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Treatment of intravaginal HSV-2 infection in mice: A comparison of CpG oligodeoxynucleotides and resiquimod (R-848)

Michael J. McCluskie [a,*], Janna L.M. Cartier [a], Amy J. Patrick [b], Dusan Sajic [b], Risini D. Weeratna [a], Kenneth L. Rosenthal [b], Heather L. Davis [a]

[a] *Coley Pharmaceutical Group, 340 Terry Fox Drive, Suite 200, Ottawa, Ont., Canada K2K 3A2*
[b] *Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ont., Canada L8N 3Z5*

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Abstract

The mammalian innate immune system recognizes pathogens via a series of pattern-recognition receptors such as the toll-like receptors (TLR) that interact with pathogen-associated molecular patterns (PAMPs) and lead to the rapid activation of innate immune cells. In this study, we compared the efficacy of CpG ODN (a TLR9 agonist) and resiquimod (R-848; a TLR7/8 agonist) for topical immunoprophylaxis or immunotherapy of vaginal herpes simplex virus type 2 (HSV-2) infection in mice. Efficacy against HSV infection was observed with CpG ODN but less so with R-848, even after repeated administrations. Intravaginal (IVAG) administration of CpG ODN resulted in strong local but relatively weak systemic immune activation, as determined by levels of the chemokines IP-10, MIG and I-TAC in vaginal tissue and plasma, respectively. In contrast, IVAG administration of R-848 resulted in high levels of plasma IP-10, similar to those seen after parenteral administration, but overall, weaker or shorter-lived local immune responses than obtained with CpG ODN. These findings suggest that differences in biodistribution and sites of immune activation between CpG ODN and R-848 after IVAG delivery account for differences in efficacy, and demonstrate the need for local mucosal innate activation for protection against HSV-2.

Keywords: CpG ODN; R-848; Innate; TLR; HSV-2; Vaginal

1. Introduction

Herpes simplex virus type 2 (HSV-2) is the major cause of genital ulcer disease worldwide and a significant co-factor in the transmission and acquisition of human immunodeficiency virus type 1 (HIV-1) (Schacker, 2001). Despite current therapies, the incidence and prevalence of HSV-2 infection continues to increase worldwide (Fleming et al., 1997; Halioua and Malkin, 1999; O'Farrell, 1999). HSV-2 viral replication starts locally in the genital epithelium and thereafter ascends sensory nerves to the sacral ganglia, where latency can be established. While many individuals experience life-long infection characterized by sporadic, sometimes frequent, ulcerative outbreaks, others have less severe, more infrequent outbreaks, with eventual long-term control of symptoms associated with development of HSV-specific adaptive immunity, in particular Th1-type cell-mediated immunity (Koelle and Corey, 2003).

The standard treatment for genital herpes infection is the use of anti-viral drugs, such as the guanosine analogues acyclovir and famciclovir, however, even with chronic use these drugs do not result in permanent viral clearance or long-term control. Moreover, clinical issues with their use include drug resistance and safety concerns in small children or pregnant women (Naesens and De Clercq, 2001). For prophylaxis against HSV-2, the main strategies are the use of topical microbicides to block attachment or prevent viral transmission (Zeitlin and Whaley, 2002), and vaccination to generate HSV-2-specific adaptive immunity (Koelle and Corey, 2003). However, microbicides require a high patient compliancy and can have an undesired contraceptive effect. In addition, microbicides can be toxic or proinflammatory and frequent use may lead to disruption of the vaginal epithelium and enhanced risk of HIV-1 transmission during sexual intercourse (Van Damme et al., 2002). While preclinical vaccination studies against HSV-2 have been encouraging, results of vaccine trials against HSV-2 in humans have

* Corresponding author. Tel.: +1 613 254 5622x3139; fax: +1 613 254 5625.
E-mail address: mmccluskie@coleypharma.com (M.J. McCluskie).

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been disappointing, possibly due to a lack of T cell responses since induction of high levels of HSV-2 neutralizing antibodies have not previously provided protection (Corey et al., 1999; Stanberry et al., 2002).

An alternate approach for treatment of chronic HSV-2 infection may be the use of immunomodulators applied directly to the site of infection. This may result in innate immune responses to provide immediate control of viral replication, as well as the induction or augmentation of adaptive immune responses, in the presence of viral antigen, to help control subsequent outbreaks and possibly provide long-term control. To be effective, such immunomodulators must be able to induce Th1-type immune responses.

Innate immune responses result from the recognition of pathogen-associated molecular patterns (PAMPs) present in infectious agents by the vertebrate innate immune system via pattern-recognition receptors, including the Toll-like receptors (TLRs). To date, 10 different human TLRs have been identified, as well as a number of naturally occurring TLR-ligands (Pasare and Medzhitov, 2005). Synthetic TLR ligands have also been identified, including imidazoquinoline compounds such as imiquimod and resiquimod (R-848), which activate human TLR7 and 8 (Gibson et al., 2002; Hemmi et al., 2002), and oligodeoxynucleotides containing CpG motifs (CpG ODN), which are TLR9 agonists (Hemmi et al., 2000; Bauer et al., 2001b). Both of these classes of immunomodulators have antiviral and antitumor properties in animal models and have been used in a number of clinical trials (Hengge et al., 2001; Krieg, 2002, 2004). Imiquimod is also sold as a topical cream (Aldara^{TM}) to treat genital warts.

When used for prophylaxis against HSV-2 in mouse or guinea pig models, both CpG ODN and imidazoquinolines have proven efficacy (Harandi et al., 2003; Pyles et al., 2002; Sajic et al., 2003; Bernstein et al., 2001; Harrison et al., 1994). However, despite similarities in their biological mechanisms and effects, a number of differences exist between these molecules that may influence their effect on the immune system. For example, the cellular distribution of the TLR receptors for CpG ODN and imidazoquinolines are different. In humans, TLR9, which recognize CpG ODN, are found exclusively on B cells and plasmacytoid dendritic cells (pDC) (Bauer et al., 2001a; Krug et al., 2001), whereas imidazoquinolines interact with TLR7 and 8 found on B cells, monocytes, and both plasmacytoid and myeloid DC (mDC) (Jarrossay et al., 2001; Kadowaki et al., 2001). While effects on a wider selection of cell types could be advantageous, it could also result in additional toxicities. In addition, there are considerable physical and chemical differences between the two molecules that could affect biodistribution and thus efficacy.

To date, an in vivo comparison of an imidazoquinoline and CpG ODN has only been made for their use as vaccine adjuvants (Vasilakos et al., 2000; Weeratna et al., 2005). However, no such comparison has been made for the ability of these two TLR ligands to activate innate immunity. In this study, we have evaluated and compared R-848 and CpG ODN for their ability to stimulate innate immune responses and to protect mice from lethal infection with HSV-2. This allowed us to study the effect of the physical and chemical differences of the two molecules on relative efficacy, but not TLR distribution since in mice TLR7 and TLR9 are both found on B cells, pDC and mDC and peripheral mononuclear cells (Hemmi et al., 2000; Wagner, 2004).

2. Materials and methods

2.1. Animals

Female C57BL/6 or BALB/c mice (Charles River Canada, Montreal, Que., Canada) 6–8 weeks old, were used for these studies, which were conducted at the Ottawa Hospital Research Institute, Ottawa, ON, Canada; Coley Pharmaceutical Group, Ottawa, Canada; and McMaster University Health Sciences Centre, Hamilton, ON, Canada. All HSV-2 challenge studies were conducted in C57BL/6 mice, since this disease model has been established and optimized in this mouse strain. However, it has been reported that DCs from C57BL/6 mice preferentially express TLR9 mRNA compared to BALB/c mice (Liu et al., 2002), therefore, additional studies were conducted in BALB/c mice to determine chemokine production after local or parenteral administration. All animal experiments were subject to approval by the Animal Care Committees of the Ottawa Hospital and Coley Pharmaceutical Group or the Animal Research Ethics Board of McMaster University, under the requirements of the Canadian Council on Animal Care (CCAC).

2.2. Test articles and experimental groups

B Class CpG (2006, TCGTCGTTTTGTCGTTTTGTCGTT; 10104, TCGTCGTTTCGTCGTTTTGTCGTT; 1826, TCCATGACGTTCCTGACGTT) and non-CpG control (1982, TCCAGGACTTCTCTCAGGTT; 2137, TGCTGCTTTTGTGCTTTTGTGCTT; 2138, TCCATGAGCTTCCTGAGCTT) ODN were synthesized with a phosphorothioate backbone, tested for purity and endotoxin contamination (Coley Pharmaceutical Group, Wellesley, MA), re-suspended in sterile, endotoxin free TE at pH 8.0 (OmniPur®; EM Science, Gibbstown, NJ) and stored and handled under aseptic conditions to prevent both microbial and endotoxin contamination. R-848 (4-amino-2-ethoxymethyl-α,α-dimethyl-1*H*-imidazo[4,5-*c*]quinoline-1-ethanol) was manufactured by GL synthesis (Boston, MA) and was dissolved in TE buffer (pH 8.0) containing 10% DMSO. Dilution of ODNs and R-848 for experiments was carried out in sterile, endotoxin free phosphate buffered saline (PBS) at pH 7.2 (Sigma Diagnostics, St. Louis, MO).

To compare single dose pre- or post-challenge treatments, six groups of 10 mice were administered CpG ODN (CpG 10104) or R-848 IVAG on a single occasion, either 24 h prior to or 4 h after HSV-2 infectious challenge (see below). Both CpG ODN and R-848 were given at doses of 1, 10 or 100 μg, which are equivalent to 0.13, 1.3, or 13 nmol CpG ODN, respectively, and 3.2, 32, or 320 nmol R-848, respectively.

To evaluate effects of multiple dose treatments, CpG ODN (CpG 2006) or R-848 (0.1, 1, 10, or 100 nmol) were administered

intravaginally (IVAG) as a single daily dose for 5 consecutive days starting 4 h after infection via a micropipette in a total volume of 25 μl of PBS. These doses are approximately equivalent to 0.8 μg (0.1 nmol), 8 μg (1 nmol), 80 μg (10 nmol) CpG ODN, and 0.3 μg (1 nmol), 3 μg (10 nmol) and 30 μg (100 nmol) for R-848.

Control groups received either PBS or non-CpG control ODN.

2.3. Cells and viruses

Vero cells (ATCC CCL81) were grown in α-MEM growth media (Life Technologies, Burlington, Ont., Canada) supplemented with 5% fetal calf serum (Life Technologies) and 50 μg penicillin/ml, 50 μg streptomycin/ml and 2 mM L-glutamine (Life Technologies) and were incubated at 37 °C and 5% CO_2. Stocks of HSV-2 (strain 333) were prepared from infected Vero monolayers, tittered by plaque assay and stored at −80 °C until used.

2.4. Intravaginal HSV-2 challenge

Five days prior to IVAG challenge, mice were injected subcutaneously (SC) with 2 mg of progesterone (Depo-Provera®; Pharmacia & Upjohn Co., Mississauga, Ont., Canada) in the upper back, using a 29-gauge needle. IVAG administrations of virus or each drug dose were via a micropipette in a total volume of 25 μl PBS.

On the day of challenge, mice were anesthetized with Ketamine (150 mg/kg) and Xylazine (10 mg/kg) by intraperitoneal injection and a lethal dose of HSV-2 strain 333 at 1×10^4 pfu per mouse was delivered IVAG in 10 μl PBS via micropipette. Genital pathology was monitored daily after IVAG HSV-2 challenge. Pathology was scored on a 5-point scale as follows: 0 = no apparent infection; 1 = slight redness of external vagina; 2 = redness and swelling of external vagina; 3 = severe redness and swelling of external vagina with hair loss of surrounding tissue; 4 = genital ulceration with severe redness; 5 = severe genital ulceration extending to surrounding tissue. Mice were euthanized upon reaching stage 5 or when paralysis of the hind limbs was observed.

2.5. Biomarker determination

Mice were administered CpG ODN or R-848 (100 nmol) either by IVAG instillation in a volume of 20 μl using a micropipette or by SC injection in a volume of 50 μl using a 29-gauge needle (Becton Dickinson, Franklin Lakes, NJ). For IVAG groups, mice were administered 2 mg of progesterone by SC injection in the lower back using a 29-gauge needle 5 days prior to IVAG administration of CpG ODN or R-848. At various times (0.25–24 h) after administration of CpG ODN or R-848, plasma was collected. Other animals (IVAG only) had vaginal tissues (vagina, cervix and uterus) collected at various times (0.25–24 h) and placed in 200 μl protease inhibitor solution prepared by dissolving 14 Complete Mini, EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN) in 100 ml PBS, pH 7.2 (Sigma Diagnostics, St. Louis, MO). Vaginal tissues were cut into small pieces using fine-point scissors, ground with a mortar and pestle, and the final volume of protease inhibitor solution brought up to 600 μl per sample of vaginal tissue. Homogenized vaginal tissues were centrifuged at 13,000 rpm for 10 min and supernatants collected. Plasma and vaginal tissue supernatants were stored at ≤−15 °C until assayed.

Plasma and vaginal tissue supernatants (five samples per group per time-point) were assayed by ELISA for the presence of interferon-gamma-inducible protein 10 (IP-10), interferon-inducible T cell alpha chemoattractant (I-TAC), and monokine induced by gamma interferon (MIG), as markers for immune activation. These ligands have been shown to be induced by IFN-γ and are important in Th1-type immune responses (Cole et al., 1998; Luster and Ravetch, 1987; Ogasawara et al., 2002). All assays were completed using 96-well flat-bottom microtiter plates (NUNC-Immuno™ Maxisorp™ plates, Nalge NUNC International, Rochester, NY). For IP-10 ELISA, microtiter plates were coated with 100 μl per well of 6 μg/ml capture antibody (purified rat anti-mouse CRG-2 monoclonal antibody; BD Pharmingen, San Diego, CA) diluted in 0.2 M phosphate buffer, pH 9.0, or 2 μg/ml capture antibody (purified rat anti-mouse CRG-2 monoclonal antibody; R&D Systems, Minneapolis, MN) diluted in PBS (Gibco, Grand Island, NY) and incubated overnight at 4 °C or room temperature, respectively. After washing with 0.05% Tween 20 (Sigma–Aldrich, St. Louis, MO) in PBS, plates were blocked with either 10% fetal calf serum (FCS, Sigma) in PBS or with 1% BSA (Sigma) in PBS containing 5% sucrose (Sigma) and 0.05% sodium azide (Sigma). After washing, 100 μl of each plasma or vaginal tissue supernatant and recombinant mouse IP-10 standard (R&D Systems) were coated onto the plate and incubated at room temperature for 2 h. After washing, 100 μl of either 4 μg/ml detection antibody (biotinylated rabbit anti-mouse CRG-2 polyclonal antibody, BD Pharmingen) diluted in assay diluent (10% FCS in PBS) or 1 μg/ml detection antibody (biotinylated goat anti-mouse CRG-2 antibody) diluted in assay diluent (1% BSA in PBS) was added to each well and samples were incubated at room temperature for 1 or 2 h, respectively. After washing, the plates were coated with 100 μl per well of either avidin-HRP conjugate (Sigma) diluted 1:250 in assay diluent (10% FCS in PBS) or streptavidin–HRP conjugate (R&D Systems) diluted 1:200 in assay diluent (1% BSA in PBS) and incubated at room temperature for 30 or 20 min, respectively. Plates were washed again and developed with 100 μl per well of tetramethylbenzadine liquid substrate system containing hydrogen peroxide (Sigma or R&D Systems). After 15–30 min in the dark, the reaction was stopped by addition of 50 μl 2N H_2SO_4 to each well. Absorbance was read at 450 nm and the concentration of IP-10 was calculated from the standard curve using an automated ELISA plate reader with MRX Revelation software (Dynex Technologies, Chantilly, VA). Assays for I-TAC and MIG were performed following the protocol in commercially available ELISA kits (R&D Systems) using 1% BSA (Sigma) in PBS (Gibco) as assay diluent and washing with 0.05% Tween 20

(Sigma) diluted in PBS. Absorbance was read at 450 nm and the concentration of I-TAC and MIG were calculated as for IP-10.

2.6. Statistical analysis

Data were analyzed using the GraphPAD Prism program (GraphPAD Software, San Diego). The statistical significances of differences in survival rates were determined by log rank analyses. Statistical significances of differences in IP-10 levels were determined by Student's 2-tailed *t*-test for two groups, or by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups. Differences were considered to be not significant with $p > 0.05$. Group geometric mean titers ± standard error of the mean (S.E.M.) are presented in the text and figures.

3. Results

3.1. Effects of prophylactic administration of CpG ODN or R-848

A single IVAG administration of R-848 24 h prior to lethal HSV-2 challenge provided no protection relative to PBS challenge controls, regardless of dose ($p > 0.05$). In contrast, a single prophylactic administration of CpG ODN resulted in a dose-dependent protection with significantly enhanced survival for 100 and 10 μg dose groups compared to PBS control mice ($p = 0.0001$ and 0.0058, respectively), with 90 and 60% of mice surviving to the study termination at 15 days for 100 and 10 μg doses, respectively, compared to 10% for control mice (Fig. 1A). A similar ranking was obtained in pathology with CpG ODN (100 or 10 μg) treated mice demonstrating a marked reduction

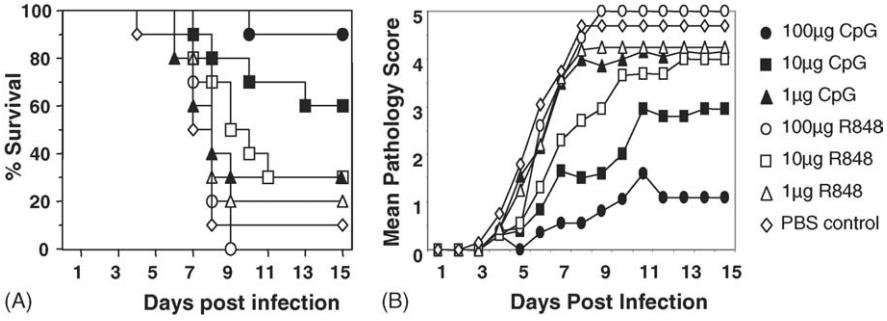

Fig. 1. Effects of local prophylactic administration of CpG or R-848 on protection from subsequent IVAG HSV-2 challenge. Groups of female C57BL/6 mice ($n = 10$ per group) were administered by the IVAG route 1, 10, or 100 μg CpG (ODN 10104) or resiquimod (R-848) 24 h prior to challenge with a lethal dose of HSV-2. Control animals received PBS alone at the same time-point. Animals were monitored daily for morbidity and mortality. Panel A shows percent survival and panel B mean pathology score.

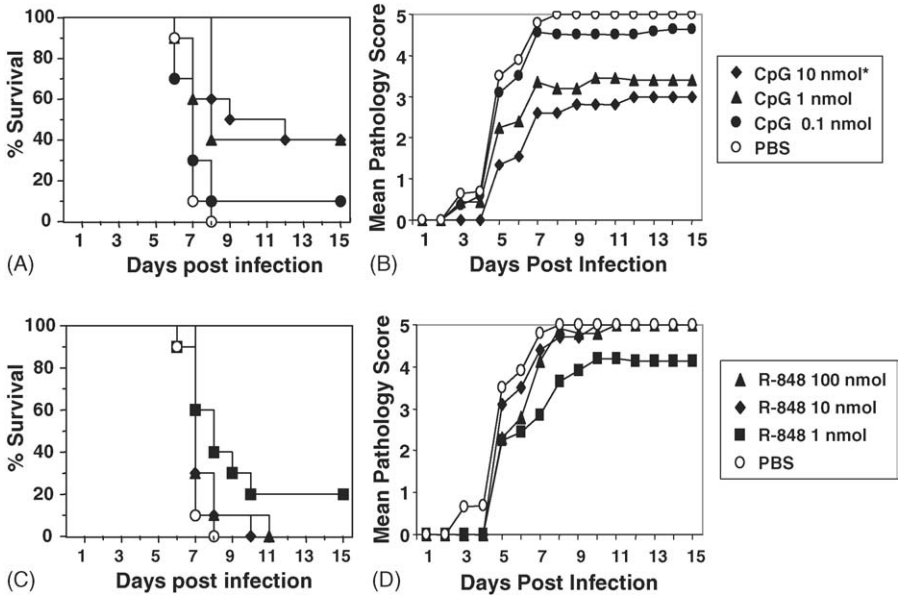

Fig. 2. Effects of local therapeutic administration of CpG or R-848 on protection from subsequent IVAG HSV-2 challenge. Groups of female C57BL/6 mice ($n = 10$ per group) were administered by the IVAG route 0.1 or 1.0 nmol CpG (ODN 2006), or 1, 10, or 100 nmol resiquimod (R-848) daily for 5 days starting 4 h after challenge with a lethal dose of HSV-2. Additional animals received a single administration of 10 nmol CpG 4 h after control (indicated by ★). Control animals were treated with PBS. Animals were monitored daily for morbidity and mortality. Panels A and C show percent survival following administration of CpG and R-848, respectively. Panels B and D show mean pathology score following administration of CpG and R-848, respectively.

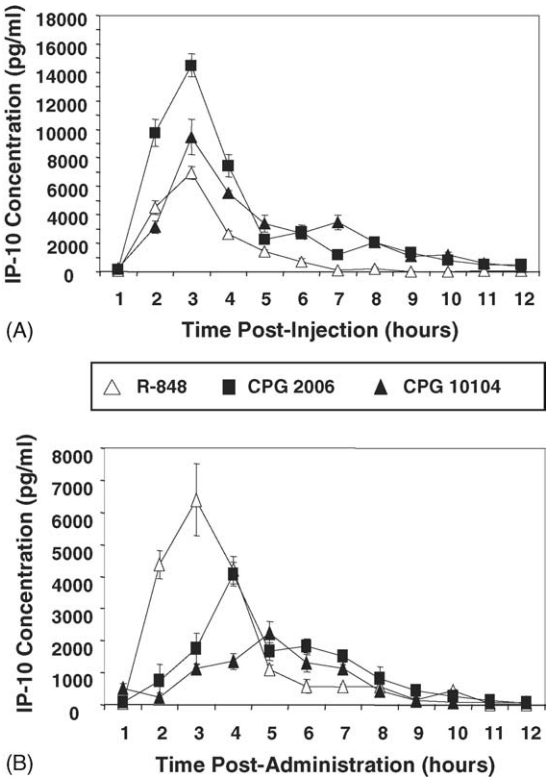

Fig. 3. Effects of local or parenteral CpG ODN or R-848 administration on plasma IP-10. Groups of female BALB/c mice ($n = 10$ per group per time-point) were administered 100 nmol CpG (ODN 2006 or 10104) or R-848 by SC injection or IVAG administration. Panel A shows IP-10 concentration in plasma of animals following SC injection. Panel B shows IP-10 concentration in plasma of animals following IVAG administration. Bars represent group geometric mean IP-10 concentration ± S.E.M.

in HSV-2 induced pathology compared to R-848 treated or PBS-treated mice (Fig. 1B). We show here (Fig. 4) and have also previously demonstrated that CpG ODN mediated protection against HSV-2 challenge is due to the presence of CpG motifs rather than a phosphorothioate backbone related effect since no protection occurs with a non-CpG ODN (Sajic et al., 2003).

3.2. Effects of therapeutic administration of CpG ODN or R-848

The therapeutic potential of CpG ODN or R-848 was compared when equimolar amounts were delivered IVAG daily for 5 days starting 4 h after IVAG HSV-2 infection. Mice treated with 1 nmol (~8 μg) CpG daily for 5 days had significantly enhanced protection against lethal HSV-2 challenge compared to PBS-treated control animals ($p < 0.0001$) (Fig. 2A). HSV-2 induced pathology was also reduced in CpG-treated animals compared to PBS-treated controls (Fig. 2B). Specifically, survival following multiple administration of 1 nmol CpG ODN was ~40%, which was equivalent to that of a 10-fold higher dose of CpG (10 nmol) given as a single administration 4 h after HSV-2 challenge ($p > 0.05$). In contrast, there was no significant protection or reduction in pathology against lethal HSV-2 challenge in mice treated with multiple administrations of R-848, even at the highest dose (100 nmol; ~31 μg) (Fig. 2C and D).

3.3. Effects of local or parenteral CpG ODN or R-848 administration on chemokine production

Following SC injection of CpG ODN or R-848 (100 nmol each) to mice, all groups exhibited high levels of plasma IP-10 that peaked at 3 h. CpG 2006 was somewhat more potent than CpG 10104, and CpG 10104 more potent than R-848 (Fig. 3A). This result confirms that despite the lack of protection associated with IVAG administration, the R-848 used in this study was biologically active. Furthermore, the R-848 used in this study has also been shown to have strong in vitro immunostimulatory effects on murine splenocytes (Weeratna et al., 2005). In con-

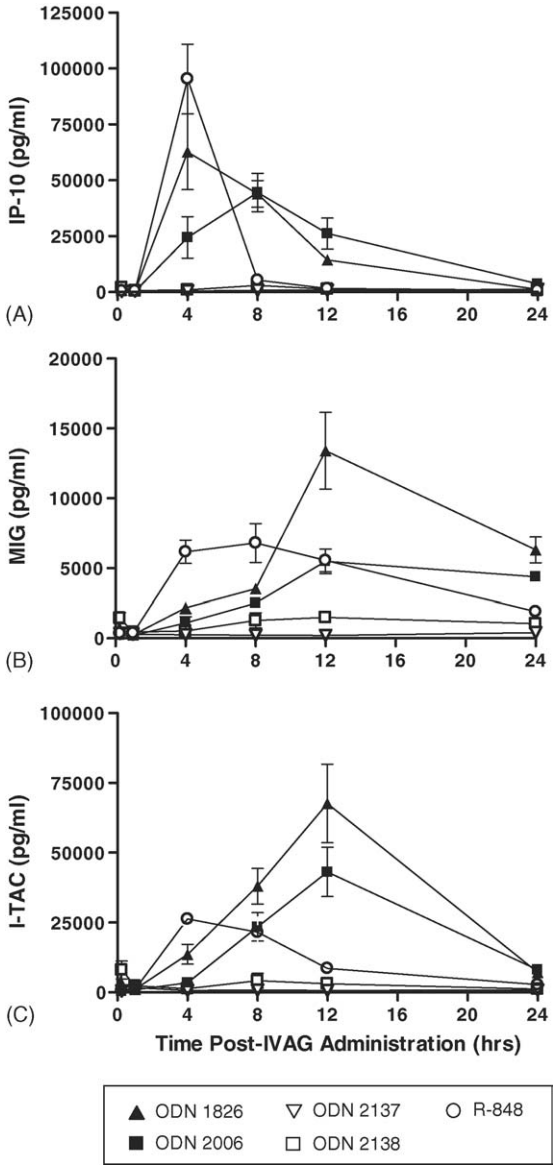

Fig. 4. Effects of local CpG ODN or R-848 administration on chemokines levels in vaginal tissue. Groups of female BALB/c mice ($n = 5$ per group per time-point) were administered 100 nmol CpG (ODN 2006, 1826), non-CpG control ODN (ODN 2137, 2138) or R-848 by IVAG administration. Vaginal tissues were collected at various times after administration. Bars represent group geometric mean IP-10 (panel A), MIG (panel B) and I-TAC (panel C) concentration in vaginal tissue extracts ± S.E.M.

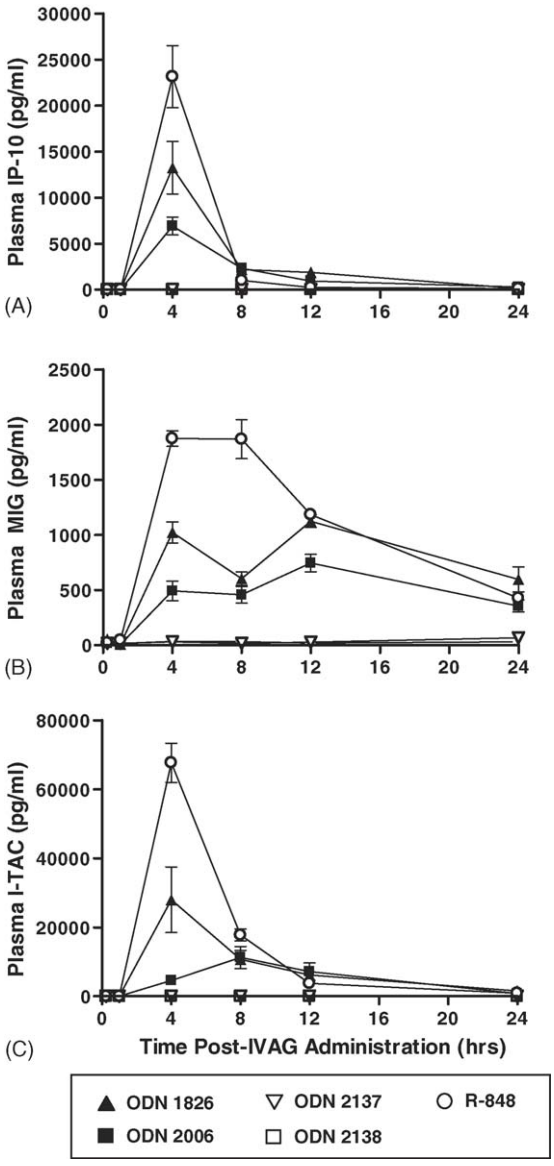

Fig. 5. Effects of local CpG ODN or R-848 administration on plasma CXC chemokines levels. Groups of female BALB/c mice ($n = 5$ per group per time-point) were administered 100 nmol CpG (ODN 2006, 1826), non-CpG control ODN (ODN 2137, 2138) or R-848 by IVAG administration. Plasma was collected at various times after administration. Bars represent group geometric mean IP-10 (panel A), MIG (panel B) and I-TAC (panel C) concentration in plasma ± S.E.M.

trast, after topical (IVAG) administration, the R-848 induced much higher plasma IP-10 levels than an equivalent (100 nmol) dose of CpG ODN. While the R-848 IP-10 response after IVAG instillation was of essentially the same magnitude ($p > 0.05$) and kinetics as after SC injection, IP-10 levels with CpG were only 20–25% as high with IVAG as with SC, and the peak with IVAG was delayed by 1–2 h (Fig. 3B). These results suggest that the biodistribution of R-848 but not CpG ODN may be similar with SC and IVAG administrations. The superior efficacy of IVAG CpG ODN against HSV-2, combined with the finding that it is inferior to SC administration for induction of systemic innate immune activation (plasma IP-10), suggests that its action when given by a mucosal route may be primarily local. Indeed, higher and/or more sustained levels of the chemokines IP-10, MIG and I-TAC were detected in vaginal tissues from animals administered IVAG CpG ODN (2006 or 1826) than in those animals administered IVAG R-848 or control non-CpG ODN (Fig. 4), whereas plasma chemokine levels were higher in animals that received R-848 compared to those that received CpG ODN (Fig. 5). This supports the notion of a better local effect by CpG ODN than R-848 after IVAG delivery, and also indicates that local but not systemic chemokine levels correlate with protection against HSV-2 challenge.

4. Discussion

Genital herpes remains one of the most common chronic viral infections worldwide. Despite progress in anti-viral therapies in recent years, alternative strategies are needed to enable the establishment of innate and adaptive mucosal immune responses against HSV-2 that can control the severity or frequency of outbreaks. One such strategy may be the use of immunomodulators that through activation of the innate immune system could reduce the severity of an outbreak and possibly lead to the generation of adaptive immune responses for long-term control.

Several reports have established the potent effect that the TLR9 agonist, CpG ODN, can have on HSV-2 infection in mice and guinea pigs when co-administered with HSV antigens (Gallichan et al., 2001; Gierynska et al., 2002). Stimulation of the innate immune system against HSV-2 infection by topical therapeutic or prophylactic administration of CpG ODN to the genital tract has also been demonstrated (Pyles et al., 2002; Harandi et al., 2003; Sajic et al., 2003). We have previously demonstrated that CpG ODN can have a protective effect against HSV-2 infection when administered 24 h prior to vaginal infection or up to 4 h after vaginal infection, i.e. during the acute phase of HSV-2 infection (Sajic et al., 2003). Similar findings have been reported by other groups in both mice and guinea pig models of HSV-2 infection (Pyles et al., 2002; Harandi et al., 2003). Intravaginal delivery of CpG ODN appears to result in a rapid recruitment of inflammatory cells to the vaginal epithelium, and a rapid proliferation and thickening of the genital epithelium that may enhance resistance to HSV-2 infection through induction of an innate anti-viral state (Ashkar et al., 2003). These events are associated with a rapid production of cytokines, in particular Th1-type cytokines such as IFN-γ, IL-12 and IL-18 and the chemokine RANTES of innate immunity (Harandi et al., 2003). More recently, we showed that local delivery of CpG to the vaginal mucosa induced IL-15 which was associated with protection against IVAG HSV-2 challenge (Gill et al., 2005). Following IVAG viral challenge with high doses of HSV-2, HSV-2-specific durable cellular and humoral immune responses can be detected in animals pre-treated locally with CpG ODN, and these can protect against subsequent challenge (Harandi et al., 2003). Therefore, it appears that local delivery of CpG ODN leads to a rapid, short-term innate anti-viral effect with a subsequent slower development of antigen-specific acquired immune responses. In all HSV-2 studies using CpG ODN to date, single doses of between 60 and 100 μg have been used (Pyles et al., 2002; Harandi et al., 2003; Sajic et al., 2003). Herein,

we now demonstrate that survival can also be enhanced to an equivalent level when considerably lower doses of CpG ODN (1 nmol; 8 μg) are given as multiple administrations for several days after infection. In our studies, CpG ODN was well tolerated with no apparent signs of adverse local events or systemic toxicity.

Therapeutic treatment of HSV-2 infection using the TLR7/8 agonist imiquimod, or the more potent resiquimod, is also associated with the production of cytokines, including interferon-alpha (IFN-α) and IL-12 (Bernstein et al., 2001; Harrison et al., 1994). Previous studies have shown that topical administration of such imidazoquinolines as immunomodulators can protect animals against HSV-2 infection when administered before or after inoculation with HSV-2 and to reduce the period of viral shedding and incidence of recurrences (Bernstein et al., 2001; Harrison et al., 1994). Protection appeared to be T cell mediated with a significant increase in IL-2 concentrations measured in these animals (Bernstein et al., 2001). However, the role of imidazoquinolines in HSV-2 therapy in humans is still uncertain. In a recent Phase II study, Aldara (cream-formulated imiquimod) failed to show an effect on the short-term natural history of genital herpes recurrences (Schacker et al., 2002), although pilot studies have indicated a reduction in recurrence of herpetic lesions with the use of resiquimod (Spruance et al., 2001).

In the present study, we compared CpG ODN and R-848 side by side in the same mouse HSV-2 challenge model as a single administration 24 h prior to challenge (prophylactic) or 4 h post-challenge (therapeutic) or as multiple (five daily doses starting at 4 h) dose treatments after infection. When applied topically in saline to the vaginal tract, moderate and high doses of CpG ODN were able to protect against lethal HSV-2 infection as demonstrated by enhanced survival and decreased pathology of disease. In contrast, there was little protective effect associated with R-848, even with multiple administrations at a high dose (100 nmol). The poor protection and therapeutic effect with R-848 in the current studies was somewhat surprising since imiquimod, a less potent analogue of R-848, has been demonstrated to be effective in prevention of HSV-2 infection in guinea pigs when administered topically (Bernstein and Harrison, 1989; Harrison et al., 1988), and R-848 has been shown to reduce recurrences in HSV-2 infected guinea pigs, albeit after parenteral administration (Bernstein et al., 2001). It is possible that imidazoquinolines may be less potent in mice than in guinea pigs or humans. These differences might also be related to the pathology of HSV-2 infection in mice and guinea pigs, in which it is acute or recurrent, respectively, and/or differences in administration protocols or dosing schedule. For example, it is possible that better results with R-848 might have been obtained in the murine model with a more extended period of treatment, since it has been previously shown in guinea pigs that daily topical administration of imiquimod starting 14 days after viral challenge reduces the incidence of later recurrence, if the treatment is continued for 21 days but not if it is stopped after 5 days (Harrison et al., 1994). In addition, doses used in guinea pigs with imiquimod have tended to be higher (5–10 mg/kg daily for 5 days) than the R-848 dose used in the present study in mice (30 μg, ∼1.5 mg/kg daily for 5 days) (Bernstein and Harrison, 1989; Harrison et al., 1988), and thus it is possible that better results with R-848 may be obtained in mice with higher doses.

Nevertheless, in this study, with identical single or multiple dosing regimes, CpG ODN was clearly superior to R-848 for protecting or treating mice in a HSV-2 challenge model. This difference is most likely due to the ability of the CpG ODN but not R-848 to stimulate strong local innate immunity in the vaginal tract.

The chemokine receptor CXCR3 is expressed on a number of different cell types including T cells, B cells and NK cells and is activated by the ligands CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC) (Murphy et al., 2000). These ligands are induced by IFN-γ and are important in Th1-type immune responses (Cole et al., 1998; Luster and Ravetch, 1987; Ogasawara et al., 2002). They are expressed in response to and are required for protection against a number of viral and bacterial infections (Liu et al., 2000; Lauw et al., 2000; Salazar-Mather et al., 2000; Cole et al., 2001; Mahalingam et al., 1999). When used as biomarkers for activation of innate immunity, either systemically (plasma) or locally (vaginal tissue), it was found that mucosally applied R-848 behaved much like SC injected drug with strong systemic but weak local innate immunity. This suggests that following IVAG administration, R-848 rapidly passes through the mucosal epithelium and passes directly into the blood where systemic activation occurs. In contrast, the systemic or local IP-10 response with CpG ODN correlated with the route of administration, suggesting that after IVAG delivery it remains in the mucosal area where it activates local immune cells. This is supported by previous findings that local, but not systemic, delivery of CpG ODN induces rapid proliferation and thickening of the genital epithelium and caused significant recruitment of inflammatory cells to the submucosa (Ashkar et al., 2003). Furthermore, IVAG administration of CpG ODN has been shown to rapidly increase levels of Th1 cytokines and chemokines in the genital tract and/or local draining lymph nodes, while systemic levels of IFN-γ, IL-18 or RANTES were unaffected (Harandi et al., 2003).

Collectively, the results indicate that, at least in mice, activation of local, but not systemic innate immunity can protect against a mucosal HSV-2 challenge. Indeed, we have previously demonstrated that parenteral delivery of CpG ODN does not protect against vaginal HSV-2 challenge (Sajic et al., 2003).

The differences in efficacy with IVAG CpG ODN and R-848 are likely related to their different molecular sizes and hence differences in biodistribution and sites of immune activation. It is also possible that the findings may reflect differences in expression of TLR7/8 and 9 in the genital tract or differences in signaling following activation of these receptors, especially for induction of a mucosal innate anti-viral response. Furthermore, in the present study, progesterone was used to synchronize mice in a diestrus-like state and to facilitate HSV infection. The administration of progesterone to mice has been shown to reduce immune responses to genital herpes infection (Kaushic et al., 2003), and therefore, it is possible that the immunosuppressive effects of progesterone may have acted on TLR7 induced responses, although this does not appear to have influenced TLR9-mediated immune responses.

If this were also the case in humans, it is possible that topical use of CpG ODN in humans chronically infected with HSV-2 would prove superior to the disappointing results already obtained with topical R-848. However, this would need to be tested in clinical studies since human vaginal epithelium, which differs from that in mice may affect drug biodistribution. As well, there are differences in the TLR 7/8 versus TLR9 cellular distributions between humans and mice that may affect outcomes.

References

Ashkar, A.A., Bauer, S., Mitchell, W.J., Vieira, J., Rosenthal, K.L., 2003. Local delivery of CpG oligodeoxynucleotides induces rapid changes in the genital mucosa and inhibits replication, but not entry, of herpes simplex virus type 2. J. Virol. 77, 8948–8956.

Bauer, M., Redecke, V., Ellwart, J.W., Scherer, B., Kremer, J.P., Wagner, H., Lipford, G.B., 2001a. Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells. J. Immunol. 166, 5000–5007.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G.B., 2001b. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U.S.A. 98, 9237–9242.

Bernstein, D.I., Harrison, C.J., 1989. Effects of the immunomodulating agent R837 on acute and latent herpes simplex virus type 2 infections. Antimicrob. Agents Chemother. 33, 1511–1515.

Bernstein, D.I., Harrison, C.J., Tomai, M.A., Miller, R.L., 2001. Daily or weekly therapy with resiquimod (R-848) reduces genital recurrences in herpes simplex virus-infected guinea pigs during and after treatment. J. Infect. Dis. 183, 844–849.

Cole, A.M., Ganz, T., Liese, A.M., Burdick, M.D., Liu, L., Strieter, R.M., 2001. Cutting edge: IFN-inducible ELR-CXC chemokines display defensin-like antimicrobial activity. J. Immunol. 167, 623–627.

Cole, K.E., Strick, C.A., Paradis, T.J., Ogborne, K.T., Loetscher, M., Gladue, R.P., Lin, W., Boyd, J.G., Moser, B., Wood, D.E., Sahagan, B.G., Neote, K., 1998. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. J. Exp. Med. 187, 2009–2021.

Corey, L., Langenberg, A.G., Ashley, R., Sekulovich, R.E., Izu, A.E., Douglas Jr., J.M., Handsfield, H.H., Warren, T., Marr, L., Tyring, S., DiCarlo, R., Adimora, A.A., Leone, P., Dekker, C.L., Burke, R.L., Leong, W.P., Straus, S.E., 1999. Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. Chiron HSV Vaccine Study Group. JAMA 282, 331–340.

Fleming, D.T., McQuillan, G.M., Johnson, R.E., Nahmias, A.J., Aral, S.O., Lee, F.K., St. Louis, M.E., 1997. Herpes simplex virus type 2 in the United States, 1976 to 1994. N. Engl. J. Med. 337, 1105–1111.

Gallichan, W.S., Woolstencroft, R.N., Guarasci, T., McCluskie, M.J., Davis, H.L., Rosenthal, K.L., 2001. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. J. Immunol. 166, 3451–3457.

Gibson, S.J., Lindh, J.M., Riter, T.R., Gleason, R.M., Rogers, L.M., Fuller, A.E., Oesterich, J.L., Gorden, K.B., Qiu, X., McKane, S.W., Noelle, R.J., Miller, R.L., Kedl, R.M., Fitzgerald-Bocarsly, P., Tomai, M.A., Vasilakos, J.P., 2002. Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. Cell Immunol. 218, 74–86.

Gierynska, M., Kumaraguru, U., Eo, S.K., Lee, S., Krieg, A., Rouse, B.T., 2002. Induction of CD8 T-cell-specific systemic and mucosal immunity against herpes simplex virus with CpG-peptide complexes. J. Virol. 76, 6568–6576.

Gill, N., Rosenthal, K.L., Ashkar, A.A., 2005. NK and NKT cell-independent contribution of interleukin-15 to innate protection against mucosal viral infection. J. Virol. 79, 4470–4478.

Halioua, B., Malkin, J.E., 1999. Epidemiology of genital herpes—recent advances. Eur. J. Dermatol. 9, 177–184.

Harandi, A.M., Eriksson, K., Holmgren, J., 2003. A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection. J. Virol. 77, 953–962.

Harrison, C.J., Jenski, L., Voychehovski, T., Bernstein, D.I., 1988. Modification of immunological responses and clinical disease during topical R-837 treatment of genital HSV-2 infection. Antiviral Res. 10, 209–223.

Harrison, C.J., Miller, R.L., Bernstein, D.I., 1994. Posttherapy suppression of genital herpes simplex virus (HSV) recurrences and enhancement of HSV-specific T-cell memory by imiquimod in guinea pigs. Antimicrob. Agents Chemother. 38, 2059–2064.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., Akira, S., 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat. Immunol. 3, 196–200.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740–745.

Hengge, U.R., Benninghoff, B., Ruzicka, T., Goos, M., 2001. Topical immunomodulators—progress towards treating inflammation, infection, and cancer. Lancet Infect. Dis. 1, 189–198.

Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F., Lanzavecchia, A., 2001. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur. J. Immunol. 31, 3388–3393.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., Liu, Y.J., 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 194, 863–869.

Kaushic, C., Ashkar, A.A., Reid, L.A., Rosenthal, K.L., 2003. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. J. Virol. 77, 4558–4565.

Koelle, D.M., Corey, L., 2003. Recent progress in herpes simplex virus immunobiology and vaccine research. Clin. Microbiol. Rev. 16, 96–113.

Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20, 709–760.

Krieg, A.M., 2004. Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. Curr. Oncol. Rep. 6, 88–95.

Krug, A., Towarowski, A., Britsch, S., Rothenfusser, S., Hornung, V., Bals, R., Giese, T., Engelmann, H., Endres, S., Krieg, A.M., Hartmann, G., 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur. J. Immunol. 31, 3026–3037.

Lauw, F.N., Simpson, A.J., Prins, J.M., van Deventer, S.J., Chaowagul, W., White, N.J., van der, P.T., 2000. The CXC chemokines gamma interferon (IFN-gamma)-inducible protein 10 and monokine induced by IFN-gamma are released during severe melioidosis. Infect. Immun. 68, 3888–3893.

Liu, M.T., Chen, B.P., Oertel, P., Buchmeier, M.J., Armstrong, D., Hamilton, T.A., Lane, T.E., 2000. The T cell chemoattractant IFN-inducible protein 10 is essential in host defense against viral-induced neurologic disease. J. Immunol. 165, 2327–2330.

Liu, T., Matsuguchi, T., Tsuboi, N., Yajima, T., Yoshikai, Y., 2002. Differences in expression of toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice. Infect. Immun. 70, 6638–6645.

Luster, A.D., Ravetch, J.V., 1987. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). J. Exp. Med. 166, 1084–1097.

Mahalingam, S., Farber, J.M., Karupiah, G., 1999. The interferon-inducible chemokines MuMig and Crg-2 exhibit antiviral activity in vivo. J. Virol. 73, 1479–1491.

Murphy, P.M., Baggiolini, M., Charo, I.F., Hebert, C.A., Horuk, R., Matsushima, K., Miller, L.H., Oppenheim, J.J., Power, C.A., 2000. International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol. Rev. 52, 145–176.

Naesens, L., De Clercq, E., 2001. Recent developments in herpesvirus therapy. Herpes 8, 12–16.

O'Farrell, N., 1999. Increasing prevalence of genital herpes in developing countries: implications for heterosexual HIV transmission and STI control programmes. Sex Transm. Infect. 75, 377–384.

Ogasawara, K., Hida, S., Weng, Y., Saiura, A., Sato, K., Takayanagi, H., Sakaguchi, S., Yokochi, T., Kodama, T., Naitoh, M., De Martino, J.A., Taniguchi, T., 2002. Requirement of the IFN-alpha/beta-induced CXCR3 chemokine signalling for CD8+ T cell activation. Genes Cells 7, 309–320.

Pasare, C., Medzhitov, R., 2005. Toll-like receptors: linking innate and adaptive immunity. Adv. Exp. Med. Biol. 560, 11–18.

Pyles, R.B., Higgins, D., Chalk, C., Zalar, A., Eiden, J., Brown, C., Van Nest, G., Stanberry, L.R., 2002. Use of immunostimulatory sequence-containing oligonucleotides as topical therapy for genital herpes simplex virus type 2 infection. J. Virol. 76, 11387–11396.

Sajic, D., Ashkar, A.A., Patrick, A.J., McCluskie, M.J., Davis, H.L., Levine, K.L., Holl, R., Rosenthal, K.L., 2003. Parameters of CpG oligodeoxynucleotide-induced protection against intravaginal HSV-2 challenge. J. Med. Virol. 71, 561–568.

Salazar-Mather, T.P., Hamilton, T.A., Biron, C.A., 2000. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. J. Clin. Invest. 105, 985–993.

Schacker, T., 2001. The role of HSV in the transmission and progression of HIV. Herpes 8, 46–49.

Schacker, T.W., Conant, M., Thoming, C., Stanczak, T., Wang, Z., Smith, M., 2002. Imiquimod 5-percent cream does not alter the natural history of recurrent herpes genitalis: a phase II, randomized, double-blind, placebo-controlled study. Antimicrob. Agents Chemother. 46, 3243–3248.

Spruance, S.L., Tyring, S.K., Smith, M.H., Meng, T.C., 2001. Application of a topical immune response modifier, resiquimod gel, to modify the recurrence rate of recurrent genital herpes: a pilot study. J. Infect. Dis. 184, 196–200.

Stanberry, L.R., Spruance, S.L., Cunningham, A.L., Bernstein, D.I., Mindel, A., Sacks, S., Tyring, S., Aoki, F.Y., Slaoui, M., Denis, M., Vandepapeliere, P., Dubin, G., 2002. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. N. Engl. J. Med. 347, 1652–1661.

Van Damme, L., Ramjee, G., Alary, M., Vuylsteke, B., Chandeying, V., Rees, H., Sirivongrangson, P., Mukenge-Tshibaka, L., Ettiegne-Traore, V., Uaheowitchai, C., Karim, S.S., Masse, B., Perriens, J., Laga, M., 2002. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet 360, 971–977.

Vasilakos, J.P., Smith, R.M., Gibson, S.J., Lindh, J.M., Pederson, L.K., Reiter, M.J., Smith, M.H., Tomai, M.A., 2000. Adjuvant activities of immune response modifier R-848: comparison with CpG ODN. Cell Immunol. 204, 64–74.

Wagner, H., 2004. The immunobiology of the TLR9 subfamily. Trends Immunol. 25, 381–386.

Weeratna, R.D., Makinen, S.R., McCluskie, M.J., Davis, H.L., 2005. TLR agonists as vaccine adjuvants: comparison of CpG ODN and resiquimod (R-848). Vaccine 23, 5263–5270.

Zeitlin, L., Whaley, K.J., 2002. Microbicides for preventing transmission of genital herpes. Herpes 9, 4–9.