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Differential roles of B cells and IFN-γ-secreting CD4$^+$ T cells in innate and adaptive immune control of genital herpes simplex virus type 2 infection in mice

Ali M. Harandi,[1] Bo Svennerholm,[2] Jan Holmgren[1] and Kristina Eriksson[1]

Departments of Medical Microbiology and Immunology[1] and Clinical Virology[2], Göteborg University, Guldhedsgatan 10A, 413 46 Göteborg, Sweden

The role of B, CD4$^+$ T and CD8$^+$ T cells in both primary genital infection with attenuated herpes simplex virus type 2 (HSV-2) and development of protective immunity to a later challenge with virulent HSV-2 using lymphocyte-deficient mice has been elucidated. Following primary inoculation with attenuated thymidine kinase-deficient (TK$^-$) HSV-2, B cell-deficient (μMT) mice developed a local viraemia and transient genital inflammation, suggesting a role for B cells in the innate control of local infection and inflammation. Natural antibodies are implicated in this process, as passive transfer of normal serum into μMT mice significantly reduced HSV-2 TK$^-$ shedding in the vaginal lumen, although it did not affect subsequent inflammation. Protection against lethal HSV-2 challenge was noted in HSV-2-vaccinated wild-type, CD8$^+$ T cell-deficient and μMT mice and was characterized by strong virus-specific IFN-γ responses *in vitro* and delayed type hypersensitivity (DTH) responses *in vivo*. In contrast, CD4$^+$ T cell-deficient (CD4$^{-/-}$) mice had impaired HSV-2-specific IFN-γ production and DTH responses and succumbed rapidly to genital HSV-2 challenge. However, protective responses to HSV-2 could be induced in HSV-2-vaccinated CD4$^{-/-}$ mice by treatment with recombinant IFN-γ. Taken together, these results suggest that CD4$^+$ T cells secreting IFN-γ are critical for immune protection against lethal genital HSV-2 re-infection, whereas B cells/natural antibodies have anti-viral and -inflammatory effects in the innate control of a primary infection.

Introduction

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects the human genital tract mucosa and is the most common cause of genital ulcer disease in humans (Kinghorn, 1994; Nahmias *et al*., 1990). HSV-2 infects the genital epithelium and can, following vaginal replication, be transmitted to the central nervous system via uptake and retrograde transport in sensory neurons. The virus may establish latency in infected ganglia and can therefore give rise to lifelong infection. Despite numerous efforts, little is known about the mechanisms involved in disease progression and generation of protective immunity. Thus, a better understanding of the immune mechanisms deployed against HSV-2 would facilitate the development of therapeutics against human genital herpes infection.

Author for correspondence: Kristina Eriksson.
Fax +46 31 820160. e-mail kristina.eriksson@microbio.gu.se

Innate immune mechanisms such as natural killer (NK) cells, neutrophils, macrophages and complement proteins participate in the natural defence against HSV infections (Adler *et al*., 1999; Benencia & Courreges, 1999; Da Costa *et al*., 1999; Milligan, 1999; Milligan *et al*., 1998). In addition, protection against genital HSV-2 infection and disease requires an adequate adaptive immune response. Acquired protective immunity can be mediated by both humoral and cell-mediated immune mechanisms. In experimental animals, protection against genital disease can be achieved by passive administration of either monoclonal or polyclonal HSV-specific antibodies (Eis-Hübinger *et al*., 1993; Parr & Parr, 1997; Whaley *et al*., 1994). Furthermore, three lines of evidence support the contention that T cells are important in both control of a primary HSV infection and protection against re-infection: (i) in humans, clearance of HSV-2 from recurrent genital lesions correlates with infiltration of HSV-2-specific cytotoxic T cells (Koelle *et al*., 1998), (ii) *in vivo* depletion of T cells from HSV-2-immunized mice abrogates their ability to

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resolve an HSV-2 vaginal re-infection (Milligan *et al*., 1998; Parr & Parr, 1998) and (iii) resistance to intravaginal challenge with HSV-2 can be transferred to naive recipient mice with genital lymph node cells from immune mice (McDermott *et al*., 1989). Both $CD4^+$ and $CD8^+$ T cell subsets have been implicated in immune protection (Martin & Rouse, 1987; Milligan *et al*., 1998).

The present study was undertaken to elucidate further the requirement for B, $CD4^+$ and $CD8^+$ T cells in control of primary infection and protection against genital re-infection, for which purpose we used mice deficient in IgM heavy chain (μMT), $CD4^+$ T cells ($CD4^{-/-}$) or $CD8^+$ T cells ($CD8^{-/-}$). We used a mouse genital infection model system in which a single vaginal inoculation of a thymidine kinase-deficient attenuated strain of HSV-2 TK^- confers protective immunity to a subsequent vaginal challenge with a lethal dose of a wild-type (WT) HSV-2 strain (McDermott *et al*., 1984; Parr *et al*., 1994). The results indicate that B cells secreting natural antibodies are involved in the innate control of local virus replication after primary genital HSV-2 TK^- infection. Furthermore, $CD4^+$ but not $CD8^+$ T cells were shown to play a crucial role in adaptive immune protection against genital HSV-2 re-infection. Moreover, we demonstrated that exogenous IFN-γ could be used to compensate for the lack of $CD4^+$ T cells during HSV-2 re-infection.

Methods

Mice. Female 8- to 10-week-old mice were used for all experiments. Naive C57BL/6 mice (WT) were purchased from B&K Universal, Stockholm, Sweden. C57BL/6 μMT (Kitamura *et al*., 1991), $CD4^{-/-}$ (Rahemtulla *et al*., 1991) and $CD8^{-/-}$ (Fung-Leung *et al*., 1991) mice were kindly provided by N. Lycke (Göteborg University, Sweden). The phenotype of each strain of mice was confirmed by FACS analysis. The animals were kept in ventilated cages under specific-pathogen-free conditions at the Department of Medical Microbiology and Immunology, Göteborg University, Sweden.

Viruses. The attenuated mutant of HSV-2, strain Lyon, which contains a partial deletion of the thymidine kinase gene (TK^-), was kindly provided by E. De-Clercq (Rega Institute for Medical Research, K. U. Leuven, Belgium) (Andrei *et al*., 1997). HSV-2 TK^-, strain Lyon and WT HSV-2, strain 333 (Seth *et al*., 1974) were grown and titrated in monolayers of African green monkey kidney cells (GMK-AH1) and prepared by one cycle of freeze–thaw and subsequent removal of cellular debris by centrifugation.

HSV-2 antigen preparation. HSV-2 strain 333 was grown in GMK-AH1 cell monolayers. One cycle of freeze–thaw and centrifugation for the removal of cellular debris was used to recover virus from the culture. Control cell extract (mock antigen) was prepared in a similar fashion from uninfected GMK-AH1 cell monolayers. Virus preparations containing 1·5 to 2×10^7 p.f.u./ml of HSV-2 and control cell extracts were inactivated by UV light for 30 min (Koelle *et al*., 1994). Inactivated HSV-2 and mock antigen were used at 1:100 final dilution for *in vitro* experiments.

Primary and secondary infections. Mice were injected subcutaneously with 2·0 mg of Depo-Provera (DP) (Pharmacia) in 100 μl PBS, anaesthetized 6 days later using methofane (Schering-Plough Animal Health) and vaccinated by intravaginal inoculation of $3{\cdot}6 \times 10^6$ p.f.u. of attenuated HSV-2 TK^- in 20 μl Hanks' balanced salt solution (HBSS). Four weeks after HSV-2 TK^- vaccination, vaccinated and unvaccinated mice were pre-treated with DP as above, followed 6 days later by an intravaginal inoculation of 4×10^4 p.f.u. (100 LD_{50}) of virulent HSV-2 strain 333 in 20 μl HBSS.

Monitoring of infection

(a) Virus replication. Following intravaginal HSV-2 infection, vaginal fluids were collected 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 immediately centrifuged to clarify the vaginal washes and then stored at −70 °C. HSV-2 titres were determined by plaque assay on GMK-AH1 cell monolayers.

(b) Inflammation and disease. Mice were examined daily for vaginal inflammation, neurological illness and death after HSV-2 infection. The severity of disease was graded as healthy (0), genital erythema (1), moderate genital inflammation (2), severe and purulent genital lesion (3), hind-limb paralysis (4) and death or sacrifice due to paralysis (5), as described previously (Morrison *et al*., 1998).

Reconstitution of μMT mice. Groups of DP-treated μMT mice were injected, 0·5 ml intraperitoneally (i.p.) and 0·2 ml intravenously, with pooled serum from naive control (C57BL/6) mice. These mice, together with non-reconstituted μMT mice, were vaginally inoculated 2 h later with $3{\cdot}6 \times 10^6$ p.f.u. of attenuated HSV-2 TK^-.

***In vivo* IFN-γ treatment.** Chinese hamster ovary (CHO) cells or CHO cells transfected with the gene encoding murine IFN-γ (kindly provided by M. Taube, Göteborg University, Sweden) were used as sources of control supernatent and recombinant IFN-γ (rIFN-γ), respectively. The supernatants were dialysed against PBS and further concentrated using polyethylene glycol (PEG 3000) as described (Zhao *et al*., 1998). HSV-2 TK^--vaccinated, DP-treated $CD4^{-/-}$ mice and naive, DP-treated WT mice were given either 6 μg of rIFN-γ or the same volume of control supernatant i.p. The mice were injected intravaginally 6 h later with either 0·4 μg of rIFN-γ in 10 μl or the same volume of control supernatant and then challenged intravaginally with 4×10^4 p.f.u. of HSV-2 strain 333. The challenged mice were treated i.p. with rIFN-γ or control supernatant as above on days 2, 4, 6 and 8 post-challenge.

Proliferation assays. Mononuclear cell suspensions of spleen cells isolated 4 weeks after vaccination with attenuated HSV-2 TK^- were seeded in triplicate wells at a concentration of 1×10^5 cells per well in Iscove's medium supplemented with L-glutamine, 50 μM 2-mercaptoethanol, gentamycin and 10% foetal calf serum (complete Iscove's medium) and incubated at 37 °C for 5 days in 96-well round-bottom plates in the presence of UV-inactivated HSV-2 or mock antigen. Cells were pulsed with 1 μCi [^{3}H]thymidine (Amersham Pharmacia) for the last 6 h of culture and the cellular DNA was harvested on glass-fibre filters and then assayed by liquid scintillation counting. Data are expressed as stimulation indices (SI), corresponding to the mean c.p.m. of UV-inactivated HSV-2-treated cultures divided by the mean c.p.m. of mock-treated cultures.

Cytokine measurements. Measurement of cytokine production in spleen cells was performed using a modified version of a celELISA method (Beech *et al*., 1997). Briefly, wells of a 96-well flat-bottom plate were coated with 50 μl anti-mouse IFN-γ (1·5 μg/ml, R&D Systems), IL-2 (2 μg/ml, Pharmingen) or IL-4 (1 μg/ml, Pharmingen) in PBS. After

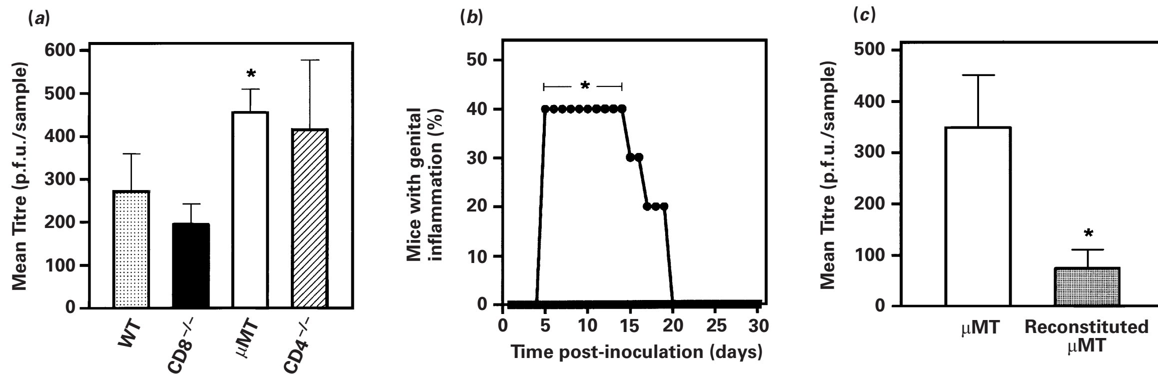

Fig. 1. Primary genital HSV-2 TK⁻ infection in lymphocyte-deficient mice. (*a*) HSV-2 replication was determined in vaginal washings obtained 2 days after vaginal HSV-2 TK⁻ inoculation. Data are expressed as the mean ± SEM virus load (p.f.u./sample washing fluid) for μMT, $CD8^{-/-}$ or $CD4^{-/-}$ mice. Each group consisted of four animals or more. (*) Statistically significant at $P < 0{\cdot}05$ compared with WT mice. (*b*) Percentages of mice with genital erythema and inflammation were determined. (■) WT, $CD8^{-/-}$ and $CD4^{-/-}$ mice. (●) μMT mice. Each group consists of ten mice. (*) Statistically significant at $P < 0{\cdot}05$ compared with WT mice. (*c*) HSV-2 titres in vaginal secretions of serum-reconstituted and untreated μMT mice. HSV-2 replication was determined in vaginal washings obtained 2 days after vaginal HSV-2 TK⁻ inoculation. Data are expressed as the mean ± SEM virus load (p.f.u./sample) for groups of five mice. (*) Statistically significant at $P < 0{\cdot}05$ compared with, untreated μMT mice. The experiments were performed twice, with comparable results.

overnight incubation at 4 °C, wells were blocked with a sterile solution of 1% BSA in PBS. Mononuclear spleen cell suspensions obtained 4 weeks post-vaccination with attenuated HSV-2 TK⁻ were seeded in duplicate at different cell densities in complete Iscove's medium containing UV-inactivated HSV-2 or mock antigen and incubated at 37 °C for 24 h (for IFN-γ and IL-2 determinations) or 48 h (for IL-4 determination). The cells were removed by extensive washing and biotinylated anti-mouse IFN-γ (1 μg/ml), IL-2 (2 μg/ml) or IL-4 (0·25 μg/ml) was added overnight at 4 °C followed by a 45 min incubation with 2 μg/ml peroxidase-labelled avidin (Sigma) at room temperature. Colour development was achieved with 100 μl peroxidase substrate containing 3,3′,5,5′-tetramethylbenzidine (0·1 mg/ml, Sigma) and 0·06% H_2O_2 in 0·05 M phosphate–citrate buffer at pH 5·0. The reaction was stopped using 25 μl 1 M H_2SO_4 and absorbance was read at 450 nm. The concentration of each cytokine was determined by extrapolation from a standard curve obtained using recombinant cytokine (IFN-γ and IL-4, R&D systems; IL-2, Pharmingen). Results are expressed as the concentration of each cytokine secreted per 1×10^6 spleen cells.

■ **Measurement of delayed type hypersensitivity (DTH) responses.** DTH reaction was elicited 4 weeks after vaccination with attenuated HSV-2 TK⁻ by injection of UV-inactivated HSV-2 (corresponding to 7×10^6 p.f.u. in 30 μl medium) in the left (experimental) footpad and 30 μl of mock antigen in the right (control) footpad. Swelling of the footpads was measured 48 h later using a calliper (Oditest) and the specific footpad swelling was determined for each animal as the mean difference between the thickness of experimental and control footpads.

■ **Antibody measurements.** Maxisorp 96-well plates (Nunc) were coated with 100 μl of a deoxycholate-solubilized membrane fraction of HSV-1-infected cells (Jeansson *et al.*, 1983) for 4 h at room temperature in 0·05 M carbonate buffer at pH 9·6. The plates were blocked with 2% BSA in PBS for 30 min at 37 °C. Serial dilutions of sera obtained 4 weeks post-vaccination with attenuated HSV-2 TK⁻ were incubated for 1 h at 37 °C. After washing with 0·05% Tween 20, the plates were incubated for 1 h at 37 °C with goat anti-mouse IgG coupled to horseradish peroxidase (1:1000, Southern Biotechnology) in 1% BSA in PBS. The plates were 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. The sample IgG titre was defined as the reciprocal of the sample dilution giving an absorbance value of 0·4 above the background value.

■ **Statistical analysis.** Statistical analyses were done by Student's *t*-test or Pearson's correlation test.

Results

Lack of B cells is associated with increased virus replication and local genital inflammation

To investigate the role of B cells and T cell subsets in control of a primary genital infection with HSV-2 TK⁻, mice deficient in B, $CD4^+$ or $CD8^+$ cells along with WT control animals were infected vaginally with HSV-2 TK⁻. Two days after infection, the virus titres in vaginal secretions were comparable in the WT and $CD8^{-/-}$ mice, whereas virus titres in $CD4^{-/-}$ mice were slightly increased (Fig. 1*a*). However, WT, $CD8^{-/-}$ and $CD4^{-/-}$ mice did not exhibit any visible signs of infection-induced inflammation. Interestingly, in μMT mice, a statistically significant enhancement in vaginal HSV-2 titre was seen following HSV-2 TK⁻ inoculation compared with that of WT control mice ($P < 0{\cdot}05$, Fig. 1*a*). Furthermore, 40% of μMT mice developed moderate vaginal inflammation (severity score 1–2) between days 5 and 14 post-infection. There was no correlation between vaginal inflammation and vaginal virus load in μMT mice inoculated with HSV-2 TK⁻. However, all μMT mice survived and were able to clear the inflammation (Fig. 1*b*). These results indicate that, in this mouse model system, B cells and/or antibodies may participate in the innate control of a local primary infection with attenuated HSV-2 TK⁻.

Table 1. HSV-2 levels in the genital tract of lymphocyte-deficient mice

Mice were vaccinated with an attenuated (TK^-) strain of HSV-2 4 weeks prior to HSV-2 challenge infection. At days 0, 2, 4, 6 and 8 after HSV-2 infection, mice were treated with IFN-γ. Vaginal fluids were collected 3 days after HSV-2 infection and virus titres were determined by standard plaque assay.

Mice	Vaccination	IFN-γ treatment	HSV-2 titre (p.f.u./sample) mean ± SEM
WT	−	−	841·5 ± 114·3
WT	+	−	< 10
$CD8^{-/-}$	+	−	< 10
μMT	+	−	48·8 ± 23·2
$CD4^{-/-}$	+	−	220·1 ± 43·8
$CD4^{-/-}$	+	+	< 10

Role of natural antibodies in protection against primary genital HSV-2 infection

In order to determine whether B cell production of antibodies is critical to the control of primary HSV-2 TK^- infection, we injected μMT mice with pooled serum from naive C57BL/6 mice. This treatment resulted in comparable levels of total IgM in the serum and vagina of recipient μMT mice compared with those in naive C57BL/6 WT animals (data not shown). Reconstituted μMT mice developed a statistically significant reduction in virus load in the vagina on day 2 post-challenge with HSV-2 (Fig. 1*c*), which was four times lower than that in the untreated μMT mice ($P < 0{\cdot}05$). However, the inflammatory response did not differ between the reconstituted and control μMT mice (40% in both groups, data not shown). This finding suggests that natural antibodies contribute to the innate control of virus replication during a primary genital infection with HSV-2 TK^-.

$CD4^+$ T cells provide protective immunity against genital HSV-2 re-infection

To examine the importance of different lymphocyte subsets for the induction of a protective immune response against a lethal vaginal HSV-2 challenge, μMT, $CD4^{-/-}$, $CD8^{-/-}$ and WT mice were vaccinated vaginally with HSV-2 TK^- 4 weeks prior to vaginal challenge with 100-fold LD_{50} dose (4×10^4 p.f.u. per mouse) of HSV-2. All unvaccinated WT, μMT, $CD4^{-/-}$, $CD8^{-/-}$ and control mice died within 12 days after HSV-2 challenge (median, day 8; range, days 7 to 12; data not shown). Vaccinated $CD8^{-/-}$ mice were similar to vaccinated WT mice in that they had no detectable HSV-2 in vaginal washings obtained day 3 post-challenge (Table 1) and no genital or neurological signs of inflammation during the 30 day observation period (Fig. 2). In vaccinated μMT mice, a low titre

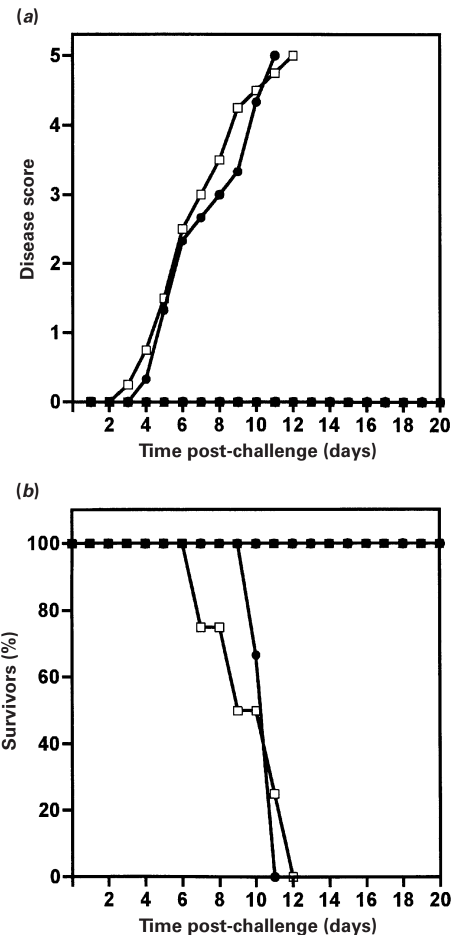

Fig. 2. Disease progression in HSV-2-challenged lymphocyte-deficient mice. C57BL/6 WT, $CD8^{-/-}$, μMT and $CD4^{-/-}$ mice were vaccinated intravaginally with $3{\cdot}6 \times 10^6$ p.f.u. HSV-2 TK^- and challenged 4 weeks later with a lethal dose of HSV-2. Animals were scored daily for (*a*) pathological symptoms and (*b*) mortality. Disease progression was scored as healthy (0), genital erythema (1), moderate genital inflammation (2), severe and purulent genital lesion (3), hind-limb paralysis (4) and death or sacrifice due to paralysis (5). (■) Vaccinated WT, $CD8^{-/-}$ and μMT mice. (●) Vaccinated $CD4^{-/-}$ mice and (□) non-vaccinated WT controls. Each group consisted of five mice or more. The experiments were repeated three times, with similar results.

of HSV-2 was seen at day 3 post-challenge (Table 1); however, these mice displayed resistance to subsequent lethal challenge with HSV-2 (Fig. 2). The vaccinated $CD4^{-/-}$ mice exhibited significantly increased titres of HSV-2 at day 3 post-challenge (Table 1). Furthermore, the vaccinated $CD4^{-/-}$ mice all succumbed to a lethal HSV-2 challenge, with vaginal symptoms starting at day 4 (Fig. 2*a*), and died as a result of neurological illness within 2 weeks, i.e. at approximately the same time as the non-vaccinated WT control mice (Fig. 2*b*). These findings indicate that $CD4^+$ but not $CD8^+$ T cells or B cells are critical for an effective acquired immune response against a lethal HSV-2 challenge in mice, even though B cells/antibodies appear to suppress the local virus replication in the genital tract.

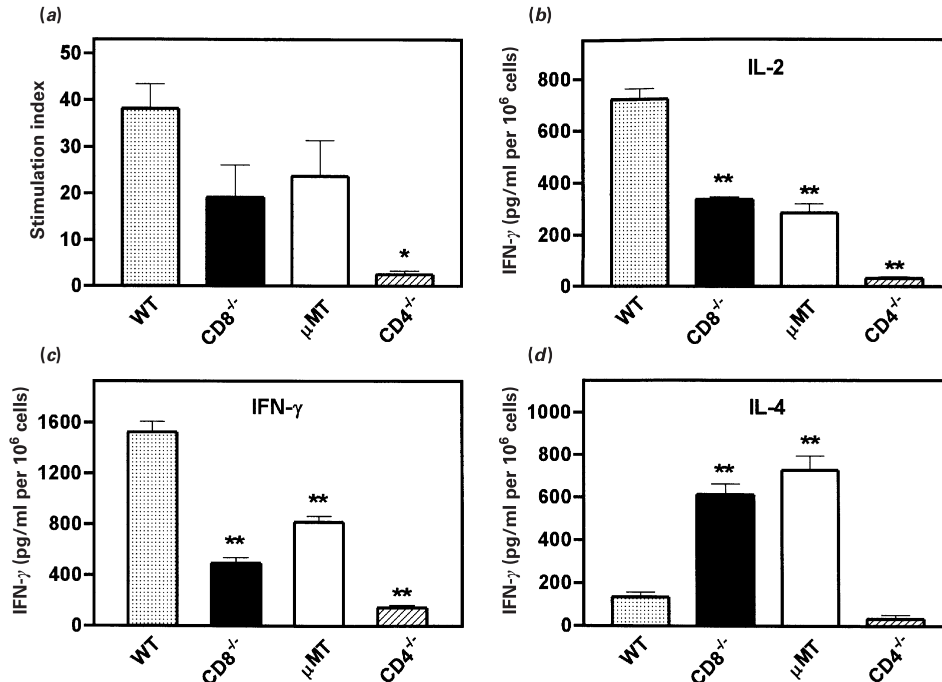

Fig. 3. HSV-2-specific T cell responses in lymphocyte-deficient mice. (*a*) HSV-2-specific *in vitro* proliferative responses by spleen mononuclear cells obtained 4 weeks after HSV-2 TK$^-$ vaccination. Data are expressed as the mean ± SEM of the SI for WT, CD4$^{-/-}$, CD8$^{-/-}$ and μMT mice. (*b*)–(*d*) IFN-γ (*b*), IL-2 (*c*) and IL-4 (*d*) cytokine production by spleen mononuclear cells obtained 4 weeks after HSV-2 TK$^-$ vaccination and cultured *in vitro* with HSV-2 antigen. Data are expressed at the mean ± SEM concentration of cytokine secreted per 1 × 10^6 spleen cells (pg/ml) for WT, CD4$^{-/-}$, CD8$^{-/-}$ and μMT mice. Differences were statistically significant at $P < 0{\cdot}05$ (*) or $P < 0{\cdot}01$ (**) by Student's *t*-test compared with vaccinated WT mice. Experiments were performed twice, with comparable results.

Immune responses in immune cell-deficient mice after HSV-2 TK$^-$ vaccination

We studied the immunological mechanisms involved in acquired immune protection against vaginal HSV-2 re-infection by analysing HSV-specific T and B cell responses in mice 4 weeks after vaginal HSV-2 TK$^-$ vaccination (i.e. at the time of challenge).

(a) T cell proliferative responses *in vitro*. Spleen cells from vaccinated WT mice developed strong HSV-2-specific proliferative responses with SI values of 30 or more. Vaccinated μMT and CD8$^{-/-}$ mice showed marked reductions in their specific proliferative responses compared with WT animals (Fig. 3*a*). Thus, all vaccinated mice that were protected against subsequent challenge had measurable proliferative response *in vitro*. In contrast, vaccinated CD4$^{-/-}$ mice, which failed to develop protective immune responses to HSV-2, showed severely impaired HSV-2-specific proliferative responses *in vitro* ($P < 0{\cdot}05$ compared with that in vaccinated WT mice) (Fig. 3*a*).

(b) *In vitro* cytokine production. HSV-2-specific production of IFN-γ, IL-2 and IL-4 was analysed in spleen cell suspensions using the celELISA method. As shown in Fig. 3(*b*, *c*), spleen cells from vaccinated WT mice, which were protected against

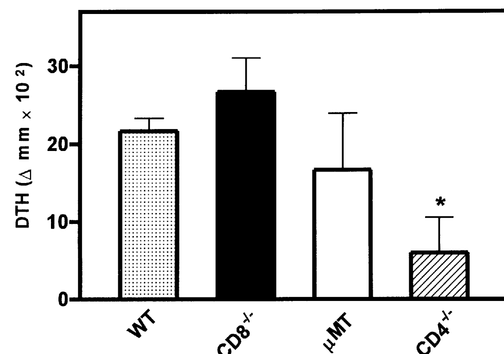

Fig. 4. HSV-2-specific DTH reaction in vaccinated WT, μMT, CD4$^{-/-}$ and CD8$^{-/-}$ mice. Four weeks after vaginal vaccination with HSV-2 TK$^-$, UV-inactivated HSV-2 and mock antigen were injected in the left and right footpads and swelling was measured 48 h later. Results are expressed as the mean ± SEM of the HSV-2-specific footpad swelling (Δ mm × 10^2) for each group of mice. (*) Statistically significant at $P < 0{\cdot}05$ by Student's *t*-test compared with vaccinated WT mice. The experiment was performed twice, with comparable results.

HSV-2 challenge, developed significant levels of HSV-2-specific IL-2 and IFN-γ compared with un-vaccinated WT animals. The CD8$^{-/-}$ and μMT spleen cells from vaccinated animals produced intermediate levels of IL-2 and IFN-γ, though, when compared with vaccinated WT spleen cells, the differences were statistically significant ($P < 0{\cdot}01$) (Fig. 4*b*, *c*). In

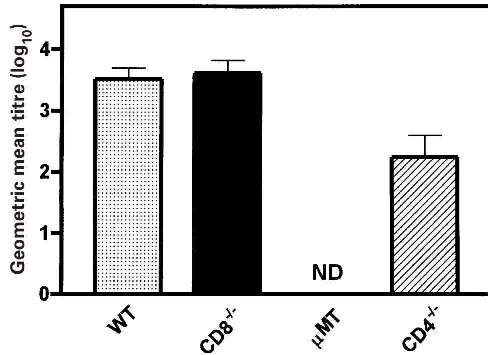

Fig. 5. HSV-specific antibody responses in lymphocyte-deficient mice. WT, μMT, CD4$^{-/-}$ and CD8$^{-/-}$ mice were vaccinated with HSV-2 TK^{-} and serum levels of HSV-specific IgG were analysed 4 weeks later. Data are expressed as the $\log_{10}$ mean$\pm$SEM of the reciprocal HSV-specific IgG titre. Each group consisted of at least five mice. Similar experiments were performed twice, with comparable results. ND, Not detected.

contrast, spleen cells from vaccinated CD4$^{-/-}$ mice, which were not protected against vaginal HSV-2 challenge, showed insignificant production of IL-2 or IFN-γ ($P < 0{\cdot}01$ compared with vaccinated WT mice) (Fig. 3*b*, *c*). Low levels of IL-4 were produced by spleen cells from vaccinated WT and CD4$^{-/-}$ mice, whereas spleen cells from vaccinated CD8$^{-/-}$ and μMT mice showed statistically significant enhancement in the levels of IL-4 compared with vaccinated WT animals ($P < 0{\cdot}01$) (Fig. 3*d*). Thus, CD8$^{-/-}$ and μMT animals vaccinated with HSV-2 TK^{-} displayed impaired IL-2 and IFN-γ production but enhanced IL-4 production, indicating a shift towards a Th-2 phenotype.

(c) DTH responses. To investigate whether HSV-2 TK^{-} vaccination elicited HSV-2-specific T cell responses *in vivo*, mice were tested for DTH responses 4 weeks post-vaccination with HSV-2 TK^{-}. No DTH reaction was observed in naive animals (data not shown). In contrast, a strong HSV-2-specific DTH response was seen in vaccinated WT as well as in CD8$^{-/-}$ and μMT mice (Fig. 4). The vaccinated CD4$^{-/-}$ mice, on the other hand, showed a significantly reduced DTH response (Fig. 4). Using Pearson's correlation test, we found a significant correlation between protection against re-infection and HSV-2-specific DTH response in vaccinated mice ($r = 0{\cdot}66$, $P < 0{\cdot}05$).

(d) Antibody responses. To investigate whether the absence of CD4^{+} or CD8^{+} T cells might affect the systemic specific IgG response, mice deficient in lymphocyte subsets were examined for specific serum IgG antibodies. Sera obtained from vaccinated WT and CD8$^{-/-}$ mice contained similar titres of HSV-specific IgG, while vaccinated μMT mice, as expected, lacked specific serum IgG antibodies (Fig. 5). Surprisingly, sera from vaccinated CD4$^{-/-}$ mice contained HSV-specific IgG, although at a tenfold lower level than that in WT animals (Fig. 5). Thus, a specific serum IgG response can be elicited in the absence of CD4^{+} T cells following HSV-2 TK^{-} inoculation.

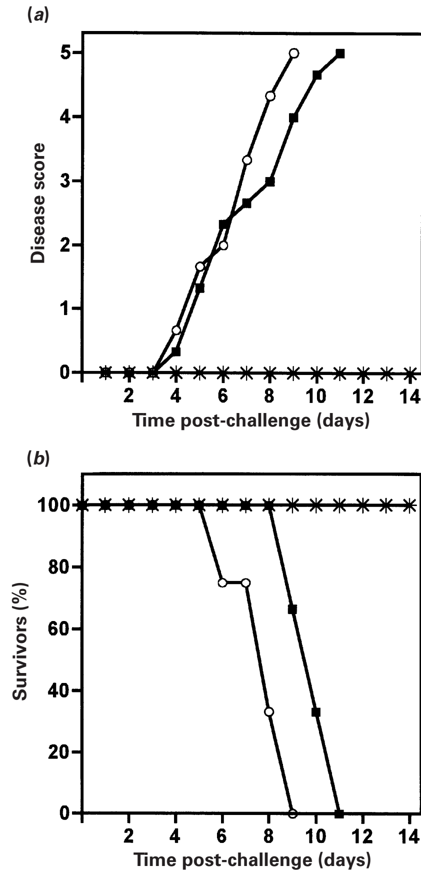

Fig. 6. IFN-γ treatment restores protection against genital HSV-2 infection in CD4$^{-/-}$ mice vaccinated with HSV-2 TK^{-}. Vaccinated mice were treated with rIFN-γ or control media 6 h prior to vaginal HSV-2 challenge and then re-treated with rIFN-γ every second day for 8 days. The mice were scored daily for pathological symptoms (*a*) and mortality (*b*) as described in Fig. 2. (○) Non-vaccinated WT mice, (■) vaccinated CD4$^{-/-}$ mice supplemented with control media and (*) vaccinated CD4$^{-/-}$ mice supplemented with rIFN-γ are represented.

Treatment with IFN-γ restored resistance to genital HSV-2 re-infection in CD4$^{-/-}$ mice

The involvement of IFN-γ in control of genital HSV-2 infection has been well documented (Milligan & Bernstein, 1997; Milligan *et al.*, 1998; Parr & Parr, 1999). To test whether IFN-γ could compensate for the lack of CD4^{+} T cells in genital HSV-2 re-infection, we supplemented the HSV-2 TK^{-}-vaccinated CD4$^{-/-}$ mice with rIFN-γ a few hours before HSV-2 challenge and then every second day during the first 8 days after re-infection. IFN-γ-supplemented HSV-2-infected CD4$^{-/-}$ mice displayed HSV-2 clearance from the genital tract (Table 1) and were protected from HSV-2-induced disease, whereas all control-treated HSV-2-infected CD4$^{-/-}$ animals succumbed to HSV-2 challenge (Fig. 6). IFN-γ treatment of unvaccinated animals failed to protect against HSV-2 infection, as all IFN-γ-treated animals died following a lethal HSV-2 challenge (data not shown). These results suggest that IFN-γ is a critical component of the adaptive immune response to HSV-2 and

that this cytokine can indeed replace the requirement for $CD4^+$ T cells.

Discussion

In the present study, we show that B cells secreting non-specific antibodies have a role in the innate control of local virus replication following genital infection with attenuated HSV-2 TK^- and that $CD4^+$ T cells are crucial for the development of protective immunity against genital re-infection with HSV-2. Interestingly, in the latter case, the administration of exogenous IFN-γ could compensate for the lack of $CD4^+$ T cells. In contrast, a lack of $CD8^+$ T cells did not result in any appreciable impairment in either innate or adaptive immunity to HSV-2 infection.

In WT mice, vaginal inoculation with HSV-2 TK^- caused a subclinical infection. In comparison, μMT mice, which lack functional B cells and antibodies, had significantly higher virus loads and 40% of the animals also displayed an apparent genital inflammation. The most likely explanation for the greater virus replication following primary HSV-2 infection is the lack of immunoglobulins including natural antibodies. We found that transfer of normal mouse serum into μMT mice significantly reduced the vaginal load of HSV-2, indicating an early role of natural antibody in control of virus replication. To our knowledge this is the first report to suggest that natural antibodies play an early and beneficial role in primary genital HSV-2 infection. These results are in line with a recent study showing a role for natural antibodies in both innate control of a cutaneous HSV-1 infection (Deshpande *et al*., 2000) and limiting of the spread of lymphocytic choriomeningitis virus during the first days of a primary infection (Ochsenbein *et al*., 1999). The beneficial effect of natural antibodies on HSV-2 replication that we observed could depend on the initial virus load, as no differences in virus replication were observed between μMT and WT mice when a low-dose HSV-2 inoculum was used (Dudley *et al*., 2000).

Despite the dramatic drop in virus shedding, the serum-reconstituted μMT mice still developed genital inflammation at the same frequency as those not receiving serum transfer. Therefore, we postulate that natural antibodies have little role to play in early control of HSV-2-induced inflammation. We cannot conclusively exclude such a role, however, since a single serum transfer would not mimic the continuous immunoglobulin production of WT mice. It is also possible that B cells themselves could have an anti-inflammatory role. Thus, B cells have been shown to be the first lymphocytes to be recruited to the vagina of mice following HSV-2 TK^- inoculation (Parr *et al*., 1994) and provide several functions in addition to antibody production, including: (i) B cells may regulate the appropriate trafficking of other cells to the site of infection via secretion of, e.g. macrophage inflammatory protein-1 α (MIP-1α) and MIP-1β (Krzysiek *et al*., 1999), (ii) B cells are one of the major sources of IFN-α (Liles & van Voorhis, 1995), which blocks virus replication and spread, and (iii) B cells may also activate NK cells (Yuan *et al*., 1994), which can suppress HSV infection (Minato *et al*., 1980).

Previous studies have shown that transfer of specific serum IgG from HSV-2 TK^--vaccinated mice to the vaginal lumen of naive mice reduced the virus load and pathological signs of disease in recipient mice following vaginal HSV-2 challenge (Parr & Parr, 1997) and also that transfusion of monoclonal antibodies to glycoprotein gB of HSV conferred protection against a subsequent challenge (Eis-Hübinger *et al*., 1991, 1993; Kino *et al*., 1985). In contrast to these findings, our study corroborates the findings of Dudley *et al*. (2000) in showing clearly that resistance to a lethal vaginal HSV-2 challenge in mice vaccinated with HSV-2 TK^- is not critically dependent on B cells and/or antibodies.

Our results in $CD8^{-/-}$ mice show that, in similarity with experiments with B cells, $CD8^+$ T cells are not crucial for control of vaginal HSV-2 re-infections in mice. These results are in agreement with recent findings in humans that show that HSV-2 recurs even in the presence of high levels of specific $CD8^+$ cytotoxic T cells in the genital tract (Posavad *et al*., 2000). Interestingly, the lack of B cells or $CD8^+$ T cells was associated with increased IL-4 responses concomitant with reduced levels of IL-2 and IFN-γ, indicating that the presence of both B cells and $CD8^+$ T cells favours Th-1 phenotype responses. However, the shift towards Th-2 phenotype responses, characterized by decreased levels of IFN-γ and increased levels of IL-4, did not affect protection.

Our data show that $CD4^+$ T cells are critical for the development of an efficient protective immune response to HSV-2. The failure of $CD4^{-/-}$ mice vaccinated with HSV-2 TK^- to combat infection was associated with an enhanced HSV-2-specific T cell-mediated immune response after the initial infection/vaccination, as evidenced by impaired HSV-2-specific cytokine production and proliferative response *in vitro* and a compromised virus-specific DTH response *in vivo*. These results support previous findings that vaccination with recombinant antigens could induce protection from genital HSV-2 infection in WT but not $CD4^{-/-}$ mice (Kuklin *et al*., 1998) and extend the observations made by others with HSV-2 infection in T cell-depleted animals (Milligan *et al*., 1998; Parr & Parr, 1998).

Interestingly, our results show that protection against genital HSV-2 re-infection in $CD4^{-/-}$ mice vaccinated with HSV-2 TK^- can be restored by administration of exogenous IFN-γ at the time of HSV-2 re-infection. It was obvious that IFN-γ was not anti-viral per se, as exogenous IFN-γ treatment could not rescue non-vaccinated WT mice from a lethal genital HSV-2 challenge. Thus, in the absence of $CD4^+$ T cells, IFN-γ acts in conjunction with other components of the adaptive immune system in combating HSV-2 infection. This latter finding suggests that $CD4^+$ T cells may be the major source of IFN-γ at the time of HSV-2 re-infection, which is supported by the virtual absense of IFN-γ in $CD4^{-/-}$ mice and by the

decreased levels of genital IFN-γ responses in $CD4^+$ T cell-depleted animals (Milligan *et al.*, 1998). Previous studies have shown an important role for IFN-γ in control of genital HSV-2 infection (Milligan & Bernstein, 1997; Milligan *et al.*, 1998; Parr & Parr, 1999) but it has not been reported previously that IFN-γ could substitute for the $CD4^+$ lymphocyte population.

Several mechanisms might account for the contribution of IFN-γ in protection against genital HSV-2 infection including: (i) IFN-γ enhanced expression of both MHC class I and II on the cell surfaces, which facilitates the recognition and subsequent destruction of infected cells by $CD8^+$ and $CD4^+$ T cells, (ii) IFN-γ inhibition of HSV replication *in vitro* by blocking transactivation-induced transcription of immediately early genes (De Stasio & Taylor, 1990) (however, since administration of exogenous IFN-γ to naive WT mice did not protect against disease and death, this direct anti-viral effect, if it occurs *in vivo*, is of relatively minor importance), (iii) IFN-γ-mediated chemoattraction and activation of macrophages and neutrophils (Fong & Mosmann, 1989), and (iv) IFN-γ up-regulation of intracellular adhesion molecules such as ICAM-1 on endothelial cells (Dustin *et al.*, 1986) to direct lymphocytes into the site of HSV infection.

In conclusion, the data presented here indicate that B cells secreting natural antibodies play an important role in innate control of local HSV-2 replication following primary genital infection with HSV-2. The protective immunity following vaginal vaccination with HSV-2 TK^- depends on $CD4^+$ T cells. Furthermore, IFN-γ could compensate for the lack of $CD4^+$ T cells during genital HSV-2 re-infection. These findings have implications for our understanding of immune mechanisms involved in protection against genital HSV-2 infection as well as for immunomodulating therapies against genital herpes infection.

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