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Intraepithelial γδ T Cells May Bridge a Gap between Innate Immunity and Acquired Immunity to Herpes Simplex Virus Type 2

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Mice depleted of γδ T cells by in vivo administration of anti-TCRγδ monoclonal antibodies showed susceptibility against an intravaginal infection with herpes simplex virus type 2 (HSV-2). The systemic Th1 response was impaired in the γδ T-cell-depleted mice. Mice deficient in the Vδ1 T subset were susceptible to an intravaginal infection with HSV-2. Intraepithelial γδ T cells bearing Vδ1 may help protect against intravaginal infection with HSV-2 through promoting the systemic Th1 response.

T-cell receptor (TCR) γδ T cells are present only in small numbers in peripheral lymphoid tissues but are abundant in intraepithelial lymphocytes in the skin, intestines, and reproductive organs such as the uterus and vagina (3, 5). The intraepithelial γδ T-cell subsets in the mouse model bear invariant TCRs, including the Vδ1 subset that comprises most of the γδ T cells in the skin and the female reproductive tract (3, 5). Animal models of infection with herpes simplex virus type 2 (HSV-2) have demonstrated that protective mechanisms against primary infection with HSV-2 are mainly mediated by class II-restricted $CD4^+$ Th1 cells secreting gamma interferon (IFN-γ) (7, 10, 11, 14, 17). TCR γδ T cells are reported to play important roles in protection against systemic infection with HSV-1 in mice (15, 16) and to respond to acute vaginal infection with HSV-2 (12, 13). However, the role of the intraepithelial γδ T cells in intravaginal infection with HSV-2 is not yet elucidated. In this study, we examined the susceptibility of mice harboring a mutated TCRδ chain constant gene or Vδ1 gene to intravaginal infection with HSV-2.

Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were used at 5 to 6 weeks of age. In order to synchronize their estrous cycle at the progesterone-dominant stage, all mice were injected subcutaneously with 0.1 μg of β-estradiol 17-cypionate (Sigma) 5 days before infection. Mice were injected intravaginally with various doses of the 186 strain of HSV-2 (9, 20) in 20 μl of phosphate-buffered saline. In some experiments, 300 μg of anti-TCRγδ monoclonal antibody (MAb) (UC7-13D5) or isotype control hamster immunoglobulin G was administered to mice 2 days before an intravaginal challenge with HSV-2. Vaginal wash fluids from six mice were individually assayed for virus infectivity. Uterine and vaginal intraepithelial lymphocytes (r-IEL) were prepared as described previously (12, 18). For flow cytometric analysis, r-IEL were stained with biotin-conjugated anti-CD3ε MAb, fluorescein isothiocyanate-conjugated anti-TCRαβ MAb, and phycoerythrin-conjugated anti-TCRγδ MAb (PharMingen, San Diego, Calif.) and then were stained with Red-613-conjugated streptavidin (Life Technologies, Gaithersburg, Md.). The stained cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.). To determine the Vδ repertoire, total RNA was extracted from r-IEL by the acid-guanidium phenol-chloroform method. cDNA synthesis and PCR were performed by using a cDNA cycle kit (Invitrogen Corp., San Diego, Calif.). RNA was primed with 6.7 pmol of δ chain C region (Cδ) primers in 20 μl of reaction mixtures for reverse transcription. The Cδ and 5′ Vδ primers were described previously (8). PCR products (4 μl) were subjected to electrophoresis on a 1.5% agarose gel (Gibco) and transferred to a Gene Screen Plus filter (New England Nuclear, Boston, Mass.). The Southern blots of PCR products were hybridized with Jδ1 or Jδ2 oligonucleotide probes, which were labeled with [γ-^{32}P]ATP by using a Megalabel 5′-labeling kit (Takara Shuzo Co. Ltd., Kyoto, Japan) according to the manufacturer's instructions. The r-IEL were cultured with anti-CD3 MAb (145-2C11; 100 μg/ml) or anti-TCRγδ MAb (100 μg/ml) that had been immobilized on the plates by prior incubation for 1 h. To estimate cytokine production, the supernatants were collected after a 48-h culture. The cytokine activity in the cell-free culture supernatants was assayed by an enzyme-linked immunosorbent assay (ELISA) with mouse IFN-γ (Genezyme Diagnostics, Cambridge, Mass.). The $CD4^+$ T cells (more than 95% purity) were purified from spleen cells by using an autoMACS cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) and were cultured in 200 μl of complete culture medium in 96-well flat-bottom plates (Falcon; Becton Dickinson Ltd., Oxford, United Kingdom) at a density of 5×10^5 cells/well, with the same number of mitomycin-treated spleen cells from C57BL/6 mice with or without 2.5×10^4 PFU of heat-inactivated HSV-2 (56°C for 1 h). The mutant mouse (F_2 interbred from 129/Ola × C57BL/6) strain deficient in the Vδ1 gene ($V\delta1^{-/-}$) was generated by gene targeting, as described previously (4), and backcrossed with C57BL/6 mice more than eight times. The C57BL/6 background mutant mouse deficient in the TCRδ chain constant gene ($TCR\delta^{-/-}$) was kindly provided by S.

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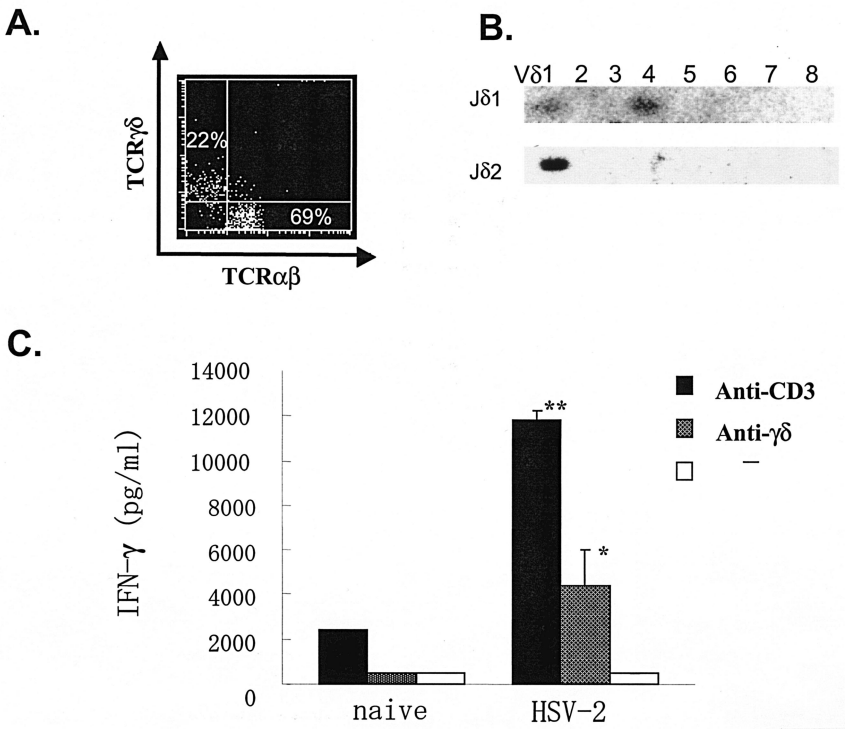

FIG. 1. γδ T cells in the uterus and vagina in C57BL/6 mice following an intravaginal infection with 250 PFU of HSV-2 strain 186. (A) Flow cytometric analysis of r-IEL on day 3 after HSV-2 infection. The r-IEL were stained with anti-CD3ε, anti-TCRγδ, and anti-TCRαβ MAbs. Cells gated on $CD3^+$ cells were analyzed for their expression of TCRαβ and TCRγδ. Representative data from three independent experiments are shown. (B) Vδ and Jδ usages of r-IEL on day 3 after HSV-2 infection. The total RNA extracted from the r-IEL was reverse transcribed into cDNA and amplified by PCR with primers for Cδ and various Vδ segments. The Southern blot of δ PCR products was hybridized with an oligonucleotide probe for Jδ1 or Jδ2. The results are representative of three independent experiments. (C) IFN-γ production by r-IEL on day 3 after HSV-2 infection. The r-IEL were cultured on plates coated with anti-TCRγδ MAb or anti-CD3 MAb for 48 h. IFN-γ levels in the supernatants were determined by ELISA. Data are means ± standard deviations (SD) for five mice in each group. There was a statistically significant difference from the value for C57BL/6 mice (*, $P < 0.05$; **, $P < 0.01$ [by Student's *t* test]). Representative data from three independent experiments are shown.

Itohara (Riken, Saitama, Japan) (6). The statistical significance of the data was determined by Student's *t* test, except for lethality data, which were analyzed by the generalized Wilcoxon's test.

Accumulation of IFN-γ-producing γδ T cells in the uterus and vagina following intravaginal HSV-2 infection. We monitored the kinetics of γδ T cells in the uterus and vagina following an intravaginal inoculation with HSV-2. The absolute numbers of γδ T cells in the uterus and vagina increased to a peak on day 3 in C57BL/6 mice after HSV-2 infection (Fig. 1A) ($1.5 \times 10^4 \pm 0.2 \times 10^4$ cells before infection versus $7.2 \times 10^4 \pm 0.6 \times 10^4$ cells after infection; $P < 0.01$). Reverse transcription-PCR analysis showed that the γδ T cells on day 3 preferentially used the Vδ1-rearranged Jδ1 gene, similar to those used by the γδ T cells in noninfected control mice (Fig. 1B). We also found Vδ4-rearranged Jδ1, which has been reported for accumulations in the uterus and vagina after infection (13). Early production of IFN-γ has an important role in determining whether naive $CD4^+$ T cells will differentiate into Th1 cells (2, 18). We next examined the IFN-γ production by γδ T cells accumulated in the uterus and vagina following HSV-2 infection. IFN-γ production by the γδ T cells in the uterus and vagina was significantly augmented on day 3 in response to immobilized anti-CD3 MAb or anti-TCRγδ MAb (Fig. 1C). There was no interleukin-4 production in response to TCR stimulation (data not shown). Thus, the number of γδ T cells with Vδ1 and with a high ability to produce IFN-γ increased in the uterus and vagina following HSV-2 infection.

Susceptibility of mice depleted of γδ T cells to intravaginal infection with HSV-2. To define the roles of γδ T cells in protection against primary intravaginal infection with HSV-2, we examined the effects of in vivo depletion of γδ T cells by anti-TCRγδ MAb on the survival rate of mice after intravaginal inoculation with HSV-2 strain 186. We confirmed that the numbers of γδ T cells were severely reduced in uterine IEL and spleen cells on day 7 after HSV-2 infection when 300 μg of anti-TCRγδ MAb was administered 3 days prior to infection (Fig. 2; data not shown). As shown in Fig. 2A, all mice given anti-TCRγδ MAb died by day 16, and 60% of the mice given control MAb survived beyond 18 days ($P < 0.01$ by the generalized Wilcoxon's test). We compared virus titers in the organs of control mice and mice given anti-TCRγδ MAb on day 7 after infection with HSV-2 wild-type strain 186 (5×10^3 PFU), at which time mice began to die. As shown in Fig. 2C, in mice given anti-TCRγδ MAb, larger amounts of infectious virus were detectable in the vaginal wash fluids of mice given anti-TCRγδ MAb ($P < 0.01$). Thus, these results indicated that mice given anti-TCRγδ MAb were susceptible to HSV-2 infection. To compare the HSV-2-specific Th1 cell responses of control mice and mice depleted of γδ T cells after an intravag-

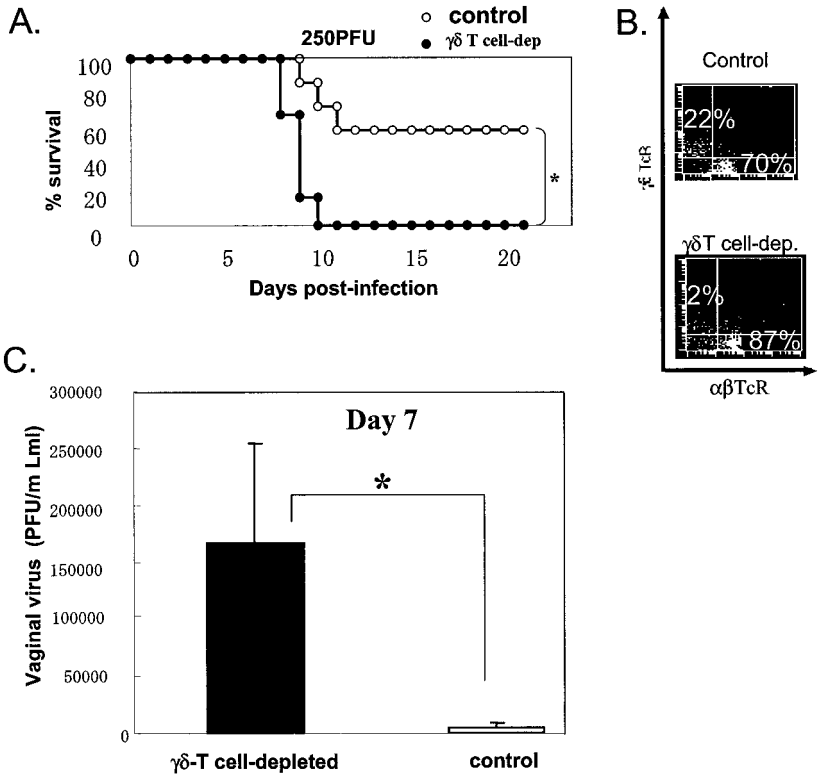

FIG. 2. Susceptibility of mice depleted of γδ T cells to intravaginal infection with HSV-2 strain 186. (A) Survival rates of C57BL/6 mice (20 mice in each group) that were injected intraperitoneally with 300 μg of anti-TCRγδ MAb (γδ T cell-dep) or control hamster immunoglobulin G (control) 3 days before an intravaginal inoculation with 250 PFU of HSV-2 strain 186 and were monitored for survival. Representative data from three independent experiments are shown. *, statistically significant difference from control group ($P < 0.01$ by the generalized Wilcoxon's test). (B) Flow cytometric analysis of r-IEL from mice depleted of γδ T cells is shown. r-IEL were collected on day 3 after infection with 250 PFU of HSV-2 strain 186 and were stained for expression of TCRαβ, TCRγδ MAb, and CD3. The dot plot analysis is gated on CD3-positive cells and presented as typical two-dimensional profiles. Numbers represent the percentages of total cells found in each quadrant. (C) Viral titers in vaginal wash fluids of mice depleted of γδ T cells on day 7 after infection with 5,000 PFU of HSV-2 strain 186. Data are means ± SD of five mice in each group. Representative data from three independent experiments are shown. *, statistically significant difference from control group ($P < 0.01$).

inal infection, we examined IFN-γ production by spleen $CD4^+$ T cells in response to heat-inactivated HSV-2 on day 7 after an intravaginal infection with HSV-2. As shown in Fig. 3, IFN-γ production by $CD4^+$ T cells from mice depleted of γδ T cells was significantly lower than that in control mice ($P < 0.05$).

Susceptibility of $V\delta1^{-/-}$ mice to intravaginal infection with HSV-2. We next examined the survival rates of $TCR\delta^{-/-}$ mice or $V\delta1^{-/-}$ mice after an intravaginal infection with HSV-2. As shown in Fig. 4, all of the $TCR\delta^{-/-}$ mice and 90% of the $V\delta1^{-/-}$ mice died by 11 days, while 40% of their littermates survived beyond 18 days ($P < 0.05$ by the generalized Wilcoxon's test). Thus, mice deficient in whole γδ T cells and those deficient in Vδ1 cells were equally susceptible to an intravaginal infection with HSV-2.

Protective mechanisms against HSV infection are mediated by two major waves of host responses. The first one, innate immunity, depends mainly on the phagocyte system and NK and NKT cells (1). The second mechanism, acquired immunity, depends on the immune response mediated by T-helper type 1 cells of $CD4^+$ αβ T cells that are indispensable in protection against systemic HSV infection (6, 14). We also confirmed that mice depleted of TCRαβ T cells by in vivo treatment with anti-TCRαβ MAb and $TCR\beta^{-/-}$ mice are highly susceptible

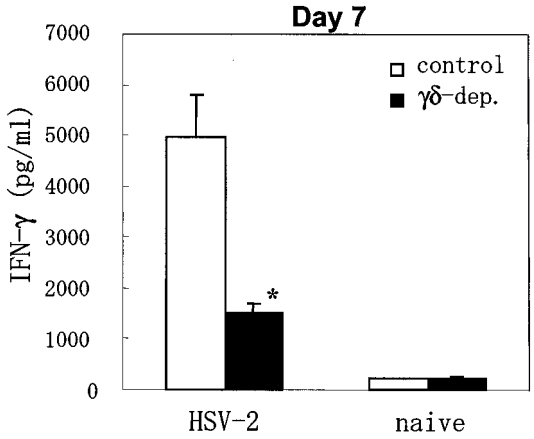

FIG. 3. IFN-γ production by spleen $CD4^+$ T cells of mice depleted of γδ T cells in response to HSV-2. $CD4^+$ T cells were obtained from the spleens of γδ T-cell-depleted (γδ-dep.) mice or control mice on day 7 after an intravaginal challenge with 250 PFU of HSV-2 strain 186. The $CD4^+$ T cells were cultured in the presence of mitomycin C-treated spleen cells with or without heat-inactivated HSV-2. IFN-γ levels in the supernatants were determined by ELISA. Data are means ± SD of five mice in each group. There was a statistically significant difference from the value for the control in response to HSV-2 antigen. (*, $P < 0.05$ by Student's t test). Representative data from three independent experiments are shown.

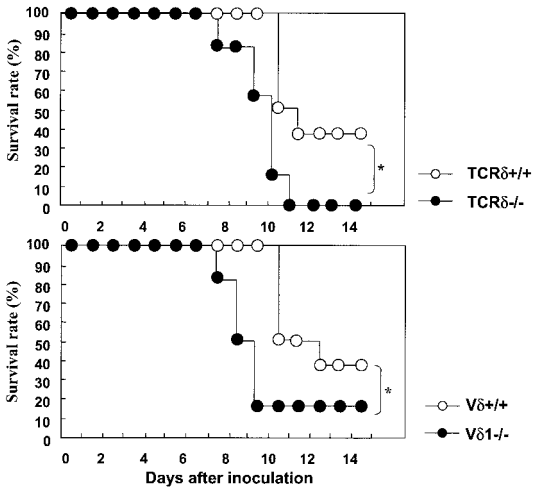

FIG. 4. Survival rates of mice deficient in whole γδ T cells or the Vδ1 subset after intravaginal infection with HSV-2 strain 186. TCRδ$^{-/-}$ or TCRδ$^{+/+}$ mice (20 mice in each group) and Vδ1$^{-/-}$ or Vδ1$^{+/+}$ mice (10 mice in each group) were inoculated with 250 PFU of HSV-2 strain 186 and were monitored for survival. *, statistically significant difference from littermates for each group ($P < 0.05$ by the generalized Wilcoxon's test).

to an intravaginal infection with HSV-2 (unpublished data). Early production of IFN-γ has an important role in determining whether naive CD4$^+$ T cells will differentiate into Th1 cells (2, 19). The results of the present study showed that epithelial γδ T cells in the uterus and vagina are activated to produce IFN-γ during the course of HSV-2 infection and that mice deficient in the intraepithelial γδ T cells bearing Vδ1 showed a high susceptibility to the infection. Furthermore, mice depleted of γδ T cells showed an impaired CD4$^+$ Th1 response following HSV-2 infection. Thus, these results suggest that the intraepithelial γδ T cells may not only function in the front lines of host defense but also may help protect against HSV-2 infection by promoting a systemic CD4$^+$ Th1 response to HSV-2.

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