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Research paper

# Detection of herpes simplex virus type 2 (HSV-2) -specific cell-mediated immune responses in guinea pigs during latent HSV-2 genital infection

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ABSTRACT

Genital infections with herpes simplex virus type 2 (HSV-2) are a source of considerable morbidity and are a health concern for newborns exposed to virus during vaginal delivery. Additionally, HSV-2 infection diminishes the integrity of the vaginal epithelium resulting in increased susceptibility of individuals to infection with other sexually transmitted pathogens. Understanding immune protection against HSV-2 primary infection and immune modulation of virus shedding events following reactivation of the virus from latency is important for the development of effective prophylactic and therapeutic vaccines. Although the murine model of HSV-2 infection is useful for understanding immunity following immunization, it is limited by the lack of spontaneous reactivation of HSV-2 from latency. Genital infection of guinea pigs with HSV-2 accurately models the disease of humans including the spontaneous reactivation of HSV-2 from latency and provides a unique opportunity to examine virus-host interactions during latency. Although the guinea pig represents an accurate model of many human infections, relatively few reagents are available to study the immunological response to infection. To analyze the cell-mediated immune response of guinea pigs at extended periods of time after establishment of HSV-2 latency, we have modified flow-cytometry based proliferation assays and IFN-γ ELISPOT assays to detect and quantify HSV-specific cell-mediated responses during latent infection of guinea pigs. Here we demonstrate that a combination of proliferation and ELISPOT assays can be used to quantify and characterize effecter function of virus-specific immune memory responses during HSV-latency.



## 1. Introduction

HSV-2 is an important human pathogen with approximately 16% of Americans and up to 80% of some populations worldwide being seropositive for this virus (Centers for Disease and Prevention, 2010; Mbopi-Keou et al., 2000). Animal models have proven useful for understanding pathogen-host interactions as well as for testing of antiviral compounds and vaccines against HSV-2. Both murine and guinea pig models of HSV-2 genital infection have been utilized in these capacities and provide complementary information about disease pathogenesis and host response. Infection of the vaginal mucosa of mice with HSV-2 requires pre-treatment with medroxy-progesterone and results in virus replication and generalized mucocutaneous disease of the vagina rather than the vesiculo-ulcerative disease commonly observed in humans. While HSV can establish a latent infection of the sensory neurons of mice, it does not reactivate spontaneously from latency as the virus does in humans. The strength of the murine model lies in the rich repository of reagents and of the availability of gene-depleted strains of mice for examination of immune cell phenotype and protective function. By contrast, few reagents for characterizing the host response of guinea pigs are available although the guinea pig model of genital HSV-2 infection more accurately mirrors the disease in humans (Fowler et al., 1992; Stanberry, 1991) and represents a unique system to examine pathogenesis and therapeutic efficacy of candidate antiviral compounds and vaccines (Bernstein et al., 2000; Bourne et al., 2005; Veselenak et al., 2012). Genital HSV-2 infection of guinea pigs can be achieved regardless of the hormonal state of the animal and results in a self-limiting quantifiable vulvovaginitis with neurologic and urologic complications mirroring those found in human disease. Primary disease in female guinea pigs involves virus replication in genital epithelial cells which is generally limited to eight days (Bourne et al., 2002). During this time, virus reaches sensory nerve endings and is transported to nerve

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cell bodies in the sensory ganglia. Following a brief period of acute replication at this site, the immune system usually resolves acute virus replication by day 15 post inoculation and the virus is maintained as a lifelong, latent infection of sensory neurons. Similar to humans, guinea pigs undergo spontaneous, intermittent reactivation of virus with virus shedding which can occur in the presence or absence of clinical symptoms.

In the current studies, we modified cell-mediated immune assays to allow detection and quantification of HSV-specific T lymphocyte function in lymphoid tissues of outbred guinea pigs experiencing a latent HSV-2 infection. We optimized a flow-cytometry-based proliferation assay to detect proliferation of both CD4 + and CD8 + lymphocytes and used a recently developed ELISPOT assay (Gillis et al., 2014; Xia et al., 2014) to quantify HSV-specific, IFN-γ secreting cells as a sensitive method for detecting virus-specific effector function. The availability of these assays should augment efforts to examine host-virus interactions during HSV-2 latency.

## 2. Materials and methods

### 2.1. Virus

HSV-2 strain MS stocks were prepared on Vero cell monolayers and stored at −80 °C as described previously (Bourne et al., 1999). The replication-defective HSV-2 strain, HSV-2 *dl5*-29, deleted of the HSV DNA replication protein genes UL5 and UL29, and the complementary cell line V529 expressing the UL5 and UL29 proteins (Da Costa et al., 2000) were a kind gift of Dr. David Knipe (Harvard Medical School, Boston, MA). Virus stocks were prepared as described previously by Xia et al. (Xia et al., 2014) and stored at −80 °C.

### 2.2. Guinea pigs

Female Hartley guinea pigs (250-300 g) were purchased from Charles River (Burlington, MA). Guinea pigs were maintained under specific pathogen free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal research center of the University of Texas Medical Branch. All animal research was humanely conducted and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch with oversight of staff veterinarians. Guinea pigs were infected by intravaginal (ivag) inoculation with 200 μl of a suspension containing $10^6$ PFU of HSV-2 strain MS as described previously (Bourne et al., 1999). Primary disease severity and frequency of spontaneous recurrent disease were scored daily as described previously (Valencia et al., 2013). Guinea pigs infected intravaginally 8–12 months previously with HSV-2 were used in this study.

### 2.3. Proliferation assay

Responder lymphocytes from uninfected or previously infected animals were obtained from single cell suspensions of spleen, inguinal lymph node (ingLN) or mesenteric lymph nodes (mLN). Responder cells were labeled with carboxy-fluorescein succinimydl ester (CFSE, Molecular Probes, Eugene, OR) in the dark as described previously (Nelson et al., 2011), washed and re-suspended in T cell medium Labeled cells were added to proliferation cultures at $1.6 \times 10^6$ cells and cultured for up to 96 h. For assays incorporating antigen-pulsed antigen presenting cells (APC), mLN cells were collected as APC and treated with Mitomycin C (Sigma-Aldrich, St. Louis, MO) for 20 min at 37 °C. APC were then washed and re-suspended in T cell medium (Iscove's Modified Dulbecco Medium, 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 50 μM 2-mercaptoethanol) and infected with HSV-2 dl5-29, pulsed with UV-killed HSV-2, or incubated in medium-only as a control. After 1 h incubation at 37 °C, cells were washed and labeled with CellTracker™ Orange CMRA (CTO) (Molecular Probes) for 15 min in the dark as described by the manufacturer. APC were washed, resuspended in T cell medium, and added at concentrations between $1 \times 10^5$ and $8 \times 10^5$ cells to each proliferation culture. Proliferation of CD4 + and CD8 + lymphoid cells was quantified by flow cytometric analysis. Red blood cells were removed from cultured cells by incubation in Red Blood Cell Lysis Buffer (Sigma-Aldrich) and cells were washed and resuspended in FACS media (10% FBS, 1% P/S, and 0.1% Na azide in RPMI). $F_c$ receptors were blocked by incubation of cells in 24G2 Antibody ($F_c$ Block, BD Biosciences, San Jose, CA) in FACS medium and stained with mouse anti-guinea pig CD8, anti-guinea pig CD4, or isotype control antibody followed by APC-labeled rat anti-mouse IgG antibody (Acris by OriGene, Herford, Germany). Cells were washed and fixed in 1% formaldehyde prior to analysis. Data were acquired on a BD FACSCanto II (BD Biosciences) at the UTMB Flow cytometry Core Facility and analyzed using FlowJo software (Tree Star, Ashland, OR).

### 2.4. ELISPOT assay

Ninety six well nitrocellulose plates were coated with protein- purified monoclonal antibodies specific for guinea pig IFNγ (V-E4.1.3 antibody, (Schafer et al., 2007), a kind gift of Dr. Hubert Schaefer, Robert Koch Institute, Berlin, Germany). After overnight incubation at 4 °C, plates were blocked with 2.5% bovine serum albumin (BSA) in PBS. APC were prepared from single cell suspensions of autologous mLN cells pulsed with UV-killed HSV-2, infected with *HSV-2 dl5*-29, or treated with medium as a control as described for proliferation assay APC and were added at $4 \times 10^5$ cells/well in T cell medium. Serial dilutions of effector lymphocytes beginning at $1 \times 10^6$ cells/well from single cell suspensions of spleen, mLN, or ingLN were added in duplicate. Plates were incubated at 37 °C for 48 h. Anti-guinea pig IFN-γ-specific monoclonal antibody N-G3.5 (Schafer et al., 2007) was purified from culture supernatants on protein A columns (Bio-Rad, Hercules, CA), and biotinylated using a biotinylation kit (Thermo Scientific, Rockford, IL) as described by the manufacturer and utilized as the detection antibody. At the end of culture incubation, plates were washed extensively to remove cells and the biotinylated anti-IFNγ detection antibody was added to plates and incubated overnight at 4 °C. Plates were then washed and streptavidin peroxidase in 2.5% BSA/PBS was added for 1 h at 37 °C. Plates were developed with aminoethylcarbamazole substrate containing $H_20_2$. HSV-specific IFN-γ secreting cells were quantified using an ImmunoSpot reader and analyzed with ImmunoSpot software (Cellular Technology Ltd., Cleveland, OH).

### 2.5. Statistics

Statistical differences for unpaired student *t*-test with Welsh's correction. Values for $p < 0.05$ were considered significant. All calculations were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. CFSE-based proliferation assay

To demonstrate the feasibility of a CFSE-based flow cytometric proliferation assay for guinea pig lymphocytes, splenocytes were labeled with CFSE, cultured with Concanavalin A (Con A), and samples were stained for CD8 or CD4 at 72 or 96 h of culture. Fig. 1 shows the gating scheme for demonstrating lymphocyte proliferation in Con A-stimulated cultures. The lymphocyte gate was selected from forward and side scatter plots and the lymphocyte subset gate was selected from allophycocyanin-CD8 or -CD4 (not shown) – stained cells. CFSE staining was measured in the selected lymphocyte populations. Very low levels of proliferation were detected in medium-stimulated cells at either the 72 or 96 h time points. However, extensive CFSE dilution indicative of