The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 15 2005

Neurokinin 1 Receptor Signaling Affects the Local Innate Immune Defense against Genital Herpes Virus Infection[1] FREE

Alexandra Svensson; ... et. al

J Immunol (2005) 175 (10): 6802–6811.
https://doi.org/10.4049/jimmunol.175.10.6802

Neurokinin 1 Receptor Signaling Affects the Local Innate Immune Defense against Genital Herpes Virus Infection[1]

Alexandra Svensson,[2]* Joanna Kaim,[2†] Carina Mallard,[‡] Annika Olsson,[§] Ernst Brodin,[§] Tomas Hökfelt,[¶] and Kristina Eriksson[3]*

We show that genital infection with neurotropic HSV type 2 (HSV-2) induced a significant increase of the neuropeptide substance P (SP) within the genital tract of mice. SP was shown to weakly interfere with the HSV-2 replication. Furthermore, lack of SP signaling through the use of mice deficient in the SP receptor, neurokinin 1 receptor (NK1R), revealed an important role for SP in the innate defense against HSV-2. NK1R-deficient mice had significantly enhanced levels of HSV-2 in the genital tract and in the CNS following infection and a significantly accelerated disease progression, which was associated with an impaired NK cell activity locally in the vagina. Lack of NK1R signaling did, however, not impair the animals' ability to mount a protective immune response to HSV-2 following vaccination with an attenuated virus. Both $NK1R^{+/+}$ and $NK1R^{-/-}$ mice developed strong HSV-2-specific Th1 T cell responses following vaccination. No genital viral replication was observed in either vaccinated NK1R-deficient or $NK1R^{+/+}$ control animals following a genital HSV-2 challenge, and all of these animals survived without any symptoms of disease. In conclusion, the present results indicate that SP and NK1R signaling contributes to the innate resistance against HSV-2 infection in mice. *The Journal of Immunology,* **2005, 175: 6802–6811.**

Herpes simplex virus can use the CNS as a hiding place where it remains dormant for extended periods of time. Following mucosal transmission and replication in epithelial cells, the virus enters local sensory nerve endings and spreads through retrograde transport to the nearest ganglia where latency is established. Latent virus can be reactivated following, e.g., stress or immune suppression, and is then transported in the sensory nerves back to the mucosal epithelium where replication can occur.

HSV type 2 (HSV-2)[4] is one of the most common sexually transmitted viral diseases with an estimated prevalence of up to 50% among the female population worldwide (1). HSV-2 infection gives rise to genital lesions and ulcers and can, in rare cases, spread to the spinal cord and meninges, causing encephalitis. In the majority of infected individuals, the virus establishes latency in the sacral ganglia, where it can be reactivated at infrequent intervals causing recurrent genital disease.

Substance P (SP) is an 11-aa neuropeptide belonging to the family of tachykinins, also called neurokinins (2). SP is abundantly expressed within the CNS and in peripheral neurons, including those innervating the female genital tract. In rodents, SP-containing nerves are present in the vagina, uterine cervix, uterine horn, and oviduct (3–5).

SP is a potent neuroimmunoregulator. It is produced not only by neuronal cells but also by endocrine cells and cells belonging to the immune system (6–8). Most immune cells, including lymphocytes, macrophages, and polymorphonuclear leukocytes, express the neurokinin 1 receptor (NK1R) and thus respond to SP. Interactions between SP and NK1R on mononuclear cells induce the secretion of both inflammatory cytokines and cytokines that regulate the direction of the adaptive immune response. SP stimulates human monocytes to produce cytokines such as IL-1, IL-6, IL-10, IL-12, and TNF-α (9–12) and T cells to secrete IL-2, IFN-γ, IL-4, and IL-10 (13). One consequence of the SP-induced cytokine secretion is a block in Th1 and Th2 polarization (13), implying that the quality of the ensuing acquired immune response might be altered by SP. SP has also been shown to influence the function of lymphocytes. It affects the killing potential of both NK cells and cytotoxic T cells and also influences the migratory potential of these cells (14–17).

SP synthesis can be induced by several viruses and can have effects on both the virus replication and on the immune response to the virus. In humans, HIV infection enhances SP expression in immune cells (18) and enhances HIV-1 replication in latently infected human immune cells (19). In rodents, respiratory syncytial virus infection induces an enhanced production of pulmonary SP (20) and an increased expression of SP receptors in lung tissue (21) and on bronchoalveolar monocytes and T cells (22). Expression of SP and its receptor are also increased in mucosal and peripheral lymphoid organs after oral infection with murine gamma-herpesvirus 68 (17). Absence of the NK1R, thus abrogating SP signaling, is associated with a reduced CTL response against gamma-herpesvirus 68 and a lowered expression of IL-12 during the viral infection, leading to viral persistence within the infected animal (17).

In this study, we have investigated whether HSV-2 infection can induce SP production within the female genital tract and also whether the absence of SP signaling would impact on the innate and acquired immune control of viral replication. For this purpose,

*Department of Rheumatology and Inflammation Research, †Department of Medical Microbiology and Immunology, and ‡Department of Physiology, Göteborg University, Göteborg, Sweden; and §Department of Physiology and Pharmacology and ¶Department of Neuroscience, Karoliska Institutet, Stockholm, Sweden

Received for publication July 22, 2004. Accepted for publication August 30, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These studies were supported by the Swedish Science Council of Medicine (Contracts 2887, 6836, and 15341), Läkatutbildningsavtalet/Avtalet om Läkarnas Fortbildning, and the Marianne and Marcus Wallenberg Foundation.

[2] A.S. and J.K. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Kristina Eriksson, Department of Rheumatology and Inflammation Research, Guldhedsgatan 10A, 413 46 Göteborg, Sweden. E-mail address: kristina.eriksson@microbio.gu.se

[4] Abbreviations used in this paper: HSV-2, HSV type 2; SP, substance P; NK1R, neurokinin 1 receptor; TK, thymidine kinase; RT, room temperature; TSA, tyramide signal amplification.

0022-1767/05/$02.00

we used a well-characterized mouse model of genital HSV-2 infection (23–26). In this model, HSV-2 deposited on the female vaginal mucosa was replicated for a few days in the vaginal epithelium, which was subsequently destroyed (23, 24). The virus also ascends the sensory neurons and can be retrieved from dorsal root ganglia 5–6 days after infection (23). There is a zosteriform spread of HSV-2 from the infected ganglia to the external area surrounding the vagina concomitant with a spread to the spinal cord (23, 24). The viral infection can thus be assessed through several parameters: 1) viral replication in the vagina during the first few days of infection; 2) viral replication in the ganglia from day 5 onward and in the spinal cord from day 7 onward; 3) ulceration in the skin surrounding the vaginal entrance from day 5 onward; and 4) neurological complications from day 7 onward, which ultimately leads to mortality. An important consideration with this mouse model is that mice, with age, become more and more resistant to genital HSV-2 infection (23). This age-dependent resistance can, however, be overcome by treating the animals with progesterone (24). It is also possible to vaccinate the mice with an attenuated strain of HSV-2 lacking the gene for thymidine kinase (TK). The attenuated virus replicates in the vaginal epithelium, which leads to sterilizing immunity in C57BL/6 mice (25, 26), but the virus is unable to replicate in the ganglia and can thus neither cause zosteriform spread nor CNS complications (23).

Materials and Methods

Mice

Female mice, 6–8 wk of age, were used for all experiments. C57BL/6 mice ($NK1R^{+/+}$ mice) were purchased from B&K Universal. $NK1R^{-/-}$ mice (27), which lack the receptor for SP and that had been backcrossed for >10 generations to a C57BL/6 background, were obtained from Dr. N. P. Gerard (Harvard Medical School, Boston, MA). The animals were kept in ventilated cages under specific pathogen-free conditions at the Department of Experimental Biomedicine at the Göteborg University. The studies were approved by the Ethical Committee for Animal Experimentation, Göteborg, Sweden.

Viruses and plaque assay

The attenuated mutant of HSV-2 strain Lyon, which contains a partial deletion of the TK gene, was provided by Dr. E. De-Clercq (Rega Institute for Medical Research, K.U. Leuven, Belgium) (28). HSV-2 (TK) strain Lyon and HSV-2 strain 333 (29) were grown and titrated in African green monkey kidney cells (GMK-AH1) and prepared by one cycle of freeze-thaw and subsequent removal of cellular debris by centrifugation. Both strains of virus have been used previously in similar studies (25, 26). To assess the impact of SP on HSV-2 replication in vitro, 500 PFU of HSV-2 was incubated for 15 min on ice, either alone or along with SP at 10^{-6} M, before adding the virus-SP solution to confluent GMK-AH1 cells in six-well plates in medium containing 1% methylcellulose. The cells were incubated for 3 days at 37°C, and the plaques were stained with crystal violet solution. Plaques were evaluated using a light microscope at ×4 magnification.

HSV-2 infection

Mice were injected s.c. with 2.0 mg of medroxyprogesterone (Depo-Provera; Upjohn Puurs-Belgium) in 100 μl of PBS. Six days later, the mice were infected by an intravaginal inoculation of 4×10^4 PFU (100 LD_{50}) of HSV-2 strain 333 in 20 μl of HBSS. This dose has been carefully titrated to achieve disease development in at least 90% of inoculated C57BL/6 mice (25). In some experiments, the animals were vaccinated 4 wk before the HSV-2 challenge with 3.6×10^6 PFU of attenuated HSV-2 (TK) given vaginally in 20 μl of HBSS.

Monitoring of infection

Evaluation of HSV-2-positive cells in the vagina and the brainstem. HSV-2-infected mice were anesthetized with Pentothal (20 mg) and perfused intracardially with 0.9% NaCl followed by 5% buffered formalin (pH 7.4; Histolab). The vaginas and the brainstems were dissected out and fixed for 24 h in 5% formalin and then stored in 70% ethanol. The tissues were dehydrated and embedded in paraffin and cut into 5-μm sections on a microtome. The sections were burned in 60°C for 30 min, rehydrated in xylene followed by ethanol, and boiled in 0.1 M citrate buffer (pH 6.0) for 10 min. Sections were blocked with goat serum (1/10 dilution) and then incubated with a mouse monoclonal IgG1 Ab specific for HSV-2 mgG-2 (01.C5.B2, 1/50 dilution of culture supernatant, gift from J.-Å. Liljeqvist, Göteborg University, Göteborg, Sweden) followed by biotinylated goat anti-mouse IgG1 (1/250; Southern Biotechnology Associates). Endogenous peroxidase was quenched with 0.6% H_2O_2 in methanol before addition of avidin-biotin enzyme complex (ABC Elite; Vector Laboratories). Slides were developed with 0.5 mg/ml 3,3-diaminobenzidine in 15 mg/ml ammonium nickel sulfate, 2 mg/ml β-D-glucose, 0.4 mg/ml ammonium chloride, and 0.01 mg/ml glucose oxidase dissolved in 0.1 M sodium acetate buffer (pH 6.0). The stained sections were dehydrated in graded series of ethanol followed by xylene, air-dried, and mounted with Mountex (Histolab Products).

Viral replication. Forty-eight hours following intravaginal HSV-2 infection, vaginal washings were performed as described previously (25) and stored at −70°C. Eight days after infection, the animals were killed and the spinal cord was excised, and then homogenized in 1 ml of PBS. HSV-2 titers in vaginal washings and in spinal cord homogenates were determined by plaque assay on GMK-AH1 cell monolayers.

Microglia activation. Resident microglia bearing galactose-containing glycoconjugates, but not other glial cells, were stained by B4 isolectin from *Griffonia simplicifolia* seeds and HRP. Following insults to the brain, microglia cells become activated and change their morphology to an amoeboid form. To detect such activated microglia, 5-μm sections from brainstems were prepared as described above and burned in 60°C for 30 min, deparaffinized, and then boiled for 10 min in 0.1 M citrate buffer (pH 6.0), and then rinsed in PBS. The sections were then incubated for 2 h at room temperature (RT) with 15 μg/ml HRP-labeled isolectin B_4 from *G. simplicifolia* (Sigma-Aldrich) and 0.2% Triton X-100 in PBS as described previously (30) and rinsed with PBS and then with 0.1 M NaAc (pH 6.0) before being developed with diaminobenzidine as described above.

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 0, healthy; 1, genital erythema; 2, mild to moderate genital inflammation; 3, genital lesions; 4, hind limb paralysis; and 5, death or sacrifice due to paralysis, as previously described (25).

SP determinations

Radioimmunoassay for SP. HSV-2-infected mice were anesthetized with Pentothal (20 mg) and perfused intracardially with 0.9% NaCl. The vaginas were dissected out, weighted, and stored at −70°C. SP was extracted as described elsewhere (31). Briefly, vaginas were boiled for 15 min in 4 ml of 0.5 M HAc, immediately cooled to 4°C, homogenized, and centrifuged at 4°C for 15 min at 3000 × *g*. Supernatants were collected and freeze-dried. Before analysis, the lyophilized tissue was dissolved in 1 ml of 0.15 M NaCl containing 0.5% BSA. 0.1 ml of tissue extract, or 0.1 ml of peptide standard (catalog no.7451, dissolved in 0.15 M NaCl containing 0.5% BSA; Peninsula Laboratories,), was incubated with 0.5 ml of ^{125}I-(Tyr^8)-SP (1000–1100 cpm in 0.02 M barbital buffer (pH 8.6) containing 0.8% BSA) and 0.5 ml of diluted antiserum (in 0.02 M barbital buffer (pH 8.6) containing 0.8% BSA). The antiserum used, SP2 (32), is highly specific and directed against the C-terminal of SP (i.e., the portion of the molecule responsible for most biological actions of SP). The final dilution of antiserum had been adjusted to produce 30–35% binding of radioligand. Following 72 h of incubation at 4°C, free and bound tracer were separated by adding 0.25 ml of decanting suspension (sheep anti-rabbit IgG coupled to Sepharose; Pharmacia Biotech) to each tube, followed by a 30-min incubation at 4°C and centrifugation (1560 × *g*, 10 min, 4°C). The supernatant was decanted, and the radioactivity in the bound fraction was counted for 10 min in a gamma counter. The smallest detectable amount of SP (antiserum SP2) was 3 fmol per assay tube.

Immunohistochemistry for SP. HSV-2-infected mice were anesthetized with Pentothal (20 mg) and perfused intracardially with 0.9% NaCl followed by 4% paraformaldehyde plus 0.2% picric acid in phosphate buffer (pH 6.9). After perfusion, vaginas were removed and immersed for 2 h in the same ice-cold fixative. The tissues were then rinsed for 48 h in ice-cold 0.1 M Sörensen's buffer containing 10% sucrose. After rinsing, the vaginas were quickly frozen in CO_2, and 14-μm-thick sections of vagina were cut in a cryostat (Microm) and processed for the highly sensitive tyramide signal amplification (TSA) method (33). Sections were rinsed in 0.01 M PBS, preincubated with 0.01% H_2O_2 (to reduce endogenous peroxidase activity) for 5 min, and rehydrated in PBS for 15 min. Sections were incubated overnight at 4°C with rabbit anti-rat antiserum to SP (34), diluted

1/8000 in PBS containing 0.3% Triton X-100 (Sigma-Aldrich), 0.02% Bacitracin (Sigma-Aldrich), and 0.01% sodium azide (Sigma-Aldrich). Briefly, the sections were then processed for the TSA method using a commercial TSA-Plus kit (NEN). After the incubation with primary rabbit anti-SP antiserum, sections were rinsed in Tris-NaCl-Tween 20 (TNT) buffer for 15 min and blocked in Tris-NaCl-blocking reagent (kit) buffer for 30 min at RT. The sections were then incubated with HRP-labeled porcine anti-rabbit Igs (DakoCytomation) diluted 1/200 in Tris-NaCl blocking reagent (kit) for 30 min at RT, and rinsed in TNT (kit) buffer for 15 min at RT. Finally, slides were incubated with fluorophore tyramide diluted 1/100 in amplification reagent (kit) for 10 min at RT, rinsed in TNT buffer for 15 min at RT, and mounted for fluorescence microscopy in 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) diluted in glycerol. For the control of specificity of immune staining, preadsorption of SP antiserum with SP (10^{-6} and 10^{-5} M; Bachem) was performed. This procedure abolished all of the staining patterns described below. Sections were analyzed using a Nikon Eclipse E600 microscope equipped with epifluorescence with appropriate filter. Kodak T-MAX 400 black and white film was used for photography and then scanned using a Nikon LS-2000 film scanner (Nikon). Scanned and digital images were imported into Adobe PhotoShop 6.0 (Adobe Systems) and optimized for brightness, contrast, and sharpness.

Cytokine measurements

The concentrations of the chemokines were determined in the vagina at various intervals after viral challenge using a modified version of a perfusion-extraction method combined with ELISA as previously described (35). Briefly, mice were sacrificed and the vaginas were excised and weighed before storage at −70°C in 300 μl of a PBS solution containing 2 mM PMSF, 0.1 mg/ml trypsin inhibitor from soybean (Sigma-Aldrich), and 0.05 M EDTA. The vaginal samples were thawed and then permeabilized with saponin (Sigma-Aldrich) at a final concentration of 2% (w/v) in PBS at 4°C overnight. The tissue samples were then centrifuged at 16,000 × *g* for 5 min, and the supernatants were analyzed for the concentrations of MIP-1α, IL-12, IL-18, and IFN-γ using ELISA (Duoset ELISAs; R&D Systems). Spleen cell suspensions from naive $NK1R^{+/+}$ and $NK1R^{-/-}$ mice were also analyzed for their production of cytokines in response to activation with live HSV-2. For this purpose, spleen cells (5×10^5 cells/well) were exposed to graded amounts of HSV-2 strain 333 for 48 h. Cell supernatants were collected, frozen at −70°C, and analyzed for cytokine content as above.

NK cell cytotoxicity assay

Spleen cell suspensions were obtained from naive $NK1R^{+/+}$ and $NK1R^{-/-}$ mice. Erythrocytes were removed by osmotic killing and the remaining mononuclear cells were incubated in 24-well plates in IMEM containing L-glutamine, 50 μM 2-ME, gentamicin, 10% FCS (complete IMEM), and 150 ng/ml recombinant murine IL-2 (R&D Systems). After 48 h, the IL-2-activated effector NK cells were retrieved and seeded in triplicates at different cell densities in round-bottom 96-well plates along with 10^4 $Na^{51}CrO_4$-labeled YAC-1 target cells, in a total of 200 μl of complete IMEM. In some experiments, SP at different concentrations was added to the cultures. The plates were incubated for 8 h at 37°C. Supernatants were collected by a tissue collecting system (Amersham Biosciences) and assayed for radioactivity in a gamma counter. Maximum ^{51}Cr release was determined in target cell cultures treated with Triton X-100. T cell cytotoxicity was calculated as percent cell killing according to the following formula: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)] = percent specific killing.

Vaginal cells were excised from $NK1R^{+/+}$ and $NK1R^{-/-}$ mice 48 h after vaginal HSV-2 infection. The tissues were cut into small pieces and single-cell suspensions were obtained by two 45-min incubations in 1 mg/ml collgenase/dispase (Roche) in complete IMEM at 37°C under constant stirring. The cells were immediately analyzed for cytotoxic killing of $Na^{51}CrO_4$-labeled YAC-1 target cells as described above. Because of the limited cell yield, the vaginal cells were only assayed at an E:T ratio of 35:1.

IFN-γ measurements

Measurement of IFN-γ production from $CD4^+$ and $CD8^+$ spleen cells was performed using a CellELISA using the Abs from the murine IFN-γ duoset ELISA (catalog no. DY485; R&D Systems). $CD4^+$ T cell activation was performed with CD8-depleted spleen cell cultures in the presence of inactivated whole virus. Depletion of $CD8^+$ T cells was done by incubating the cells in complete IMEM with 5 μg/ml mouse anti-mouse CD8 Ab (catalog no. 553027; BD Pharmingen) for 45 min at 4°C. This was followed by anti-mouse magnetic beads (Dynal Biotech) for 30 min at 4°C and then separation on a magnet. $CD8^+$ T cells activation was analyzed in whole spleen cell cultures using the MHC I-restricted peptide SSIEFARL.

Flat-bottom Grainer 96F microwell plates (Labora Chemicon) were coated with 50 μl of anti-mouse IFN-γ Ab diluted 1/180 in PBS and incubated in 4°C overnight. Plates were blocked with 150 μl of BSA in PBS for 1 h at 37°C. For $CD8^+$ T cell analysis, mononuclear spleen cell suspensions were seeded in duplicate at different cell densities in complete IMEM containing 10 μg/ml SSIEFARL peptide. Spleen cells depleted of $CD8^+$ T cells were seeded in duplicate at different cell densities in complete IMEM containing UV-inactivated HSV-2 Ag diluted 1/50 corresponding to 2×10^7 PFU/ml. Plates were then incubated at 37°C for 24 h. Plates were washed in 0.05% Tween 20 and biotinylated anti-mouse IFN-γ was added (1 μg/ml) and incubated at 4°C overnight. This was followed by a 45-min incubation with peroxidase-labeled avidin (Sigma-Aldrich) (diluted 1/200 in 0.5% BSA in PBS) at RT. Plates were developed for 20 min using 50 μl of 0.1 mg/ml tetramethylbenzidine in 0.05% phosphate-citrate buffer (pH 5.0) and 0.04% H_2O_2. Development was stopped with 25 μl of 1 M H_2SO_4, and absorbance was measured at 450 nm. The concentration of IFN-γ was determined by extrapolation from a standard curve obtained using recombinant IFN-γ. The sensitivity of the assay was 50 pg/ml. Results are expressed as the concentration of IFN-γ secreted per 1×10^6 spleen cells.

FACS analysis

The number of SSIEFARL-specific $CD8^+$ T cells was determined in vaccinated $NK1R^{-/-}$ and $NK1R^{+/+}$ mice. Spleen cells were stained with FITC-labeled anti-mouse-CD8 Ab (BD Biosciences), diluted 1/100, and PE-labeled SSIEFARL pentamer (ProImmune) used undiluted. Cells were stained for 30 min in 4°C and then washed two times in PBS with 1% FCS. To determine the frequency of NK cells in vaginal cell suspensions, the cells were stained with a PerCP-Cy5.5-labeled anti-NK1.1-specific Ab (BD Biosciences).

Statistical analysis

Statistical analyses were done by Student's *t* test.

Results

Detection of virus-infected cells in the genital tract and CNS of HSV-2-infected mice

The presence and distribution of HSV-2-infected cells in the vagina was examined daily during the first 7 days of HSV-2 infection. HSV-2-infected vaginal epithelial cells were detected days 1–3 after viral inoculation. Virus was present most abundantly day 1 after infection. During days 2 and 3, the infected epithelium was gradually destroyed, and no HSV-2- infected cells remained from day 4 onward (Fig. 1).

We also examined microglia activation and the presence of virus in the brainstem on days 0, 3, 6, and 9 after genital HSV-2 infection. Low expression of microglia was detected on days 0 (Fig. 2*A*) and 3 (Fig. 2*B*) after infection, which represent resident, nonactivated cells. Low levels of activated microglia were detected by day 6 (Fig. 2*C*), and both the numbers of activated microglia and the degree of activation of each individual microglia (evident as a more rounded appearance) were increased by day 9 (Fig. 2*D*). We could not detect any HSV-2-infected cells in the brainstem on days 0–6 (Fig. 2*E* and data not shown), whereas high numbers of HSV-2-infected cells were evident on day 9 (Fig. 2*F*).

Increased levels of SP in the genital tract of HSV-2-infected mice

The levels of SP were investigated in vaginal tissue extracts obtained during the first 3 days of HSV-2 infection, when virus-infected cells could be detected within the vaginal epithelium (see Fig. 1). The levels of SP tended to increase already 1 and 2 days after genital HSV-2 infection, and the levels of SP were significantly elevated by day 3 after infection (Fig. 3). To assess the distribution of SP in the vagina, we performed immunohistochemistry staining of vaginas obtained before or 3 days after genital HSV-2 infection. Numerous SP-immunoreactive axons and small

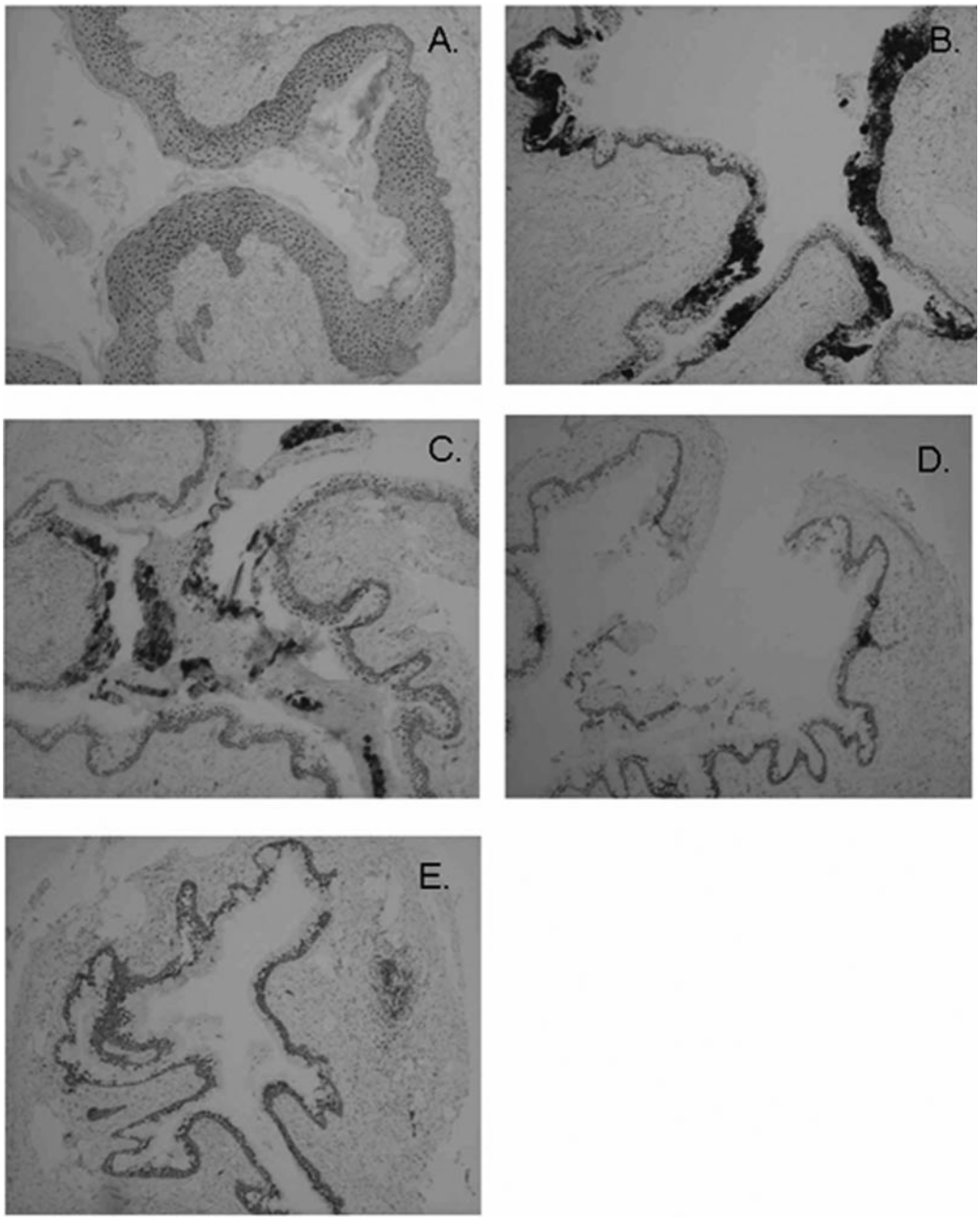

FIGURE 1. Detection of virus-infected cells in the genital tract of HSV-2-infected mice. HSV-2-infected cells in the genital tract of C57BL/6 mice were examined by immunohistochemistry either before infection (*A*) or day 1 (*B*), day 2 (*C*), day 3 (*D*), and day 4 (*E*) after infection (original magnification, ×10).

nerve bundles were observed in the wall of vaginas of normal mice (Fig. 4*A*). Similar results were seen after infection with HSV-2 (Fig. 4*B*). No certain difference with regard to the number of fibers or fluorescence intensity was seen between controls and HSV-2-infected vaginas.

SP reduces HSV-2 replication in vitro

To assess whether SP has a direct ability to interfere with HSV-2 replication, we added SP to GMK-AH1 cells at the time of in vitro HSV-2 infection. We found that SP reduced the in vitro plaque formation (Fig. 3*B*) without affecting the viability of the GMK cells. The reduction observed was modest but statistically significant and implies that SP has a weak antiviral effect.

Enhanced viral replication in HSV-2-infected mice lacking NK1R, the receptor for SP

Next, we investigated whether lack of NK1 receptors would impact on local vaginal HSV-2 replication. For this purpose, $NK1R^{+/+}$ and $NK1R^{-/-}$ mice were infected with HSV-2 vaginally and the levels of virus were examined in genital secretions obtained 1, 2, and 4 days after infection (Fig. 5*A*). The viral titers peaked 24 h after infection in both strains of mice and then gradually decreased with time. Even though the virus initially replicated to the same extent in $NK1R^{+/+}$ and $NK1R^{-/-}$ mice, the $NK1R^{-/-}$ mice had a significantly impaired ability to clear the virus. Thus, the levels of infectious virus retrieved in vaginal washings from $NK1R^{+/+}$ and $NK1R^{-/-}$ mice were identical at

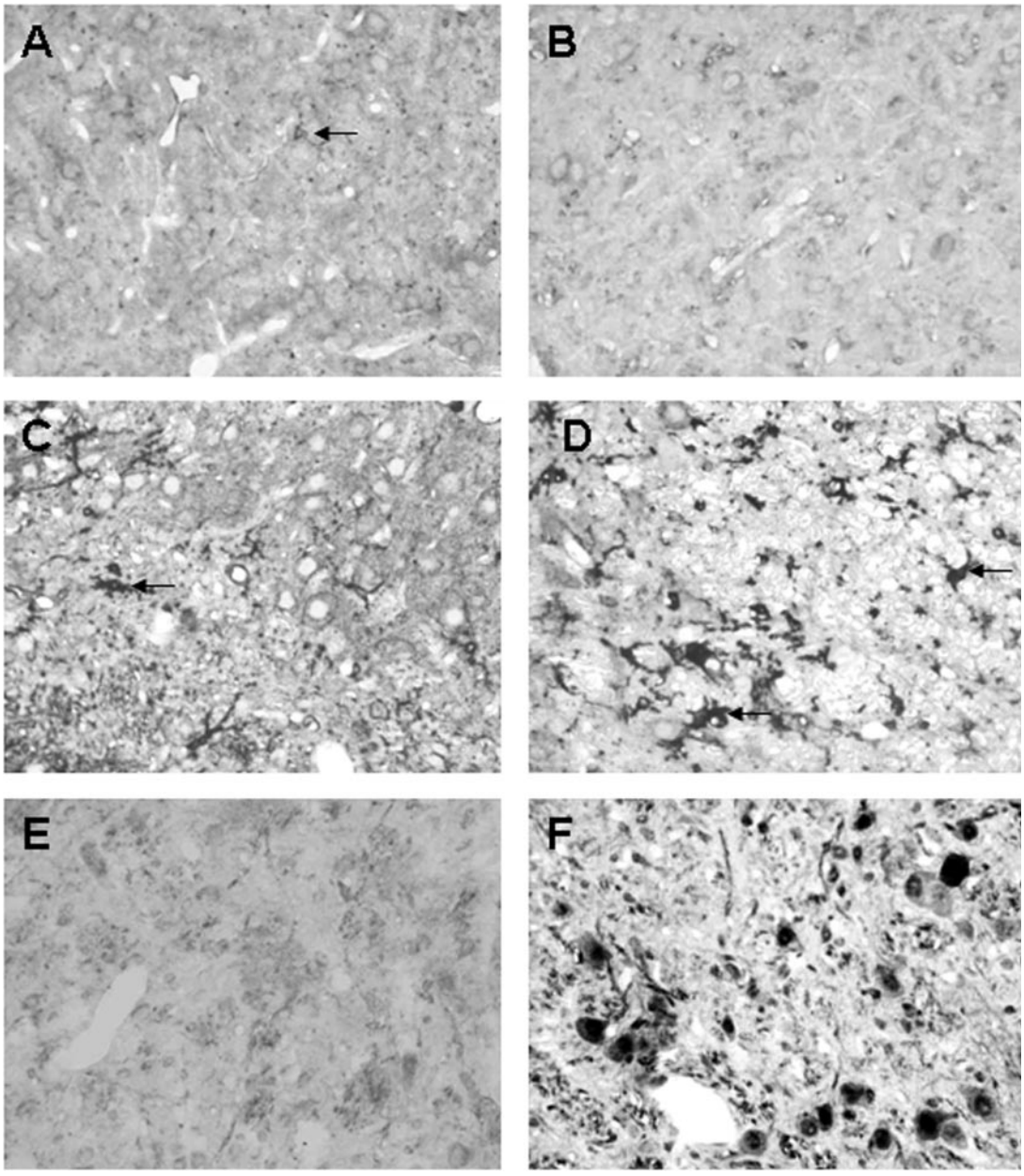

FIGURE 2. CNS engagement following genital HSV-2-infection. Microglia activation in the brainstem of C57BL/6 mice was detected by immunohistochemistry either before infection (*A*) or day 3 (*B*), day 6 (*C*), and day 9 (*D*) after infection. Arrows indicate microglia cells. HSV-2-infected cells were detected by immunohistochemistry either before infection (*E*) or day 9 after infection (*F*) (original magnification, ×40).

24 h after infection. At 48 h after infection, $NK1R^{-/-}$ mice had an 11-fold increase in infectious virus in the genital secretions compared with $NK1R^{+/+}$ mice, and at 4 days after infection this increase remained (3-fold increase; Fig. 5*A*). Similarly, when we analyzed the levels of HSV-2 in the CNS 8 days after infection, we found that $NK1R^{-/-}$ mice had a 3-fold increase in infectious virus in homogenates of the spinal cord compared with $NK1R^{+/+}$ mice (Fig. 5*B*). Furthermore, $NK1R^{-/-}$ mice developed symptoms of disease significantly faster than the $NK1R^{+/+}$ controls (Fig. 5*C*).

Increased levels of MIP-1α in the genital tract of HSV-2-infected $NK1R^{-/-}$ mice

We compared the local accumulation of IFN-γ, IL-12, IL-18, and MIP-1α in genital tissue extracts from $NK1R^{-/-}$ and $NK1R^{+/+}$ mice during the first 48 h of HSV-2 infection. Even though the levels of MIP-1α were significantly increased in both $NK1R^{+/+}$ and $NK1R^{-/-}$ mice at 24 and 48 h after infection ($p < 0.05$), these increases were significantly more pronounced in the HSV-2-infected $NK1R^{-/-}$ mice ($p < 0.05$; Fig. 6*A*). The levels of IL-18 increased 48 h after infection in both $NK1R^{-/-}$ and $NK1R^{+/+}$ animals (Fig. 6*B*). The levels of IFN-γ and IL-12 remained below the detection limits throughout the time period examined (data not shown). To determine whether the differences in MIP-1α responses in $NK1R^{+/+}$ and $NK1R^{-/-}$ mice were due to an intrinsic difference in MIP-1α responsiveness or rather a reflection of the higher viral load in $NK1R^{-/-}$ mice, we compared the MIP-1α response to graded amounts of HSV-2 in spleen cell suspensions. We found that MIP-1α was induced in an HSV-2 dose-dependent fashion in both $NK1R^{+/+}$ and $NK1R^{-/-}$ mice and that spleen cells from $NK1R^{-/-}$ were not superior in their ability to produce MIP-1α in response to HSV-2 (Fig. 6*C*). Furthermore, both IL-10 and IL-12 were induced in a dose-dependent fashion in response to

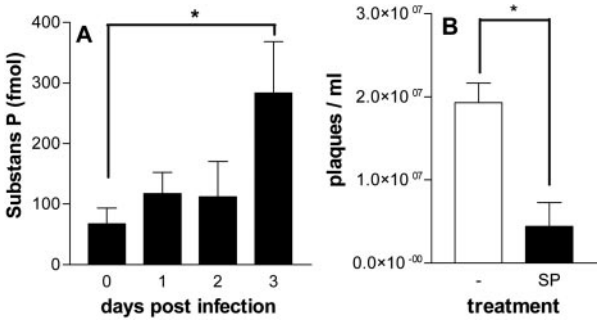

FIGURE 3. Levels of SP in the genital tract of HSV-2-infected mice and the effect of SP on viral replication. *A*, The levels of SP were measured by radioimmunoassay in vaginal tissue extracts obtained at different time points following a genital infection with HSV-2. Data are presented as the mean ficomoles SP per vaginal sample + SEM with five animals analyzed for each time point. *B*, The effect of SP (10^{-6} M) on viral replication in vitro was analyzed in a conventional plaque assay using GMK cells. $n = 3$; *, $p < 0.05$.

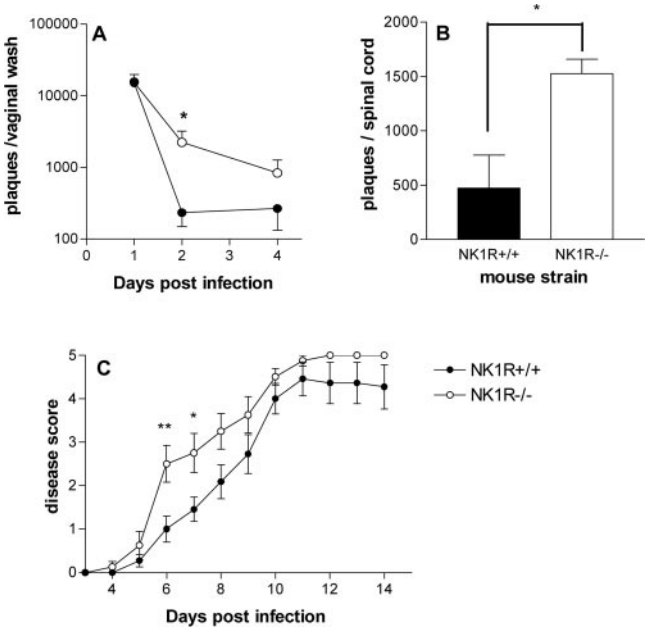

FIGURE 5. Enhanced viral replication in HSV-2-infected mice lacking NK1R, the receptor for SP. NK1R$^{-/-}$ mice and NK1R$^{+/+}$ mice were challenged intravaginally with HSV-2. HSV-2 replication was determined (*A*) in vaginal fluids obtained 1, 2, and 4 days after vaginal HSV-2 challenge ($n = 3$ for days 1 and 4; $n = 14$ for day 2), and (*B*) in the spinal cord obtained 8 days after vaginal HSV-2 challenge ($n = 6$). Data are expressed as the mean virus load + SEM. *C*, Animals were scored daily for pathological symptoms. Disease progression was scored as healthy (0), mild inflammation (1), moderate genital inflammation (2), genital lesion and/or generally bad condition (3), hind limb paresis (4), or death or sacrifice due to paralysis (5). Data represent the mean score from two experiments with a total of 11 mice per group. *, $p < 0.05$; **, $p < 0.01$ using Student's *t* test.

HSV-2, and these responses did not differ significantly in-between spleen cell cultures from NK1R$^{+/+}$ and NK1R$^{-/-}$ mice, even though the IL-12 responses appeared to be lower in NK1R$^{-/-}$ mice (data not shown).

NK cell function in NK1R-deficient mice

We assessed the killing potential of NK cells from NK1R$^{+/+}$ and NK1R$^{-/-}$ mice in a conventional cytotoxicity assay. We found that spleen NK cells from NK1R$^{-/-}$ mice could kill YAC-1 target cells equally as efficiently as NK cells from NK1R$^{+/+}$ mice (Fig. 7*A*). Addition of SP at 10^{-5}–10^{-7} M to the spleen NK cells immediately before their use in the cytotoxicity assay had no impact on the NK cell killing of YAC-1 cells (Fig. 7*B*). The NK cell activity in freshly isolated vaginal cells from HSV-2-infected NK1R$^{-/-}$ mice was, however, impaired. Vaginal cell suspensions from NK1R$^{+/+}$ mice killed YAC-1 cells seven times more efficiently than vaginal cell suspensions from NK1R$^{-/-}$ mice (Fig. 7*C*). The frequency of NK cells in the vaginal cell suspensions was slightly lower in NK1R$^{-/-}$ mice compared with NK1R$^{+/+}$ mice (Fig. 7*D*), but these differences were not as prominent as the differences observed in killing potential (Fig. 7*C*). When comparing the specific killing obtained to the actual numbers of NK cells present, we found that the relative lytic unit per NK cell was eight times higher in NK1R$^{+/+}$ mice compared with NK1R$^{-/-}$ mice (4.8 lytic units in NK1R$^{+/+}$ mice compared with 0.6 lytic units in NK1R$^{-/-}$ mice). The rationale for using vaginal cells from HSV-2-infected mice is that NK cells do not normally reside in the murine vagina (36, 37).

SP is not required for acquired protection against genital HSV-2 infection

Finally, we investigated whether SP signaling is important for the development of a protective acquired immunity. NK1R$^{-/-}$ and NK1R$^{+/+}$ mice were vaccinated vaginally with an attenuated strain of HSV-2 lacking the gene for viral TK (28) and then challenged 4 wk later with virulent HSV-2. NK1R$^{-/-}$ mice were, similar to NK1R$^{+/+}$ mice, completely protected against the HSV-2

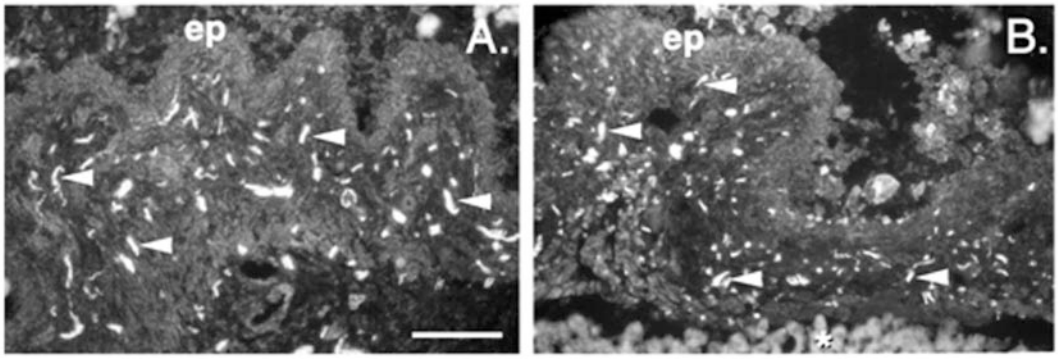

FIGURE 4. Distribution of SP-reactive cells in the vagina. SP-containing cells in the vagina before (*A*) and 3 days after (*B*) infection with HSV-2. There are numerous SP-positive structures, both single nerves but mostly small nerve bundles (arrowhead) both before and after infection. ep, Epithelium. *, Liver tissue used to separate the different vaginas. Scale bar, 100 μm.

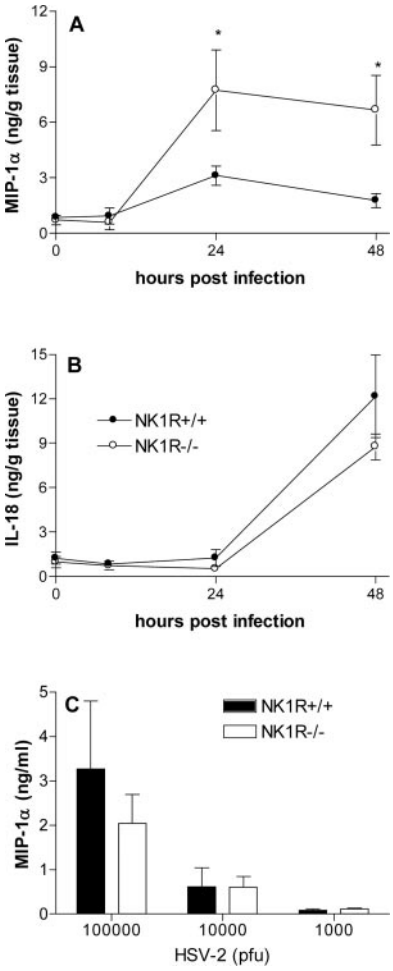

FIGURE 6. Increased levels of MIP-1α in the genital tract of HSV-2-infected NK1R$^{-/-}$ mice. The levels of (*A*) MIP-1α and (*B*) IL-18 were analyzed in vaginal tissue extracts obtained at various time points after HSV-2 infection from NK1R$^{-/-}$ (○) and NK1R$^{+/+}$ (●) mice. Data are presented as the mean concentration of cytokine per g of tissue ± the SEM. *, $p < 0.05$ using Student's *t* test. *C*, Levels of MIP-1α secreted in response to different doses of HSV-2 was analyzed in 48-h supernatants of spleen cell suspensions from NK1R$^{+/+}$ (■) and NK1R$^{-/-}$ (□) mice.

challenge. None of 13 NK1R$^{-/-}$ mice and none of 14 NK1R$^{+/+}$ mice had any detectable virus in the genital secretions obtained 48 h after viral challenge, and all animals survived the infection without any signs of disease (data not shown). Furthermore, there were no detectable levels of IFN-γ, IL-12, IL-18, or MIP-α in vaginal tissue extracts obtained 8, 24, and 48 h after the viral challenge in either NK1R$^{+/+}$ or NK1R$^{-/-}$ mice (data not shown).

Protective immunity to genital HSV-2 infection of mice is mediated mainly by T cells secreting IFN-γ (25, 38). We therefore measured the T cell immune responses to HSV-2 in vitro 4 wk after vaccination in NK1R$^{+/+}$ and NK1R$^{-/-}$ mice. We detected strong HSV-2-specific T cell responses in both NK1R$^{+/+}$ and NK1R$^{-/-}$ mice. There were no statistical differences in the HSV-2-specific IFN-γ responses from either CD4$^+$ (Fig. 8*A*) or CD8$^+$ (Fig. 8*B*) T cells, nor were there any differences in the HSV-2-specific CD4$^+$ T cell proliferative responses or the frequencies of HSV-2-specific CD8$^+$ T cells between vaccinated NK1R$^{+/+}$ and NK1R$^{-/-}$ mice (data not shown), which indicates that SP/NK1R is not required for the induction of IFN-γ-secreting HSV-2-specific effector T cells.

Discussion

In this study, we show that genital infection with HSV-2 induces increased levels of SP in the vagina. Absence of SP signaling in mice deficient for the NK1R receptor was associated with a marked reduction in the local vaginal NK cell cytotoxic killing potential and a reduced ability to control local virus replication. SP was also found to have a direct inhibitory effect on viral replication in vitro. NK1R deficiency, however, did not impact on the animal's ability to mount a protective immune response following vaccination.

Levels of SP were increased in the vagina of HSV-2-infected mice. This is to our knowledge the first report to show a pathogen-induced accumulation of SP in the reproductive tract. We found that the bulk of the SP present in the vagina was localized in nerve fibers and we therefore propose that the HSV-2-induced increase in SP in the vagina is derived mainly from the sensory nerves in response to the neuronal HSV-2 infection. These data are in accordance with other studies showing that most SP present within the female reproductive organ originates from primary afferent fibers (39). However, leukocytes can also produce SP, albeit at a much lower level, which makes it difficult to detect with immunohistochemistry, and we cannot, therefore, fully exclude that leukocytes contribute to the increased SP levels day 3 after infection. HSV-2 infection of the vaginal epithelium attracts neutrophils, which can produce SP, already day 1 after infection (24). This coincides with the early low increase in SP within the vagina. The significant increase in SP day 3 after HSV-2 infection occurs simultaneously with an accumulation of B cells (24), but it is to our knowledge not known whether B cells can produce SP.

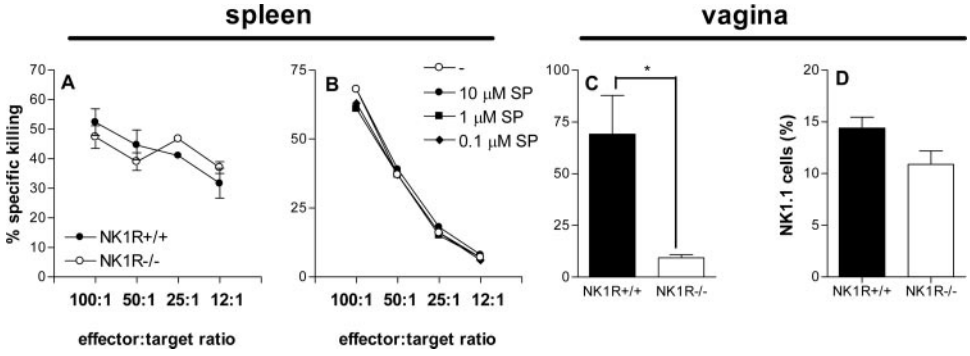

FIGURE 7. Effect of SP/NK1R on NK cell function. Cytotoxic killing of YAC-1 cells by IL-2-activated spleen cell suspensions (*A* and *B*) and freshly isolated vaginal cell suspensions (E:T ratio of 35:1) (*C*) obtained from NK1R$^{-/-}$ and NK1R$^{+/+}$ mice. *B*, Different concentrations of SP were added to IL-2-activated spleen cell suspensions immediately before the 8-h cytotoxicity assay. *D*, The frequency of NK1.1-positive cells was evaluated in freshly isolated vaginal cell suspensions obtained 48 h after HSV-2 infection. Data represent three animals per group. *, $p < 0.05$.

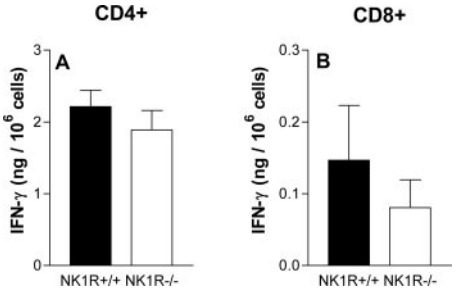

FIGURE 8. T cell function in NK1R$^{-/-}$ mice. *A*, HSV-2-specific IFN-γ production by CD8$^+$ T cell-depleted spleen cell suspensions from vaccinated NK1R$^{-/-}$ and NK1R$^{+/+}$ mice was analyzed by a CellELISA using whole UV-inactivated HSV-2 as specific Ag. *B*, HSV-2-specific IFN-γ production by CD8$^+$ T cells was analyzed by a CellELISA using SSIEFARL peptide as specific Ag. Data are presented as the mean + SEM of IFN-γ produced per 10^6 cells with three animals per group.

No obvious difference was observed with regard to the number of fibers or fluorescence intensity between controls and HSV-2-infected vaginas. However, it should be emphasized that immunohistochemistry is only semiquantitative and a rather insensitive tool to detect changes in peptide levels. A 4-fold increase in SP in the vagina, as indicated by the immunochemical measurements, may thus very well be undetectable by immunohistochemistry unless dilution curves and measurements based on stereology are performed.

NK1R signaling appears to be an important component of the local innate response to genital HSV-2 infection. There was a significantly increased vaginal viral replication in NK1R-deficient mice leading to an accelerated disease progress. In this context, it is interesting to note that SP, at least in our hands, interfered with HSV-2 replication in vitro. Whether these effects are mediated through NK1R or through a direct effect on HSV-2 cannot be discerned by these studies. Israel et al. (40) have previously reported that SP and inhibitors of the NK1R receptor do not interfere with HSV-1 plaque formation. However, we disagree with their conclusion as they, in their article, show that SP at 10^{-5} M reduces the plaque formation by 42% (40). We propose that the antiviral properties of SP, albeit weak, could contribute to the innate defense against HSV-2 by reducing the viral infectivity in the vaginal epithelium. Similar observations have been made with respect to measles virus replication where SP blocks viral fusion (41) and for HSV with a number of different peptides of disparate origin (42, 43).

During the first days of a primary HSV-2 infection, IFN-γ and NK cells appear to be especially important in the local vaginal immune response. Both IFN-γ-deficient mice and mice lacking NK and NKT cells (IL-15$^{-/-}$) are highly susceptible to genital HSV-2 infection and display an accelerated viral accumulation and disease development (26, 44). In line with these data are our previous results showing that the IFN-γ-inducing cytokines IL-12 and IL-18 are important in innate defense (26), most likely by their ability to induce IFN-γ production and to enhance NK cell activity (45, 46). In this study, we show that NK1R deficiency affects local vaginal NK cell functions but not Th1 cytokine production. NK1R$^{-/-}$ mice had an 8-fold lower relative cytotoxic capacity per NK cell locally in the vagina which most likely contributes to their impaired ability to control the viral replication. In contrast, the spleen NK cells were not affected by NK1R deficiency. These data are in accordance with previous studies (16) showing that NK cells from different anatomical compartments have a differential sensitivity to SP. NK cells from the spleen of mice do not respond to SP, in contrast to NK cells from mucosal compartments such as the intestine (16). We could not document any impact of NK1R deficiency on the levels of Th1 cytokines locally in the vagina. The level of IL-18 produced local in the vagina was intact in NK1R$^{-/-}$ mice and similarly low (below detection limit) levels of IFN-γ and IL-12 were detected in the vaginal tissues of NK1R$^{+/+}$ and NK1R$^{-/-}$ mice.

Both NK1R$^{+/+}$ and NK1R$^{-/-}$ mice produced MIP-1α locally in the vagina following a primary HSV-2 infection. However, NK1R$^{-/-}$ mice had significantly enhanced increases in their vaginal levels of the CC chemokine MIP-1α compared with NK1R$^{-/-}$ mice. MIP-1α is typically induced in response to infection and recruit effector cells to the site of pathogen entry (47). Our in vitro studies show that MIP-1α is induced in a HSV-2 dose-dependent manner and that spleen cells from NK1R$^{+/+}$ and NK1R$^{-/-}$ have the same propensity to produce MIP-1α in response to HSV-2. We therefore propose that the enhanced induction of MIP-1α in NK1R$^{-/-}$ mice reflects the increased viral load in these animals. MIP-1α can be secreted by most mature hemopoietic cells, fibroblasts, and epithelial cells (48). NK cells do not normally reside in nonlymphoid organs including the murina vagina (36, 37). Studies of other nonlymphoid organs show that local production of MIP-1α is a prerequisite for NK cell inflammation (49). The importance of MIP-1α in NK cell-mediated antiviral defense has been validated in mice that are completely deprived of MIP-1α due to deletions in the MIP-1α coding gene (49). However, the increased local MIP-1α concentrations observed in NK1R$^{-/-}$ mice did not induce an enhanced numbers of infiltrating NK cells as compared with NK1R$^{+/+}$ mice.

Lack of SP signaling did not impair the animals' ability to mount a protective immune response to HSV-2 despite the well-documented effect of SP on T cell activation and IFN-γ secretion (50). We and others have previously shown that CD4$^+$ T cells in combination with IFN-γ comprise the most important components in acquired protective immunity to genital HSV-2 infection. Mice lacking either the gene for IFN-γ or CD4$^+$ T cells cannot mount a protective immune response to HSV-2 following vaccination (25, 26, 51–53). Accordingly, in humans, an impaired HSV-2-specific IFN-γ response by CD4$^+$ T cells correlates with recurrent clinical disease, whereas high levels of IFN-γ are produced in T cells from individuals with an asymptomatic HSV-2 infection (54, 55). In this study, we show that vaccinated NK1R$^{-/-}$ mice have similar levels of HSV-2-reactive CD4$^+$ and CD8$^+$ T cells as NK1R$^{+/+}$ mice and that T cells from NK1R$^{-/-}$ and NK1R$^{+/+}$ mice produce comparable amounts of IFN-γ in response to HSV-2. Other studies have shown that mice lacking NK1R have an increased viral burden in combination with a reduced CTL response following infection with murine gamma-herpesvirus 68 (17). Whether the cytotoxic function of HSV-2-specific CD8$^+$ T cells was affected by NK1R deficiency cannot be deduced from these studies because we did not perform any functional studies of CD8$^+$ T cell cytotoxicity due to the redundant role of CD8$^+$ T cells in protective immunity to HSV-2 in mice (25, 51, 53).

It should be emphasized that these studies were conducted in NK1R$^{-/-}$ mice and not in mice deficient for SP (SP$^{-/-}$ mice). The phenotype of NK1R$^{-/-}$ mice show similarities to those of SP$^{-/-}$ mice, but there are differences. The main members of the mammalian tachykinin family are SP, neurokinin A, and neurokinin B, which preferentially bind to and induce effects via NK1, NK2, and NK3 receptors, respectively. SP may also activate NK2 receptors and NK1 receptors may also be activated by neurokinin A, but these interactions have a much lower affinity (56). From the immunological point of view, SP acting on NK1 receptors seems

to be the most important signaling path in leukocytes (57). However, it is still possible that SP-deficient and NK1R-deficient mice would respond differently to a HSV-2 infection.

In summary, we show that SP is induced in the vagina following genital HSV-2 infection of mice and that NK1R signaling contributes to the innate resistance against the infection.

Acknowledgments

We thank Inger Nordström, Margareta Fredriksson, and Patrik Molander for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Kinghorn, G. R. 1994. Epidemiology of genital herpes. *J. Intern. Med. Res.* 22: 14A–23A.
2. Hökfelt, T., B. Pernow, and J. Wahren. 2001. Substance P: a pioneer amongst neuropeptides. *J. Intern. Med.* 249: 27–40.
3. Alm, P., J. Alumets, E. Brodin, R. Hakanson, G. Nilsson, N. O. Sjoberg, and F. Sundler. 1978. Peptidergic (substance P) nerves in the genito-urinary tract. *Neuroscience* 3: 419–425.
4. Mitchell, B. S., and E. Ahmed. 1992. An immunohistochemical study of the catecholamine synthesizing enzymes and neuropeptides in the female guinea-pig uterus and vagina. *Histochem. J.* 24: 361–367.
5. Papka, R. E., J. P. Cotton, and H. H. Traurig. 1985. Comparative distribution of neuropeptide tyrosine-, vasoactive intestinal polypeptide-, substance P-immunoreactive, acetylcholinesterase-positive, and noradrenergic nerves in the reproductive tract of the female rat. *Cell Tissue Res.* 242: 475–490.
6. Lambrecht, B. N., P. R. Germonpre, E. G. Everaert, I. Carro-Muino, M. de Veerman, D. de Filipe, S. P. Hunt, K. Thielmans, G. F. Joos, and R. A. Pauwels. 1999. Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. *Eur. J. Immunol.* 29: 3815–3825.
7. Ho, W.-Z., J. P. Lai, X. H. Zhu, M. Uvaydova, and S. D. Douglas. 1997. Human monocytes and macrophages express substance P and neurokinin-1 receptor. *J. Immunol.* 159: 5654–5660.
8. de Giorgio, R., P. L. Tazzari, G. Barbara, V. Stanghellini, and R. Corinaldesi. 1998. Detection of substance P immunoreactivity in human peripheral leucocytes. *J. Neuroimmunol.* 82: 175–181.
9. Lotz, M., J. H. Vaughan, and D. A. Carson. 1988. Effect of neuropeptides on production of inflammatory cytokines. *Science* 241: 1218–1221.
10. Kincy-Cain, T., and K. L. Bost. 1997. Substance P-induced IL-12 production by murine macrophages. *J. Immunol.* 158: 2334–2339.
11. Laurenzi, M. A., M. A. Persson, C. J. Dalsgaard, and A. Haegerstrand. 1990. The neuropeptide substance P stimulates production of interleukin-1 in human blood monocytes: activated cells are preferentially influenced by the neuropeptide. *Scand. J. Immunol.* 31: 529–533.
12. Ho, W.-Z., D. Kaufman, M. Uvaydova, and S. D. Douglas. 1996. Substance P augments interleukin-10 and tumor necrosis factor-α release by human cord blood monocytes and macrophages. *J. Neuroimmunol.* 71: 73–80.
13. Levite, M. 1998. Neuropeptides, by direct interaction with T cells, induce cytokine secretion and break the commitment to a distinct T helper phenotype. *Proc. Natl. Acad. Sci. USA* 95: 12544–12549.
14. Lang, K., T. L. Drell, B. Niggemann, K. S. Zanker, and F. Entschladen. 2003. Neurotransmitters regulate the migration and cytotoxicity in natural killer cells. *Immunol. Lett.* 90: 165–172.
15. Flageole, H., M. Senterman, and J. L. Trudel. 1992. Substance P increases in vitro lymphokine-activated-killer (LAK) cell cytotoxicity against fresh colorectal cancer cells. *J. Surg. Res.* 53: 445–449.
16. Croitoru, K., P. B. Ernst, J. Bienenstock, I. Padol, and A. M. Stanisz. 1990. Selective modulation of the natural killer activity of murine intestinal intraepithelial leucocytes by the neuropeptide substance P. *Immunology* 71: 196–201.
17. Elsawa, S. F., W. Taylor, C. C. Petty, I. Marriott, J. V. Weinstock, and K. L. Bost. 2003. Reduced CTL response and increased viral burden in substance P receptor-deficient mice infected with murine γ-herpesvirus 68. *J. Immunol.* 170: 2605–2612.
18. Ho, W. Z., J. P. Lai, Y. Li, and S. D. Douglas. 2002. HIV enhances substance P expression in human immune cells. *FASEB J.* 16: 616–618.
19. Li, Y., S. D. Douglas, L. Song, S. Sun, and W.-Z. Ho. 2001. Substance P enhances HIV-1 replication in latently infected human immune cells. *J. Neuroimmunol.* 121: 67–75.
20. Tripp, R. A., D. Moore, J. Winter, and L. J. Anderson. 2000. Respiratory syncytial virus infection and G and/or SH protein expression contribute to substance P, which mediates inflammation and enhanced pulmonary disease in BALB/c mice. *J. Virol.* 74: 1614–1622.
21. Piedimonte, G., M. M. Rodriguez, K. A. King, S. McLean, and X. Jiang. 1999. Respiratory syncytial virus upregulates expression of the substance P receptor in rat lungs. *Am. J. Physiol.* 277: L831–L840.
22. Tripp, R. A., A. Barskey, L. Goss, and L. J. Anderson. 2002. Substance P receptor expression on lymphocytes is associated with the immune response to respiratory syncytial virus infection. *J. Neuroimmunol.* 129: 141–153.
23. McDermott, M. R., J. R. Smiley, L. J. Brais, H. E. Rudzroga, and J. Bienenstock. 1984. Immunity in the female genital tract after intravaginal vaccination of mice with an attenuated strain of herpes simplex virus type 2. *J. Virol.* 51: 747–753.
24. Parr, M. B., L. Kepple, M. McDermott, M. D. Drew, J. J. Bozzola, and E. L. Parr. 1994. A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2. *Lab. Invest.* 70: 369–380.
25. Harandi, A. M., B. Svennerholm, J. Holmgren, and K. Eriksson. 2001. Differential roles of B-cells and IFN-γ secreting $CD4^+$ T-cells in innate and adaptive immune control of genital HSV-2 infection in mice. *J. Gen. Virol.* 82: 845–853.
26. Harandi, A. M., B. Svennerholm, J. Holmgren, and K. Eriksson. 2001. Interleukin-12 (IL-12) and IL-18 are important in innate defense against genital herpes simplex virus type 2 infection in mice but are not required for the development of acquired γ interferon-mediated protective immunity. *J. Virol.* 75: 6705–6709.
27. Bozic, C. R., B. Lu, U. E. Höpken, C. Gerard, and N. P. Gerard. 1996. Neurogenic amplification of immune complex formation. *Science* 273: 1722–1725.
28. Andrei, G., R. Snoeck, and E. De Clerq. 1997. Differential susceptibility of several drug-resistant strains of herpes simplex virus type 2 to various antiviral compounds. *Antivir. Chem. Chemother.* 8: 457–461.
29. Seth, P., W. E. Rawls, R. Duff, F. Rap, E. Adam, and J. L. Melnick. 1974. Antigenic differences between isolates of herpesvirus type 2. *Intervirology* 3: 1–14.
30. Streit, W. J. 1990. An improved staining method for rat microglial cells using the lectin from *Griffonia simplicifolia* (GSA I-B4). *J. Histochem. Cytochem.* 38: 1683–1686.
31. Brodin, E., A. Rosen, E. Theodorsson, A. Jonczyk, B. E. Sandberg, and K. Brodin. 1994. Multiple molecular forms of tachykinins in rat spinal cord: a study comparing different extraction methods. *Regul. Pept.* 52: 97–110.
32. Brodin, E., N. Lindefors, C. J. Dalsgaard, E. Theodorsson-Norheim, and S. Rosell. 1986. Tachykinin multiplicity in rat central nervous system as studied using antisera raised against substance P and neurokinin A. *Regul. Pept.* 13: 253–272.
33. Adams, J. C. 1992. Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J. Histochem. Cytochem.* 40: 1457–1463.
34. Christensson-Nylander, I., M. Herrera-Marschitz, W. Staines, T. Hokfelt, L. Terenius, U. Ungerstedt, C. Cuello, W. H. Oertel, and M. Goldstein. 1986. Striato-nigral dynorphin and substance P pathways in the rat. I. Biochemical and immunohistochemical studies. *Exp. Brain Res.* 64: 169–192.
35. Harandi, A. M., B. Svennerholm, J. Holmgren, and K. Eriksson. 2001. Protection against genital HSV-2 infection in mice is associated with a rapid induction of local IFN-γ-dependent RANTES production following a vaginal viral challenge. *Am. J. Reprod. Immunol.* 46: 420–424.
36. Nandi, D., and J. P. Allison. 1993. Characterization of neutrophils and T lymphocytes associated with the murine vaginal epithelium. *Reg. Immunol.* 5: 332–338.
37. Parr, M. B., and E. L. Parr. 1991. Langerhans cells and T lymphocyte subsets in the murine vagina and cervix. *Biol. Reprod.* 44: 491–498.
38. Blaney, J. E., Jr., E. Nobusawa, M. A. Brehm, R. H. Bonneau, L. M. Mylin, T. M. Fu, Y. Kawaoka, and S. S. Tevethia. 1998. Immunization with a single major histocompatibility complex class I-restricted cytotoxic T-lymphocyte recognition epitope of herpes simplex virus type 2 confers protective immunity. *J. Virol.* 72: 9567–9574.
39. Traurig, H., A. Saria, and F. Lembeck. 1984. Substance P in primary afferent neurons of the female rat reproductive system. *Naunyn Schmiedebergs Arch. Pharmacol.* 326: 343–346.
40. Israel, B. A., K. T. Schultz, and C. J. Murphy. 1998. Lack of detectable interaction between HSV-1 and integrins or tachykinins. *Intervirology* 41: 132–134.
41. Schroeder, C. 1986. Substance P, a neuropeptide, inhibits measles virus replication in cell culture. *Acta Virol.* 30: 432–435.
42. Yasin, B., W. Wang, M. Pang, N. Cheshenko, T. Hong, A. J. Waring, B. C. Herold, E. A. Wagar, and R. I. Lehrer. 2004. 32 θ Defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J. Virol.* 78: 5147–5156.
43. Jenssen, H., J. H. Andersen, D. Mantzilas, and T. J. Gutteberg. 2004. A wide range of medium-sized, highly cationic, α-helical peptides show antiviral activity against herpes simplex virus. *Antiviral Res.* 64: 119–126.
44. Ashkar, A. A., and K. L. Rosenthal. 2003. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J. Virol.* 77: 10168–10171.
45. Tomura, M., X. Y. Zhou, S. Maruo, H. J. Ahn, T. Hamaoka, H. Okamura, K. Nakanishi, T. Tanimoto, M. Kurimoto, and H. Fujiwara. 1998. A critical role for IL-18 in the proliferation and activation of $NK1.1^+CD3^-$ cells. *J. Immunol.* 160: 4738–4746.
46. Wolf, S. F., P. A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, et al. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.* 146: 3074–3081.

47. Moser, B., and P. Loetscher. 2001. Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2: 123–128.
48. Menten, P., A. Wuyts, and J. Van Damme. 2002. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev.* 13: 455–481.
49. Salazar-Mather, T. P., and K. L. Hokeness. 2003. Calling in the troops: regulation of inflammatory cell trafficking through innate cytokine/chemokine networks. *Viral Immunol.* 16: 291–306.
50. Lambrecht, B. N. 2001. Immunologists getting nervous: neuropeptides, dendritic cells, and T cell activation. *Respir. Res.* 2: 133–138.
51. Milligan, G. N., and D. I. Bernstein. 1995. Analysis of herpes simplex virus-specific T cells in the murine female genital react following genital infection with herpes simplex virus type. *Virology* 212: 481–489.
52. Milligan, G. N., and D. I. Bernstein. 1997. Interferon-γ enhances resolution of herpes simplex virus type 2 infection of the murine genital tract. *Virology* 229: 259–268.
53. Milligan, G. N., D. I. Bernstein, and N. Bourne. 1998. T lymphocytes are required for protection of the vaginal mucosae and sensory ganglia of immune mice against reinfection with herpes simplex virus type 2. *J. Immunol.* 160: 6093–6100.
54. Eriksson, K., L. Bellner, G.-B. Löwhagen, P. Tunbäck, K. Rydberg, and J.-Å. Liljeqvist. 2004. $CD4^+$ T-cell responses to herpes simplex virus type 2 (HSV-2) glycoprotein G are type-specific and differ in symptomatic and asymptomatic HSV-2 infected individuals. *J. Gen. Virol* 85: 2139–2147.
55. Singh, R., A. Kumar, W. D. Creery, M. Ruben, A. Giulivi, and F. Diaz-Mitoma. 2003. Dysregulated expression of IFN-γ and IL-10 and impaired IFN-γ-mediated responses at different stages in patients with genital herpes simplex virus-2 infection. *Clin. Exp. Immunol.* 133: 97–107.
56. Almeida, T. A., J. Rojo, P. M. Nieto, F. M. Pinto, M. Hernandez, J. D. Martin, and M. L. Candenas. 2004. Tachykinins and tachykinin receptors: structure and activity relationships. *Curr. Med. Chem.* 11: 2045–2081.
57. van Hagen, P. M., L. J. Hofland, A. M. ten Bokum, E. G. Lichtenauer-Kaligis, D. J. Kwekkeboom, D. Ferone, and S. W. Lamberts. 1999. Neuropeptides and their receptors in the immune system. *Ann. Med.* 31(Suppl.) 2: 15–22.