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Gr-1$^+$ cells, but not neutrophils, limit virus replication and lesion development following flank infection of mice with herpes simplex virus type-1

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ABSTRACT

Neutrophils are prominent in epidermal and dermal layers of human herpetic lesions and are rapidly recruited into the skin follow epidermal abrasion and infection of mice with herpes simplex virus type-1 (HSV-1). Herein, we demonstrate that early production of neutrophil-attracting chemokines KC/MIP-2 is associated with transient recruitment of neutrophils into the skin of HSV-1-infected mice in temporal association with the development of herpetic lesions. Treatment of HSV-1-infected mice with a Ly6G-specific mAb induced systemic neutropenia, but surprisingly did not alter virus replication or lesion development. In contrast, depletion of Gr-1$^+$ cells with mAb RB6-8C5 led to enhanced virus growth and lesion severity. Thus, while neutrophils are prominent in zosteriform lesions of HSV-1-infected mice, they do not appear to play a major role in controlling virus replication or lesion development and/or healing. In contrast, Gr-1$^+$ cells limit both virus replication and lesion development in the zosteriform model.

Introduction

Herpes simplex virus type-1 (HSV-1) and HSV-2 are double-stranded DNA viruses of the family *Herpesviridae* that infect mucosal surfaces, usually via epithelial tissue that has been damaged by abrasion or trauma (Cunningham et al., 2006). HSV-1 infection is accompanied by virus replication and destruction of epithelial cells, producing a primary lesion at the initial site of infection before establishment of latent infection in sensory neurons. Periodic reactivation results in shedding of virus at epithelial surfaces in the presence or absence of clinical symptoms.

HSV-1 pathogenesis has been investigated *in vivo* using several different routes of infection, including ocular, vaginal, footpad, intranasal, peritoneal and flank epidermal inoculation of mice (Hill et al., 1975; Hurd and Robinson, 1977; Reading et al., 2006; Simmons and Nash, 1984; Sydiskis and Schultz, 1965; Tullo et al., 1982; Walz et al., 1977). In the epidermal model, the flank is scarified or abraded in the presence of HSV-1 and 2–3 days later, a primary lesion forms at the site of inoculation. Within 4–7 days, secondary lesions form at sites distant from the inoculation site resulting from retrograde transport of virus from the inoculation site to the associated ganglion and subsequent anterograde transport to secondary sites in the epidermis (Simmons and Nash, 1984; Sydiskis and Schultz, 1965; van Lint et al., 2004). Thus, the timing and histopathology of disease in the murine flank model effectively mimics HSV-1 infection in humans (Simmons and Nash, 1984; Sydiskis and Schultz, 1965).

Protective immunity to HSV-1 requires a coordinated response by both innate and adaptive immune systems. During the early phase of HSV-1 infection, the antiviral activities of macrophages (Bauer et al., 2000; Sarmiento, 1988) and natural killer (NK cells) (Adler et al., 1999; Habu et al., 1984; Reading et al., 2006) have been implicated in limiting early virus replication and spread. Neutrophils are the first and predominant cell type observed in lesions associated with the development of herpetic stromal keratitis (Chen et al., 1996; Stumpf et al., 2002; Thomas et al., 1997) and in the brain after intranasal infection with neurovirulent HSV-1 (Marques et al., 2008). Following cutaneous footpad infection with HSV-1, neutrophils localized to both dermal and epidermal layers of the skin (Watanabe et al., 1999) and showed a similar distribution in the dermis and epidermis of human herpetic lesions (Patel et al., 2009).

Despite their prominence in HSV-1-induced lesions, the precise role neutrophils play in host defense and/or development of inflammatory lesions has not been clearly defined. Several studies have attempted to identify neutrophils in HSV-1-infected tissues using mAb RB6-8C5 (Bauer et al., 2007; Divito and Hendricks, 2008; Stumpf et al., 2002; Thomas et al., 1997; Yan et al., 1998), a mAb originally defined by its ability to bind to granulocyte receptor-1 (Gr-1) (Fleming et al., 1993). Moreover, antibody-mediated depletion of neutrophils with mAb RB6-

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8C5 has been associated with enhanced virus replication in ocular (Thomas et al., 1997; Tumpey et al., 1996) or intravaginal models (Milligan, 1999) of HSV-1 and HSV-2, respectively. In addition to the Ly6G antigen expressed at high levels by neutrophils, mAb RB6-8C5 binds to Ly6C which is expressed by a range of other leukocyte populations, including plasmacytoid dendritic cells (DC) (Nakano et al., 2001), monocytes (Geissmann et al., 2003) and CD8$^+$ T cells (Matsuzaki et al., 2003; Tumpey et al., 1996). While clear differences exist in the nature of the inflammatory responses elicited to HSV-1/HSV-2 at different sites of infection, studies using mAb RB6-8C5 to deplete Ly6G^{high} neutrophils in each of these murine models are likely to be complicated by the unwanted depletion of additional Ly6C$^+$ leukocyte subsets.

Recent studies have used Ly6G-specific mAb 1A8 to induce systemic depletion of Ly6G^{high} neutrophils, but not Gr-1^{high} blood monocytes (Daley et al., 2008) and antibody-mediated depletion of Ly6G$^+$ cells during influenza infection was associated with enhanced virus replication, pulmonary inflammation and death (Tate et al., 2009). Few neutrophils were recruited to the airways following intranasal infection with HSV-1 and treatment of mice with mAb RB6-8C5, but not mAb 1A8, was associated with exacerbated virus replication and disease (Wojtasiak et al.). Herein, we have used mAb 1A8 to identify and enumerate neutrophils in skin lesions and dorsal root ganglia (DRG) from C57BL/6 (B6) mice at various times after flank scarification with HSV-1. Moreover, we demonstrate that treatment of mice with either mAb 1A8 or mAb RB6-8C5 induced systemic neutropenia, however only depletion of Gr-1$^+$ cells was associated with increased virus replication and enhanced lesion severity in the zosteriform model. Together, these findings suggest that neutrophils do not play a major role in controlling HSV-1 infection in the skin. Furthermore, they further highlight the problems associated with the widespread use of antibody-mediated depletion of Gr-1$^+$ cells to define the role of neutrophils *in vivo*.

Results

Flank infection of C57BL/6 mice with HSV-1

HSV-1 typically initiates infection of mucosal membranes or skin, where the virus replicates in epithelial cells before establishing a latent infection in the associated sensory ganglia. In order to investigate the role of neutrophils during HSV-1 infection, we first characterized the parameters of disease that could be quantified following flank zosteriform infection of B6 mice with HSV-1. Epidermal abrasion and subsequent zosteriform lesion development in mice is an established model for the development of recrudescent lesions in humans (Simmons et al., 1992). During zosteriform disease, virus spreads from the primary inoculation site in the skin to sensory dorsal root ganglia (DRG) and subsequently reappears in the distal flank. This progression is seen in Fig. 1A, where inoculation of 10^6 PFU onto abraded skin of the flank (primary site) was associated with subsequent detection of infectious virus in DRG and skin (secondary site) after day 3 post-infection and mice developed an erythematous skin lesion that was graded daily over an 11 day observation period (Fig. 1B). No paralysis or death was observed over a 30-day monitoring period.

Recruitment of neutrophils to skin following intranasal or flank infection with HSV-1

We next assessed the cellular inflammatory response following flank infection of mice with 10^6 PFU of HSV-1, with a particular focus on characterizing the neutrophil response to infection. Based on previous findings (Daley et al., 2008), we have utilized mAb 1A8, specific for Ly6G, to identify neutrophils from naïve and HSV-1-infected mice. We have used mAb 1A8 to identify a distinctive population of Ly6G^{high} cells in the blood of uninfected mice and in the skin from HSV-1-infected mice (Fig. 2A) and cell sorting demonstrated that >95% of Ly6G^{high} blood leukocytes displayed the characteristic nuclear morphology of neutrophils when examined using light microscopy (data not shown).

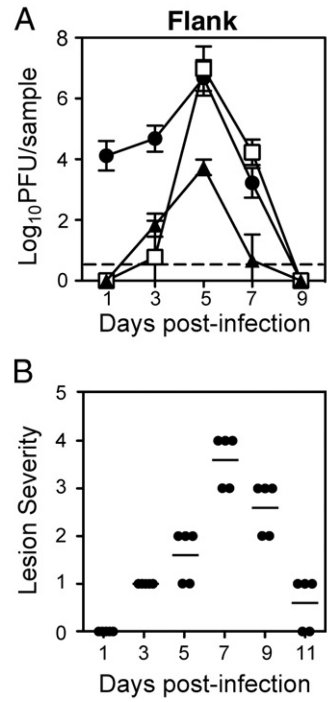

Fig. 1. Clinical disease and virus replication following flank infection of B6 mice with HSV-1. Groups of 5 mice were infected with 10^6 PFU of HSV-1 by flank scarification. (A) At the time-points indicated, skin samples (corresponding to the primary (black circles) and secondary (white squares) sites) and DRG (black triangles) were removed and titres of infectious virus were determined in clarified homogenates by plaque assay. Data represent mean virus titres ± 1 SD. The detection limit for the assay (0.6 Log_{10} PFU/sample) is indicated by the dotted line. (B) Lesion severity. Lesion severity was determined on following flank infection using the scoring system described in Materials and methods. Lesion scores from individual mice are shown are as circles and horizontal bars represent the mean lesion scores. Data are representative of 3 independent experiments.

Previous studies utilizing immunohistochemical staining with mAb RB6-8C5 have demonstrated neutrophil recruitment and accumulation in murine skin following inoculation of HSV-2 onto the abraded skin of the footpad (Watanabe et al., 1999). In the current study, collagenase/DNase treatment was used to prepare single cell suspensions of skin samples from HSV-1-infected mice and these were analyzed by flow cytometry for expression of CD45 and Ly6G. Fig. 2B demonstrates the accumulation of CD45$^+$ cells in the skin following flank infection of HSV-1, with neutrophils comprising 18 ± 6.5, 34 ± 7.2, 35 ± 6.4 and 9.0 ± 10.5% of CD45$^+$ leukocytes at days 3, 5, 7 and 10, respectively. Total leukocyte numbers recovered from the skin of uninfected mice were very low ($9.7 \times 10^3 \pm 1.8 \times 10^3$ cells/mouse) and neutrophils comprised 6.5 ± 0.5% of these cells. In contrast to results obtained in the skin, neutrophils comprised <5% of leukocytes infiltrating the DRG 3, 5, 7 and 10 days after flank infection with HSV-1 in 2 independent experiments.

Compared to skin from naïve animals, enhanced levels of the neutrophil chemoattractants KC and MIP-2 (Fig. 2C) were detected in homogenates of skin from HSV-1-infected mice at day 3 and day 7 post-infection, consistent with the timing of neutrophil infiltration. Compared to day 3 post-infection, levels of both KC and MIP-2 were significantly reduced at day 7, in agreement with our findings that neutrophil numbers had waned in skin lesions after this time (Fig. 2B). It should be noted that levels of MIP-2/KC in the skin of mice that