $^{**}P<0.01$ versus C57BL/6-$Kit^{W/W\text{-}v}$ mice. Data are representative of three independent experiments; $n=10$ mice/group.

we conclude that enhanced TNF-α and IL-6 production in MCs by supernatants from HSV-2-infected keratinocytes is, at least in part, mediated by IL-33 signaling.

MCs do not contribute to the induction of HSV-specific $CD8^+$ T cells

Previous studies revealed a significant role of $CD8^+$ T cells in controlling HSV infections (Simmons and Tscharke, 1992; Chew *et al.*, 2009). Recent studies in mice demonstrated that migrating dermal $CD103^+$ and langerin-expressing DCs are the major transporters of HSV antigens out of skin and, together with resident $CD8^+$ DCs, are the major antigen-presenting cells of HSV antigens to $CD8^+$ T cells in draining lymph nodes (DLNs) (Allan *et al.*, 2003; Bedoui *et al.*, 2009). Therefore, we assessed the number of HSV-gB-specific $CD8^+$ T cells by using tetramer staining, as well as $CD8\alpha^+$ DCs and $langerin^+$ DCs, in DLNs of $Kit^{+/+}$ and $Kit^{W/W\text{-}v}$ mice after intradermal infection with HSV. The proportions of HSV-gB-specific $CD8^+$ T cells in the DLNs of HSV-infected $Kit^{+/+}$ and $Kit^{W/W\text{-}v}$ mice at 6 days after infection were significantly increased when compared with that of uninfected mice (Figure 5a). However, the numbers of HSV-gB-specific $CD8^+$ T cells in $Kit^{+/+}$ and $Kit^{W/W\text{-}v}$ mice were comparable (Figure 5b), suggesting that MCs are not essential for the generation of HSV-specific $CD8^+$ T cells in primary cutaneous HSV-2 infection. Consistent with these findings, there was no significant difference in the frequency of $CD8\alpha^+$ DCs or $langerin^+$ DCs in DLNs of $Kit^{+/+}$ and $Kit^{W/W\text{-}v}$ mice at 2 or 5 days after infection, respectively (Figure 5c). Similarly, no significant difference was detected in the number of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells found in DLNs (Figure 5d). These results suggest that the impaired clearance of HSV-2 at the infection site observed in $Kit^{W/W\text{-}v}$ mice (Figure 1e) may not be attributed to the impaired induction of HSV-specific cytotoxic T lymphocytes (CTLs).

DISCUSSION

In this study, we have identified previously unknown functions of skin MCs in host protection against HSV-2. The contribution of MCs to host defense against HSV, one of the most common viral infections in the world, had been less clear, probably because MCs are resistant to HSV-2 infection. Indeed, recent

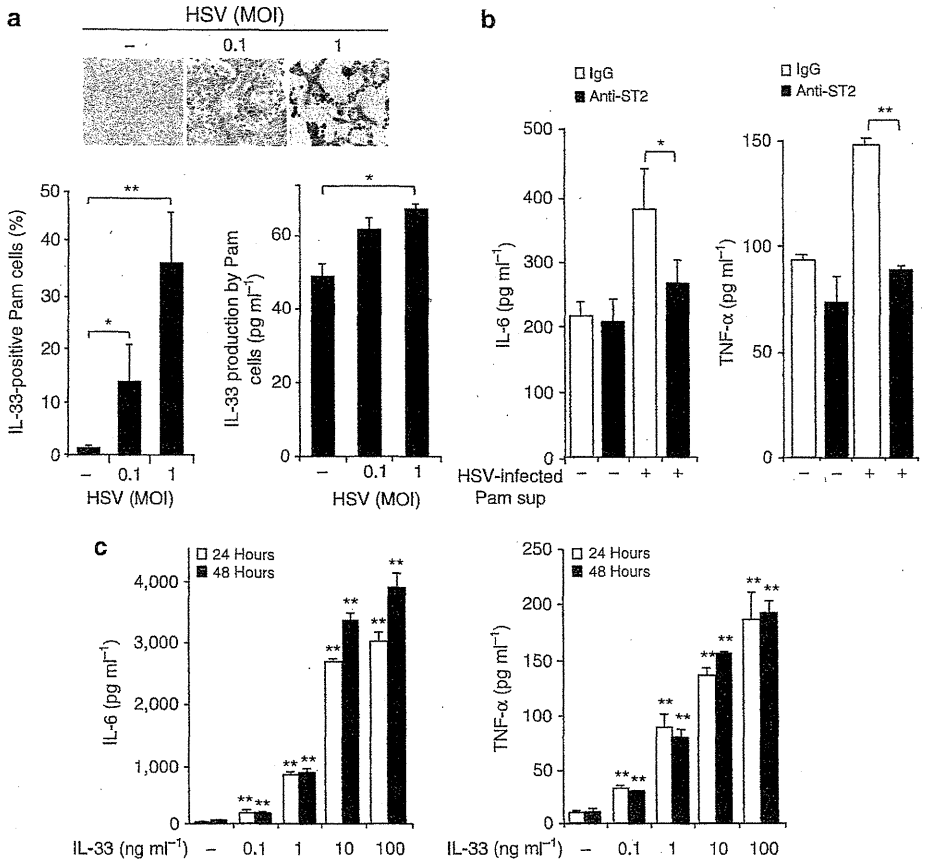


Figure 4. IL-33 is upregulated in herpes simplex virus 2 (HSV-2)–infected keratinocytes, and promotes IL-6 and tumor necrosis factor-α (TNF-α) production by mast cells (MCs). (**a**) Immunohistochemical staining for IL-33 in HSV-infected (multiplicities of infection (MOIs) 0.1 and 1) or uninfected Pam-212 cells 24 hours after infection (original magnification ×400). The percentage of IL-33-positive staining cells in the total population of Pam-212 cells was assessed throughout five high-power fields of view. IL-33 production by HSV-infected (MOIs 0.1 and 1) or uninfected Pam-212 cells 24 hours after infection. (**b**) Bone marrow–derived MCs (BMMCs) were pretreated with anti-T1/ST2 antibody or control IgG for 30 minutes, and then stimulated with culture supernatants (sup) from HSV-infected (MOI 30) or uninfected Pam-212 cells for 24 hours. (**c**) BMMCs were stimulated with control IgG (100 ng ml^{-1}), IL-33 (0.1–100 ng ml^{-1}), or lipopolysaccharide (LPS; 10 ng ml^{-1}) for 24 or 48 hours. (**b**, **c**) IL-6 and TNF-α production by BMMCs was assessed by ELISA. *P<0.05 and **P<0.01 versus results for control IgG. Data are representative of three independent experiments, showing the means (n=3) ± SD.

studies have shown MC involvement in viral host defense, in which the infection of MCs with the virus, such as dengue virus or vaccina virus, is required for MC-mediated immune responses (St John *et al.*, 2011; Wang *et al.*, 2012). Unlike these viral infection models, we demonstrated that MCs are critically involved in viral host defense against HSV-2, even though relatively few HSV-2-infected MCs were detected *in vitro* and *in vivo* (Supplementary Figure S3a online and data not shown). We also report that MC deficiency resulted in impaired HSV-2 clearance at the infection sites during the first 72 hours after infection, suggesting that MCs have key roles as the first line of defense against HSV-2 rather than contributing to acquired immunity. Using "MC knock-in mouse" model, we also demonstrated the crucial contribution of MC-produced TNF-α and IL-6 to protective antiviral responses to HSV-2. Several previous studies suggested an antiviral mechanism involving TNF-α and IL-6 that is responsible for a direct reduction in viral replication or for increasing local infiltration of innate immune cells at the site of HSV infection (Feduchi *et al.*, 1989; Rossol-Voth *et al.*, 1991; Heise and Virgin, 1995; Lundberg *et al.*, 2007; Murphy *et al.*, 2008). In addition, the infiltrating cells, including plasmacytoid DCs, neutrophils, and NK cells, and their products including key antiviral cytokines IFN-α/β, may also provide other means of limiting HSV replication and eliminating virus-infected cells (Melchjorsen *et al.*, 2009).

A recent study revealed that MC-dependent CTL responses are important for an optimized host defense, such as protection against intracellular bacteria, because MCs can internalize, process, and present bacterial antigens and induce antigen-specific activation and proliferation of CD8$^+$ T cells upon infection with *Listeria monocytogenes* (Stelekati *et al.*, 2009). In our model of cutaneous HSV-2 infection, however, HSV-specific CTL generation in MC-deficient mice was not impaired. It is possible that differences in pathogens or their infection levels in MCs might explain the differences in MC

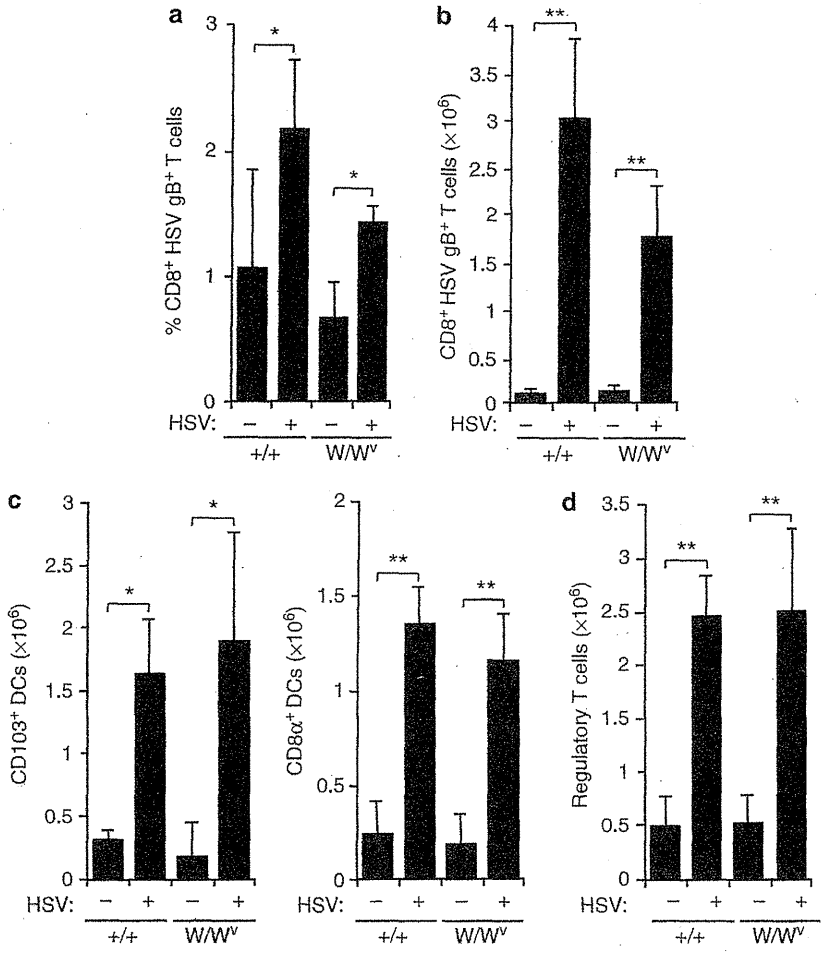


Figure 5. Mast cells (MCs) are not essential for the generation of herpes simplex virus 2 (HSV)–specific CD8$^+$ T cells. Kit$^{W/W\text{-}v}$ and Kit$^{+/+}$ mice were injected with HSV-2, as described in Figure 1. Draining lymph nodes (DLNs) were harvested at (**a**, **b**) 6, (**c**) 2 or 5, and (**d**) 3 days after infection. (**a**) The percentages of CD8$^+$ HSV gB$^+$ T cells in DLNs of HSV-2-infected and -uninfected mice were analyzed by flow cytometry using major histocompatibility complex (MHC) class I tetramer specific for HSV peptide glycoprotein B (gB) or tyrosinase-related protein 2 (TRP2) as a negative control (data not shown). (**b**) The number of CD8$^+$ HSV gB$^+$ T cells in DLNs. (**c**) The number of CD103$^+$ CD205$^+$ dendritic cells (DCs) at 5 days after infection and CD8α$^+$ DCs at 2 days after infection in DLNs. (**d**) The number of CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T-cell population in DLNs. *P<0.05 and **P<0.01 versus the corresponding uninfected mice. Data are representative of two independent experiments, showing the means (n=5 mice/group) ± SD.

contribution for CTL generation among these infection models. Nevertheless, in our model, it is still possible that MCs are involved in the recruitment of CTLs to the sites of HSV infection, because CD8$^+$ T-cell recruitment to sites of infection is facilitated by MCs during infection with Newcastle disease virus (Orinska *et al.*, 2005). Further detailed analysis under different viral infection is needed to reveal the functions and significance of MCs for CTL responses against viral infection.

The results of our study show that IL-33 derived from epidermal keratinocytes damaged by viral infection has a critical role in triggering the production of inflammatory cytokines by MCs. Although several viruses have been shown to infect and/or activate MCs *in vitro*, we observe neither significant HSV-2 infection nor HSV-induced direct activation of BMMCs monitored by cytokine production and degranulation. In contrast, the culture supernatants from HSV-2-treated keratinocytes, as well as IL-33 alone, induced TNF-α and IL-6 production by MCs independent of degranulation. The effect of the supernatants from HSV-2-treated keratinocytes was reduced consistently and most effectively by blockade of IL-33–IL-33R signaling in MCs. In addition, although we have recently reported that extracellular adenosine 5′-triphosphate also mediates "danger signal" derived from the damaged keratinocytes (Kawamura *et al.*, 2012), hydrolyzing extracellular adenosine 5′-triphosphate contained in the supernatants by soluble ecto-nucleoside triphosphate diphosphohydrolase (NTPDase; apyrase) did not affect TNF-α and IL-6 production by MCs (data not shown). These results clearly indicate the importance of IL-33 as an "alarmin" in cutaneous HSV infection. Our findings also suggest that IL-33 may be important for other cutaneous virus infections, such as varicella, hand–foot–mouse disease, warts, molluscum, and so on, which also induce the damage or degeneration of

keratinocytes. Further studies are needed to determine whether there is a significant *in vivo* role for IL-33 as an "alarmin" in viral infections other than HSV-2.

MATERIALS AND METHODS

Mice

Female c-Kit-mutant genetically MC-deficient C57BL/6-Kit$^{W/W\text{-}v}$ (Kit$^{W/W\text{-}v}$) mice and the congenic normal C57BL/6-Kit$^{+/+}$ (Kit$^{+/+}$) mice were purchased from Japan SLC (Hamamatsu, Japan). C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ (Kit$^{W\text{-}sh/W\text{-}sh}$) mice were obtained from Sankyo Labo Service (Tokyo, Japan). C57BL/6-Tnf$^{-/-}$ mice and C57BL/6-Il-6$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were carried out in strict accordance with the recommendations in Guidelines for Proper Conduct of Animal Experiments of Science Council of Japan. The protocol was approved by University of Yamanashi Animal Care and Use Committee (permit number: 19–50).

Virus

The WT HSV-2 strain 186 was prepared in Vero cells as described previously (Ushijima *et al.*, 2009), and stored at −80 °C with an approximate titer of 1×10^7 PFU per ml.

HSV inoculation of mice

Kit$^{+/+}$ mice, Kit$^{W/W\text{-}v}$ mice, and Kit$^{W\text{-}sh/W\text{-}sh}$ mice were injected intradermally with HSV-2 (7.5×10^4–7.5×10^5 PFU in 50 μl of EMEM; Nissui, Tokyo, Japan) on the right lower flank after shaving under diethyl ether anesthesia. Mice were then monitored for survival and scored for skin lesions and paralysis. Clinical score was assessed using the experimental autoimmune encephalomyelitis score, and skin lesions were scored as described previously (Takasaki *et al.*, 2000).

Measurement of viral titers and cytokines in HSV-2-inoculated skin

Kit$^{W/W\text{-}v}$ mice or Kit$^{+/+}$ mice were intradermally infected with 7.5×10^4 PFU of HSV-2 186 strain ($n=3$) on the right back. The primary inoculation site of skin (8 × 8 mm) was excised at 1, 3, 5, or 7 days after infection, respectively. Skin samples were thawed and homogenized in 2 ml of Dulbecco's phosphate-buffered saline (Invitrogen, Carlsbad, CA) containing protease inhibitors (Thermo Scientific, Waltham, MA) with sea sand on ice, centrifuged at 7,000 r.p.m. for 5 minutes, and the supernatants were added on Vero cell monolayers. After 24 hours, cultures were fixed with 5% formalin, stained with 0.05% crystal violet, and plaques were counted using a dissecting microscope. For measurement of cytokines, skin samples were excised and homogenized in 250 μl of Tper EDTA-free lysis buffer (Pierce) containing protease inhibitors (Thermo Scientific). Cytokine screening was performed in the supernatants using the Multi-Analyte ELISArray Kit (SABiosciences, Fredrick, MD) according to the manufacturer's instructions.

Preparations of BMMCs

BMMCs were prepared from B6 mice BM cell suspensions, as described previously (Matsushima *et al.*, 2004). Briefly, crude BM cells (4×10^5 cells per ml) were cultured in complete RPMI-1640 (Invitrogen) in the presence of murine recombinant IL-3 (10 ng ml^{-1}) and recombinant stem cell factor (10 ng ml^{-1}; PeproTech, Boston, MA). Nonadherent and loosely adherent cells were recovered twice a week, and further expanded in fresh medium for 4 to 6 weeks. The resulting MC preparations contained >95% CD45$^+$ CD117$^+$ cells.

In vitro HSV infection of BMMCs and keratinocytes

For HSV-2 infection of keratinocytes, 1×10^5 Pam-212 keratinocytes were exposed to HSV-2 strain 186 at an MOI of 0.1, 1, 10, or 30 for 1 hour and washed three times. At 24 hours after incubation, the culture supernatants were collected and passed through a filter (Minisart high flow, pore size 0.1 μm; Sartorius Stedim Biotech, Goettingen, Germany) to remove free virus. For HSV-2 infection of MCs, 2×10^5 BMMCs were infected for 1 hour with HSV-2 at an MOI of 0.1, 1, or 10. Following infection, cells were washed three times and then incubated for an additional 24 hours. In some experiments, BMMCs were stimulated with the culture supernatants from Pam-212 cells, treated with or without HSV for 1 hour, or were stimulated with lipopolysaccharide from *E. coli* serotype 0111:B4, containing <1% protein and <1% RNA (10–100 ng ml^{-1}, Sigma-Aldrich, St Louis, MO) for 24 hours, as control. In the blocking experiments, the culture supernatants from HSV-infected (MOI 30) Pam-212 cells were pretreated with 20 μg ml^{-1} of anti-IL-1α (R&D Systems, Minneapolis, MN), IFN-β (Abcam, Cambridge, UK), and thymic stromal lymphopoietin (R&D Systems) mAbs or isotype-matched control IgG mAbs (BD Pharmingen, San Diego, CA) for 15 minutes before addition to BMMCs. For blocking IL-33-mediated signals, BMMCs were preincubated with 40 μg ml^{-1} of anti-T1/ST2 mAb (MABioproducts, Walkerville, MD) or isotype control mAb for 30 minutes before addition of the culture supernatants from HSV-infected Pam-212 cells. BMMCs were stimulated for 6 hours with the supernatants, washed three times, and incubated in their growth medium for an additional 24 hours. In some experiments, BMMCs were stimulated with control IgG (100 ng ml^{-1}), recombinant IL-33 (0.1–100 ng ml^{-1}, R&D Systems), or lipopolysaccharide (10 ng ml) for 24 or 48 hours. The levels of IL-1α, IL-6, TNF-α, IFN-α, IL-12, and IL-17 in the culture supernatants were determined using an ELISA kit (BD Pharmingen and eBiosciences, San Diego, CA) or the Multi-Analyte ELISArray Kit (SABiosciences) according to the manufacturer's instructions.

Degranulation assay

Degranulation was assessed by β-hexosaminidase assay, as described previously (Matsushima *et al.*, 2004). Briefly, the supernatants were incubated with 2.5 mM *p*-nitrophenyl-*N*-acetyl β-D glucosaminide (Sigma-Aldrich) for 90 minutes. The reactions were terminated by 0.4 M glycine, and the colored products were measured using an ELISA plate reader (Molecular Devices, Sunnyvale, CA).

Local MC reconstitution in Kit$^{W/W\text{-}v}$ mice

Four-week-old Kit$^{W/W\text{-}v}$ mice were transfused by intradermal injection with 2×10^6 BMMCs in 50 μl of RPMI derived from Kit$^{+/+}$, Tnf$^{-/-}$, or Il-6$^{-/-}$ mice as previously described (Grimbaldeston *et al.*, 2007). At 9 weeks after intradermal transfer, the BMMC-reconstituted Kit$^{W/W\text{-}v}$ mice were used for experiments.

Flow cytometry

Cells were stained with allophycocyanin (APC)-labeled anti-CD3, FITC- or phycoerythrin (PE)-anti-CD8, APC-anti-CD11c, FITC-anti-CD205, and PE-anti-CD103 mAbs (10 μg ml^{-1}, Pharmingen, San Diego, CA) for 30 minutes at 4 °C and analyzed on a FACSCalibur

(Becton Dickinson, Franklin Lakes, NJ). For tetramer staining, inguinal and axillary lymph nodes at 6 days after infection were stained with PE-H-2K^b HSV-1gB498-505 (SSIEFARL) tetramer, or PE-H-2K^b TRP2 tetramer (5 μg ml^{-1}, MBL, Nagoya, Japan) as control, for 20 minutes at 4 °C. To detect regulatory T cells or HSV-2-infected BMMCs, cells were fixed and permeabilized with Cytofix/Cytoperm reagents (BD Biosciences-Pharmingen), and then stained with APC-anti-Foxp3 mAb using the Foxp3 Staining Set (eBioscience) or FITC-anti-HSV glycoprotein D mAb (Argene, Verniolle, France), respectively.

Immunohistochemistry

Skin tissues were collected from infected sites at 1, 3, or 5 days after HSV infection and fixed in formalin. Paraffin-embedded sections were dewaxed and rehydrated through graded concentrations of ethanol. Tissue sections were stained with hematoxylin–eosin. Otherwise, the sections were preincubated with 3% hydrogen peroxide in methanol for 10 minutes to inactive endogenous peroxidase. Sections were incubated with rabbit anti-HSV-2 polyclonal antibody or control rabbit Ig for 1 hour at room temperature using the Dako envision kit (Dako, Glostrup, Denmark). The sections were washed and incubated with a goat anti-rabbit Ig conjugated to peroxidase-labeled dextran polymer for 1 hour at room temperature. After wash, they were treated with the chromogenic indicator dye 3,3′-diaminobenzidine for 5 minutes, and then counterstained with Mayer's hematoxylin. For IL-33 staining, plated Pam-212 cells exposed to HSV for 24 hours were washed and stained with goat anti-mouse IL-33 polyclonal antibody (R&D Systems) at 10 μg ml^{-1} for 3 hours at room temperature using the Dako LSAB kit (Dako). Cells were incubated with biotinylated anti-goat secondary antibody, and then incubated with peroxidase-conjugated streptavidin for 30 minutes. After development with 3,3′-diaminobenzidine substrate, cells were counterstained with Mayer's hematoxylin.

Statistical analyses

The GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) was used to determine the statistical significance of survival data. Log-rank test was used for comparison of survival curves. Other data were analyzed by Student's *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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