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Rectal immunization generates protective immunity in the female genital tract against herpes simplex virus type 2 infection: Relative importance of myeloid differentiation factor 88

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Abstract

The present study was undertaken to examine the potential of rectal route of immunization for induction of protective immunity in the female genital tract against genital herpes infection in mice. A single rectal immunization of female C57Bl/6 mice with live attenuated herpes simplex virus type 2 lacking thymidine kinase (HSV-2 TK$^-$) was shown to confer HSV-specific cellular and humoral immune responses as well as protection against an otherwise lethal vaginal challenge with a virulent HSV-2 strain. The immunity afforded by rectal immunization with HSV-2 TK$^-$ was shown to be independent of sex hormonal influence and the usage of the adaptor protein myeloid differentiation factor 88 (MyD88). Next, the impact of rectal immunization with HSV-2 glycoprotein D (gD) in combination with CpG oligodeoxynucleotide (ODN) or cholera toxin (CT) on induction of immunity against HSV-2 was investigated. Rectal immunization of mice with gD + CpG failed to generate gD specific immune responses and protection against genital herpes infection. Conversely, rectal immunization with gD + CT elicited potent gD-specific cellular immune responses and protection against genital herpes infection through a MyD88-dependent manner. These results highlight the potential of rectal route for the development of novel immunization strategies to elicit immunity in the female genital tract against genital herpes and presumably other sexually transmitted diseases.

Keywords: Rectal immunization; Genital herpes; Sex hormones; MyD88; Mucosal vaccine; Adjuvant

1. Introduction

Genital herpes infection caused by herpes simplex virus type 2 (HSV-2), is the leading cause of genital ulcer diseases worldwide (Kinghorn, 1994; Whitley, 2002). The socioeconomic burden and morbidity associated with genital herpes, as well as the alarming association of genital herpes with HIV transmission (Corey et al., 2004) underline the need for an efficient vaccine. Over the last decades, numerous efforts have been made to develop vaccines against genital herpes infection and disease, and several candidates have reached advanced phase clinical trials. However, intramuscular vaccination with recombinant HSV-2 glycoprotein B (gB) and/or HSV-2 glycoprotein D (gD) in combination with different adjuvants, conferred no (Straus et al., 1997) or only limited protection against genital herpes (Stanberry et al., 2002) in recent clinical trials. Thus, development of novel strategies to elicit immunity against genital herpes is needed.

Vaginal inoculation of mice with live thymidine-kinase deficient HSV-2 (HSV-2 TK$^-$) elicits complete protective immunity against a subsequent vaginal challenge with virulent strain of HSV-2 (Parr et al., 1994). However, the use of live attenuated HSV-2 TK$^-$ is precluded in humans due to safety concerns. Our laboratory and others have recently reported that vaginal immunization of mice with gB or gD in combination with synthetic CpG ODN, a Toll-like receptor 9 (TLR9) ligand, confers protection against genital herpes in mice (Kwant and Rosenthal, 2004; Tengvall et al., 2006). While the genital tract is an attractive site for immunizations against sexually transmitted pathogens, genital tract immunity resulting from vaginal immunization appears

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to be greatly influenced by sex hormones (Gillgrass et al., 2003, 2005a; Parr et al., 1994), which in turn may limit the use of vaginal immunization in humans. Therefore, the assessment of an alternative strategy for generation of immunity in the female genital tract against genital herpes should be considered.

Rectal immunization is an intriguing concept as lymphoid tissue in the large bowel has been suggested as a possible induction site for genital tract immunity (Brandtzaeg, 1997). The rectal mucosa of mice and humans are rich in antigen-transporting M cells and organized mucosal lymphoid follicles with characteristics of an immune-inductive site (Mahajan et al., 2005). Long-term presence of vaginal influenza-specific IgG antibodies has been demonstrated in women after rectal administration of influenza vaccine (Crowley-Nowick et al., 1997). Furthermore, targeted iliac lymph node immunization and in vivo labeling experiments in non-human primates indicate that T and B cells from the iliac lymph node preferentially home to the genital and rectal mucosa (Mitchell et al., 1998). Thus, rectal immunization appears to be a promising route for vaccination against sexually transmitted pathogens targeting the genital mucosa.

In this study we examined the protection against genital herpes as well as the humoral and cellular immune responses induced by rectal immunization of mice with HSV-2 TK$^-$ or gD combined with mucosal adjuvants cholera toxin (CT) and CpG oligodeoxynucleotide (ODN). We also examined whether induction of female genital tract immunity following rectal immunization is under the influence of sex hormones, and requires the usage of the adaptor molecule myeloid differentiation factor 88 (MyD88). Our results indicate that rectal immunization is an effective way to generate immunity against genital herpes infection, and that such immunity can be achieved during both estrus and diestrus phases of the estrus cycle in mice. These results support the consideration of the rectal route for the development of new immunization strategies against genital herpes in humans.

2. Materials and methods

2.1. Mice

Six- to 8-week-old female C57Bl/6 (M&B) mice and myeloid differentiation factor 88 (MyD88$^{-/-}$) knockout mice with C57Bl/6 background (gift from Nils Lycke and Mary-Jo Wick, Göteborg University) were housed in microisolators under specific pathogen-free conditions at the EBM Animal Facility, Sahlgrenska Academy at Göteborg University. All experiments were carried out with the approval from the Ethical Committee for Laboratory Animals in Göteborg, Sweden. The studies were performed twice (gD vaccination studies) or three times (HSV-2 TK$^-$ studies).

2.2. Immunization studies

Before each immunization, mice were deeply anesthetized by Isoflouran (Baxter Medical AB) and mucosal immunizations were performed by gentle administration with a fine micropipette tip.

For HSV-2 TK$^-$ immunization studies, groups of female C57Bl/6 mice ($n = 12$–16) were injected subcutaneously with 3.0 mg of Depo-Provera (DP) (Upjohn s.a., Puurs, Belgium) in PBS or left untreated. Six days later, the DP treated mice were inoculated intravaginally (ivag) with 9×10^4 PFU of thymidine kinase lacking herpes simplex virus type 2 (HSV-2 TK$^-$) or UV-inactivated HSV-2 TK$^-$ in 10 μl of Hank's balanced salt solution (HBSS). The DP untreated mice were inoculated intrarectally (ir) with equivalent amount of HSV-2 TK$^-$ or UV-inactivated HSV-2 TK.

For studies on the effect of sex hormones on rectal vaccination, groups of female C57Bl/6 mice ($n = 4$–12) were injected subcutaneously with 3.0 mg of DP in 150 μl PBS or 0.1 mg 17-beta-estradiol (Sigma) in 100 μl oil, 6 days prior rectal immunization with live HSV-2 TK$^-$. Vaginal lavage for reproductive cycle staging was collected by pipetting 2× 40 μl of phosphate-buffered saline (PBS) in and out of the vagina several times for a few hours prior to immunization. Vaginal fluids were smeared on glass slides and following staining with methylene blue examined by light microscopy to determine the stage of the estrous cycle. The predominance of cornified epithelial cells in the vaginal smears was identified as estrus, whereas the predominance of leukocytes in the vaginal smears was used as a hallmark for diestrus. Mice pre-treated with DP were all at the diestrus phase, whereas all estradiol-treated mice were at the estrus phase.

For rectal immunization studies with gD (Novartis Vaccines, Emeryville) in mixture with CpG oligodeoxynucleotides (ODNs) (1826, a 20-mer containing two copies of a CpG motif; Operon Biotechnologies GMBH) or cholera toxin (CT) (Sigma), groups of female C57Bl/6 and MyD88$^{-/-}$ mice ($n = 10$–14) were immunized (without prior hormone treatment) twice at 7–10 days interval with 5 μg gD, 5 μg gD in a mixture with 30 μg CpG ODN or 5 μg gD in a mixture with 10 μg CT. Control groups were left untreated. One day prior rectal immunization, vaginal smear analysis was performed for reproductive cycle staging, and results showed that mice were not cycling together.

2.3. Tissues and immunolabeling

The rectum and the vagina were removed from control uninfected and infected animals at different time points post rectal or intravaginal inoculation of HSV-2 TK$^-$ virus, frozen in liquid nitrogen and sectioned using cryostat. Histological sections were blocked in 2% fetal bovine serum, incubated in rabbit anti-HSV-2 (1/2000, 60 min, 37 °C; Dako, Carpinteria, CA), washed in PBS, treated with 0.5% hydrogen peroxide in methanol, washed with PBS, incubated with goat anti-rabbit IgG (1/300, 30 min, room temperature, abcam), followed by peroxidase staining (ABC kit, Vector Laboratories) and exposure to substrate (DAB kit, Vector Laboratories) according to the Manufacturer's recommendations. The specificity of HSV-2 specific labeling was verified by the absence of positively stained cells in the mucosal tissues of control mice, and also in the rectal tissue of mice inoculated ir with HSV-2 TK$^-$ following staining with an irrelevant rabbit IgG antibody. Sections were counterstained with Mayer hematoxylin (HistoLab, Sweden) and mounted in Mountex (HistoLab, Sweden).

2.4. Proliferation assay

In vitro proliferation studies were performed 4 weeks after the vaccination. $CD11c^+$ cells, representing conventional dendritic cells, were purified from spleen cells isolated from naïve mice by using MACS-beads from Miltenyi Biotec (Gladbach, Germany) according to the Manufacturer's recommendations and cultured overnight in the presence of inactivated HSV-2 TK^- (for HSV-2 TK^- vaccination studies) or gD (for gD vaccination studies). The $CD11c^+$ cells (10^4/well) were washed and then co-cultured with 2×10^5 freshly isolated genital lymph node (gLN) cells or splenic $CD4^+$ T cells (purified by using MACS-beads (Miltenyi Biotec, Gladbach, Germany) collected from the vaccinated animals in Iscove's medium supplemented with L-glutamine, 50 μM 2-mercaptoethanol, gentamycin and 10% fetal calf serum. After 96 h of incubation at 37 °C, culture supernatants were collected and assayed for cytokine contents, and the cells were pulsed with 1 μCi of [^{3}H]-thymidine (Amersham Pharmacia) for the last 6–8 h of incubation. The cellular DNA was harvested on glass-fibre filters and then assayed by liquid scintillation counting. Data are expressed as the mean count per minute (cpm).

2.5. Cytokine and chemokine quantification

Concentrations of cytokines in the cell culture supernatants were determined by using Duoset chemokine ELISA kits from R&D Systems (Abingdon, United Kingdom) according to the Manufacturer's recommendations.

2.6. Analysis of antibody response

Maxisorp 96-well plates (Nunc) were coated with recombinant glycoprotein D (3 μg/ml) diluted to a final concentration in 50 mM carbonate buffer (pH 9.6) for 4 h at room temperature, followed by overnight incubation at 4 °C. The plates were blocked with 2% BSA in PBS for 30 min at 37 °C. Serial dilutions of sera or tissue extract from the vagina and the rectum obtained 4 weeks after vaccination were incubated for 1 h at 37 °C. After being washed with 0.05% Tween 20, the plates were incubated for another 1 h at 37 °C with horseradish peroxidase-conjugated goat-anti-mouse IgA, IgG, IgG1 or IgG2c (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The plates were then washed with 0.05% Tween 20 and developed using 100 μl of 1 mg/ml *o*-phenylenediamine dihydrochloride (Sigma) in 0.1 M citrate buffer (pH 4.5) containing 0,04% H_2O_2. After 20 min incubation at room temperature, the absorbance was read at 450 nm. Results are shown as OD values of 1/10 pre-diluted samples to appreciate gD specific IgG antibody levels of all immunized groups of which some showed low but detectable levels of gD specific IgG antibody.

2.7. Viral preparation and viral challenge

HSV-2 strain 333 was grown and titrated in monolayers of African green monkey kidney cells (GMK-AH1) and prepared by one cycle of freezing and thawing followed by removal of cellular debris by centrifugation. For virus challenge experiments, groups of 5-to-8 vaccinated as well as naïve control mice were injected subcutaneously with 3.0 mg of Depo-Provera (Upjohn s.a., Puurs, Belgium) in PBS. Six days later, the mice were anesthetized with Isoflouran (Baxter Medical AB) and challenged by an intravaginal administration of 9×10^4 PFU (200 LD_{50}) of HSV-2 strain 333 in 10 μl of Hank's balanced salt solution (HBSS).

2.8. Monitoring of infection

2.8.1. Viral replication

Following intravaginal HSV-2 challenge, vaginal fluids were collected 72 h after challenge by pipetting 40 μl of sterile HBSS in and out of the vagina until a discrete clump of mucus was retrieved. A second wash was then performed. The two washes were pooled and stored at −70 °C. HSV-2 titres were determined by plaque assay on GMK-AH1 cell monolayers. Briefly, vaginal fluids were diluted in Iscove's medium containing 2% newborn calf serum and 0.75% penicillin–streptomycin (all from Sigma–Aldrich) and added to GMK-AH1 cell monolayers in duplicates. After 1 h at room temperature, methylcellulose (Sigma–Aldrich) solution was added to the wells and the plates were incubated at 37 °C/5% CO_2 for 3 days. Wells were stained with 1% solution of crystal violet (Sigma–Aldrich) and plaques were counted using light microscope.

2.8.2. Inflammation and disease

Mice were monitored daily for genital pathology and survival after HSV-2 challenge for at least 4 weeks. The severity of the disease was scored as healthy (score of 0), genital erythema (score of 1), moderate genital inflammation (score of 2), severe and purulent genital lesions (score of 3), signs of hindlimb paralysis (score of 4), and death or sacrifice due to severe paralysis (score of 5).

2.9. Statistical analyses

One way ANOVA was used for statistical analyses using Graph Pad Prism 4 software (CA).

3. Results

3.1. Live attenuated HSV-2 TK^- strain infects cells in the rectal mucosa

We first investigated the presence of HSV-2 in the rectal and genital mucosa of mice 24 h and 7 days after rectal or vaginal administration of live or UV-inactivated HSV-2 TK^-. As expected, no HSV-2 infected cells could be detected in the mucosa of mice given inactivated HSV-2 TK^- ivag or ir (Fig. 1A and B). Mice administered with live virus vaginally showed numerous labeling of HSV-2 infected cells in their vaginal epithelium (not shown). Similarly, mice rectally immunized with live HSV-2 TK^- showed labeling of many HSV-2 infected

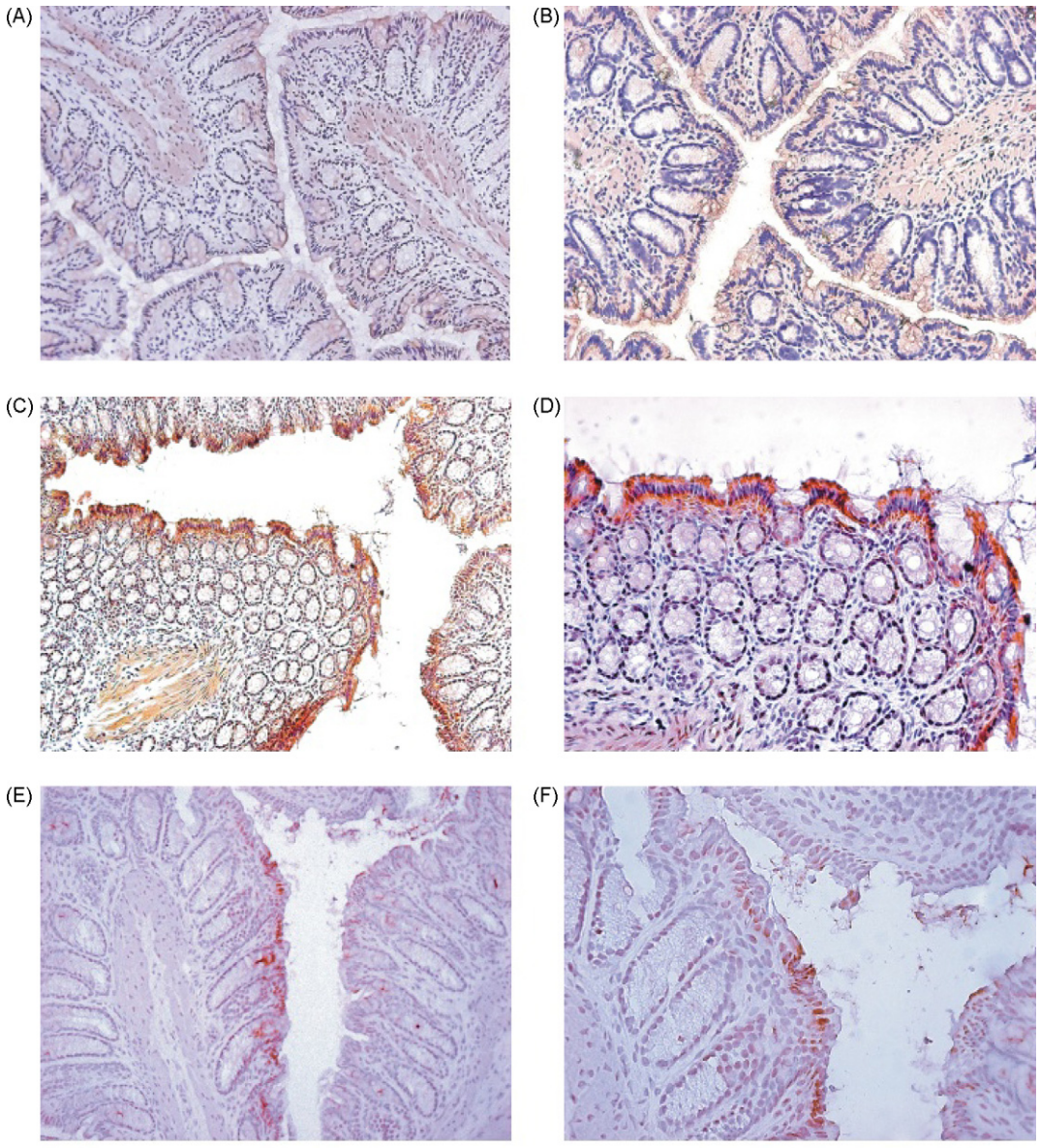

Fig. 1. HSV-2 infects the rectal mucosa. Groups of female C57Bl/6 mice were intrarectally (ir) inoculated with inactivated or live HSV-2 TK$^-$. At 24 h (A, B, C and D) and 7 days (E and F) following inoculation, the rectums were collected and examined by immunohistochemistry for the presence of HSV-2 infected cells. After 24 h, while no HSV-2 labeling could be detected in the rectums of mice inoculated with inactivated HSV-2 TK$^-$ (A: 200× and B: 400×), many HSV-2 infected cells were detected in the rectums of mice inoculated with live HSV-2 TK$^-$ after 24 h (C: 200× and D: 400×) and 7 days (E: 200× and F: 400×).

cells in their rectal tissues (Fig. 1C and D). HSV-2-infected cells could be detected in the rectal mucosa of the HSV-2 TK$^-$/ir mice for up to 7 days after rectal inoculation (Fig. 1E and F). However, mice inoculated rectally with HSV-2 TK$^-$ did not show any macroscopic signs of anorectal or vaginal inflammation up to 1 month after inoculation. Further, no HSV-2 labeling could be detected in the vaginal tissues of rectally infected animals on day 1 and 7 (not shown), excluding the possibility of leakage of the virus inoculum from the rectum to the vagina. Accordingly, the vaginal washes collected on day 2-to-7 after rectal inoculation of HSV-2 TK$^-$ contained no detectable levels of HSV-2 (not shown). These results suggest that live attenuated HSV-2 TK$^-$ can infect the murine rectal mucosa.

3.2. Rectal immunization with live attenuated HSV-2 TK$^-$ elicits antigen specific mucosal as well as systemic immune responses

We next examined whether rectal immunization with HSV-2 TK$^-$ could elicit mucosal or systemic HSV-2 specific immune responses. Groups of female C57Bl/6 mice were immunized once ir or ivag with live HSV-2 TK$^-$ or UV-inactivated HSV-2 TK$^-$ (inac HSV-2 TK$^-$). One month after immunization, CD11c$^+$ dendritic cells pulsed overnight with inac HSV-2 TK$^-$ was co-cultured with genital lymph node (gLN) cells obtained from the immunized mice, and the HSV-specific proliferative response was analyzed after 96 h. Similar to control mice, gLNs

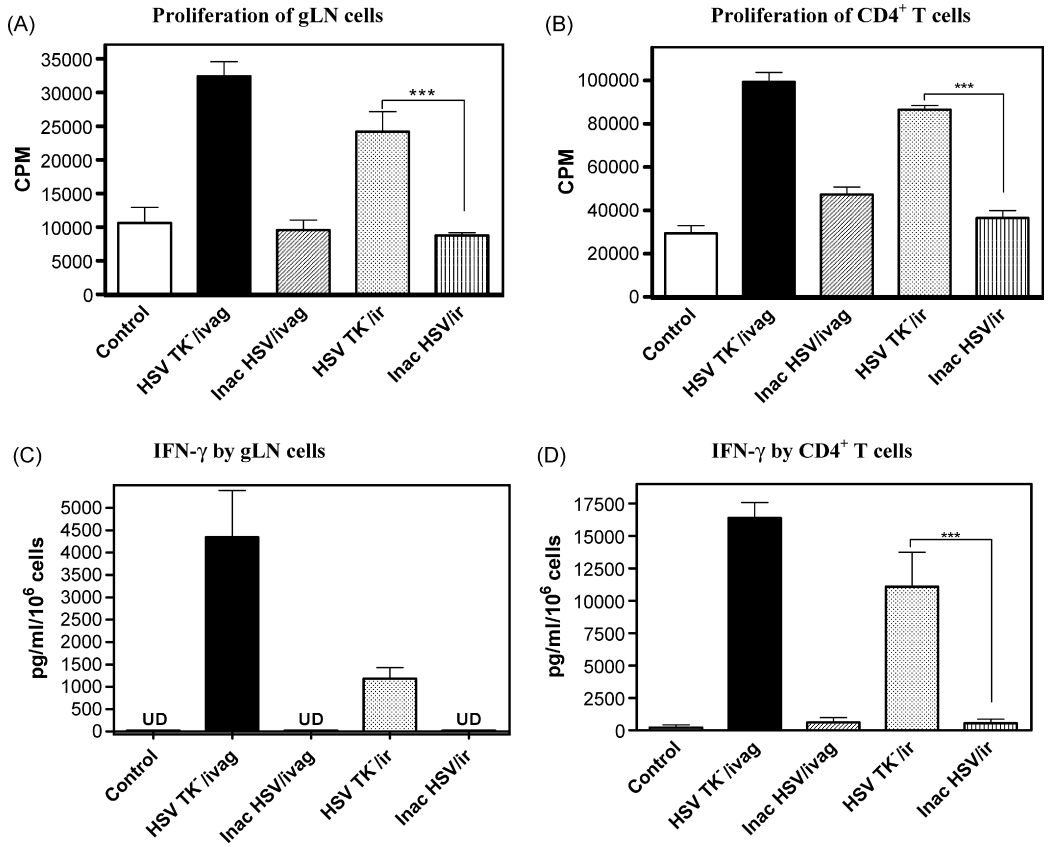

Fig. 2. HSV-2-specific proliferative and IFN-γ responses in splenic CD4$^+$ T cells and gLN cells after rectal or vaginal immunization with live or inactivated HSV-2 TK$^-$. Groups of female C57Bl/6 mice ($n = 12$–16) were ivag or ir vaccinated with live or inactivated HSV-2 TK$^-$. Four weeks after immunization, genital lymph node cells (10^6/ml) (A and C) and splenic purified CD4$^+$ T cells (10^6/ml) (B and D) were co-cultured with CD11c$^+$ cells (10^5/ml) pulsed overnight with inactivated HSV-2 TK$^-$ for 96 h. The results are expressed as the mean + S.E.M. of counts per minute (CPM) for proliferation and pg/ml/million cells for IFN-γ responses. Statistical analysis was performed using one-way ANOVA. ***$p < 0.001$ and UD: undetectable.

cells from inac HSV-2 TK$^-$/ivag and inac HSV-2 TK$^-$/ir immunized mice showed low HSV-specific proliferative responses (Fig. 2A). In contrast, gLNs isolated from live HSV-2 TK$^-$/ivag as well as HSV-2 TK$^-$/ir vaccinated mice showed potent HSV-2 specific proliferative responses (Fig. 2A). Given the importance of IFN-γ in acquired immune protection against genital herpes (Parr and Parr, 1999; Harandi et al., 2001), we next examined IFN-γ responses in gLN cells isolated from the immunized mice upon recall antigen stimulation *in vitro*. While gLN cells from control as well as inac HSV-2 TK$^-$/ivag and HSV-2 TK$^-$/ir vaccinated mice produced no detectable levels of IFN-γ, gLN cells from the HSV-2 TK$^-$/ivag vaccinated mice showed potent IFN-γ response (Fig. 2C). The HSV-2 TK$^-$/ir immunized group displayed a moderate IFN-γ response. Thus, rectal immunization of mice with HSV-2 TK$^-$ elicited HSV-2 specific proliferative and IFN-γ responses in the genital lymph nodes (Fig. 2A and C).

We then examined the impact of rectal immunization with HSV-2 TK$^-$ on induction of cell-mediated immune responses in the spleen. To this end, purified splenic CD4$^+$ T cells were co-cultured with antigen-pulsed CD11c$^+$ dendritic cells. Low levels of proliferative and IFN-γ responses were observed in CD4$^+$ T cells isolated from mice immunized with inac HSV-2 TK$^-$/ivag or inac HSV-2 TK$^-$/ir (Fig. 2B and D). CD4$^+$ T cells from the HSV-2 TK$^-$/ivag mice, on the other hand, showed high proliferative and IFN-γ responses (Fig. 2B and D). Splenic CD4$^+$ T cells from the HSV-2 TK$^-$/ir mice showed significantly higher levels of proliferative ($P < 0.001$) and IFN-γ ($P < 0.001$) responses than those of the inac HSV TK$^-$/ir mice (Fig. 2B and D). Thus, rectal immunization of mice with live attenuated, but not inactivated, HSV-2 TK$^-$ confers strong systemic HSV-specific cell-mediated immune response.

Next, we examined the level of HSV-specific IgG and IgA antibodies in the sera and saponin-extracted vagina and rectum of the immunized mice. None of the immunized mice showed any detectable levels of HSV-2 specific IgA in their sera, rectal or vaginal extracts (data not shown). While mice inoculated intravaginally with HSV-2 TK$^-$ had high levels of HSV-2 specific IgG antibody in their sera, rectally immunized mice displayed detectable although low levels of HSV-2-specific serum IgG antibody (Fig. 3).

3.3. Rectal immunization with live attenuated HSV-2 TK$^-$ confers protection against genital herpes

Vaginal inoculation of mice with live attenuated HSV-2 TK$^-$ provides complete protection against vaginal challenge with

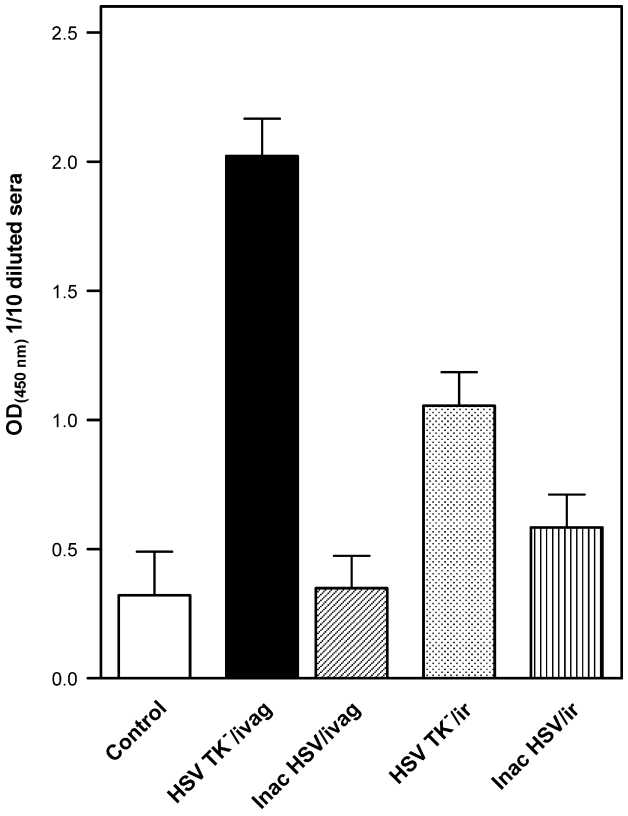

Fig. 3. HSV-specific IgG antibody response after rectal immunization with live attenuated HSV-2 TK$^-$. Groups of female C57Bl/6 mice (n = 12–16) were ivag or ir vaccinated with live or inactivated HSV-2 TK$^-$. Four weeks after the immunization, the mice were sacrificed and the gD-specific IgG antibody levels in the sera were determined using a gD-specific ELISA. The data are expressed as the mean + S.E.M. of the optical density (OD) value of 1/10 prediluted serum samples measured at 450 nm. The results are pooled from two independent experiments.

wild-type HSV-2 (Parr et al., 1994). We thus examined whether rectal immunization with HSV-2 TK$^-$ could confer protection against a subsequent vaginal challenge with a lethal dose of a virulent HSV-2 strain. Groups of immunized mice were vaginally challenged with 200 LD_{50} (9×10^4 PFU) of HSV-2 strain 333, 4 weeks after a single vaginal or rectal immunization with HSV-2 TK$^-$. As depicted in Fig. 4A, all mice except for those vaccinated with live HSV-2 TK$^-$/ivag or HSV-2 TK$^-$/ir showed significant levels of viral titer in the vagina 3 days after challenge. All these mice developed rapidly progressing disease requiring euthanization within 9–10 days after challenge (Fig. 4B and C). While the HSV-2 TK$^-$/ivag immunized mice had no detectable levels of virus after a lethal vaginal HSV-2 challenge, very low levels of replication were observed in the HSV TK$^-$/ir immunized mice following challenge (Fig. 4A). In line with the viral replication results, the HSV-2 TK$^-$/ir immunized mice survived the challenge with no or very mild symptoms of the disease (vaginal redness) (Fig. 4B and C). Thus, rectal immunization with HSV-2 TK$^-$ provided protection against an otherwise lethal vaginal HSV-2 challenge in mice.

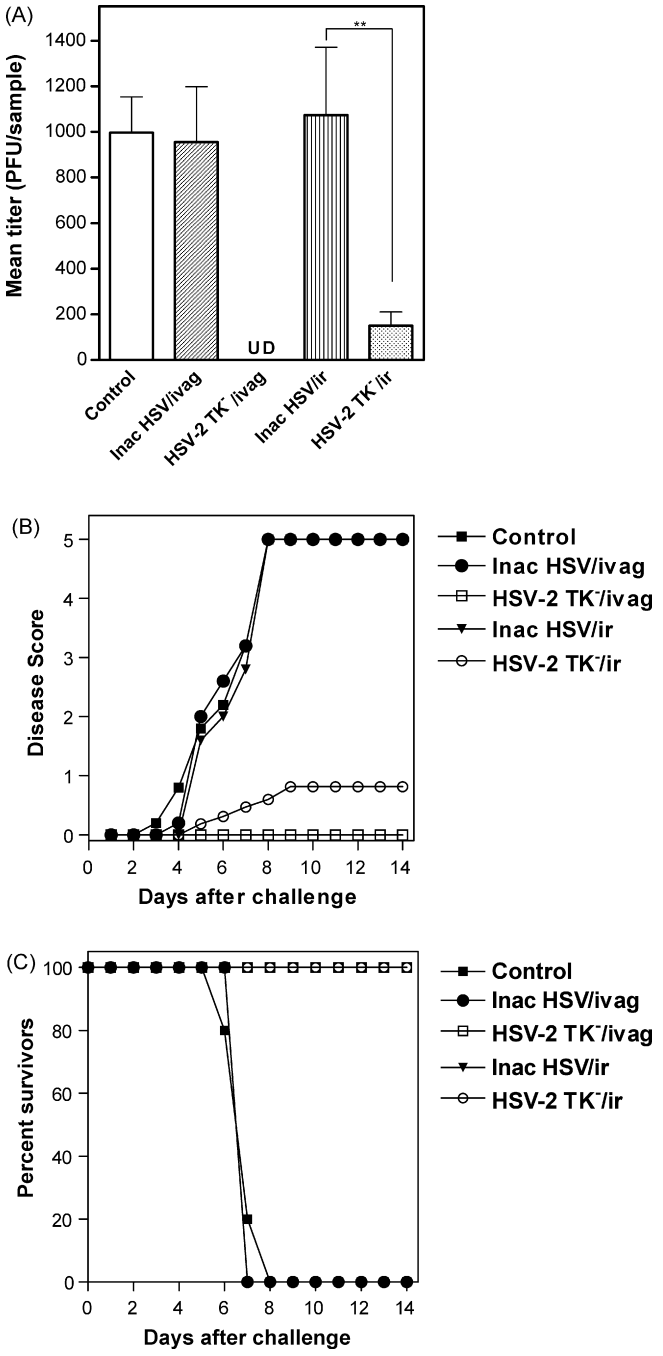

Fig. 4. Rectal immunization with live attenuated HSV-2 TK$^-$ confers protection against genital herpes in mice. Groups of 12-to-16 female C57Bl/6 mice were ivag or ir immunized with live or inactivated HSV-2 TK$^-$. Four weeks after immunization, the mice were challenged vaginally with a lethal dose of a virulent strain HSV-2. (A) The challenged mice were examined for the vaginal viral titer on day 3. The data are expressed as mean + S.E.M. of virus load (plaque forming units (PFU)/sample). The macroscopic signs of the disease (B) and survival (C) were also examined in these mice. The results are pooled from two independent experiments.

3.4. Immune protection induced by rectal immunization with live HSV-2 TK$^-$ is not influenced by the sex hormones progesterone and estrogen

Previous studies have shown that treatment of mice with the sex hormones estrogen and progesterone strongly influences

induction of immunity in the female genital tract following ivag immunization with live HSV-2 TK$^-$ (Gillgrass et al., 2003; Parr et al., 1994). We hence sought to examine the role of sex hormones on induction of protective immunity following ir immunization with HSV-2 TK$^-$. Groups of mice were injected subcutaneously with DP, a long-lasting progesterone, or estradiol (E2) 1 week prior to immunization with live HSV-2 TK$^-$/ir. In addition, one group was immunized intrarectally with HSV-2 TK$^-$ without any prior hormonal treatment. On the day of immunization, vaginal fluids were collected and the stage of estrus was verified by vaginal smears (results not shown). Four weeks after the immunization, the immunized as well as control mice were ivag challenged with a lethal dose of HSV-2 strain 333. While control mice displayed high levels of viral replication in vaginal washings and developed rapidly progressing disease requiring euthanization within 9 days after challenge, no viral titers could be detected in vaginal washes from DP-treated and HSV-2 TK$^-$/ivag immunized mice, and these mice were completely protected against the challenge (Table 1). In the group DP-treated 1 week before rectal HSV-2 TK$^-$ immunization, only one mouse showed viral titers in vaginal washings and developed mild symptoms of the disease, and all mice survived the challenge. Mice given estrogen before ivag HSV-2 TK$^-$ immunization all showed viral replication and 75% of them showed signs of the disease and 50% succumbed to the challenge. By contrast, none of the mice treated with estrogen before rectal HSV-2 TK$^-$ vaccination showed viral replication or signs of disease and all mice survived the challenge (Table 1). These results show that the sex hormones progesterone and estrogen did not provide protection in the female genital tract after rectal immunization with HSV-2 TK$^-$.

3.5. MyD88 is not required for induction of HSV-2 specific humoral immunity and protection following rectal vaccination with live HSV-2 TK$^-$

A recent study has demonstrated that HSV can engage several TLRs (Sato et al., 2006). We next addressed the question of whether the usage of MyD88, which is a common adaptor molecule used by most TLRs, is required for induction of adaptive immunity in the female genital tract after rectal immunization with HSV-2 TK$^-$. Thus, MyD88$^{-/-}$ as well as C57Bl/6 mice were rectally immunized with live HSV-2 TK$^-$. MyD88$^{-/-}$ mice immunized with HSV-2 TK$^-$/ir mounted a significantly higher gD-specific IgG response in their sera as compared to the HSV-2 TK$^-$/ir C57Bl/6 mice (Fig. 5A). While both the immunized C57Bl/6 and MyD88$^{-/-}$ mice had comparable levels of HSV-specific IgG1, the immunized MyD88$^{-/-}$ had higher serum IgG2c antibody levels as compared to the immunized C57Bl/6 mice (data not shown). The latter finding suggests the development of a more Th1 biased immune response in MyD88$^{-/-}$ mice after HSV-2 TK$^-$ immunization was comparable with the HSV-2 TK$^-$ immunized C57Bl/6 mice. Moreover, while MyD88$^{-/-}$ control mice had high levels of replicating virus in their genital secretions (Fig. 5B) and rapidly succumbed to vaginal challenge with a lethal dose of wild type HSV-2 (Fig. 5C and D), the HSV-2 TK$^-$/ir immunized MyD88$^{-/-}$ mice survived the challenge without showing any symptoms of the disease or any detectable level of HSV-2 in their genital secretions (Fig. 5 B, C and D). Thus, the usage of TLR/MyD88 signaling is not required for induction of protective immunity in the female genital tract following rectal immunization with live HSV-2 TK$^-$.

3.6. Rectal immunization with HSV-2 gD in a mixture with CT induces gD-specific immunity

We have recently shown that twice, but not a single, vaginal immunization with gD together with CpG ODN could confer potent HSV-2 specific immune protection against genital herpes infection (Tengvall et al., 2006). We next examined if rectal immunization with gD in mixture with CpG ODN or cholera toxin (CT) could induce immunity against HSV-2. Groups of mice were immunized twice rectally with recombinant gD, a mixture of gD and CpG ODN or a mixture of gD and CT. One day prior to immunization, mice were examined for their estrus phase status by vaginal smear analysis. Results showed that mice were not cycling together. Four weeks after the last immunization, gD-specific cell mediated immune responses in gLNs and spleens, as well as gD-specific IgG antibody responses in sera were analyzed.

Table 1
The impact of sex hormones on protective immunity against genital herpes after rectal HSV-2 TK$^-$ immunization

Immunization group	Route of immunization	No. of mice with detectable HSV-2 titer	Vaginal HSV-2 titer PFU/sample	No. of mice with herpetic signs	No. of mice surviving
Control	–	10/10	2204 ± 187	10/10	0/10
DP	Ivag	0/10	UD	0/10	10/10
DP	Ir	1/12	<100	1/12	12/12
E2	Ivag	4/4	270 ± 21	3/4	2/4
E2	Ir	0/12	UD	0/12	12/12
–	Ir	0/6	UD	0/6	6/6

Groups of female C57Bl/6 mice were intrarectally (ir) or intravaginally (ivag) immunized with live HSV-2 TK$^-$ 1 week after progesterone (DP) or estrogen (E2) treatment. One month after vaccination, the mice were challenged vaginally with a lethal dose of HSV-2. The mice were examined for vaginal viral titer on day 3 and were also monitored daily for signs of disease and survival. Vaginal HSV-2 titer data are expressed as mean of virus load (plaque forming units (PFU)/sample) from mice that had detectable levels of viral replication ± standard deviation. Results shown are pooled from two independent experiments, except for the E2 ivag group that was performed once. UD: undetectable.

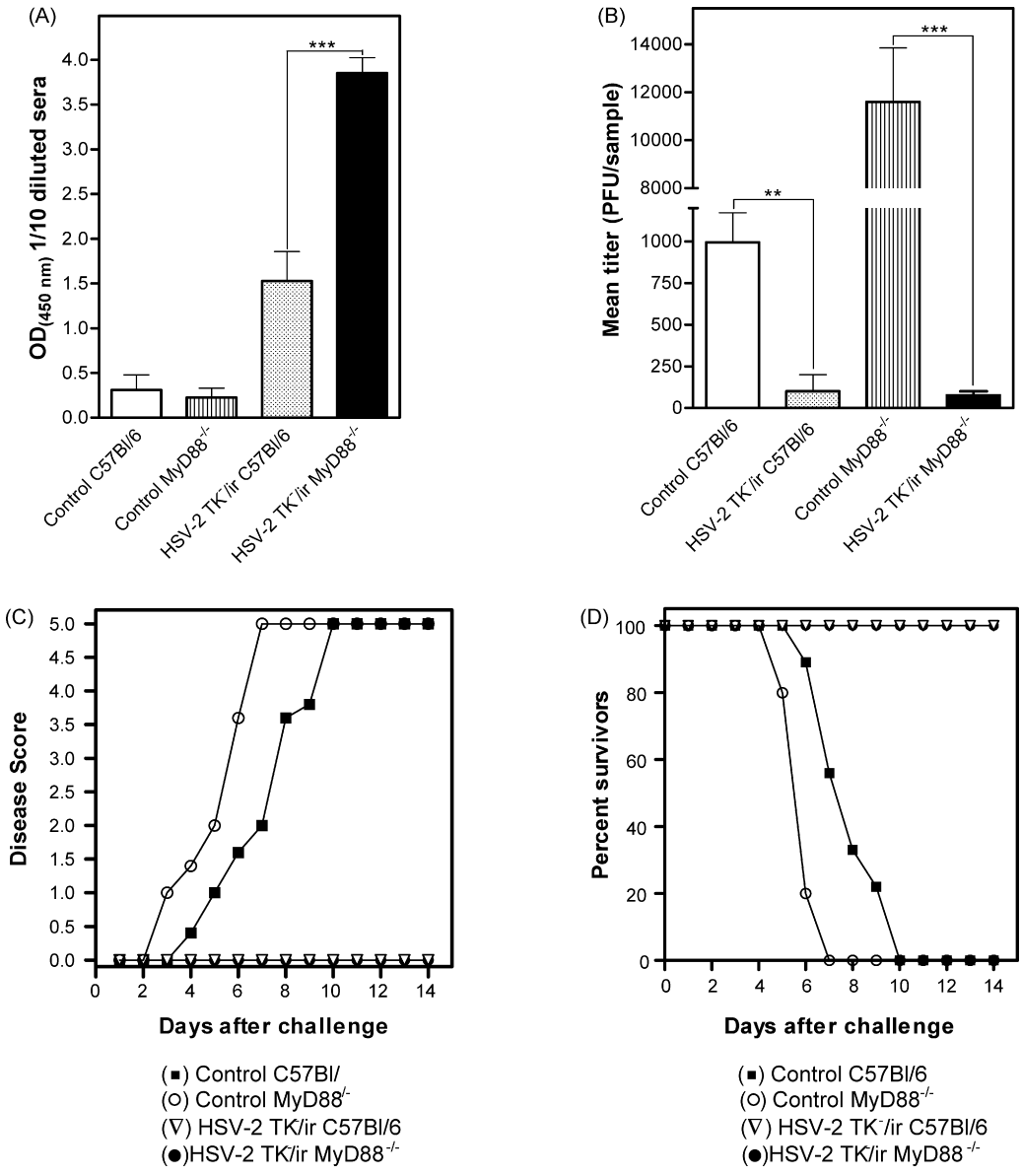

Fig. 5. MyD88 is not required for induction of HSV-2 specific IgG antibody response and protective immunity against genital herpes following rectal immunization with live HSV-2 TK$^-$. Groups of 5-to-6 female C57Bl/6 or MyD88$^{-/-}$ mice were immunized ir with live HSV-2 TK$^-$. Four weeks after the immunization, the mice were sacrificed and the gD-specific IgG (A) antibody levels were determined in their sera using a gD-specific ELISA. The data are expressed as the mean + S.E.M. of the optical density (OD) value of 1/10 prediluted serum samples measured at 450 nm. In addition, mice were challenged with a lethal dose of virulent HSV-2. Challenged mice were examined for viral replication in vaginal fluids 3 days after viral inoculation (B) and the resulting disease progression (C) and survival (D) were determined. $^{**}p < 0.01$, $^{***}p < 0.001$ by using one-way ANOVA. (■) Control C57Bl/6, (◯) Control MyD88$^{-/-}$, (▽) HSV-2 TK$^-$/ir C57Bl/6 and (●) HSV-2 TK$^-$/ir MyD88$^{-/-}$. The results are representative for two independent experiments.

CD11c$^+$ cells pulsed overnight with gD were co-cultured with either gLN or splenic CD4$^+$ T cells purified from the immunized mice. Similar to naïve control group, gLN from gD and gD + CpG ODN mice showed very low gD-specific proliferative responses (Fig. 6A). However, the gLN cells from gD + CT immunized group displayed a significantly higher gD-specific proliferative response compared to the gD group ($p < 0.001$) (Fig. 6A). In addition, while gLN cells from the gD and the gD + CpG ODN immunized mice produced undetectable levels of IFN-γ, gLN cells obtained from the gD + CT immunized mice produced high amounts of IFN-γ (Fig. 6B). Next, we tested if rectal vaccination with gD + CT could elicit HSV-2 specific cell-mediated immune response in the spleen. While control mice as well as the gD and the gD + CpG ODN immunized mice showed very low gD-specific CD4$^+$ T cell proliferative and IFN-γ responses (Fig. 6C and D), CD4$^+$ T cells from the gD + CT immunized mice developed higher proliferative and IFN-γ responses than those of the gD group (Fig. 6C and D) ($p < 0.001$).

We then examined the levels of gD-specific IgG antibody in the sera of the immunized mice 4 weeks after the last immunization. Low levels of gD-specific IgG antibody were detected

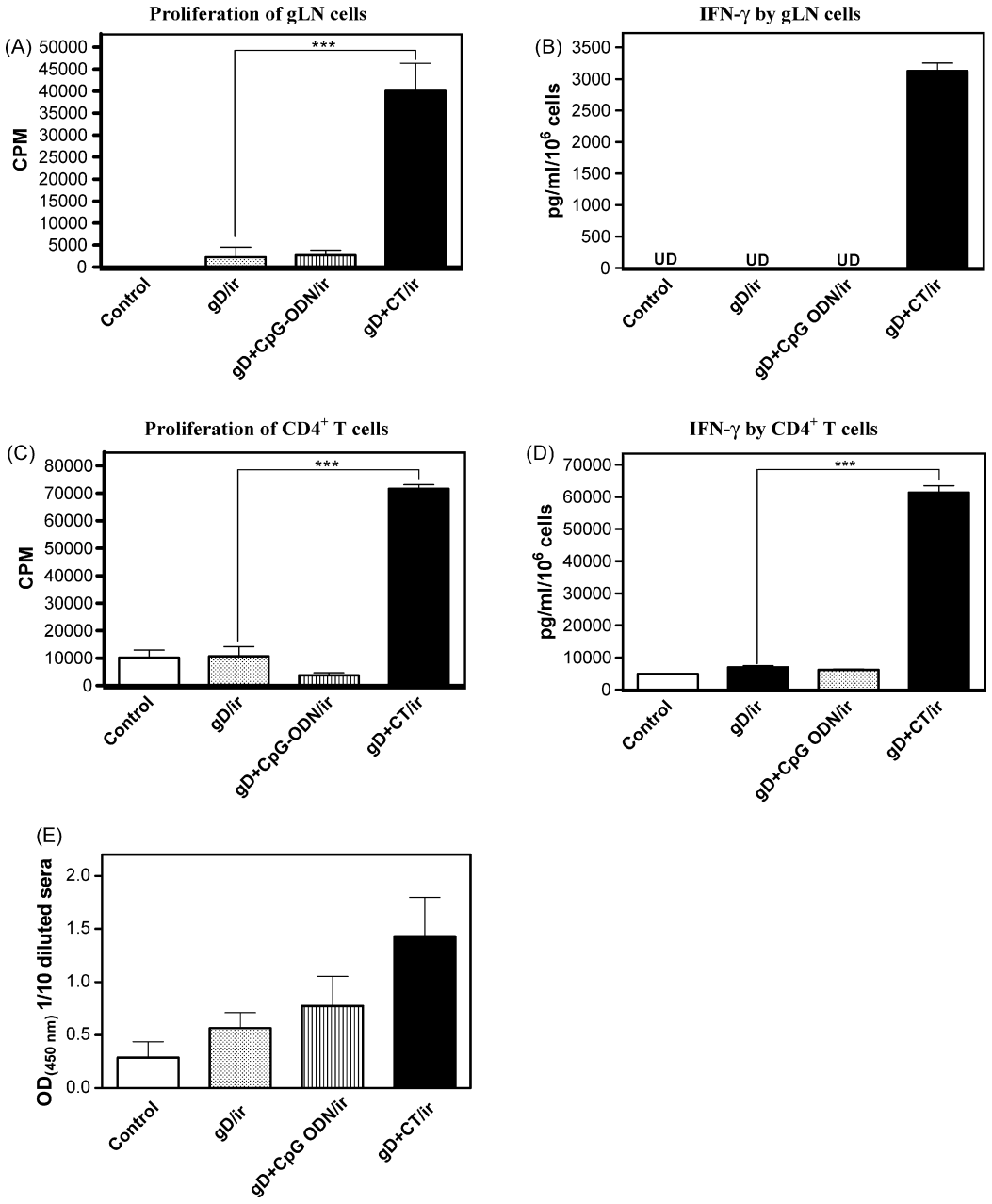

Fig. 6. Rectal immunization with gD in combination with CT elicits gD-specific cell-mediated and IgG antibody responses. Groups of female C57Bl/6 mice ($n = 10$–14) were intrarectally immunized twice with gD, gD + CpG ODN or gD + CT. One month after the last immunization gLN cells (A) and splenic $CD4^+$ T cells (C) were isolated and co-cultured with $CD11c^+$ cells pulsed overnight with gD for 96 h. The data are expressed as mean + S.E.M. of counts per minute (CPM). Statistical analysis were performed using one-way ANOVA and (***) means $p < 0.001$. IFN-γ production in the gLN cells supernatants (B) and in the $CD4^+$ T cells supernatants (D) in co-culture with dendritic cells at 96 h. UD: undetectable. Also, gD-specific IgG levels (E) in animal sera were determined using a gD-specific ELISA. The data are expressed as the mean + S.E.M. of the optical density (OD) value of 1/10 prediluted serum samples measured at 450 nm. The results are representative of two independent experiments.

in the gD and gD + CpG ODN immunized groups. The gD + CT group had somewhat higher levels of gD-specific IgG antibody in their sera compared with those of the gD and the gD + CpG immunized mice (Fig. 6E). Together, these data imply that rectal immunization with gD in combination with CT elicits potent gD-specific cellular immune response. However, gD specific IgG antibody response in the gD + CT immunized mice appeared to be weak.

3.7. Rectal immunization with HSV-2 gD plus CT confers protection against genital herpes

Finally, we investigated whether rectal immunization with gD + CpG ODN or CT could confer protective immunity against a subsequent vaginal challenge with a lethal dose of HSV-2. Four weeks after the last immunization, groups of immunized mice were ivag challenged with 9×10^4 PFU of HSV-2 strain 333.

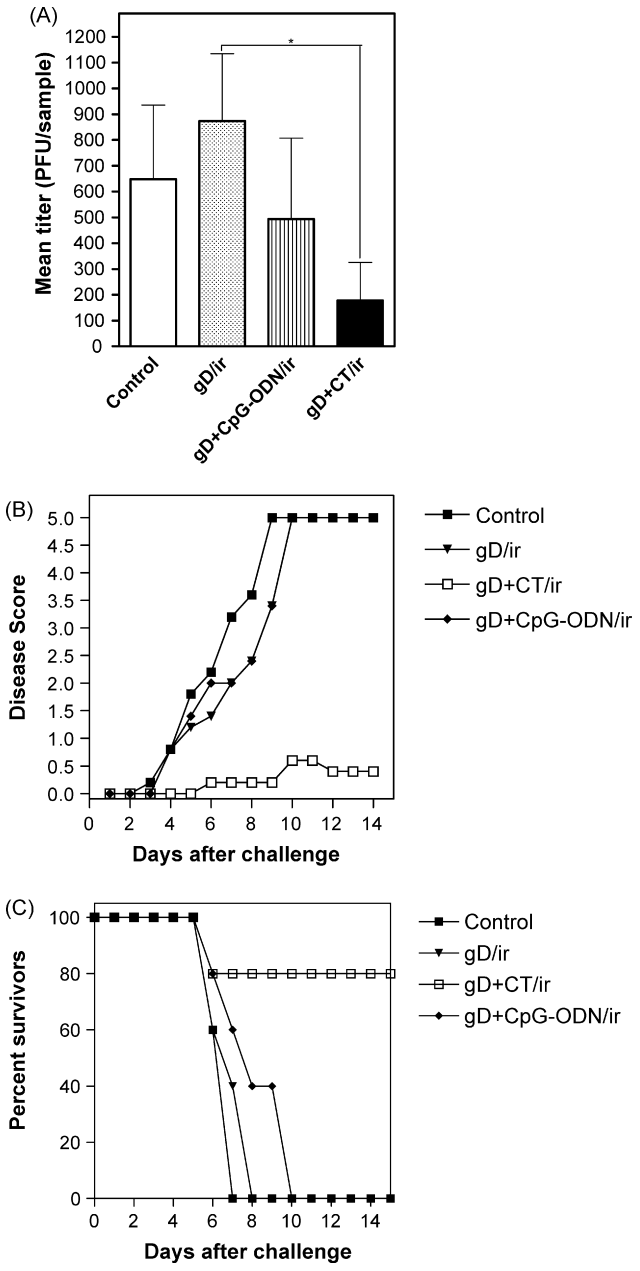

Fig. 7. Rectal immunization with gD plus CT confers protection against genital herpes in mice. Groups of 10-to-14 female C57Bl/6 mice were intrarectally immunized twice with gD, gD + CpG ODN or gD + CT. One month after the last immunization, the mice were intravaginally challenged with a lethal dose of HSV-2. The animals were examined for vaginal viral titer on day 3 (A). The data are expressed as mean + S.E.M. of virus load (plaque forming units (PFU)/sample). Animals were monitored daily for macroscopic signs of the disease (B) and survival (C). The results are pooled from two independent experiments. (■) Control, (▼) gD/ir, (△) gD + CpG ODN/ir and (□) gD + CT/ir. (*) represents $p < 0.05$.

Upon vaginal challenge, control mice as well as the gD and the gD + CpG ODN immunized mice showed high levels of viral replication in the vagina, and all showed signs of the disease and died within 2 weeks after challenge (Fig. 7A, B and C). The gD + CT vaccinated mice, on the other hand, had low levels of virus in the genital secretions and no or very mild symptoms of the disease and 80% survived the challenge (Fig. 7A, B and C). Hence, rectal immunization with gD in combination with CT, but not CpG ODN, elicits strong protective immunity against genital HSV-2 infection in mice.

3.8. MyD88 is required for induction of gD-specific humoral immunity and protection against genital herpes following rectal vaccination with gD plus CT

Next, we investigated if the adaptor molecule MyD88 is required for induction of adaptive immunity following rectal immunization with gD + CT. To this end, C57Bl/6 and MyD88$^{-/-}$ mice were rectally immunized twice, at 7–10 days interval, with gD + CT, and 4 weeks after that last immunization the resulting immunity was evaluated. Unexpectedly, rectal immunization of MyD88$^{-/-}$ mice resulted in lower gD-specific IgG antibody than the gD + CT/ir immunized C57Bl/6 mice (Fig. 8 A), and also the gD + CT/ir immunized MyD88$^{-/-}$ mice had high levels of viral replication in their vaginal fluids after vaginal challenge (Fig. 8B) and the majority of these mice developed progressive disease and died (Fig. 8C and D). The gD + CT/ir immunized C57Bl/6 mice, on the other hand, showed low levels of replicating virus and 90% of these mice survived without displaying symptoms of the disease (Fig. 8 B, C and D). Thus, the usage of MyD88 is necessary for induction of effective HSV-2 specific immune protection following rectal immunization with gD plus CT.

4. Discussion

Previous studies have shown that vaginal inoculation of mice with live attenuated HSV-2 TK$^-$ confers complete protective immunity against a subsequent vaginal challenge with a virulent strain of HSV-2 (Parr and Parr, 1998; McDermott et al., 1990). The safety concerns associated with the use of live attenuated HSV-2 in humans has availed several investigators to develop HSV-2 subunit vaccines. However, recent clinical trials showed that intramuscular vaccination with adjuvanted recombinant HSV-2 gB and gD offered no (Straus et al., 1997) or only limited protection against genital herpes (Stanberry et al., 2002) in humans. It is therefore desirable to develop novel immunization strategies to be used in the context of recombinant HSV-2 protein to counter genital herpes in humans.

We and others have recently shown that ivag immunization with recombinant HSV-2 gB or gD in combination with CpG ODN could generate a protective immunity comparable to that afforded by HSV-2 TK$^-$ (Kwant and Rosenthal, 2004; Tengvall et al., 2006). However, immunity resulting from vaginal immunization has been reported to be greatly influenced by sex hormones (Gillgrass et al., 2005a) and this may limit the use of the vaginal route for immunization in humans. The present study was undertaken to investigate whether the rectal mucosa can be an alternative site for induction of protective immunity in the female genital tract against genital HSV-2 infection.

We could document that HSV-2 TK$^-$ could establish infection in the rectal mucosa as HSV-2 infected cells were detected in the rectal mucosa at 24 h and for up to 7 days following rectal inoculation of HSV-2 TK$^-$. It has previously been shown

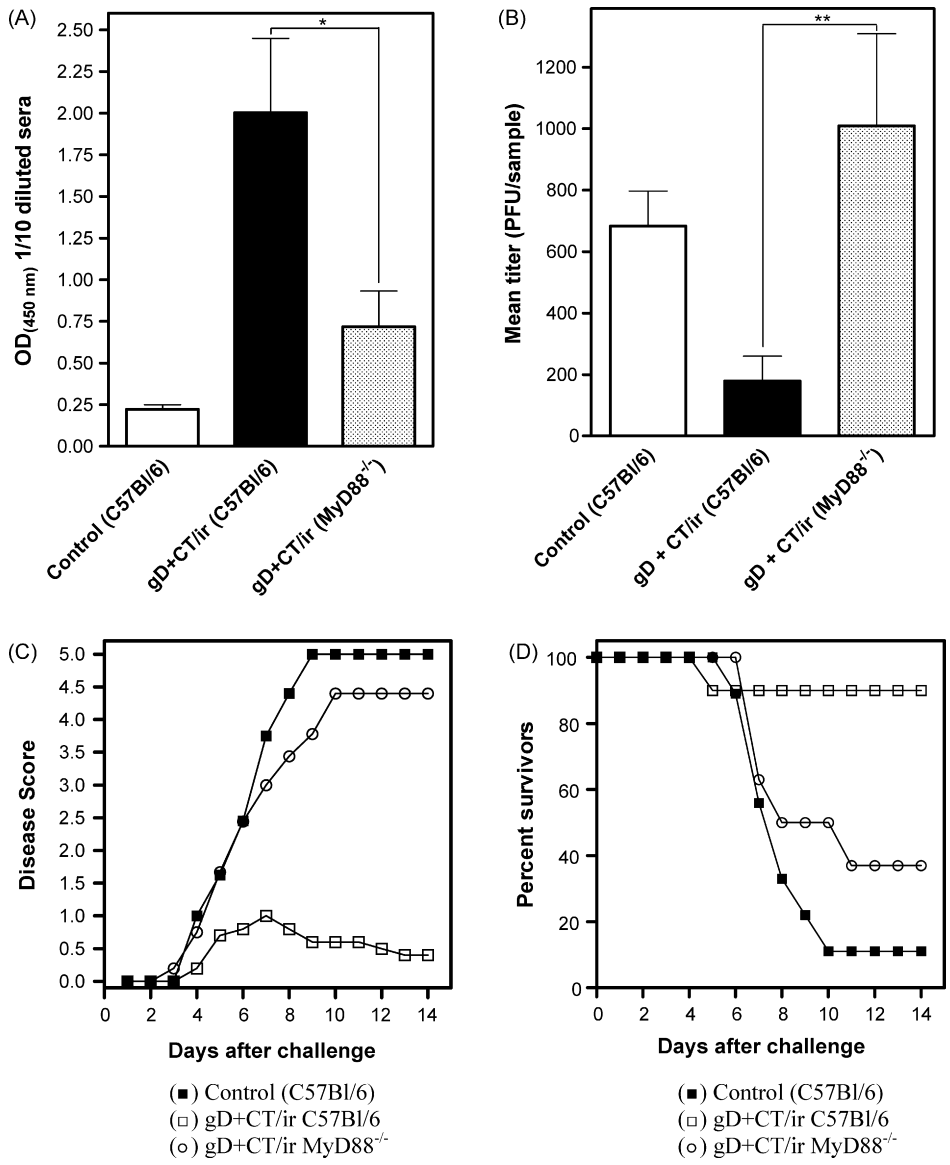

Fig. 8. Induction of protective immunity against HSV-2 following rectal immunization with gD + CT is dependent on MyD88. Groups of 8-to-10 female C57Bl/6 and MyD88$^{-/-}$ mice were intrarectally immunized twice with gD + CT. One month after the last immunization, gD-specific IgG antibody in sera was examined (A) and the mice were intravaginally challenged with a lethal dose of HSV-2. Three days after challenge the animals were examined for vaginal viral titer (B). The data are expressed as mean + S.E.M. of virus load (plaque forming units (PFU)/sample). Mice were monitored daily for macroscopic signs of the disease (C) and survival (D). (*) and (**) represent $p < 0.05$ and $p < 0.01$, respectively, as determined by one-way ANOVA. The results are pooled from two independent experiments. (■) Control, (□) gD + CT/ir C57Bl/6 and (◯) gD + CT/ir MyD88$^{-/-}$.

that HSV-2-infected cells could be detected in the vagina 24 h after vaginal HSV-2 inoculation (Parr and Parr, 1998, 2003a). We also showed that rectal immunization of mice with HSV-2 TK$^-$ elicits strong HSV-2 specific lymphoproliferative and IFN-γ responses both in the gLNs and the spleen. We and others have previously documented the critical importance of Th1-type immunity, including IFN-γ response for protective immunity against genital herpes infection in mice (Milligan and Bernstein, 1997; Harandi et al., 2001; Parr and Parr, 2003b). Importantly, the rectally HSV-2 TK$^-$ immunized animals were completely protected against an otherwise lethal vaginal challenge with a virulent strain of HSV-2.

Moreover, we show that pre-treatment of mice with the sex hormones estrogen and progesterone could not influence the protective immunity in the vagina afforded by the rectal immunization with HSV-2 TK$^-$. Conversely, previous studies have demonstrated that the sex hormones could substantially influence the protective immunity elicited by vaginal immunization with HSV-2 TK$^-$. Thus, pre-treatment of mice with estrogen prior to HSV-2 TK$^-$ immunization was shown to hinder the development of protection against a vaginal challenge with a virulent strain of HSV-2. Short term exposure to progesterone, on the other hand, has been shown to facilitate vaginal infection and development of immune protection in the female genital tract against HSV-2 (Parr et al., 1994). The normal vagina lacks organized lymphoid tissues resembling intestinal Peyer's patches where mucosal immune responses are initiated and disseminated to distant effector sites. Interestingly, progesterone-treated

mice have been shown to develop vagina-associated lymphoid tissue in their vaginal mucosa upon an ivag HSV-2 TK$^-$ inoculation (Gillgrass et al., 2005b). The terminal rectum of mice and humans constantly contain lymphoid follicles with characteristics of an immune inductive site (Langman and Rowland, 1986; Owen et al., 1991). Further, a connection between rectal and genital mucosa has previously been demonstrated (Shen et al., 2000). It is therefore likely that following rectal immunization, the rectal lymphoid aggregates, which are constantly present in the rectum and presumably operate irrespective of the sex hormonal status, serve as inductive site of immunity in the rectal mucosa and in close communication with the iliac (genital) lymph nodes disseminate immunity to the female genital tract.

We could also document that the usage of the adaptor protein MyD88 is not required for the development of immunity in the female genital tract after rectal HSV-2 TK$^-$ immunization. Thus, similar to the immunized C57Bl/6 mice the immunized MyD88$^{-/-}$ mice resisted the lethal vaginal HSV-2 challenge. To our surprise, the HSV-2 specific IgG antibody response after rectal HSV-2 TK$^-$ inoculation was more potent in MyD88$^{-/-}$ mice than that of the HSV-2 TK$^-$ immunized C57Bl/6 mice. These results are surprising as TLR2 and TLR9, the only known TLRs to be involved in HSV detection by innate immunity (Kurt-Jones et al., 2004; Lund et al., 2003; Krug et al., 2004), function exclusively through a MyD88-dependent signaling pathway. It is however possible that other potential PAMPs in HSV-2 may signal through a MyD88-independent pathway and contribute to initiation of innate immune response following rectal inoculation of HSV-2 TK$^-$. Our finding that the absence of MyD88 did not abrogate and rather enhanced the development of specific antibody response is in line with the recent observation that an adjuvant-enhanced antibody response in a parental immunization setting could be mounted even in the absence of TLR signaling (Gavin et al., 2006).

We could also show that rectal immunization of mice with recombinant gD in combination with potent mucosal adjuvant CT can elicit potent HSV-2 specific cellular immune response; however, such immunization elicited a weak antibody response. Importantly, this immunization resulted in protection against HSV-2 in the vagina comparable to those resulting from the rectal immunization with live attenuated HSV-2 TK$^-$. The potent adjuvant activity of CT is at least in part attributed to the ability of CT in increasing mucosal permeability, stimulating the influx of antigen presenting cells, and enhancing antigen presentation by dendritic cells (Holmgren et al., 2003). We could also demonstrate that induction of immune protection in the female genital tract following rectal immunization with gD + CT is dependent on usage of adaptor molecule MyD88 contrary to rectal immunization with HSV-2 TK$^-$. It has been shown that CT induces IL-1 response in epithelial as well as antigen presenting cells (Bromander et al., 1991, 1993; Lycke et al., 1989). It is therefore likely that the IL-1 signaling that is mediated via the adaptor molecule MyD88 contributes to the immunostimulatory/adjuvant effect of CT. However, this issue remains to be further elucidated.

Conversely, our data indicate that rectal immunization with gD combined with the TLR/MyD88 targeting adjuvant CpG ODN failed to achieve HSV-2 specific immune responses and protection. Several recent reports have demonstrated that CpG ODN is a potent inducer of innate and acquired immunity in both systemic and mucosal compartments (Krieg, 2002; Harandi, 2004). We and others have previously documented the ability of CpG ODN combined with recombinant HSV-2 envelop glycoproteins for generation of immune protection in the murine female genital tract against genital herpes (Gallichan et al., 2001; Kwant and Rosenthal, 2004; Tengvall et al., 2006). However, the strength of immunity resulting from immunization may differ depending on HSV-2 glycoprotein used among other factors involved, including type of adjuvant and route of immunization. Our observation that CpG ODN together with gD failed to elicit protective immunity in the female genital tract is in agreement with a recent report indicating that rectal immunization with rotavirus virus-like particles plus CT or *Escherichia coli*-derived heat-labile toxins, but not TLR targeting adjuvants CpG ODN and resiquimod, could elicit a potent antigen specific immunity against enteric rotavirus challenge in mice (Parez et al., 2006). Thus, the rectal mucosa in mice appeared to respond differently to the rectally administered antigens depending upon the type of co-administered adjuvant. This may have important implications for the development of vaccine adjuvants for rectal immunization against mucosally transmitted pathogens.

In conclusion, the present study demonstrates that rectal immunization with a live attenuated HSV-2 TK$^-$ induces potent mucosal and systemic HSV-specific cell-mediated immune responses and confers protection against an otherwise lethal HSV-2 challenge in mice. The observed protective immunity was shown to be independent of sex hormones and the usage of MyD88. Importantly, we could show that rectal immunization with gD in mixture with the mucosal adjuvant CT generates MyD88-dependent immune protection comparable to that of live attenuated HSV-2. These results support the consideration of the rectal-mucosal route for the development of new immunization strategies to generate immunity in the female genital tract against genital herpes and presumably other sexually transmitted infections.

Acknowledgments

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