Depletion of Gr-1⁺, but not Ly6G⁺, immune cells exacerbates virus replication and disease in an intranasal model of herpes simplex virus type 1 infection

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In the absence of a viable 'knockout' mouse, researchers have relied extensively on monoclonal antibody (mAb) RB6-8C5 [anti-granulocyte receptor 1 (Gr-1)] to deplete neutrophils in murine models of inflammation and infection. Using an intranasal model of herpes simplex virus type 1 (HSV-1) infection, we demonstrate that mAb RB6-8C5 also binds to plasmacytoid dendritic cells, F4/80⁺ macrophages/monocytes and CD8⁺ T cells recovered from the airways of HSV-1-infected mice. In contrast, mAb 1A8 (anti-Ly6G) bound specifically to Ly6G high neutrophils. Following intranasal infection of C57BL/6 mice with HSV-1, few Ly6G high neutrophils were recruited to the airways and treatment of mice with purified mAb 1A8 induced systemic neutropenia, but did not alter virus replication or disease progression. In contrast, treatment of HSV-1-infected mice with mAb RB6-8C5 led to exacerbated virus replication, disease severity and mortality. These findings highlight the limitations associated with widespread use of antibody-mediated depletion of Gr-1⁺ cells to define the role of neutrophils in vivo. Furthermore, we use mAb 1A8 to demonstrate that specific depletion of neutrophils does not modulate disease or alter virus replication following intranasal infection with HSV-1.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is an important human pathogen that causes a variety of diseases ranging from mild skin lesions to life-threatening encephalitis. HSV-1 entry via the skin or mucosal membranes is accompanied by local virus replication before the virus ascends sensory neurons by retrograde axonal transport and establishes a latent infection within the neurons of the sensory ganglia. Periodic reactivation results in shedding of virus at epithelial surfaces in the presence or absence of clinical symptoms. Experimental murine models of corneal, flank and intranasal (i.n.) inoculation have been developed to gain insight into the immunopathological processes underlying herpetic stromal keratitis (HSK), herpetic skin lesions and virus-induced pneumonia, respectively, during natural HSV-1 infections.

Neutrophils are a prominent feature of the cellular infiltrate at sites of HSV-1 infection and numerous studies have attempted to elucidate their role in early host defence and/or in HSV-1-induced immunopathology. Inflammatory-cell infiltration has been particularly well-characterized following infection of the murine cornea with HSV-1. Here, an early and transient influx of neutrophils into the corneal stroma is followed by a ‘second wave’ of infiltration into both the cornea and the eyelids, which is associated with the development of HSK (Chen et al., 1996; Stumpf et al., 2002; Thomas et al., 1997). Infiltrating cells produce inducible nitric oxide synthase, tumour necrosis factor alpha (TNF-α) and interleukin (IL)-12 (Daheshia et al., 1998), and neutrophil-derived chemokines and cytokines have been proposed to contribute to control of early virus replication as well as the induction of subsequent CD4⁺ T-cell-mediated corneal pathology (Thomas et al., 1997; Tumpey et al., 1996). Neutrophils were also prominent in the cellular infiltrate at other sites of HSV-1 infection, including the skin following cutaneous footpad infection (Watanabe et al., 1999), the brain following i.n. infection with neurovirulent strains of HSV-1 (Marques et al., 2008) and in human cutaneous and mucosal herpesvirus vesicles (Worrell & Cockerell, 1997). Of interest, neutrophils were virtually absent in cellular exudates recovered from the trigeminal ganglion (TG) after corneal infection of mice (Liu et al., 1996), indicating that different factors are likely to regulate

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cellular recruitment to sensory ganglion during HSV-1 infections.

Monoclonal antibody (mAb) RB6-8C5, originally described as binding to granulocyte receptor 1 (Gr-1) (Fleming et al., 1993), has been used extensively to deplete Ly6G+ neutrophils in murine models of infection and inflammation. In the context of HSV-1 infection, Gr-1 expression has been used to identify neutrophil infiltration in immunohistological (Banerjee et al., 2004; Bauer et al., 2007; Stumpf et al., 2002; Thomas et al., 1997; Yan et al., 1998) and flow-cytometric (Divito & Hendricks, 2008) analysis of cellular infiltrates from virus-infected tissues. Moreover, antibody-mediated depletion of Gr-1+ cells (with mAb RB6-8C5 to induce neutropenia) led to enhanced virus replication following ocular infection with HSV-1 (Thomas et al., 1997; Tumpey et al., 1996; Zheng et al., 2008) or intravaginal inoculation with virulent or attenuated HSV-2 (Milligan, 1999; Milligan et al., 2001).

Whilst Ly6G has been shown to be the major antigen detected by mAb RB6-8C5 (Fleming et al., 1993), there is growing evidence to suggest that this mAb also binds to the Ly6C antigen expressed on other leukocytes, including monocytes (Geissmann et al., 2003; Henderson et al., 2003), plasmacytoid dendritic cells (pDCs) (Nakano et al., 2001; Palamara et al., 2004) and CD8+ T cells (Matsuzaki et al., 2003; Tumpey et al., 1996). Thus, caution must be exercised when interpreting studies using mAb RB6-8C5 to induce neutropenia, as the results may be complicated by the unwanted depletion of other Gr-1+ cells. A recent study by Daley et al. (2008) illustrated that the Ly6G-specific mAb 1A8 (anti-Ly6G) could deplete neutrophils from mice, but that this treatment had no impact on Gr1high blood monocytes. Thus, use of anti-Ly6G mAbs could allow for the specific depletion of neutrophils without the problematic effect of binding to Ly6C+ cells associated with the use of anti-Gr-1 mAbs.

We present studies aimed at defining the role of neutrophils following i.n. inoculation of C57BL/6 (B6) mice with HSV-1. Flow cytometry demonstrated that few Ly6Ghigh neutrophils were recruited to the lungs following i.n. inoculation with HSV-1. Treatment of mice with purified mAb 1A8 induced systemic and local neutropenia, but did not alter virus replication or disease progression. In contrast, treatment of mice with mAb RB6-8C5 led to exacerbated virus replication and severe disease in the i.n. model of HSV-1 infection. These findings highlight the limitations of the widespread use of antibody-mediated depletion of Gr-1+ cells to define the role of neutrophils in vivo.

RESULTS

I.n. infection of B6 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 infection of the murine respiratory tract with HSV-1. I.n. inoculation of B6 mice with 106 p.f.u. HSV-1 strain KOS was accompanied by virus replication in the lung and nasal tissues (Fig. 1a), as well as mild and transient weight loss (Fig. 1b). Sporadic virus replication was detected in TG 3–5 days post-infection (p.i.) (data not shown) and latent infection was established in TG of all infected animals (Reading et al., 2006, 2007).

Recruitment of neutrophils to the airways following i.n. infection with HSV-1

We next assessed the cellular inflammatory response following inoculation of mice with 106 p.f.u. HSV-1 by i.n. infection, with a particular focus on characterizing the neutrophil response to infection. Based on previous

![Fig. 1. Virus replication and clinical disease following intranasal infection of B6 mice with HSV-1. Groups of five mice were infected with 10^6 p.f.u. HSV-1 via the i.n. route. (a) At various times p.i., mice were euthanized and nasal tissue (□) and lungs (●) were removed. Titres of infectious virus in clarified homogenates were determined by standard plaque assay on Vero cells. Data represent mean ± SD virus titres. The detection limit of the assay is 1.6 log_{10} p.f.u. per sample, indicated by the dashed line. (b) Weight change of HSV-1-infected mice. Mice were weighed daily and results are expressed as the mean ± SEM percentage weight change of each group, compared with the weight immediately prior to infection. ▲, PBS-treated mice; ●, HSV-1-infected animals.](http://vir.sgmjournals.org)
findings (Daley et al., 2008), we have utilized mAb 1A8, specific for Ly6G, to identify neutrophils from naïve and HSV-1-infected mice. In preliminary experiments, a distinctive population of Ly6G<sup>high</sup> cells (i.e. mAb 1A8<sup>high</sup>) was observed in the blood of uninfected mice or in bronchoalveolar lavage (BAL) from HSV-1-infected mice (Fig. 2a), and cell sorting demonstrated that >95% of Ly6G<sup>high</sup> blood leukocytes were neutrophils when examined microscopically (data not shown). Cytospin analysis (Fig. 2a) confirmed that neutrophils were present in BAL at low numbers 3 days after HSV-1 infection.

I.n. infection with HSV-1 was associated with the rapid recruitment of leukocytes to the airways, with peak numbers recorded between 3 and 7 days p.i. in BAL and on day 3 p.i. in nasal tissues (Fig. 2b). Using mAb 1A8, we found that Ly6G<sup>high</sup> neutrophils comprised only a small percentage (10%) of cells recruited to the airways after i.n. HSV-1 infection (Reading et al., 2006, 2007). Neutrophils comprised <10% of leukocytes in cell suspensions from pooled TG samples at days 3, 5 and 7 p.i. in two independent experiments (data not shown). Neutrophils also comprised a small percentage (<10%) of cells recruited to the airways 3 or 7 days after i.n. infection with an equivalent dose of HSV-1 strain SC-16 (data not shown).

**Selective expression of Ly6G by murine neutrophils from naïve or HSV-1-infected mice**

mAb RB6-8C5 (anti-Gr-1) has been used extensively to detect and deplete neutrophils from mice (reviewed by Egan et al., 2008); however, in addition to Ly6G<sup>high</sup> neutrophils, this mAb cross-reacts with the Ly6C antigen expressed by pDCs and subsets of monocytes and CD8<sup>T</sup> T cells (Geissmann et al., 2003; Matsuzaki et al., 2003; Nakano et al., 2001). Thus, mAb 1A8, which is specific for Ly6G, may offer significant advantages over mAb RB6-8C5 for both identification and specific depletion of neutrophils in murine models of infection and inflammation.

We therefore compared the ability of mAbs 1A8 and RB6-8C5 to bind to specific leukocyte subsets in lung-cell suspensions prepared from naïve or HSV-1-infected mice. mAb RB6-8C5 (Ly6C/G or Gr-1) bound strongly to pDCs, F4/80<sup>+</sup> cells and some CD8<sup>T</sup> T cells from naïve mice (Fig. 3a). Of interest, Ly6C/G expression was upregulated markedly on some CD8<sup>T</sup> T cell, pDC and conventional DCs (cDCs) leukocyte populations in the lung 7 days after i.n. infection of mice with HSV-1 (Fig. 3b). In recent studies, enhanced binding of mAb RB6-8C5 (Ly6C/G) to CD8<sup>T</sup> T cells, pDCs and cDCs in the lungs of influenza virus-infected mice was shown to correlate with enhanced expression of Ly6C, as detected by mAb AL-21 (Tate et al., 2008). Ly6C/G expression, as detected by binding of mAb RB6-8C5, increased markedly on CD8<sup>T</sup> T cells following HSV-1 infection (34% of cells from uninfected mice expressed Ly6C/G compared with 78% from HSV-1-infected mice), suggesting that upregulated expression may be associated with CD8<sup>T</sup> T-cell activation. In contrast, mAb 1A8 (Ly6G) did not bind to any of the additional leukocyte populations examined on lung cells recovered from naïve (Fig. 3a) or HSV-1-infected (Fig. 3b) mice.

**mAb RB6-8C5, but not mAb 1A8, binds to and depletes virus-specific CD8<sup>T</sup> T cells from mice**

Based on the findings described above, treatment of mice with mAb RB6-8C5 is likely to deplete neutrophils and additional leukocyte populations, including CD8<sup>T</sup> T cells, thereby complicating the interpretation of studies during...
the latter phase of infection. Virus-specific CD8\(^+\) T cells proliferate and gain effector function in local lymph nodes before their release and subsequent appearance at sites of infection 5–7 days following i.n. infection with HSV-1 (Reading et al., 2006). Therefore, it was of critical importance to determine the ability of mAb 1A8 to bind to virus-specific CD8\(^+\) T cells. The HSV-1-specific CD8\(^+\) T-cell response in B6 mice is largely directed towards the immunodominant SSIEFARL epitope in the glycoprotein B (gB) of the virus (Wallace et al., 1999). mAb RB6-8C5 (Ly6C/G or Gr-1) bound to gB-specific CD8\(^+\) T cells from the BAL of mice infected via the i.n. route with HSV-1 (Fig. 4a, b).
We next compared the ability of mAb RB6-8C5 and mAb 1A8 to deplete CD8+ T cells directly during HSV-1 infection. Mice were infected with HSV-1 and 9 days later received a single treatment with purified mAb RB6-8C5, mAb 1A8 or IgG; CD8+ T-cell responses were examined 1 day later. The rationale behind these studies was that, by day 9 p.i., the neutrophil response had peaked and subsided; thus, any effects observed on the numbers of CD8+ T cells were likely to reflect direct depletion by antibody, rather than a role for neutrophils in modulating CD8+ responses. A single treatment with mAb RB6-8C5 at day 9 p.i. led to a significant reduction in numbers of total and gB-specific CD8+ T cells in the BAL (Fig. 4c) and spleen (data not shown) of animals infected via the i.n. route. Equivalent treatment with mAb 1A8 did not alter the magnitude of CD8+ T-cell responses. Thus, antibody-mediated depletion of neutrophils using mAb 1A8 may be utilized to examine the role of neutrophils during both early and later phases of infection, while studies using anti-Gr-1 antibodies to deplete neutrophils should be interpreted with caution, particularly following the expansion and release of CD8+ effector T cells in the periphery.

**Treatment of HSV-1-infected mice with mAb RB6-8C5, but not mAb 1A8, alters virus replication, airway inflammation and disease severity**

To address the role of neutrophils following HSV-1 infection of the respiratory tract, we first compared virus titres in clarified homogenates prepared from lungs, nasal tissues, TG or brain of 1A8-treated mice with those in equivalent samples from IgG-treated controls. A third group was treated in an identical manner with purified equivalents from IgG-treated controls. A third group was treated in an identical manner with purified mAb RB6-8C5 (Ly6C/G) to determine the effect of depleting Gr-1+ cells during HSV-1 infection. Mean titres of infectious HSV-1 were similar in lungs (Fig. 5a) and nasal tissues (Fig. 5b) of 1A8-treated animals and IgG-treated controls at day 3 p.i. and lytic virus was cleared from all animals by day 7 p.i. In contrast, treatment of mice with purified mAb RB6-8C5 was associated with elevated virus titres in the nasal tissues and lungs at day 3 p.i. and delayed virus clearance, with infectious virus recovered from RB6-treated animals at day 7 p.i. (Fig. 5a, b). Virus titres in TG were not significantly different between IgG-, 1A8- or RB6-treated animals at day 3 or 7 p.i. (P<0.05, one-way ANOVA; data not shown) and infectious virus was not recovered from the brain at either time point.

In addition, at day 7 p.i., we observed reduced levels of gamma interferon (IFN-γ) in BAL from RB6-8C5-treated mice (1622±406, 1877±509 and 506±156 pg ml⁻¹ for IgG-, 1A8- and RB6-8C5-treated mice, respectively; P<0.05, RB6-8C5-treated mice compared with either IgG- or 1A8-treated mice, one-way ANOVA) and increased levels of monocyte chemotactic protein-1 (MCP-1) (41±17, 35±21 and 171±109 pg ml⁻¹ for IgG-, 1A8- and RB6-8C5-treated mice, respectively; P<0.05, RB6-8C5-treated mice compared with either IgG- or 1A8-treated mice directly during HSV-1 infection.

**Fig. 5.** Treatment of mice with anti-Gr-1, but not anti-Ly6G, antibodies exacerbates virus replication and disease following i.n. infection with HSV-1. Groups of five B6 mice were depleted of neutrophils via i.n. and i.p. administration of purified mAb 1A8 (‘1A8’) or mAb RB6-8C5 (‘RB6’) 24 h prior to infection and every 48 h thereafter. Control groups received an equivalent concentration of rat IgG (‘IgG’). Mice were euthanized at day 3 or 7 p.i. and virus titres in (a) lungs and (b) nasal tissues were determined by standard plaque assay. The dashed line represents the detection limit of the assay. Bars represent mean±SD viral titre. Virus titres from mice treated with anti-Gr-1 antibodies were significantly greater than those from Ly6G-treated or IgG-treated mice (*P<0.05; **P<0.01; one-way ANOVA followed by Tukey’s multiple comparison test). (c) Mice infected by the i.n. route with 10⁶ p.f.u. HSV-1 were weighed daily and results are expressed as the mean±SEM percentage weight change of each group, compared with original body weight. Data for mice treated with RB6-8C5 (▲), 1A8 (■) or control IgG (○) are shown from one experiment and are representative of two or more independent experiments. (d) Survival of HSV-1-infected mice treated with mAb 1A8 or RB6-8C5. Mice infected via the i.n. route with 10⁶ p.f.u. (■, □) or 5x10⁶ p.f.u. (▲, △) of HSV-1 were treated with RB6-8C5 (filled symbols) or 1A8 (empty symbols). Mice displaying evidence of pneumonia and/or having lost >25% of their original body weight were euthanized. Data shown are from two independent experiments.
mice, one-way ANOVA) and IL-6 (24 ± 8, 35 ± 18 and 147 ± 89 pg ml⁻¹ for IgG-, 1A8- and RB6-8C5-treated mice, respectively; P<0.05, RB6-8C5-treated mice compared with either IgG- or 1A8-treated mice, one-way ANOVA). Levels of TNF-α, IL-10 and IL-12 were not statistically significantly different in BAL from IgG-, 1A8- or RB6-8C5-treated mice at day 7 p.i. (data not shown).

Next, we compared weight loss in mice treated with IgG, 1A8 or RB6 as a measure of the severity of the clinical disease following i.n. HSV-1 infection. Antibody-mediated depletion of neutrophils with mAb 1A8 did not exacerbate weight loss during infection; however, animals treated with mAb RB6-8C5 lost more weight than IgG-treated controls (Fig. 5c). I.n. inoculation with 10⁶ p.f.u. HSV-1 was not associated with any mortality in 1A8- or RB6-8C5-treated animals (Fig. 5d); however, infection with a higher inoculum dose (5 × 10⁶ p.f.u.) led to 100% mortality in mice treated with RB6-8C5, whereas no animals treated with mAb 1A8 succumbed to infection (Fig. 5d). Thus, in the i.n. model, marked differences were observed in virus replication and clinical disease following antibody-mediated depletion of Gr-1⁻ (Ly6C/G) cells. Selective depletion of Ly6G⁺ cells with mAb 1A8 indicated that neutrophils do not play a critical role in control or clearance of HSV-1 from the respiratory tract in this model.

**DISCUSSION**

Neutrophils are a prominent feature of the cellular infiltrate associated with a number of experimental models of HSV-1 infection; however, their roles in host defence and/or in HSV-1-induced immunopathology have not been clearly defined. Underlying this are concerns arising from the widespread use of mAb RB6-8C5 (anti-Gr-1) to identify and deplete neutrophils in murine models of infection and inflammation, including HSV-1 (Thomas et al., 1997; Tumpey et al., 1996; Zheng et al., 2008). Herein, we demonstrate widespread expression of Gr-1 (Ly6C/G) on leukocyte populations, and show Gr-1 to be upregulated on certain leukocyte subsets during HSV-1 infection. In contrast, Ly6G was expressed at high levels in the i.n. model of HSV-1 infection, but antibody-only by neutrophils in naïve or HSV-1-infected mice.

Neutrophils comprise a small proportion of inflammatory leukocytes recruited to the airways following HSV-1 infection, consistent with our previous findings that activated NK cells and CD8⁺ T cells predominate during the acute phase of pulmonary HSV-1 infection (Reading et al., 2006). Following i.n. infection, immunohistochemical staining localized HSV-1 antigens to epithelial cells lining the airways (M. Wojtasiak & P. C. Reading, unpublished observations). Moreover, *in vitro* studies have shown that viruses such as respiratory syncytial virus readily induce chemokines, including the neutrophil-attracting chemokine KC, from airway epithelial cells (Miller et al., 2004). Whilst HSV-1-induced chemokine responses are yet to be clearly defined in the i.n. model of infection, it is likely that the very different cellular infiltrates observed in the lung compared with neutrophil-rich exudates observed in HSV-1-infected cornea (Chen et al., 1996; Stumpf et al., 2002; Thomas et al., 1997) or skin (Watanabe et al., 1999) will reflect differential profiles of chemokine production at the respective sites of infection and inflammation. Inoculation with different virus strains has been shown to induce different inflammatory infiltrates in the HSV-1-infected cornea (Hendricks & Tumpey, 1990), suggesting the possibility that particular HSV-1 strains might potentiate a more robust recruitment of neutrophils to the airways.

Following HSV-1 infection of the cornea, virus is transmitted to the TG where, after a brief period of virus replication, latency infection is established. Neutrophils represent the major cell type infiltrating the cornea, but were virtually absent from the TG during acute or latent phases of infection (Liu et al., 1996). Herein, we demonstrate that neutrophils were a minor component of the cellular infiltrate in TG following i.n. infection. Whilst there is considerable evidence that neutrophils contribute to inflammatory injury in rodent models of neuronal injury (Ryu et al., 2007; Taoka et al., 1997; Yamasaki et al., 1997), neutrophil invasion of dorsal root ganglia (DRG) occurs rarely (McLachlan et al., 2007; Morin et al., 2007). Neutrophils can, however, promote neurotoxicity of DRG *in vitro* by release of metalloproteinases, reactive oxygen species and cytokines (Nguyen et al., 2007; Shaw et al., 2008), suggesting that regulatory mechanisms may limit infiltration of neutrophils into ganglia during acute HSV-1 infection to avoid immunopathological destruction of sensory neurons. Previous studies have described early infiltration of NK cells and γδ-TCR⁺ cells, and subsequently CD8⁺ T cells and macrophages, into TG following corneal infection (Liu et al., 1996). In addition, CD8⁺ T cells have been implicated in eliminating lytic virus from neurons and maintaining the integrity of the sensory nervous system during HSV-1 infections (Simmons et al., 1992; van Lint et al., 2004). Together, these observations suggest that neutrophils play little role in modulating HSV-1 infection in the peripheral nervous system.

HSV-1 can infect a variety of mucosal tissues, including the lung, and herpetic respiratory infections can lead to a range of pathological conditions, including pneumonia. HSV
encephalitis can occur following virus dissemination and is the major cause of sporadic fatal encephalitis (Sköldenberg, 1996; Whitley & Roizman, 2001). Given the low levels of neutrophil recruitment observed following i.n. infection with HSV-1, our findings that specific depletion of Ly6G<sup>high</sup> neutrophils did not exacerbate virus replication or clinical disease were not surprising. In contrast, neutrophils are prominent in the airways following i.n. infection of mice with influenza viruses (Perrone et al., 2008; Tate et al., 2008; Tumpey et al., 2005), and treatment of influenza virus-infected mice with purified mAb 1A8 was associated with development of viral pneumonia, rapid weight loss and death (Tate et al., 2009). Our findings that treatment of HSV-1-infected mice with mAb RB6-8C5 led to exacerbated weight loss and replication of infectious virus in the airways indicate that, in addition to neutrophils, this treatment led to depletion of additional Gr-1<sup>+</sup> leukocytes that play an important role in early host defence following HSV-1 infection of the airways.

To date, a number of studies have addressed the role of neutrophils during the early and late phases of HSV-1 infection (Thomas et al., 1997; Tumpey et al., 1996; Zheng et al., 2008); however, the use of mAb RB6-8C5 to eliminate Gr-1<sup>+</sup> cells is likely to affect critical cell types associated with both innate (pDCs, cDCs and F4/80<sup>+</sup> cells) and adaptive (cDCs and CD8<sup>+</sup> T cells) antiviral responses and will inevitably yield an ambiguous result. Treatment of mice with low doses (<50 μg) of mAb RB6-8C5 has been used in an attempt to induce selective depletion of neutrophils with minimal impact on other leucocyte populations (Han & Cutler, 1997; Tvinnereim et al., 2004); however, in our hands such doses did not induce sufficient levels of neutropenia and the neutrophils that escaped depletion were found to accumulate in the airways or in the skin during infection (M. D. Tate & P. C. Reading, unpublished observations). Elimination of Gr-1<sup>+</sup> cells from immunedeficient animals lacking particular components of cell-mediated immunity represents an additional approach to address the importance of neutrophils (Tate et al., 2008; Tumpey et al., 1996; Wipke & Allen, 2001); however, deciphering their role remains complicated by the widespread expression of Gr-1. Selective depletion of Ly6G<sup>+</sup> cells using mAb 1A8 will allow the role of neutrophils to be more clearly defined in early host defence against a range of microbial infections. Furthermore, recent interest in the ability of neutrophils not only to participate in innate defence, but also to provide antigens for presentation by DCs (Abadie et al., 2005; Beauvillain et al., 2007; Tvinnereim et al., 2004) and/or immunomodulatory molecules to shape CD8<sup>+</sup> T-cell responses (Molesworth-Kenyon et al., 2005; Seiler et al., 2003; Xiaoxiao et al., 2007) could be investigated via specific depletion of Ly6G<sup>+</sup> neutrophils.

**METHODS**

**Mice and viruses.** C57BL/6 (B6) mice were bred and housed in specific-pathogen-free conditions at the Department of Microbiology and Immunology, University of Melbourne, Australia. Male mice (6–10 weeks old) were used in all experiments. The KOS strain of HSV-1 was grown and titrated on Vero cells. Vero cells were grown in minimal essential medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS; CSL Ltd), 4 mM L-glutamine (Sigma-Aldrich), 5 × 10−5 M 2-ME (MP Biomedicals) and antibiotics [100 μg benzylpenicillin ml<sup>−1</sup> (Csl Ltd) and 30 μg streptomycin sulphate ml<sup>−1</sup> (Gibco-BRL)]. Virus stocks were stored at −70 °C until required.

**Infection and treatment of mice.** For respiratory-tract infection, mice were anaesthetized and infected with 10<sup>5</sup> p.f.u. HSV-1 via the i.n. route in 50 μl PBS. Mice were weighed daily and assessed for visual signs of clinical disease, including inactivity, ruffled fur, laboured respiration and huddling behaviour. Animals that lost ≥20% of their original body weight and/or displayed evidence of pneumonia or neurological abnormalities were euthanized. All research complied with the University of Melbourne’s Animal Experimentation Ethics guidelines and policies.

At various times after infection, mice were euthanized and the lungs, nasal tissues and TG were removed, homogenized in PBS and clarified by centrifugation at 650 <i>g</i> at 4 °C until required. Differential leucocyte counts (described below) were performed at the completion of each experiment and confirmed >90% depletion of blood neutrophils using these treatment regimes.

**Neutrophil depletions.** For the depletion of neutrophils in vivo, purified anti-Ly6G rat mAb (1A8, a gift from Professor Thomas Malek, Department of Microbiology and Immunology, University of Miami, Florida, USA) or mAb RB6-8C5 were administered to mice. Control animals received a similar dose of purified whole rat IgG (Jackson Laboratories). To deplete neutrophils during i.n. infection, mice were treated with intraperitoneal (i.p.) (0.5 mg in 0.2 ml) and i.n. (0.2 mg in 0.05 ml) inoculations of purified antibodies. Mice were treated 24 h prior to infection and every 48 h thereafter. Differential leucocyte counts (described below) were performed at the completion of each experiment and confirmed >90% depletion of blood neutrophils using these treatment regimes.

**Recovery, differential counts and flow cytometry of leucocytes from mice.** BAL cells, heparinized blood and nasal tissues cells (nasal cavity and nasal turbinates) were obtained as described previously (Tate et al., 2008).

To obtain single-cell suspensions, lung and nasal tissues were minced finely with scissors. Tissues were incubated for 30 min at 37 °C with 2 mg collagenase A ml<sup>−1</sup> (Roche Diagnostics). Samples were passed through a wire mesh and treated with Tris-NH₄Cl (0.14 M NH₄Cl in 17 mM Tris, adjusted to pH 7.2) to lyse erythrocytes and washed in RPMI 1640 medium supplemented with 10% FCS (RF10). Cell numbers and cell viability were assessed via trypan blue exclusion using a haemocytometer.

For flow-cytometric analysis, single-cell suspensions prepared from BAL, lung and nasal tissues were incubated on ice for 20 min with supernatants from hybridoma 2.4G2 antibody to block Fc receptors and then stained with appropriate combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin (APC)-conjugated or biotinylated monoclonal antibodies to Ly6G (1A8), Gr-1 (RB6-8C5), Ly6C (AL-21), CD45.2 (104), CD8α (53-6.7), CD4 (GK1.5), NK1.1 (PK136), CD3e (145-2C11), MHC class II (I-A<sub>b</sub>, AF6-120.1), CD11c (HL3) (all from BD PharMingen), F4/80 (BM8; Caltag Laboratories) and mPDCA-1 (JF05-IC2.4.1; Miltenyi Biotec). Dead cells were excluded from analysis following the addition of propidium iodide (PI; 10 μg ml<sup>−1</sup>) to each sample and cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson). A minimum of 50 000 live cells (PI<sup>−</sup>) were collected. Leucocyte populations were sorted using a MoFlo cell sorter (DakoCytomation).

To obtain samples for the identification of cDCs and pDCs, lungs were digested for 20 min at room temperature with collagenase/
DNase (1 mg collagenase type II ml⁻¹; Worthington Biochemicals; and 1 µg grade II bovine pancreatic DNase I ml⁻¹; Boehringer Mannheim) as described previously (Vremec et al., 2000). Samples were then treated for a further 5 min with EDTA to disrupt T cell–DC complexes. Pulmonary macrophages were identified as CD11c⁺, MHC class IIhigh (Kirby et al., 2006), cDCs as CD11chigh, MHC class IIhigh (I–A⁺) (Kirby et al., 2006) and pDCs as CD11c⁺, PDCA-1⁺ cells (Beatty et al., 2007; Smit et al., 2006).

Cytokine bead array for the detection of inflammatory mediators. The levels of IFN-γ, TNF-α, IL-6, IL-10, IL-12; p70 and MCP-1 in BAL supernatants and serum were determined with the use of a cytokine bead array mouse inflammation kit (Becton Dickinson) according to the manufacturer’s instructions. Inflammatory mediator concentrations were calculated from a standard curve and expressed as pg ml⁻¹.

Statistical analysis. When comparing three or more sets of values, data were analysed by one-way ANOVA (non-parametric) followed by post-hoc analysis using Tukey’s multiple comparison test. A P value of ≤0.05 was considered statistically significant.

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