Infection of macrophages by a lymphotropic herpesvirus: a new tropism for Marek’s disease virus

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Marek’s disease virus (MDV) is classified as an oncogenic lymphotropic herpesvirus of chickens. MDV productively and cytolytically infects B, αβT and γδT lymphocytes and latently infects T-helper lymphocytes. The aims of this study were to identify whether MDV infects macrophages in vivo and, if so, whether quantitative differences in macrophage infection are associated with MDV strain virulence. Chickens were infected with either virulent MDV (HPRS-16) or ‘hypervirulent’ MDV (C12/130). Flow cytometry with monoclonal antibodies recognizing MDV pp38 antigen and leucocyte antigens was used to identify MDV lytically infected cells. Macrophages from HPRS-16- and C12/130-infected chickens were pp38+. It is demonstrated that macrophages are pp38+ because they are infected and not because they have phagocytosed MDV antigens, as assessed by confocal microscopy using antibodies recognizing MDV antigens of the three herpesvirus kinetic classes: infected cell protein 4 (ICP4, immediate early), pp38 (early) and glycoprotein B (gB, late). Spleen macrophages from MDV-infected chickens were ICP4+, pp38+ and gB+, and ICP4 had nuclear localization denoting infection. Finally, MDV pp38+ macrophages had high inherent death rates, confirming cytolytic MDV infection, although production of virus particles has not been detected yet. These results have two fundamental implications for understanding MDV pathogenesis: (i) MDV evolved to perturb innate, in addition to acquired, immunity and (ii) macrophages are excellent candidates for transporting MDV to primary lymphoid organs during the earliest stages of pathogenesis.

INTRODUCTION

Marek’s disease virus (MDV) is an oncogenic chicken alphaherpesvirus and a member of the genus Marek’s disease-like viruses within the subfamily Alphaherpesvirinae of the family Herpesviridae (van Regenmortel et al., 1999). MD is a commercially important poultry disease, a recognized biomedical lymphoma model (Epstein, 2001) and a model for the vaccine-driven evolution of pathogen virulence (Gandon et al., 2001; Witter, 2001).

According to paradigm, MDV infection occurs by inhalation of cell-free MDV in feather dander. At 4–6 days post-infection (p.i.), an obligate cytolytic and cell-associated productive infection of B and αβT lymphocytes is established (Calnek et al., 1984a, b). From 7 to 14 days p.i., MDV establishes life-long latency in ‘activated’ CD4+ T-helper (Th) lymphocytes (Schat et al., 1991). In genetically susceptible chickens, MDV neoplastically transforms ‘activated’ CD4+ Th lymphocytes and gross lymphomas form (Burgess & Davison, 2002). Recently, MDV has been demonstrated also to lytically infect γδT lymphocytes (Burgess & Davison, 2002). MDV is thus insidious, infecting and neoplastically transforming the acquired immune cells evolved to destroy it.

Although phagocytes are postulated to transport MDV from the lungs to primary lymphoid organs, i.e. sites of productive lymphoid infection (Calnek, 2001), this has never been demonstrated. Little work has investigated the question of MDV macrophage infection. Although MDV infects non-lymphoid cells in vitro [chicken embryo fibroblasts (CEF), chick kidney cells and feather follicle epithelium] (Payne, 1985), neither in vitro macrophage infection with MDV nor in vitro MDV isolation from macrophages taken from infected chickens has been possible (Haffer & Sevoian, 1979; Haffer et al., 1979; von Bülow & Klasen, 1983).

Regardless, in vivo lymphocyte infection alone does not account for the total number of MDV-infected splenocytes during the cytolytic stage of Marek’s disease (MD) (Baigent
& Davison, 1996; Baigent et al., 1998). Furthermore, although administration of the T lymphocyte immuno-
suppressive drug cyclosporin after the establishment of
MDV latency results in reappearance of cytolitic infection
in primary lymphoid organs, many of the MDV antigen-
positive cells are not B or T lymphocytes (Buscaglia et al.,
1988). We hypothesize that macrophages are likely to be
MDV-infected in vivo.

In addition to their suspected role as MDV transporters,
macrophages are critical to innate immunity. Specifically
for MD, macrophages probably restrict initial MDV repli-
cation in vivo (Lee et al., 1978a, b; Haffer & Sevoian, 1979;
Lee, 1979; Powell et al., 1983; Gupta et al., 1989; Schat &
Xing, 2000; Xing & Schat, 2000a, b; Djeraba et al., 2000). In
this work, our two specific aims were to identify whether (i)
MDV infects macrophages in vivo and (ii) if quantitative
differences in macrophage infection in vivo correlate with
MDV virulence. We demonstrate for the first time, directly
ex vivo, that MDV infects macrophages. Our work has
two fundamentally implications for understanding MDV
pathogenesis. Firstly, MDV evolved to perturb innate, in
addition to acquired, immunity. Secondly, and as hypo-
thesized previously by others (Calnek, 2001), macrophages
are excellent candidate cells for transporting MDV to
primary lymphoid organs during the earliest stages of
pathogenesis.

METHODS

Viruses. Plaque-purified stocks of MDV C12/130 and HPRS-16
were used either as cell-free or as tissue culture stocks prepared
from CEF or chicken kidney cells (Barrow & Venugopal, 1999). All
tested negative by PCR using specific primers for chicken infectious
anaemia and avian leukosis viruses.

Chickens, MDV infection and spleen leukocyte isolation.
Because the spleen is a site of primary MDV infection, all work was
done using splenic leukocytes. Specific-pathogen-free, 2-week-old,
Houghton Poultry Research Station Rhode-Island Red (HPRS-RIR)
chickens bred and maintained at the Institute for Animal Health
were infected by intraperitoneal injection of 10^3 p.f.u. MDV in
medium, as described (Barrow & Venugopal, 1999). Chickens
infected with the different MDVs, and uninfected control chickens
(injected with medium excluding MDV), were housed in separate
rooms. At different days p.i. (see below), spleens were removed
post-mortem and leukocyte suspensions prepared by density gradient
centrifugation (specific gravity 1.077, 500 g, 4°C, 30 min; Ficoll-
Paque, Amersham Pharmacia Biotech). All cells were maintained in
PBS with 0.5 % BSA and 0.1 % sodium azide. Experiments followed
UK Home Office guidelines.

Antibodies. All antibodies used in this study are described in
Table 1. All monoclonal antibody (mAb) dilutions were determined
previously, or were established experimentally, as described (Burgess
& Davison, 2002). mAb BD1 is of isotype IgG2a and recognizes a
complex of three antigenically related early phosphoproteins (of 24,
38 and 41 kDa) termed the pp38 complex (Li et al., 1994). mAb
HB3 identifies the glycoprotein B (gB) antigen from all three MDV
serotypes and is of isotype IgG2b (Ross et al., 1997). All other mAbs
used were isotype IgG1. CT4 and CT8 identify the chicken T cell
antigens CD4 and CD8, respectively (Chan et al., 1988). mAb TCR1
recognizes the chicken γδ T cell receptor (Sowder et al., 1988). mAb
AV20 recognizes the chicken pan B cell antigen, chB6 (Rothwell et al.,
1996). mAb KULO1 identifies chicken macrophages and monocytes
(Mast et al., 1998; Mast & Goddeeris, 1999; Van Immerseel et al.,
2002a, b). Rabbit polyclonal antibody against the MDV ICP4 homo-
logue was a gift from R. Morgan (University of Delaware, Newark,
DE, USA). RSVG— mAbs 29 and 30 were isotype-matched controls

<p>| Table 1. mAbs and antibody conjugates |
|--------------------------------------|---------------------------------|-------------------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Monoclonal</th>
<th>Isotype</th>
<th>Target antigen</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>AV20</td>
<td>IgG1</td>
<td>Chicken B6 (Bu-1)</td>
<td>Rothwell et al. (1996)</td>
</tr>
<tr>
<td>BD1</td>
<td>IgG2a</td>
<td>MDV pp38 antigen</td>
<td>Li et al. (1994)</td>
</tr>
<tr>
<td>KULO1</td>
<td>IgG1</td>
<td>Chicken macrophages</td>
<td>Mast et al. (1998)</td>
</tr>
<tr>
<td>CT4</td>
<td>IgG1</td>
<td>Chicken CD4</td>
<td>Chan et al. (1988)</td>
</tr>
<tr>
<td>CT8</td>
<td>IgG1</td>
<td>Chicken CD8 x-chain</td>
<td>Chan et al. (1988)</td>
</tr>
<tr>
<td>HB3</td>
<td>IgG2b</td>
<td>MDV gB</td>
<td>Taylor et al. (1984)</td>
</tr>
<tr>
<td>RSVG-mAb29</td>
<td>IgG2a</td>
<td>BRSV gp90</td>
<td>Taylor et al. (1984)</td>
</tr>
<tr>
<td>RSVG-mAb30</td>
<td>IgG1</td>
<td>BRSV gp90</td>
<td>Taylor et al. (1984)</td>
</tr>
<tr>
<td>RSVG-mAb11</td>
<td>IgG2b</td>
<td>BRSV F protein</td>
<td>Taylor et al. (1984)</td>
</tr>
<tr>
<td>Polyclonal</td>
<td>Source</td>
<td>MDV ICP4</td>
<td>R. Morgan, personal communication</td>
</tr>
<tr>
<td>Anti-MDV ICP4</td>
<td>Rabbit</td>
<td>Mouse IgG1</td>
<td>SBA</td>
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<tr>
<td>IgG1-TRITC</td>
<td>Goat</td>
<td>Mouse IgG1</td>
<td>SBA</td>
</tr>
<tr>
<td>IgG1-ITTC</td>
<td>Goat</td>
<td>Mouse IgG1</td>
<td>SBA</td>
</tr>
<tr>
<td>IgG2a-ITTC</td>
<td>Goat</td>
<td>Mouse IgG2a</td>
<td>SBA</td>
</tr>
<tr>
<td>IgG2b-Texas red</td>
<td>Goat</td>
<td>Mouse IgG2b</td>
<td>SBA</td>
</tr>
<tr>
<td>IgG2b-PE</td>
<td>Goat</td>
<td>Mouse IgG2b</td>
<td>SBA</td>
</tr>
<tr>
<td>IgG2b-FITC</td>
<td>Goat</td>
<td>Rabbit IgG</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>IgG-Alexa 568</td>
<td>Goat</td>
<td>Rabbit IgG</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>IgG-Alexa 488</td>
<td>Goat</td>
<td>Rabbit IgG</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

BRSV, bovine respiratory syncytial virus; SBA, Southern Biotechnology Association.
for IgG2a and IgG1, respectively, and have been described before (Baigent & Davison, 1996; Baigent et al., 1998). RSVG– mAb 11 recognizes the F protein from respiratory syncytial virus and was used as an IgG2b-matched control (Taylor et al., 1984). Fluorescent-labelled secondary antibodies goat anti-mouse IgG-FITC, goat anti-mouse IgG1-TRITC, goat anti-mouse IgG2a-FITC, goat anti-mouse IgG2a-Texas red and goat anti-mouse IgG2a-PE were purchased from Southern Biotechnologies. Goat anti-mouse IgG2b-FITC was purchased from Caltag Laboratories. Goat anti-rabbit IgG-Alexa 488 and goat anti-rabbit IgG-Alexa 568 were purchased from Molecular Probes.

**Flow cytometry.** Splenic leukocytes, taken at 2, 4, 6, 8 and 10 days p.i., were analysed for co-expression of the MDV lytic-cycle antigen pp38+ and mAbs AV20, CT4+, CT8+ for ICP4 and KULO1. Immunostaining was done on ice in 96-well U-bottomed plates (106 cells per well; Nunc), leukocytes were washed after each incubation and then fixed and permeabilized for intracellular antigen and DNA detection, exactly as described (Burgess & Davison, 2002). Because BD1 is an IgG2a antibody and the leukocyte antigen mAbs are IgG1 antibodies, BD1 and leukocyte mAb incubations were concurrent. Incubations with the fluorescently labelled secondary antibodies, IgG1-FITC and IgG2a-PE, were likewise concurrent. A FACScalibur flow cytometry was used for data collection (Becton Dickinson). AV20+, CT4+, CT8+, TCR1+ and KULO1+ cells were compared for pp38 expression as a proportion of total pp38+ cells (e.g. no. of splenocytes mAb+pp38+ cells/total pp38+ cells×100) to compare the number of pp38+ cells in different splenocyte subpopulations between HPRS-16- and C12/130-infected chickens.

**Confocal microscopy.** Immunostaining of cells for confocal microscopy was done as described for flow cytometric analyses, except that incubations with mAbs recognizing MDV and leukocyte antigens were done separately for 1 h. MDV antigen expression was examined in B lymphocytes and macrophages. The secondary antibodies used in combination were: (i) for pp38 expression, IgG1-FITC+IgG2a-Texas red; (ii) for gB expression, IgG1-TRITC+IgG2b-FITC; and for ICP4 expression, IgG1-FITC+IgG-Alexa 568. Propidium iodide (PI) (10 μg ml−1; Sigma) was used to stain DNA to identify the nucleus. PI was used in combination with anti-MDV ICP4 and IgG-Alexa 488.

To detect ICP4 and pp38 co-expression, leukocytes were incubated with anti-MDV ICP4 and BD1 concurrently, washed and then incubated with IgG-Alexa 568+IgG2a-FITC. As a confocal microscopy control, gB expression was analysed in C12/130-infected CEF that had been removed using PBS and 1 mM EDTA from infected flask and transferred to microtitre plates for immunostaining. Unpermeabilized and permeabilized CEF were incubated with HB3, washed and then incubated with IgG2b-FITC. Following immunostaining, both CEF and leukocytes were incubated (30 min at room temperature) on poly-L-lysine (Sigma)-coated glass coverslips (Merck). The coverslips were then mounted with Vectashield (Vector Laboratories) and examined by laser scanning confocal microscopy (Leica). Transmitted light images were collected with a transmitted light detector on the confocal microscope.

**Ex vivo analysis of leukocyte death.** Surface antigen expression and death were analysed in leukocytes isolated from HPRS-16- and C12/130-infected, and mock-infected, chickens by flow cytometry using an adaptation of the technique described by Nicoletti et al. (1991). The technique was used as described (Burgess & Davison, 2002) with the following changes: (i) BD1 was conjugated to ‘long-armed’ biotin (‘BD1-biotin’; Sigma); (ii) leukocytes were incubated concurrently with BD1-biotin and either AV20 or KULO1, washed and then incubated with IgG1-FITC+allophycocyanin (APC)-streptavidin conjugate (Caltag Laboratories)+PI (10 μg ml−1).

Flow cytometric analysis was performed after time-delay calibration (according to the manufacturer’s instructions) using APC-conjugated beads and the FACScalibur. At least 500 positive events were collected for the following: (i) pp38+AV20+ and pp38+KULO1+ leukocytes from HPRS-16- and C12/130-infected spleens; (ii) pp38+AV20+ and pp38+KULO1+ leukocytes from HPRS-16- and C12/130-infected spleens; and (iii) pp38+AV20+ and pp38+KULO1+ leukocytes from uninfected spleens. All flow cytometry data were analysed using the WinMDI 2.8 analysis package (http://facs.scripps.edu/software.html).

**RESULTS**

**Macrophages express the MDV lytic-cycle antigen pp38 after HPRS-16 and C12/130 infection**

Cellular expression of the MDV-specific pp38 antigen is indicative of de novo cytolytic infection (Ross et al., 1997; Burgess & Davison, 2002; Reddy et al., 2002). Data from other workers suggests MDV infects non-lymphoid leukocytes during the cytolytic stage of MDV infection (Buscaglia et al., 1988; Baigent et al., 1998). We measured the MDV pp38 antigen in HPRS-16- and C12/130-infected splenocytes by flow cytometry at 2, 4, 6, 8 and 10 days p.i. AV20+, CT4+, CT8+, TCR1+ and KULO1+ cells were compared for pp38 expression as a percentage of total pp38+ cells to see if MDV infection of these leukocyte subpopulations differed between HPRS-16 and C12/130. We used mAb KULO1 to identify macrophages because KULO1 recognizes monocytes, macrophages and interdigitating cells (probably dendritic cells) but not lymphocytes (Mast et al., 1998; Mast & Goddeeris, 1999; Van Immerseel et al., 2002a, b).

The pp38 antigen was detected in splenocytes from either HPRS-16- or C12/130-infected chickens at 4 and 6 days p.i., but was never detected in splenocytes isolated from mock-infected control chickens (data from 4 days p.i.; Fig. 1). At 4 days p.i., there were 16·8-fold more pp38+ splenocytes in C12/130 (15·1±4·3%) compared with HPRS-16 (0·9±0·3%) infected chickens (P<0·05) (Table 2). At 6 days p.i., two of five C12/130-infected chickens did not express pp38, i.e. these chickens were latently infected. The mean numbers of pp38+ splenocytes from C12/130-infected chickens were 10±7±0·7% compared with 6·3±2·8% from HPRS-16-infected chickens at 6 days p.i. (Table 2).

It is accepted that the majority of infected splenocytes during cytolytic infection are B lymphocytes (Calnek et al., 1984a, b). In agreement, most pp38+ splenocytes were AV20+ in both HPRS-16 (78·9±7·2%) and C12/130 (67·2±5·0%) infected chickens at 4 days p.i. (P<0·05) (Fig. 1 and Table 2). However, at 6 days p.i., C12/130-infected chickens (31·3±2·3%) had less than half (P<0·05) the proportion of pp38+AV20+ splenocytes compared to HPRS-16-infected chickens (69·9±5·6%).

According to the generally accepted paradigm for MDV pathogenesis (Calnek, 2001), the initial B lymphocytolytic infection results in T lymphocyte activation. These T
lymphoblasts may then become cytolytically or latently MDV infected, although cytolytic infection is, to a lesser degree, compared with B lymphocytes. Following this paradigm, we found pp38\(^{+}\)CT4\(^{+}\) and pp38\(^{+}\)CT8\(^{+}\) lymphocytes in both HPRS-16- and C12/130-infected chickens at 4 and 6 days p.i. (Fig. 1 and Table 2). There were differences between the proportions of pp38\(^{+}\)CT4\(^{+}\) and pp38\(^{+}\)CT8\(^{+}\) lymphocytes at 6 days p.i.; pp38\(^{+}\)CT8\(^{+}\) lymphocytes were more frequent (\(P < 0.05\)) in C12/130 (26.6 ± 2.7\%) compared with HPRS-16-infected chickens (16.6 ± 3.9\%).

Recently, γ\(\delta\)T lymphocytes in MDV lymphomas have been demonstrated to be cytolytically MDV infected (Burgess & Davison, 2002). Here we show pp38\(^{+}\)TCR1\(^{+}\) splenocytes during the cytolytic phase of MD at 4 and 6 days p.i. (Fig. 1 and Table 2). Notably, pp38\(^{+}\)TCR1\(^{+}\) lymphocytes were less frequent (\(P < 0.05\)) in HPRS-16 (2.3 ± 2.3\%) at 4 days p.i.; 2.2 ± 1.1\% at 6 days p.i.) compared with C12/130-infected chickens (8.8 ± 1.0\% at 4 days p.i.; 12.3 ± 1.9\% at 6 days p.i.).

Our most notable and novel finding was pp38\(^{+}\)KULO1\(^{+}\) splenocytes in both HPRS-16- and C12/130-infected chickens at 4 and 6 days p.i. (Fig. 1 and Table 2). Again, pp38\(^{+}\)KULO1\(^{+}\) splenocytes were less frequent (\(P < 0.05\)) in HPRS-16 (3.0 ± 3.0\% at 4 days p.i.; 2.4 ± 1.0\% at 6 days p.i.) compared with C12/130-infected chickens (9.6 ± 1.2\% at 4 days p.i.; 22.6 ± 1.6\% at 6 days p.i.).

Collectively, our data emphasize that C12/130 MDV causes a greater cytolytic infection; 16.8-fold more pp38\(^{+}\) splenocytes were detected at 4 days p.i. compared with HPRS-16-infected chickens. Significantly, our data shows for the first

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**Fig. 1.** Identification of pp38\(^{+}\) MDV-infected splenocytes by flow cytometry. Two-colour analysis of splenocytes from MDV-infected and uninfected chickens was performed to identify intracellular pp38 (\(y\)-axis) and leukocyte differentiation antigens (\(x\)-axis). (A) Uninfected, (B) HPRS-16- and (C) C12/130-infected chickens. Results are expressed as dot plots, where each dot represents one cell (10\(^6\) per dot plot). mAb controls were mAb 30 (\(x\)-axis) and mAb 29 (\(y\)-axis). Splenocytes expressing leukocyte antigens are located to the right of the \(x\)-axis marker bar and those expressing pp38 are located above the \(y\)-axis marker bar. Double-positive cells are located in the upper-right quadrant. Representative dot plots from 4 days p.i. are shown.
Table 2. Percentage of pp38+ leukocyte subpopulations as a proportion of total pp38+ cells from MDV-infected chickens

<table>
<thead>
<tr>
<th>Strain (4 days p.i.)</th>
<th>Phenotype</th>
<th>pp38+ cells (%)</th>
<th>Strain (6 days p.i.)</th>
<th>Phenotype</th>
<th>pp38+ cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRS-16</td>
<td>Total pp38+</td>
<td>0.9 ± 0.3</td>
<td>HPRS-16</td>
<td>Total pp38+</td>
<td>6.3 ± 2.8</td>
</tr>
<tr>
<td>AV20+</td>
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<td></td>
<td>AV20+</td>
<td>69.9 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>CT4+</td>
<td>12.9 ± 5.4</td>
<td></td>
<td>CT4+</td>
<td>17.6 ± 8.0</td>
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</tr>
<tr>
<td>CT8+</td>
<td>13.1 ± 6.2</td>
<td></td>
<td>CT8+</td>
<td>16.6 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>TCR1+</td>
<td>2.3 ± 2.3</td>
<td></td>
<td>TCR1+</td>
<td>2.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>KULO1+</td>
<td>3.0 ± 5.0</td>
<td></td>
<td>KULO1+</td>
<td>2.4 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>C12/130</td>
<td>Total pp38+</td>
<td>15.1 ± 4.3</td>
<td>C12/130</td>
<td>Total pp38+</td>
<td>10.7 ± 0.7</td>
</tr>
<tr>
<td>AV20+</td>
<td>67.2 ± 5.0</td>
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<td>AV20+</td>
<td>31.3 ± 2.3</td>
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</tr>
<tr>
<td>CT4+</td>
<td>13.4 ± 3.6</td>
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<td>13.4 ± 0.9</td>
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</tr>
<tr>
<td>CT8+</td>
<td>14.4 ± 3.1</td>
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<td>8.8 ± 1.0</td>
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<tr>
<td>KULO1+</td>
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<td>KULO1+</td>
<td>22.6 ± 1.6</td>
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</table>

Macrophage tropism of Marek’s disease virus

To investigate further the possibility that the pp38+ macrophages we measured by flow cytometry were MDV infected and not pp38+ because they had phagocytosed MDV antigens from other infected lymphocytes, we measured the expression of representative MDV antigens from the three herpesvirus kinetic classes: ICP4 (immediate early), pp38 (early) and gB (late). We compared directly the presence and distribution of these MDV proteins in macrophages with that in B lymphocytes because these cells are primarily lytically infected by MDV. ICP4 localizes to the nucleus of MDV-infected cells (Knipe et al., 1987; Xing et al., 1999) and is definitively diagnostic of herpesvirus infection (Roizman, 1996); cells that have only phagocytosed infected cells will not have nuclear ICP4 (Aderem & Underhill, 1999).

Splenocytes from uninfected chickens were ICP4−, pp38− and gB− (Fig. 2B, panel i). ICP4 localized to the nucleus of splenocytes from C12/130 MDV-infected chickens (Fig. 2A, panels ii–iii). However, a novel, unexpected finding was that ICP4 was also diffusely present within the cytoplasm of some MDV-infected cells (Fig. 2A, panel ii). This pattern was not an artefact because neither the nucleus nor the cytoplasm of leukocytes from uninfected control chickens was ICP4+ (Fig. 2A, panel iv).

Nuclear and cytoplasmic ICP4 immunostaining was also observed in KULO1+ cells (Fig. 2B, panel iii). This staining was similar in distribution to that seen in a proportion of PI/ICP4-stained cells that also had nuclear and cytoplasmic staining (compare Fig. 2B, panel iii, with Fig. 2A, panel ii). The phenomenon was not restricted to KULO1+ cells; AV20+ lymphocytes also had nuclear and cytoplasmic ICP4 immunostaining (Fig. 2C, panel i). ICP4 was not restricted to discrete cytoplasmic areas, as would be expected in cells that had phagocytosed dead or dying MDV-infected cells (Fig. 2B, panels iii and iv) (Aderem & Underhill, 1999).

Indeed, the same ICP4+ KULO1+ cell did not appear to have phagocytosed any dead or dying cells (Fig. 2B, panel iv). In our work, the MDV lytic antigen-positive KULO1+ cells were larger than lymphocytes (~20 μM diameter; lymphocytes ~6 μM) and reticular or elliptically shaped with non-dendritic morphology, characteristic of macrophages but not dendritic cells (Fig. 2B, C) (Mast et al., 1998; Mast & Goddeeris, 1999; Van Immershein et al., 2002a, b).

Typical of MDV lytically infected cells (Ross et al., 1997; Burgess & Davison, 2002; Reddy et al., 2002), pp38 was...
strongly expressed and strictly distributed throughout the cytoplasm of both AV20+ and KULO1+ leukocytes from C12/130-infected chickens (Fig. 3, panels i, ii and v). This widespread distribution suggests the pp38+ immunostaining was not due to phagocytosis of MDV-infected cells. Indeed, these same pp38+ KULO1+ cells showed no evidence of having phagocytosed any cells or cellular debris at all by transmitted light confocal microscopy (Fig. 3, panels iii and iv).

gB, reported as both a cell surface and a cytoplasmic antigen in CEF (Ross et al., 1989), was present mainly in the cytoplasm of AV20+ (Fig. 4, panel i) and KULO1+ leukocytes from C12/130-infected chickens (Fig. 4, panel ii). Small areas of co-localization with the FITC-labelled cell surface marker KULO1 were detected in one infected macrophage (Fig. 4, panel ii). In comparison with pp38 immunostaining in AV20+ and KULO1+ cells, gB staining was relatively weak and diffuse. Again, the gB+ KULO1+ cells showed no evidence of having phagocytosed any cells or cellular debris by transmitted light confocal microscopy (Fig. 4, panel iii). gB was detected on the cell surface and cytoplasm of C12/130-infected CEF (Fig. 4, panels iv and v), as expected.

Immunostaining, for all three MDV proteins, was highly similar in distribution and intensity between AV20+ and KULO1+ splenocytes of C12/130-infected chickens (Figs 2, 3 and 4). Collectively, the detection and localization of MDV antigens from each herpesvirus kinetic class emphasizes that MDV infection occurs in macrophages similar to B lymphocytes. The detection of ICP4 localizing to the nucleus of both B lymphocytes and macrophages identifies MDV infection definitively.

Macrophages and B lymphocytes expressing pp38 show increased cell death

The only known consequences after any herpesvirus infection of a cell are: lytic infection and death, latent infection, or (more rarely) neoplastic transformation (Roizman, 1996).

Fig. 2. KULO1+ and AV20+ cells express nuclear and cytoplasmic ICP4. Splenocytes were isolated from either C12/130-infected or uninfected chickens to view the subcellular localization of ICP4. (A) Panels: (i) PI (red fluorescence) and anti-ICP4 antisera (green fluorescence) from infected splenocytes; ICP4 staining in nucleus (co-localization of green with red fluorescence appears yellow); (ii) PI (red) and ICP4 (green) from infected splenocytes; both cytoplasmic (green) and nuclear ICP4 staining (yellow); (iii) BD1 (anti-pp38, green) and ICP4 (red) from infected splenocytes; widespread cytoplasmic pp38 staining; (iv) PI (red) and anti-ICP4 antisera from uninfected splenocytes, no ICP4 staining is detected. (B) Panels: (i) KULO1 (green) and anti-ICP4 antisera (uninfected splenocytes), no ICP4 staining is detected; (ii) transmitted light micrograph of same ICP4+ KULO1+ cells; (iii) KULO1 (green) and ICP4 (red) from infected chicken; nuclear and cytoplasmic ICP4 staining similar to (A, panel ii); and (iv) transmitted light micrograph of same ICP4+ KULO1+ cell, no phagocytosed material is present in the cytoplasm. (C) Panels: (i) AV20 (green) and ICP4 (red) from infected splenocytes and (ii) transmitted light micrograph of same AV20+ICP4+ cell. In all cases shown, nuclear ICP4 staining (N, nuclear; C, cytoplasm) is indicated by arrows. Bars, 10 μM.
Lytically MDV-infected macrophages would, by default, die. In contrast, macrophages that phagocytose cell debris do not die; they continue to remove dying cells and remain alive to process the phagocytosed antigens for presentation to acquired immune cells. We compared the proportions of dying pp38$^+$ B lymphocytes and macrophages from HPRS-16- and C12/130-infected chickens at 6 days p.i. by flow cytometry. Since the DNA profiles of cells in G1 or S/G2M phases could be distinguished clearly (Fig. 5A, panel i), cells that had fragmented DNA profiles because of cell death were therefore evident as subG1 events (Fig. 5A). More pp38$^+$ AV20$^+$ lymphocytes were dying (HPRS-16, 15.8 ± 1.9%; C12/130, 31.9 ± 3.9%) compared with either pp38$^-$ AV20$^+$ lymphocytes from the same infected chicken (HPRS-16, 2.1 ± 0.8%; C12/130, 2.1 ± 0.9%) or pp38$^+$ AV20$^+$ lymphocytes from uninfected chickens (2.0 ± 0.2%) (Fig. 5A, B). Similarly, more pp38$^+$ KULO1$^+$ cells (HPRS-16, 20.0 ± 1.9%; C12/130, 37.2 ± 3.2%) were dying compared with either pp38$^-$ KULO1$^+$ (HPRS-16, 2.8 ± 0.7%; C12/130, 1.8 ± 0.3%) from the same infected chicken or KULO1$^+$ cells from uninfected chickens (1.6 ± 0.2%) (Fig. 5A, B). The percentages of cell death between pp38$^+$ AV20$^+$ and pp38$^+$ KULO1$^+$ cells in

Fig. 3. AV20$^+$ and KULO1$^+$ cells express the MDV early antigen pp38. Splenocytes were isolated from either uninfected or C12/130-infected chickens and stained with mAb BD1 to identify pp38$^+$ cells by flow cytometry. Panels: (i) and (ii) KULO1$^+$-stained cells (green fluorescence) from infected chicken expressing widespread cytoplasmic pp38 (N, nuclear; C, cytoplasm) staining (red fluorescence); (iii) and (iv) transmitted light micrograph of the same pp38$^+$ KULO1$^+$ cells showing 'unactivated' appearance of KULO1$^+$ cells, with scant cytoplasm and no presence of phagocytosed dead or dying MDV-infected cells; (v) AV20$^+$ cell (green) from an infected chicken expressing pp38 (red), note the high similarity of pp38 distribution between KULO1$^+$ and AV20$^+$ cells. Bar, 10 μM.

Fig. 4. AV20$^+$ and KULO1$^+$ cells express the MDV late gB antigen. Splenocytes were isolated from either uninfected or C12/130-infected chickens and stained with mAb HB3 to identify gB$^+$ cells by flow cytometry. Panels: (i) AV20$^+$ cells (red fluorescence) expressing gB (green fluorescence) from an infected chicken; (ii) KULO1$^+$-stained cell (red) from an infected chicken expressing cytoplasmic pp38 (N, nuclear; C, cytoplasm) gB (green); (iii) transmitted light micrograph of the same gB$^+$ KULO1$^+$ cell showing scant cytoplasm and no presence of phagocytosed dead or dying MDV-infected cells; (iv) unpermeabilized C12/130-infected CEF expressing cell-surface gB (green); and (v) permeabilized C12/130-infected CEF showing both cytoplasmic and cell-surface gB staining (green). Bar, 10 μM.
chickens infected with either HPRS-16 or C12/130 were similar (Fig. 5B). However, in C12/130-infected chickens, the percentage of dying pp38\(^+\) AV20\(^+\) and pp38\(^+\) KULO1\(^+\) cells were significantly higher (\(P < 0.05\)) than in HPRS-16-infected chickens (Fig. 5B).

Our results indicate that B lymphocytes and macrophages expressing pp38 undergo a cytolytic infection but pp38\(^-\) macrophages or pp38\(^+\) B lymphocytes from the same host or those from uninfected chickens do not. pp38\(^+\) B lymphocytes have similar levels of death when compared with pp38\(^+\) macrophages from the same MDV-infected chickens. However, pp38\(^+\) B lymphocytes and pp38\(^+\) macrophages from C12/130-infected chickens have higher rates of cell death compared with pp38\(^+\) B lymphocytes and pp38\(^+\) macrophages from HPRS-16-infected chickens. Our results emphasize further the greater cell death after infection with C12/130 compared with HPRS-16.

**DISCUSSION**

Until now, MDV has been categorized as a lymphotropic herpesvirus, infecting B, Th, T killer (Calnek *et al.*, 1984a, b) and TCR\(\gamma\delta\) (Burgess & Davison, 2002) lymphocytes. However, long held but never proven dogma contends that macrophages are critical to the very earliest stages of MD pathogenesis (Calnek, 2001). Although some circumstantial experimental evidence suggests cells other than lymphocytes must be MDV infected (Buscaglia *et al.*, 1988; Baigent *et al.*, 1998), no direct experimental evidence for a
Macrophage role in MDV pathogenesis has been published. Our novel findings, made directly ex vivo, are that macrophages contain MDV lytic-cycle antigens distributed consistent with infection and MDV antigen expression and death rates are comparable with B lymphocytes. Our work is of fundamental interest to herpesvirus host cell tropism as well as the mechanisms, and evolution of, herpesvirus pathogenesis and virulence. Specifically for understanding MDV pathogenesis: firstly, macrophages are excellent candidates for transporting MDV to primary lymphoid organs during the earliest stages of pathogenesis and, secondly, MDV has evolved to perturb innate, in addition to acquired, immunity.

MDV expresses ICP4, pp38 and gB, representing the immediate-early, early and late temporal classes of herpesvirus gene expression, respectively. To identify MDV infection definitively, we demonstrate expression of MDV ICP4 in the nucleus of KULO1+ cells (Knipe et al., 1987; Roizman, 1996). Furthermore, ICP4 will not localize to the nucleus of cells that have phagocyted dead or dying MDV-infected cells. Such phagocytosis would result in localized degradation of the engulfed cell in phagolysosomes and not ICP4 expression in the nucleus (Adern & Underhill, 1999).

The MDV lytic-cycle antigens pp38 and gB are expressed in the cytoplasm and not the nucleus of MDV-infected cells (Ross et al., 1989, 1997; Reddy et al., 2002). In our work, the distributions of pp38 and gB were very similar between both AV20+ and KULO1+ splenocytes. pp38 was expressed throughout the cytoplasm of AV20+ and KULO1+ splenocytes but was not present in discrete phagocytic vacuoles. Any MDV antigens present in macrophages as a result of phagocytosis of dead/dying MDV-infected cells will lose their antigenicity (and therefore mAb reactivity) due to rapid degradation in phagolysosomes (Adern & Underhill, 1999). Furthermore, the widespread ICP4 and pp38 cytoplasmic staining we observed would not be expected if these proteins were localized in phagolysosomes. Indeed, transmitted light microscopy showed that the MDV antigen* KULO1+ cells had not phagocyted any dead or dying cells at all. The representative MDV proteins from all three herpesvirus kinetic classes shared highly similar subcellular distributions between B lymphocytes and macrophages, fulfilling the current antigenic criteria for MDV infection specifically (Ross et al., 1989, 1997; Burgess & Davison, 2002; Reddy et al., 2002) and herpesvirus infection in general (Roizman, 1996).

In addition to our primary results, we make two interesting observations about the MDV antigens gB and ICP4. Firstly, we demonstrate in B lymphocytes and macrophages that ICP4 localizes both to the nucleus and to the cytoplasm. This phenomenon is not unique to MDV; it also occurs after herpes simplex virus type 1 (HSV-1) infection (Knipe et al., 1987; Zhu & Schaffer, 1995). MDV ICP4 has a number of spliced polyadenylated sense transcripts, which are not fully characterized yet (R. Morgan, personnel communication). The ‘anti-MDV ICP4’ we used recognizes amino acids 534–1415 of ICP4. Although identifying the 140 kDa ‘native’ form of IPC4, anti-MDV ICP4 also identifies an 80 kDa antigen (probable ICP4 isoform) in MDV-infected, but not uninfected, cells (R. Morgan, personnel communication). Either isoform may be in the nucleus or cytoplasm and be functionally significant and this issue deserves further analysis.

Secondly, cell-surface gB expression, by both B lymphocytes and macrophages, was lower, or non-existent, compared with MDV-infected CEF. Notably, human herpesvirus type 6-infected lymphocytes display similar phenomena (Cirone et al., 1994; Torrisi et al., 1999). In MDV infection, downregulation of gB is reported in response to interferon-γ and nitric oxide (Xing & Schat, 2000b; Djeraba et al., 2000) as well as an undefined ‘latency maintaining factor’ (Buscaglia & Calnek, 1988; Volpini et al., 1995, 1996). These factors have been reported during the cytolitic stage of infection in MDV-infected chickens and could be responsible for the unexpected gB surface staining of B lymphocytes and macrophages isolated ex vivo.

All herpesvirus infections result in cell death (Roizman, 1996). Lytically MDV-infected macrophages would, by default, die. In contrast, macrophages that phagocytose cell debris do not die; they continue to remove debris and remain alive to process phagocyteded antigens for expression to acquired immune cells. The numbers of cells in each cell population with subdiploid DNA, resulting from cell death (most likely as a result of cytolitic infection), were analysed. pp38±AV20+ and pp38±KULO1+ leukocytes die, whereas pp38±AV20+ and pp38±KULO1+ leukocytes from the same infected individual do not. These results support our suggestion that pp38+ macrophages are lytically infected by MDV and undergo cell death.

To examine whether the MDV cytolitic infection of macrophages resulted in a productive infection, we have carried out electron microscopic examination of splenocytes from infected birds. Despite repeated efforts, after isolating KULO1+ splenocytes from HPRS-16- and C12/130-infected chickens by cell-sorting and in frozen spleen sections from MDV-infected chickens, we have been unable to identify any MDV particles in KULO1+ splenocytes by electron microscopy (EM) (data not shown). The chicken macrophage antigen recognized by mAb KULO1 is labile under EM fixative conditions (4 % paraformaldehyde ± 0.05 % gluteraldehyde), making it impossible to definitively identify macrophages in EM sections of spleen from MDV-infected chickens. Despite this, we have MACS-sorted macrophages to ≥95 % purity using mAb KULO1. Of these sorted cells, ~10 % are pp38+ by flow cytometry. Because the cells were sorted before EM fixation, the KULO1+ macrophages can be clearly identified from contaminating cells via the electron-dense MACS beads and still virus particles cannot be found in these purified fractions, despite EM evidence that these cells do not contain phagocyteded lymphocytes.
MDV infection is cell-associated and there is no in vitro model for macrophage infection using conventional coculture methods (Haffer et al., 1979; von Bülow & Klasen, 1983). It is because of these early co-culture studies that MDV is considered not to infect macrophages. Despite this, we have also tried our own in vitro co-culture experiments using MDV-infected lymphocytes on either bone marrow-derived chicken macrophages or macrophage cell lines and cannot detect any antigen expression, leading us to believe that MDV macrophage infection clearly requires in vivo conditions. On the contrary, cells are rapidly phagocytosed and degraded and no nuclear ICP4 expression can be detected in vitro, unlike the nuclear ICP4 expression we find in macrophages isolated ex vivo from MDV-infected chickens, consistent with previous studies (von Bülow & Klasen, 1983). We have also cell-sorted KULO1 + macrophages from MDV-infected chickens and co-cultured them with permissive fibroblasts and chick kidney cells. In the instances where we have obtained infectious foci from cell-sorted KULO1 + and AV20 + cells (used as a positive control), the infectious foci are infrequent (less than one or two foci per T25 flask) from 1 × 10^6 sorted KULO1 + or AV20 + cells. These cell-sorted populations are not 100% pure and the small number of infectious foci we do see could also be critically attributed to the small fraction of cells that often contaminate even MoFlo cell-sorted populations (routinely ≥ 98% pure), leaving a possibility that the low number of foci obtained arose possibly from the ~1-2% contaminants of 1 × 10^6 sorted macrophages and B cells (~ 20,000 contaminating cells).

It is possible that MDV infection of macrophages may result in an abortive infection, with no virus particle production, as occurs when HSV-1 infects macrophages (Morahan et al., 1989; Tenney & Morahan, 1991; Wu et al., 1993). In view of these results, further research should define whether MDV induces either an abortive or a productive infection of macrophages. Nevertheless, our ex vivo data showing the nuclear localization of ICP4, widespread cytoplasmic pp38 and gB localization, which is not localized to discrete phagosomes and highly similar distribution to MDV-infected B lymphocytes and death of pp38 + but not pp38 − KULO1 + cells (from the same infected bird), demonstrate infection of macrophages by MDV clearly. Our results are the first data, which support previous suggestions (Calnek, 2001), that macrophages are the proposed ‘carrier cells’ responsible for the early transport of MDV to lymphoid organs.

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