The functional significance of CD4+ and CD8+ T lymphocytes in the immune response to murine gammaherpesvirus 68

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A thesis submitted for the degree of Doctor of Philosophy
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University of Edinburgh
UK

1999
Declaration

I hereby declare that this thesis entitled 'The functional significance of CD4+ and CD8+ T lymphocytes in the immune response to murine gammaherpesvirus 68' is not substantially the same as any that I have submitted for a degree, diploma or any other qualification at any other university. I further state that no part of this thesis has already been, or is being concurrently submitted for any such degree, diploma or other qualification. This thesis is the result of my own work and does not include any work that is the outcome of a collaboration, except where duly acknowledged in the text.

Signed

University of Edinburgh

March, 1999.
ABSTRACT OF THESIS

Name of candidate: Kevin Alasdair Robertson

Degree: Ph.D. Date: March 1999

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Murine gammaherpesvirus 68 (MHV-68) is a natural pathogen of murid rodents and is closely related to Human Herpesvirus 8, Herpesvirus saimiri and Epstein Barr virus. Intranasal infection of inbred mouse strains with MHV-68 results in the lungs of these animals becoming productively infected with virus. In immunocompetent mice, MHV-68 is cleared from the lungs by day 10 after infection. By this time the virus has reached the spleen and has adopted a latent form of infection in B lymphocytes. Depletion of CD8+ T cells from mice, prior to infection, results in uncontrolled MHV-68 replication in the lungs and death of the animal by day 12 post-infection. Such evidence indicates that CD8+ T cells play an important role in the immune response of mice to primary infection with MHV-68.

In this study T lymphocyte responses to MHV-68 were examined in vitro and in vivo.

In vitro work focused on developing a conventional assay to measure MHV-68-specific CTL activity. It was found that splenocytes from MHV-68-infected mice consistently lysed 'S11' cells, a B cell lymphoma line originally isolated from an MHV-68-infected mouse. All S11 cells are latently infected with MHV-68 and around 5% of these cells support viral replication. Treatment of S11 cells with the anti-viral drug, 4'-S-EtdU, is known to prevent lytic MHV-68 protein expression. 4'-S-EtdU-treated S11 target cells were killed by lymphocytes from infected mice. This indicates that the T lymphocytes responsible for killing S11 cells were specific for a latent antigen of MHV-68. No measurable cytolitic activity against any other target cell line, infected in vitro with MHV-68, was detected.

S11 has been shown to divide and expand when implanted subcutaneously into nude (T cell-deficient) mice. S11 does not expand when implanted into normal, immunocompetent mice, implying that T lymphocytes play a key role in inhibiting tumour formation. To investigate this theory, S11 cells were injected subcutaneously into nude mice. This was followed by transfer of re-stimulated lymphocyte populations, enriched for either CD4+ or CD8+ T cells, into the animals. This protocol consistently resulted in regression of S11 tumours. Splenocyte populations depleted of CD8+ T lymphocytes were most effective in preventing tumour formation. This suggests that CD4+ T cells play a major role in preventing B cell lymphoma outgrowth. Immunohistochemical analyses highlighted populations of macrophages and CD4+ T cells in regressing tumours. It may be hypothesised that CD4+ T cells elicit tumour regression via the initiation of a delayed-type hypersensitivity (DTH) response.

Further in vivo work involved depleting BALB/c mice T lymphocyte subsets (CD4 and CD8) at a range of times before or after MHV-68 infection. Subsequently, virus titre in the lungs was assessed by plaque assay. Latently-infected B cells in the spleen were quantified by infectious centre assay and visualised by in situ hybridisation. Using these techniques it was demonstrated that both CD4+ and CD8+ T lymphocytes contribute to clearance of MHV-68 from the lungs. CD8+ T cells also appear to limit the extent of B lymphocyte infection in the spleen. However, CD8+ T cells do not appear to be responsible for the decline in latently infected B cells which occurs in MHV-68 infected mice 2 to 3 weeks after infection.

In conclusion, MHV-68 infection of inbred laboratory mouse strains has potential to model immunological responses to primary gammaherpesvirus infection in humans. In addition, histopathology induced by the S11 cell line implanted into nude mice could provide insight into gammaherpesvirus-associated B lymphoproliferative disease in immunocompromised individuals.
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Thanks very much to Professor AA Nash and Dr Edward Usherwood for their time, patience, guidance and overall help during this project.

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<td>AIDS</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>BAL</td>
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<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolylphosphate</td>
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<td>BL</td>
<td>Burkitt's lymphoma</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>Con A</td>
<td>Concanavalin A</td>
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<td>CPM</td>
<td>Counts per minute</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<td>DMEM</td>
<td>Dulbecco's minimum essential medium</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTH</td>
<td>Delayed type hypersensitivity</td>
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<td>E</td>
<td>Early</td>
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<td>EA</td>
<td>Early antigen</td>
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<td>EBNA</td>
<td>Epstein Barr virus nuclear antigen</td>
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<td>EBV</td>
<td>Epstein Barr virus</td>
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<td>E:S</td>
<td>Effector to stimulator ratio</td>
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<tr>
<td>E:T</td>
<td>Effector to target ratio</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
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<td>FITC</td>
<td>Flourescein isothiocyanate</td>
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<td>4’-S-EtdU</td>
<td>2’deoxy-5’-ethylβ-4’thiouridine</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>GI</td>
<td>Gastrointestinal</td>
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<td>GCR</td>
<td>'G' protein-coupled receptor</td>
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<td>GMEM</td>
<td>Glasgow's minimum essential medium</td>
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<td>GvHD</td>
<td>Graft versus host disease</td>
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<td>HHV</td>
<td>Human Herpesvirus</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HSV</td>
<td>Herpes simplex virus</td>
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<td>HVS</td>
<td>Herpesvirus saimiri</td>
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<td>IC</td>
<td>Infectious centre</td>
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<td>IE</td>
<td>Immediate Early</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IM</td>
<td>Infectious mononucleosis</td>
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<td>INOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<td>IV</td>
<td>Intravenous</td>
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<td>Kbp</td>
<td>Kilobase pair(s)</td>
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<td>KO</td>
<td>Knockout</td>
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<td>KS</td>
<td>Kaposi's sarcoma</td>
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<td>L</td>
<td>Late</td>
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<tr>
<td>LAK</td>
<td>Lymphokine activated killer</td>
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<tr>
<td>LAT</td>
<td>Latency-associated transcript</td>
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<td>LCL</td>
<td>Lymphoblastoid cell line</td>
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<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
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<td>LPD</td>
<td>Lymphoproliferative disease</td>
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<tr>
<td>MA</td>
<td>Membrane antigen</td>
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<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MHV-68</td>
<td>Murine gammaherpesvirus 68</td>
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<tr>
<td>MLN</td>
<td>Mediastinal lymph nodes</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
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<tr>
<td>NBCS</td>
<td>New-born calf serum</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
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<tr>
<td>OHL</td>
<td>Oral hairy leukoplakia</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PI</td>
<td>Post infection</td>
</tr>
<tr>
<td>PLP</td>
<td>Paraformaldehyde, lysine, periodate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDW</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TPB</td>
<td>Tryptose phosphate broth</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCA</td>
<td>Viral capsid antigen</td>
</tr>
<tr>
<td>Vhs</td>
<td>Viral host shutoff</td>
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<td>VZV</td>
<td>Varicella zoster virus</td>
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# Chapter 3

## 3.0 The in vitro assessment of cytotoxic T lymphocyte responses to MHV-68

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Chapter 1

Introduction
1.1 Introduction

The Herpesviridae are highly disseminated in nature and most animal species have at least one herpesvirus associated with them (Roizman, 1996). Herpesviruses are classified into three sub-families termed alpha-, beta- and gamma-, based on the biological properties of those currently recognised. Briefly, alpha-herpesviruses display a variable host range in cell culture, have a short growth cycle and efficiently destroy infected cells. These viruses establish latent infections in neuronal cells and periodically reactivate to infect cells at the nerve ending. During latency there is very little gene transcription (Whitley, 1996). Beta-herpesviruses have a restricted host range. It is unclear whether they adopt a persistent or latent infection and in which site this occurs in the host. They have a long reproductive cycle and cells which they infect display a classic cytomegalic appearance (Britt and Alford, 1996). Gamma-herpesviruses also display a relatively limited host range. In vivo, they generally replicate during acute infection in epithelioid sites and are subsequently tropic for T or B lymphocytes in which a latent state is adopted (Rickinson and Moss, 1997). Some argument reigns over whether gamma-herpesviruses can persistently replicate and/or adopt a latent form in epithelial cells.

As new herpesviruses are identified, classification by their biological characteristics of infection is largely being superseded by a classification system based upon genetic content. Nearly 100 herpesviruses have been recognised in species as diverse as horses, cows, monkeys and chickens and, currently, there are 8 recognised as infecting humans. In the alpha-herpesvirus subfamily, Herpes simplex types 1 and 2 (HSV 1 and 2) and Varicella zoster infect humans. In the beta-herpesvirus subfamily Cytomegalovirus (CMV) and human herpesviruses 6 and 7 (HHV 6 and 7) infect humans and in the gamma-herpesvirus family Epstein Barr virus (EBV) and Human herpesvirus 8 (HHV8) have been isolated from individuals (Roizman, 1996, Mesri et al., 1996). Table 1.1 lists
a number of different gammaherpesvirus family members and the species from which they were first isolated.

Herpesviruses elicit a broad range of diseases. The characteristic symptoms of the disease are determined by whether it is a primary infection, re-infection or reactivation of the virus from latency (Borysiewicz and Sissons, 1994). Viruses such as EBV are also associated with the formation of tumours. Significantly, the herpesviruses have a tendency to reactivate and produce disease when the host individual is immunosuppressed. This is an enormously important observation since it suggests the immune system, particularly the T lymphocyte arm, is critical in controlling normal infections (Borysiewicz and Sissons, 1994, Roizman, 1996).

1.2 Structural features of the Herpesvirus family

Members of the Herpesviridae are large double-stranded DNA viruses. A typical herpes virus consists of a core containing a linear double-stranded DNA genome of between 120 and 230 kilobase pairs (Kbp). DNA is packed into the core at a high density without any internal protein associations. Surrounding the core is an icosadeltahedral capsid of around 100 to 110nm in diameter consisting of 162 capsomeres. The protein components of the capsid are highly conserved between Herpesvirus family members. It has been suggested that the same basic mechanism for capsid maturation is maintained throughout the family (Davison and Clements, 1997). The capsid of Herpes simplex virus 1 (HSV-1) has been extensively studied. These studies have revealed that a single protein ‘VP5’ is the key component of the virus’s hexameric and pentameric capsomeres alongside subsidiary components such as VP23, VP190, VP24 and VP26. The capsid is surrounded by an amorphous electron-dense tegument. The structure of the tegument is currently poorly understood though it is recognised as complex, consisting of several different proteins. Within the Herpesviruses, components of the tegument are often
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Table 1.1  Representatives of the gammaherpesvirus family are found in a wide range of animal species.

Table adapted from:
specific to a particular subfamily or genus. This suggests the tegument is probably not just a passive component of the virion but probably plays an important role in the virus-host interaction (Davison and Clements, 1997). Components of the tegument which have been characterised to date include the 'viral host shutoff' protein UL-41 of HSV-1. UL-41 reduces the cytoplasmic stability of both cellular and viral mRNAs and switches off the synthesis of host cell proteins (O’ Hare, 1993). The tegument is bordered by a phospholipid membrane or envelope with glycoprotein ‘spikes’ protruding through it. These glycoproteins are embedded via one or more hydrophobic domains. Functionally, these virion surface molecules are associated with initial virus/host interactions and are, therefore, critically important in pathogenicity. Glycoproteins have also been implicated in the process of viral egress from the host cell (Davison and Clements, 1997). Overall, herpesvirus particles are between 120 and 300nm in diameter (Roizman, 1996, Borysiewicz and Sissons, 1994).

As previously noted, Herpesviruses all have double-stranded linear DNA genomes which vary a great deal in size and structure between members of different sub-families with genomes ranging in size from around 120Kbp to 230Kbp in length (Honess, 1984). 6 different types of genome have been identified to date. These differ in the number, position and orientation of internal and terminal repeat regions. A significant number of Herpesviruses have now had their genomes fully sequenced including HSV-1, Varicella zoster virus (VZV), human Cytomegalovirus (HCMV) and Murine gammaherpesvirus 68 (MHV-68) (McGeoch and Dalrymple, 1988, Davison and Scott, 1986, Chee et al., 1990, Baer et al., 1984 and Virgin et al., 1997). Sequencing is a critical step in the investigation of a virus as it allows experimental studies to be carried out on individual genes of the organism (Davison and Clements, 1997). Herpesviruses encode between 70 and 200 genes and studies have shown that in most members of the Herpesvirus family the majority of DNA codes for functional proteins.
Chapter 1 – Immune responses to Herpesviruses

The functions of virally encoded gene products have been investigated by direct genetic and biochemical studies based on sequence information. Such studies have revealed that the genes encoded by Herpesviruses fall broadly into 6 different functional categories. These categories are outlined in Table 1.2.

1.3 Herpesvirus Replication and Latency

Numerous studies have investigated the replication of Herpes simplex virus and this is now a relatively well understood area. Unfortunately, less information is available on other Herpesviruses as many have proven very difficult to propagate in vitro. For example, little success has been achieved in the development of a fully permissive cell culture system for Epstein Barr Virus (EBV). In this field, many studies have been restricted to the artificial induction of EBV reactivation from latently infected semi-permissive cell lines. Despite problems with in vitro studies, all investigations of Herpesvirus replication to date have demonstrated 3 major phases of transcription and protein synthesis. It is thought, therefore, that results obtained with viruses such as HSV can be extrapolated to more enigmatic members of the Herpesviridae family. A schematic representation of EBV cellular entry and replication is presented in Figure 1.1.

Herpes virus infection of a host cell begins with the adsorption of the virus particle to the cell surface. The attachment of individual Herpesviruses to specific cell types is covered in more depth later in this chapter. However, attachment generally occurs via some form of viral glycoprotein-cell surface molecule interaction. Adsorption is followed by the virus particle penetrating the cell surface. Again virus glycoproteins play a major role in this process. Opinion over the last few years has favoured the theory that, in the case of HSV, a fusion occurs between the viral envelope and the cell membrane leading to capsid and tegument release into the cell cytoplasm. EBV, in contrast, appears to enter
Figure 1.1

Adorption
Virus Particle
Cytoplasmic membrane
Gp350 (EBV)
CD21/CD21 homologue (EBV)
LMP 1 & 2
Smooth membrane vesicle
Cytoskeleton mediated transport of capsid to nucleus
Endocytosis (EBV)
EBV envelope fuses with vesicle membrane - probably mediated by Gp85
Immediate-Early proteins
Early proteins
Late proteins
Cytoplasmic membrane
Gp350
DNA replication (Rolling circle)
Assembly
EBV may acquire envelope via budding through cytoplasmic membrane
EBNA 1, 2, 3a, b, c, LP EBER-1 EBER-2 LMP1, LMP-2
Circularisation
Episome
α mRNA
β mRNA
γ mRNA
Nucleus
Release
Figure 1.1 Schematic composite representation of Gammaherpesvirus (EBV) replication. Epstein-Barr virus binds the cell via an interaction between the virion surface glycoprotein Gp350 and the cell surface molecule CD21. The virus then enters the cell by endocytosis. A fusion event occurs between the viral envelope and the smooth membrane vesicle resulting in the release of the capsid which is transported to the nucleus via the cellular cytoskeleton. Viral DNA is then released into the nucleus where it circularises. In the majority of infected B lymphocytes a latent form of infection is adopted where the viral genome remains in an episomal form and several genes associated with latent infection are expressed. In cells in which replication can occur a sequential expression of immediate-early, early and late genes occurs. The expression of the IE, E and L genes results in viral DNA replication (which occurs via a rolling circle mechanism), structural protein expression and ultimately assembly of the capsid and genome packaging. Herpes simplex virus is thought to obtain its envelope by budding through the inner nuclear membrane (illustrated). In contrast EBV is thought to obtain its envelope by budding through the plasma membrane of the cell after release from a cytoplasmic vesicle.

Diagram adapted from:


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<td>VP16 or UL48 of HSV which act to stimulate selective transcription of Immediate Early (IE) genes</td>
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<td>Nucleotide metabolism</td>
<td>Thymidine kinase involved in the phosphorylation of thymidine and deoxycytidine in the biosynthesis of DNA precursors</td>
</tr>
<tr>
<td>DNA replication</td>
<td>DNA polymerase involved in the synthesis of DNA</td>
</tr>
<tr>
<td>DNA packaging</td>
<td>UL15 involved in the insertion of DNA into the capsid</td>
</tr>
<tr>
<td>Virion structural</td>
<td>VP5 which makes up the majority of the viral capsid</td>
</tr>
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<td>Pathogenesis</td>
<td>The IL-10 homologue of Epstein-Barr Virus</td>
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Table 1.2 The functional groups into which Herpes virus genes are broadly classified

its preferred cell type by endocytosis (Kieff and Liebowitz, 1990). Following entry, the viral capsid and tegument components migrate to the nucleus. This migration most likely occurs via the cellular cytoskeletal network (Elliot and O' Hare, 1998).

Once viral DNA has been released into the nucleus the virus can either adopt a latent form of infection or it can proceed to replicate its DNA and produce new virus particles. Herpesvirus latent infections are characterised by the presence of circularised, non-integrated episomal genomes in the nucleus of an infected cell. Gene expression during a latent infection varies between Herpes family members. HSV expresses latency associated transcripts (LATs) during latent infections of neurones. LATs are not believed to be polyadenylated or translated in vivo but are thought to play a role in reactivation of the virus. In contrast, EBV can adopt 3 different forms of latent infection, each with a different profile of gene expression (see Table 1.3).

If the Herpesvirus replicates itself within a cell, viral DNA is transcribed into mRNA by the enzyme host polymerase II. A range of viral factors participate in all stages of this process (Wagner, 1985).

At the outset of replication a series of immediate-early (IE) or α genes are expressed. Transcription at this stage is independent of viral protein synthesis but is greatly enhanced by components of the tegument (O' Hare, 1993). IE gene expression produces a number of transacting regulators of viral genes which initiate a cascade of early (E) and late (L) gene expression (Honess and Roizmann, 1974). Early genes are described as those which are expressed in the presence of IE gene products prior to DNA replication. These encode a number of DNA replication enzymes, some proteins responsible for nucleotide metabolism and a few glycoproteins (Davison and Clements, 1997).
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</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>EBNA-1, EBERs</td>
<td>Burkitt’s lymphoma</td>
</tr>
<tr>
<td>Type 2</td>
<td>EBNA-1, LMP-1, LMP-2a, LMP-2b, EBERs</td>
<td>Hodgkin’s lymphoma; Nasopharyngeal carcinoma; T cell lymphoma</td>
</tr>
<tr>
<td>Type 3</td>
<td>EBNA-1, EBNA-2, EBNA-3a, EBNA-3b, EBNA-3c, EBNA-LP, LMP-1, LMP-2a, LMP-2b, EBERs</td>
<td>EBV-associated Lymphoma (EBV-LPD of immunosuppressed individuals; Lymphoblastoid cell lines (LCLs used for in vitro studies)</td>
</tr>
</tbody>
</table>

Table 1.3 Different patterns of latent gene expression in Epstein Barr virus infections. Abbreviations; EBERs - Epstein-Barr virus (EBV) small non-polyadenylated RNAs, EBNA - Epstein-Barr virus nuclear antigens, EBNA-LP - EBNA leader protein, LMP - latent membrane protein.

It should be noted that these categories of latency are useful but within an individual all may occur during the course of a normal infection.

Table adapted from;
Herpesvirus DNA replication is initiated from one or more origins of replication and results in the production of a number of head to tail concatameric genomes. This is thought to occur via a rolling circle mechanism (Jacob et al., 1977, 1979, Siegel et al. 1981). DNA concatamers are cleaved and packaged into pre-formed capsids. To date, this mechanism has only been extensively characterised for HSV. It is assumed, since there is significant homology between different Herpesviruses, that the mechanism may be very similar throughout the family.

It is unclear whether tegument addition occurs at the nuclear envelope or in the cytoplasmic compartment. Further, there is some debate over the origin of the Herpesvirus envelope and how the virus leaves the infected cell. In the case of HSV it appears that immature glycoproteins embed themselves in the nuclear membrane. Mature capsids can then bud through this membrane and so obtain both an envelope and a complement of immature glycoproteins. It is thought HSV subsequently moves through the Golgi apparatus where glycoprotein maturation occurs and particles are released by a process of reverse endocytosis (Kousoulas et al., 1983, Desai et al., 1988, Gong and Kieff, 1990). EBV, in contrast, appears to bud from the plasma membrane of infected cells whilst human Herpesvirus 6 (HHV-6), murine Cytomegalovirus (MCMV) and Herpesvirus saimiri (HVS) appear to bud into cytoplasmic vacuoles prior to release from the cell (Tralka, 1977, Papadimitrion, 1984, Biberfield, 1987).

1.4 The study of Herpesviruses

From a human viewpoint, many clinically important diseases are first recognised on the presentation of disease symptoms in a clinical situation, however, from a scientists point of view humans are far from 'ideal' experimental models. They have highly variable
genetic, physiological and immunological backgrounds, are difficult to obtain in large
cohorts and usually present themselves when infectious processes are well established.
Therefore, it is clear that animal models are important to the investigation and
understanding of infectious disease. Obviously, whether an animal will provide a useful
model of a disease depends on the questions to be answered but of primary importance
is; ‘does the animal infection closely mimic the human disease clinically or
experimentally?’ (Stanberry, 1992). Often the choice of model is dependent on the
availability of immunological reagents or a particular inbred strain. It should be
remembered, however, that some relatively outbred animal species whose clinical
manifestations of infection bear little resemblance to the human disease have proven
very useful in the study of viral pathogenesis. A variety of models may be used, if
available, which could reduce the risk of drawing misleading conclusions (Aurelian et al.,
1991). Generally, small rodents prove to be inexpensive, genetically well defined and
relatively malleable hosts for repeatable analytical studies. Obviously, drawbacks do
exist, not least that the pathogenesis of infectious diseases may differ significantly
between humans and animals (Mester and Rouse, 1991).

Despite the drawbacks, animal models have the potential to be enormously useful. In
particular, the development of mice with selective genetic immunodeficiencies has been
a great advance as has the use of animals in the development of vaccines and adoptive
transfer protocols.

1.5 Immune responses to viral infection

The immune system is a multi-component complex whose individual arms, the innate
immune system and the adaptive immune system usually work synergistically to rid an
individual of infection (Oldstone, 1994).
The innate immune system acts to control viral infections at a very early stage and innate immunity is thus potentially very important in preventing the systemic spread of pathogens. Innate immunity comprises 3 major components: the interferons, NK cells and macrophages.

The interferons (type I - ‘α’ & ‘β’ and type II ‘γ’) act at the onset of a viral infection to limit viral replication through interference with; target cell entry, virus replication and infectious particle release. Interferons are also critically important in the up-regulation of anti-viral effector cells such as macrophages and NK cells.

NK cells are large granular lymphocytes with the ability to kill virally infected cells and provide an important link between the innate and adaptive immune systems through their ability to participate in antibody dependent cell cytotoxicity (ADCC) and to produce cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNFα). These cells are predominantly active 2 to 3 days post infection (pi). Their importance in human herpesvirus infections was demonstrated in the clinical observations of a seminal and often quoted study of 1989 (Biron et al., 1989).

The importance of macrophages in antibacterial immunity has been widely studied, however, these cells can also exhibit potent antiviral properties. Macrophages are major producers of IFN-γ and TNF-α and can also phagocytose and kill antibody coated viruses. Alternatively, viruses may sometimes manipulate these cells to their own end, a point which is amply demonstrated in cases of macrophage infection by human immunodeficiency virus and CMV (Nash and Usherwood, 1997).

Other components of the innate immune system which may have significant antiviral effects include soluble proteins such as the collectins and complement.
The adaptive immune system has both humoral (antibody mediated) and cellular components, T lymphocytes being essential for the efficient induction of a full blown immune response. In particular the anti-viral effector functions of T lymphocytes are often highly significant. This has been demonstrated in studies in which suppression of T lymphocyte-led immune responses leads to a predisposition of an individual to severe viral infection and disease (Oldstone, 1994).

CD4+ T lymphocytes recognise, via their T cell receptor, virally derived peptides presented in the context of class II MHC on the surface of an antigen presenting cell (APC) such as a dendritic cell or B cell. Once a CD4+ T cell has been activated it can exert antiviral effector functions of its own or promote the functional activities of CD8+ T cells, NK cells or B cells. It has been demonstrated that mice lacking functional CD4+ T cells have difficulty in generating responses to infection, be they T cell or antibody mediated.

CD8+ ‘cytotoxic’ T lymphocytes (CTL) recognise short peptide sequences derived from structural or non-structural viral proteins. These peptides are presented in the context of cell surface glycoproteins coded for by the class I MHC. Once recognition of the MHC classI/ viral peptide complex has occurred CTL can exert their antiviral effects on the infected cell by a number of different means. Antiviral effector mechanisms include, granzyme/ perforin and/ or fas mediated cell killing or the induction of an antiviral state via cytokine release. An enormous amount of research is currently proceeding in this field and it is an area too broad to review here. The field has been comprehensively reviewed recently by Doherty (1996b) and Kagi et al. (1995). CTL play a ‘commanding’ role in the control of viral infections, including those of lymphocytic choriomeningitis virus, influenza virus, respiratory syncitial virus and CMV (see later). The ability of T
lymphocytes to lyse infected cells was first quantified in vitro in the late 1960's by Brunner et al. (1968). They employed radioactive $^{51}$Cr uptake and release from 'target' cells as an indicator of cytotoxicity. This quantitative assay enabled advances in the investigation of CTL specificity, MHC recognition and the cloning of virus-specific effector cells (Zinkernagel and Doherty, 1974). More sophisticated molecular techniques in the 1980s enabled the demonstration of the recognition of viral peptides in the context of MHC molecules by a receptor on the surface of T lymphocytes (Townshend et al., 1986). In recent years, the identification of specific viral peptide or 'epitope' sequences involved in stimulating immune responses, has become relatively common. CTL, then, are a critically important component of immune responses to a variety of viral infections (Oldstone, 1994). It should be noted, however, that historically defined roles for CD4+ and CD8+ T lymphocytes are becoming increasingly blurred as evidence evolves that both cell subsets can exert cytolytic or cytokine mediated antiviral effects.

The first antibody responses occur around 5 to 7 days pi. In the first instance low affinity IgM can often control the systemic spread of viral infections via the circulation. As the infection proceeds IgG becomes more prominent in the control of infection. IgG plays a role in virus neutralisation, the inhibition of fusion events, the blocking of virus uncoating and ADCC. Antibodies play an important role in increasing the effective range of the innate immune system by coating infectious particles or infected cells and allowing macrophages, NK cells or granulocytes to exert antiviral effects (Nash and Usherwood, 1997).

The fundamental objective of studies into antiviral immune responses is to enable researchers and clinicians to manipulate, interfere with, boost or even reduce immune responses to promote recovery from infection. In the future this may occur via improved vaccination strategies and reagents, adoptive transfer of virus specific lymphocytes,
passive antibody therapy, recombinant cytokine therapy and anti-inflammatory treatments. These ‘treatments’ may prove useful to a greater or lesser extent in humans, however, all are eminently open to investigation in animal models.

In this review, immune responses to prototypic members of the α, β and γ Herpesvirus subfamilies - HSV, CMV and EBV respectively - will be considered. Many of the most revealing studies of these viruses have been made in animal models. Where possible, conclusions from these studies will be included. When considering the gammaherpesvirus subfamily, overviews of the newly recognised human Herpesvirus 8 and the mouse derived MHV-68 will also be included. To conclude this review, the broad aims of current Herpesvirus research will be considered briefly alongside a concise summary of where research is aiming to proceed in the future.

1.6 The Immune response to Herpes simplex virus

Herpes simplex types 1 and 2 (HSV 1 & 2) are closely related viruses that share approximately 50% DNA identity and are responsible for both primary and recurrent infections (Stanberry, 1992). HSV-1 & 2 display the typical characteristics of herpesviruses and have genomes of around 150 kilobase-pairs (Kbp) which encode around 70 proteins. Infections with Herpes simplex virus have been recorded for thousands of years, however, it was not until the late 1800s that transmission of the virus between individuals was established. The infectious nature of the virus was confirmed beyond doubt in the early 20th century with the first recorded animal study being made. This involved corneal lesion induction in rabbits (Whitley, 1996). Between the early 1920s and 1960s a range of animals was used to study the biology of this virus. These ‘models’ enabled the demonstration that HSV infects the skin and central nervous system (CNS). Immune responses to HSV were first recognised in the 1930s in parallel with the recognition of recurrent infections and reactivation from latency
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(Whitley, 1996). In the last 30 years, major advances in this field include the description of antigenic and biological differences between HSV types 1 and 2 and the successful clinical use of antiviral agents.

1.6.1 Infection with Herpes simplex viruses

HSV is transmitted via intimate contact between a susceptible individual and someone secreting the virus. Infection begins with the virus contacting a mucosal surface or abraded skin on the susceptible recipient. Typically, this is the oropharyngeal epithelium in HSV-1 infection and a genital epithelial site in HSV-2 infection. It is thought that 3 virus particle glycoproteins are involved in binding and penetration at this site, gB, gD and gH. Attachment on the host may occur via a combination of heparin sulphate and perhaps fibroblast growth factors (Stanberry, 1992, Whitley, 1996). Following attachment, the virus undergoes penetration of the cellular membrane, loses its envelope and the nucleocapsid migrates into the nucleus where DNA replication occurs. Progeny viruses are assembled in the nucleus and then bud through the inner nuclear membrane. Mature virus particles are released from the cell surface after modification in the Golgi apparatus (Stanberry, 1992). Typically, replication at the site of primary infection can occur for around 3 to 4 weeks and is associated with the formation of vesicular lesions that heal to form scabs that rarely scar. After replication of HSV at its site of infection, either an intact virion or simply its nucleocapsid is transported via retrograde axonal transport to the dorsal root ganglion. The patch of skin innervated by the dorsal root ganglion is termed the dermatome. The dermatome, sensory ganglion dorsal root and spinal nerve comprise the neurodermatome. This combination is sufficient to propagate HSV and provides an environment for persistence in the host (Simmons et al., 1992). Unusually, primary infection spreads beyond the dorsal root ganglion to become systemic. On reaching the dorsal root ganglion HSV undergoes replication for a limited period and adopts a latent state. During latency there is only a
limited region of viral genome transcriptionally active. The function of the latency-associated transcripts is still unclear but it is known they are essential for the establishment and maintenance of latency (Stanberry, 1992). 'Stimuli' such as immune suppression, UV irradiation, stress and fever can lead to a reactivation of the virus from the dorsal root ganglion. This can lead to recurrent infections which are characterised by the virus becoming evident at mucocutaneous sites in the form of vesicles or mucosal ulcers. Very rarely, HSV infections of the brain can occur leading to encephalitis.

1.6.2 The outcomes of HSV infection

There is much variability in the clinical outcomes of primary HSV infection. Generally, a very small proportion of those infected with HSV present with clinical symptoms. Severe disseminated disease is a very rare result of infection. Usually symptoms of infection are restricted to the region of viral entry and the nervous system innervating that area. Clinically, HSV-1 and 2 are indistinguishable and in the immunocompetent individual can cause: stomatitis/pharyngitis, keratoconjunctivitis in the eye and primary oral/genital herpes infection, characterised by vesicular lesion formation (Doymaz and Rouse, 1992, Stanberry, 1992, Whitley, 1996). During the past 20 years, the prevalence of genital infection caused by HSV-2 has increased significantly. Around 5 to 20 million people in the United States (US) are infected although rarely is this severe or fatal (Aurelian et al., 1991). In the majority of cases, if T cell immunity is intact in the individual, clearance of HSV will be rapid and injury will be insignificant. However, if infected individuals are very young or immunosuppressed, severe and disseminated HSV infection and disease can result. This observation is very significant since it points towards immune mechanisms being critically important in controlling HSV infection (Schmid and Rouse, 1992).
Neonatal infection with HSV occurs in around 400 births per year (in the US) and is invariably symptomatic and frequently lethal. The infection can result in symptoms ranging from localised lesions to systemic complications such as encephalitis. The outlook for neonates infected with HSV is poor with fifty percent of those who survive infection having motor function retardation.

Primary infection or HSV reactivation in immuno-compromised individuals, e.g. those who are entering the late stages of HIV infection, can produce devastating systemic injury (Whitley, 1996).

In contrast, if inflammatory responses to the virus are prolonged, immunopathological sequelae may result. For example, Herpes simplex keratitis is the leading non-traumatic cause of blindness in the US with more than 1/2 a million sufferers (Doymaz and Rouse, 1992).

1.6.3 The study of HSV infection

A wide variety of animal models have been used to study HSV infection. The rabbit was the first animal used successfully and, more importantly, scientifically to study HSV-induced ocular disease (Stanberry, 1992, Whitley, 1996). Despite early promise from the rabbit model, the majority of information about HSV pathogenesis has been obtained from the study of HSV infection in mice and guinea pigs.

A variety of different inoculation routes have been employed for the infection of mice with HSV. The route employed has often been determined by the aspect of infection or immune response being studied. Intraperitoneal inoculation bears little resemblance to a natural route of infection and has revealed little concerning specific immune responses to the virus. On the other hand, intradermal inoculation of the virus into the ear flaps of
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mice has proven enormously useful in the study of epithelial immune responses. Rather different to this is the 'zosteriform spread inoculation model'. This employs a flank inoculation of mice and has been useful for studying the spread of virus in the peripheral nervous system, recurrent infections and some aspects of antibody-mediated protection (Simmons et al., 1992). Inoculation of HSV into the footpads of mice has been used to gather information on intra-neural virus transport. In the late 1980s this route of inoculation also proved critical to the discovery of the latency-associated transcripts produced by latent virus in the sensory ganglion (Stevens et al., 1987). Further, neonatal infection of mice has been of use in the investigation of HSV infections in the young and the importance of ADCC (Kohl, 1991).

In recent years experimental studies of host resistance to genital herpes infections have been carried out in mice. Intravaginal inoculation of BALB/c mice with a mutant HSV-2 lacking thymidine kinase induces a protective immune response to subsequent lethal challenge with wild type HSV-2. This model has proven important in the confirmation that mucosal virus-specific IgG is critical in protection in the vagina (Parr and Parr, 1998a, Milligan and Bernstein, 1997).

Female guinea pig inoculation with HSV has been employed to study experimental genital infections since genital HSV infections of guinea pigs share many similarities with those that occur in humans. Following inoculation via a natural route of infection, the animals develop herpetic lesions 3 to 4 days pi. These lesions scab over by days 8 to 10 pi and heal around 2 weeks after exposure to the virus. Perhaps the most important feature of the infection is that the virus adopts a latent state and spontaneously undergoes reactivation at intervals after inoculation. Unfortunately, guinea pigs are more expensive to rear than mice and there is a dearth of immunological reagents available for these rodents. Despite these drawbacks, guinea pigs have been used
succesfully to study neutralising antibody responses, DTH responses, ADCC, NK and LAK activity and cytokine responses to HSV (Stanberry, 1992, Whitley, 1996).

Currently, several groups around the world are planning to extend the number of animal species used to study experimental HSV infections. For example, Jugovic et al. are currently looking to identify an animal model to assess the effects of the HSV protein ICP-47 on infection. This protein does not efficiently inhibit the mouse transporter associated with antigen processing (TAP). Their studies with HSV have extended to infections of several primates including macaques and chimpanzees as well as infections of dogs, cats and pigs (Jugovic et al., 1998).

1.6.4 Immune Responses to HSV

1.6.5 Natural/ Innate immunity

The cellular innate immune system consists of monocytes and macrophages, NK cells, polymorphonuclear cells and the cytokines that they produce. These diverse components play a variety of roles during HSV infection. It has been suggested that the final extent of HSV infection can be determined by the efficacy of the initial innate immune response (Nash and Cambouropoulos, 1993). The importance of individual components in such a response has been studied by selectively depleting and then replacing cellular subsets or soluble effectors in the context of in vitro and in vivo animal studies.

Understanding of the importance of the monocyte and macrophage in anti-HSV immunity is rudimentary (Wu and Morahan, 1992). In theory, macrophages can 'extrinsically' inactivate extracellular virus, inhibit virus replication in surrounding cells or destroy infected cells. 'Intrinsically’ they have the ability to inhibit intraphagolysosomal replication via a combination of classical destructive processes coupled to an inhibition
of replication. In reality, there is only limited evidence to confirm the importance of these mechanisms in humans. For example, macrophages or monocytes from neonates are less resistant to infection with HSV than those from adults. It is thought this resistance arises as a block in HSV replication before early gene expression (Wu and Morahan, 1992). In animals, the importance of macrophages in anti-HSV immunity has long been recognised from cell transfer and protection studies in mice (Wu and Morahan, 1992). Unfortunately, exactly how the transferred macrophages confer protection remains unclear. Potentially the most important effector role for these cells is in CD4+ T cell led DTH responses to epithelial HSV infection. Evidence from HSV infection of IFN-γ knockout mice has shown that IFN-γ is a critical mediator in the natural immune response to the virus. It has been suggested that macrophages may be the critical source of this cytokine (Yu et al., 1996). Further macrophages make up a significant proportion of the inflammatory cells found in the genital tracts of mice inoculated with HSV-2 thymidine kinase negative mutants. These cells could be releasing molecules such as TNF-α and nitric oxide which may contribute to the resolution of infection in this model (Milligan and Bernstein, 1997).

The role of NK cells in HSV infection is poorly understood and remains controversial (Nash and Cambouropoulos, 1993, Wu and Morahan, 1992). It is well recognised that HSV infections are more common and severe in several immunocompromised states where NK cell function is affected (Biron et al., 1989). However, it is difficult to attribute increased HSV led disease solely to NK cell dysfunction when other immune components may be functionally awry. It has been suggested that NK cells may limit systemic HSV spread, however, the severity of HSV infection undoubtedly depends on other factors such as dose of virus received, age and site of infection (Nash and Cambouropoulos, 1993). Bukowski and Welsh demonstrated a normal HSV-led disease progression in beige mice which lack NK cell activity (Bukowski and Welsh, 1986).
Similarly, antibody depletion of NK1.1 or asialo-GM1 bearing NK cells in mice does not lead to more severe HSV elicited disease (Nash and Cambouropoulos, 1993, Wu and Morahan, 1992). Adoptive transfer experiments have demonstrated that NK cells are not required for resistance to HSV infection and disease in mice. Studies in the mouse model of HSV reactivation after UV irradiation suggest NK cells are unlikely to play an initial role during reactivation but do appear to infiltrate ganglia sometime later (Shimeld et al., 1996). Further, investigations of murine HSV-2 vaginal infections and recurrent human HSV-2 genital lesions have found no direct association between NK functional activity and viral clearance. These investigations have, however, provided some evidence for NK involvement in T lymphocyte recruitment into infected mucosal sites (Milligan and Bernstein, 1997, Koelle et al., 1998).

A role for polymorphonuclear granulocytes (PMN) in anti-HSV resistance has been suggested. In the mouse model of Herpetic stromal keratitis (HSK) the prominent cell type in HSK lesions is the neutrophil (a cell type not commonly associated with the resolution of viral infections). Thomas et al. (1997) have shown that neutrophil infiltration into the cornea of HSV infected immunocompromised mice is a biphasic event. Initial PMN infiltration into the cornea is clinically inapparent, triggered by replicating virus and clears detectable virus from the site. A second more intense PMN invasion then occurs. It is this influx of cells, orchestrated by CD4+ T lymphocytes which elicits HSK. Results indicate the PMN not only contribute to injury in this situation but also expose potentially auto-reactive antigens in the cornea (Thomas et al., 1997) Unfortunately, the role of PMN in 'normal' infections remains elusive although they have been functionally implicated in ADCC responses.

Interferon production by an infected host is a well recognised anti-viral response. It is thought that, in the context of HSV infection, interferons most likely work in concert with
macrophages. Interferons are typically pleiotropic and can initiate the degradation of viral mRNA and inhibit viral protein synthesis. A number of studies have been made of the role that HSV-induced interferon plays in the resistance of human monocytes to the virus. Albers et al. (1989) demonstrated that monocytes which were highly resistant to HSV produced high levels of interferons α and β. Unfortunately, it was eventually concluded that these were induced later than the occurrence of the anti-viral replicative block. In the mouse, the importance of interferon production in response to HSV infection has been demonstrated in a couple of ways. Injection of neutralising antibodies into infected mice rendered the animals more susceptible to infection, perhaps by dampening overall macrophage activity (Sit et al., 1988). More recently, data from interferon α, β and γ knockout mice has suggested these molecules play a major role in limiting viral replication in the cornea and peripheral nervous system (Lieb et al., 1998).

From the studies carried out to date on innate immune responses to HSV it would seem the system is of importance in humans and animals. Unfortunately, exact roles for individual components of the system remain unclear.

1.6.6 The responses of CD4+ T lymphocytes to HSV infection

It is well established that a profound suppression of T cell immunity can severely compromise the ability of humans and experimental animals to control HSV infection. Studies over the years have demonstrated that the number of potential protective immune mechanisms to HSV mediated by T lymphocytes is great. Further, investigation of distinct T cell lineages has demonstrated enormously complex interactions between cellular ‘species’ (Mester and Rouse, 1991). In vivo, studies provide several lines of evidence which implicate CD4+ T cells in anti-HSV immune responses. Cunningham and Noble (1989) demonstrated that CD4+ T lymphocytes, along with Langerhans cells and macrophages are the predominant cells inside primary and recurrent herpetic
lesions during the first few days after their appearance. CD8+ T lymphocytes often do not appear until after lesions have begun to resolve (Schmid and Mawle, 1991). More recent work, involving the detailed analysis of sequential biopsy material, has confirmed these findings. These studies have found that infiltrating CD4+ T lymphocytes with proliferative, cytokine producing and cytotoxic activities predominate in the first few days of lesion formation. It is thought these cells induce or restore the expression of MHC class I or II molecules on the surface of infected epidermal cells or keratinocytes (overriding the effects of the viral IE protein ICP-47). As a result these cells become viable targets for lesion infiltrating CD4 and/or CD8 cytotoxic T cells (Mikloska and Cunningham, 1998, Posovad et al., 1998a).

In animals, studies on the roles of CD4+ and CD8+ T lymphocytes in the resolution of HSV-led disease were very popular in the early to mid 1980's. Initially, it was demonstrated that cells from the spleen and lymph nodes of infected mice were able to protect naive animals from HSV-led disease and accelerate viral clearance (Simmons et al., 1992). At this stage some controversy arose over which T lymphocyte subset was most important in viral clearance. Nash et al. (1981) suggested that an interaction between class I and II restricted cells was required for clearance of infectious virus from the skin of mice. Later they demonstrated the simultaneous transfer of delayed type hypersensitivity (DTH) and protection from an HSV challenge inoculation of the ear pinna (Nash and Gell, 1983). HSV immune class-II-restricted T-helper (Th)/DTH cells were implicated in this protective response. The protective capacity of Th cells was further demonstrated in the zosteriform spread model. In fact, both CD4+ and CD8+ T cell subsets can exert anti-HSV effects in infected animals (Nash et al., 1987). The role of CD8+ T lymphocytes will be considered later. In 1987, Nash et al. showed that a cloned CD4+ T cell line was very effective at resolving local ear pinna infection with HSV. Transfer of the clone also reduced the level of viral latency occurring in the mice
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(Nash et al., 1987). It is thought that CD4+ T lymphocytes probably act to clear HSV from epidermal sites by recruitment and activation of macrophages. Macrophages have the capacity to eliminate HSV via the production of TNF$\alpha$, IFN-\(\alpha\) and \(\beta\), arginase and cationic protein production in a DTH-like response (Nash and Cambouropoulos, 1993).

In a recent model of genital HSV-2 infection, TH1-type CD4+ T cells have been implicated in the clearance of infectious virus from the vagina via the production of IFN-\(\gamma\) (Milligan and Bernstein, 1997, Parr and Parr, 1998b).

As well as CD4+ T cells being important in protective DTH responses to HSV infection, cytotoxic CD4+ T cells are significant in the control of infection with this virus. Early attempts to clone and characterise human HSV-specific cytotoxic T cells led to the observation that the majority of these were CD4+. It is now well established that the response of human CTL to HSV is characterised by a large component of CD4+ class II MHC restricted cells. These cells are most likely significant because HSV is capable of down regulating class I MHC molecule expression. There has, however, been some widespread reluctance to accept an important role for CD4+ CTL. This is because there are relatively few cells in the body which express class II MHC. It has been suggested that CD4+ CTL act to dampen immune responses by lowering the frequency of viral antigen presenting cells (Yasukawa et al., 1996). Evidence from animal models has suggested these CTL may be important in the control of recurrent lesions and acute ganglionic infections (Mester and Rouse, 1991). The mechanisms by which CD4+ CTL exert their effects were, until recently, unclear. However, it is now known that CD4+ CTL can express both perforin and membrane bound lymphotoxin simultaneously. Further, these cells can exert both antigen specific and non-specific cytotoxicity via two distinct mechanisms, necrosis and apoptosis (Yasukawa et al., 1996). By using an in vitro autologous human system it has been demonstrated that HSV-specific CD4+CTL from
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Peripheral blood recognise target cells presenting peptides derived from glycoproteins B, C, D and H of HSV. CD4+ CTL from this source also recognised cells presenting peptide derived from the tegument protein VP16 (Mikloska and Cunningham, 1998).

1.6.7 The responses of CD8+ T lymphocytes to HSV infection

The majority of studies into the roles of CD8+ T lymphocytes in control of HSV infection have been carried out in animals, particularly mice. As mentioned previously, early adoptive transfer studies implicated both CD4+ and CD8+ T cells in the protective responses of mice to HSV infection (Nash et al. 1981, Larsen et al., 1983). On transfer, both types of cells prevented ganglionic infection but could not contain established disease. Mice depleted of CD8+ T cells by antibody treatment displayed an inability to recover from ganglionic infection yet retained their ability to mount normal DTH and antibody responses (Cobbold et al., 1984, Nash et al., 1987). In mice the removal of CD8+ T cells impairs the clearance of virus from the dorsal root ganglion as effectively as total T cell depletion. The implication of this result is that CD8+ T lymphocytes are critical in controlling neuronal HSV infection. Significantly, infection with HSV leads to very few neurones being killed. These cells must survive very advanced stages of viral replication. It has been demonstrated that the CD8+ T cell response is critical for the survival of infected neuronal cells. In the absence of CD8+ T cells uncontrolled infection occurs in the mouse characterised by widespread neuronal death. CD8+ T lymphocytes must be capable of terminating non-cytolytic HSV replication via a non-cytolytic mechanism. It has been suggested that the antigen presenting cell for CD8+ T cells in the peripheral nervous system is the capsular cell of the neurone and that in mice CD8+ cells exert their antiviral effects via cytokine release (Simmons et al., 1992).

In contrast, recent studies of CD8+ T lymphocyte responses to HSV infection have been carried out in human systems. Prior to 1996, many reports stated that HSV specific
CD8+ CTL represented only a small fraction of the total cytolytic activity detectable in bulk cultures. This evidence was consistent with the identification of viral proteins such as ICP-47 and viral host shutoff (vhs) protein which can downregulate MHC class I expression on infected cells. However, in recent years the refinement of culture techniques has enabled the demonstration of high frequencies of HSV specific CD8+ CTL precursors in the peripheral blood of sero-positive individuals (Posovad et al., 1996). This finding raised an important question; 'how are CD8+ CTL generated if HSV infected cells have down-regulated MHC class I molecules'? To answer this question it has been suggested that MHC class I molecule down-regulation only occurs in distinct cell types such as keratinocytes whilst other cells can act to prime a CD8+ T cell response. Further, the host has a number of strategies to counteract viral immunoregulation. For example, the early production of cytokines such as IFN-γ within lesions may counteract the downregulatory effects of the protein ICP-47. Evidence for the functional significance of CD8+ CTL comes from two sources. Firstly HIV infected individuals with low frequencies of CD8+ CTL precursors suffer from frequent, severe HSV infections. Secondly, viral clearance from HSV-2 lesions has been directly correlated with increased infiltration of CD8+ CTL into this site (Posovad et al., 1998a).

In the past it was argued that in the majority of situations, CD8+ T lymphocytes are secondary to CD4+ lymphocytes in the clearance of epidermal infections. In the light of recent evidence this theory may have to be revised since it has been shown that functional HSV-specific CD8+ T cells can persist for at least two years following the reactivation of HSV at a mucosal surface (Posovad et al., 1998b).

Studies have suggested that the antigenic epitopes encoded by the virus, to which mice can respond, are determined by the MHC haplotype of the responding animal. Various reports have been made on the importance of the glycoproteins gB, gC and gD as
targets of CTL. However, antigens derived from the immediate early proteins of HSV are also thought to be of great significance, eliciting around 30% of detectable CTL responses (Nash and Cambouropoulos, 1993, Borysewicz and Sissons, 1994). In humans, CD8+ T cells from recurrent lesions have been shown to have specificity for peptides derived from Early HSV proteins (Mikloska and Cunningham, 1998).

1.6.8 Antibody responses to HSV infection
In humans, the role of antibody in protection against HSV infection is controversial. Two functional groupings of antibody are recognised; those which can neutralise virus (which specifically bind to the viral cellular receptor) and those which are capable of participating in ADCC. It is known that ADCC function is deficient in human neonates and this may be responsible for the increased severity of infections in these subjects (Kohl, 1991).

In mice, early passive transfer experiments showed that immune serum can provide a limited degree of protection against lethal HSV challenge. As long ago as 1967 it was demonstrated, however, that passively transferred antibody given 18 hours post infection does not influence the progression of virus from the peripheral to the central nervous system. Further, it was demonstrated that there is no apparent correlation between an established antibody titre and protection from virus colonisation of the sensory ganglion. Under natural conditions antibody is not detected in an infected host until several days after skin inoculation. By this time the sensory ganglion is usually already infected. It is arguable how relevant studies are involving large antibody adoptive transfers since, when physiological titres of immunoglobulin are administered to HSV infected mice, there is often no apparent effect on disease progression (Mester and Rouse, 1991).
In spite of this argument, a number of studies into primary epithelial infection, ganglionic colonisation and death have implicated a role for antibody in restricting the flow of HSV from the initial site of epithelial infection to the local sensory ganglion (Mester and Rouse, 1991). Highly specific monoclonal antibodies for viral glycoproteins such as gB, gC, gD and gE have been indispensable in these experiments. Similarly, B cell-suppressed mice have proven powerful tools in the study of endogenously produced antibody responses to HSV. These studies have shown that the elimination of the antibody response in mice has no effect on the healing of the vesicular lesions evident in primary infections. Mice lacking antibody, however, have more extensive infections of the peripheral nervous system although virus clearance is not affected and no increase in mortality is observed (Simmons et al., 1992). The suggestion has been made that locally synthesised antibodies may reduce the spread of HSV in the nervous system by neutralising extra-cellular virus moving between neurones (Simmons et al., 1992). In the mouse model of HSV-2 genital tract infection, data has indicated that virus-specific immunoglobulin (Ig) G in vaginal secretions can provide an early protective effect against low-dose viral challenge. It is thought this antibody probably acts to neutralise virus in the vaginal lumen before it can infect epithelial cells. IgA in this model appears to contribute little to protection (Parr and Parr, 1998a).

It is thought that antibody plays an important role in protecting against HSV infection via ADCC. The importance of ADCC has been highlighted by studies involving neonatal HSV infection. ADCC function is markedly deficient in neonatal mice. The adoptive transfer of antibody or human mononuclear cells to HSV-infected neonatal mice does not protect against a lethal virus challenge. In contrast, if antibody and the mononuclear cells are transferred together a protective response is generated (Kohl, 1991).
Therefore, antibody can interfere with entry and exit of HSV from the CNS/ PNS and also help to control the extent of viral infections in animal models. Its definitive function in humans, however, remains something of a mystery.

1.6.9 HSV subversion of the immune system

Herpes simplex viruses can interfere with both innate and specific immune responses in a number of ways.

1.6.10 Interference with innate immunity

Herpes simplex viruses encode a virion envelope glycoprotein, gC, which has been implicated in protection against complement activation. If gC is inactive, complement is capable of lysing enveloped viral particles and also preventing infection at a post HSV-attachment step, possibly viral entry to the cell (Wang et al., 1998).

1.6.11 Interference with the specific immune response

Herpes simplex viruses have several mechanisms for subverting specific immune responses. HSV-1 has been found to encode a receptor for the Fc portion of Ig. This receptor is a complex of two viral proteins gE and gl. It has been suggested the function of this complex is to enable the virus to mask the surface of infected cells and thus protect against specific antibodies binding. Recent evidence, however suggests that binding the Fc region of virus-specific antibodies by this receptor may result in 'bipolar bridging'. Bipolar antibody bridges can inhibit classical complement activation and ADCC (Davis-Poynter and Farrell, 1996).

Sieg et al. (1996), demonstrated that HSV-2 and not HSV-1 can potently inhibit the cell surface expression of Fas on infected cells and, therefore, suppress Fas-ligand mediated death. This mechanism enhances the viruses ability to replicate and seems to
involve HSV-2 disruption of intracellular transport mechanisms. It was proposed that the ability of HSV-2 to regulate Fas ligand activity may contribute to the viruses greater propensity for recurrence and enhanced severity of infection compared to HSV-1.

HSV infection of cells leads to the rapid degradation of host mRNA. This is largely caused by a structural component of the virus known as virion host shutoff protein (vhs). Vhs is thought to play a role in blocking de novo synthesis of MHC class I molecules at late stages of infection (Johnson and Hill, 1997).

Perhaps the most widely studied immunomodulatory product of HSV is the IE protein ICP-47. This is expressed shortly after virion cellular entry and tightly binds the cellular transporter associated with antigen binding (TAP). This results in the inhibition of TAP mediated translocation across the ER. ICP-47 can block the presentation of the vast majority of the 75 HSV polypeptides and can lower surface MHC class I molecule expression by almost 99% on infected cells. Significantly, ICP-47 is a very poor inhibitor of TAP function in murine cells yet it can still alter the course of HSV pathogenesis in these animals. The effects of ICP-47 are not absolute as IFN-γ can restore MHC class I molecule/TAP function in infected cells, overriding the viral protein. As discussed earlier, this may explain why CD8+ T lymphocytes become functionally significant within HSV lesions only after CD4 T cell release of IFN-γ (Johnson and Hill, 1997).

It has recently been shown that laboratory strains of HSV-1 but not HSV-2 can inhibit the oligonucleosome DNA fragmentation characteristic of apoptosis. HSV-1 does not, however, inhibit phosphatidylserine (PS) exposure at the cell surface. It has been suggested this is because PS exposure may facilitate virus transmission to adjacent cells (Jerome et al., 1998).
1.7 The Immune Response to Cytomegalovirus

Cytomegalovirus (CMV) is a prototypic betaherpesvirus. CMV is ubiquitous and highly species-specific, commonly infecting many animals including humans. The virus has a genome of around 230Kbp which codes for approximately 200 proteins. As with many of the herpesviruses, human CMV (HCMV) is a successful infectious agent as it undergoes widespread infection yet rarely kills its host (Britt and Alford 1996, Grundy, 1990). Primary CMV infection is followed by persistence in the infected individual probably via a latent infection of macrophages (Pollock et al., 1996).

Currently the complex natural history of CMV is poorly understood in humans. In vitro HCMV can be difficult to study as it can generally only be propagated in cells of human origin. The term cytomegalovirus was coined by Weller et al. in 1957 to reflect the changes produced by this virus in cells of the parotid and salivary glands. These changes had first been noted in the late 19th century. Humans are the only reservoir for HCMV. Transmission occurs by person to person contact through oropharyngeal/vaginal secretions, transplanted organs, semen, breast milk or blood (Britt and Alford, 1996, Merigan and Resta, 1990). On entry to the body, CMV usually infects ductal epithelial cells. From this site of initial replication, the virus can spread to the kidneys, GI tract, liver, respiratory tract and CNS. Inclusion bearing cells in the circulation suggest blood borne spread occurs in the majority of cases. Despite the fact that HCMV is lytic in tissue culture, only rarely do those infected with the virus exhibit clinical evidence of infection. In a small percentage of cases HCMV infection results in a mononucleosis-like condition. HCMV becomes clinically significant in a number of well defined situations in which some form of immunosuppression of the host is evident.

HCMV is the most common congenitally acquired viral infection and is the leading infectious cause of CNS maldevelopment in children. Infection at a young age can also
result in chorioretinitis, hepatitis and often life threatening organ dysfunction (Britt and Alford, 1996). The immune status of the mother can not only affect the likelihood of transmission but also the outcome of infection in the child (Merigan and Resta, 1990). Generally, it is felt that primary HCMV infection during pregnancy has a poor prognosis for the child.

HCMV is also a critically important pathogen in allograft recipients. Between 60% and 100% of all renal allograft recipients develop HCMV infection. The source of this virus is thought to be the transplanted organ or blood products received during the operation. HCMV causes high rates of mortality in bone marrow transplant recipients, most commonly from HCMV pneumonia. The pneumonia is thought to be a result of uncontrolled viral replication and/or an outcome of graft versus host disease (GvHD). HCMV is also one of the most important opportunistic infections of HIV infected individuals with AIDS. Disease can develop in all the organs of this infected group but is particularly destructive in the lung, CNS and GI system (Britt and Alford, 1996).

1.7.1 The Study of CMV

Since HCMV is so difficult to study, a number of animal models of CMV infection have been developed. CMV infections of the rat, guinea pig and non-human primates such as Rhesus macaques have all been attempted in order to study restricted aspects of CMV-induced disease. Unfortunately, most of these models have severe limitations and are imprecise replicas of the human infection.

Over the last decade, infection of the mouse with murine CMV (MCMV) has provided a relatively amenable model for CMV study. In order for this infection to conform to a clinical reality, the mouse strain selected for use had to be susceptible to infection yet resistant to clinical disease in an immunocompetent state. Importantly, lethal CMV-
induced disease should develop in the mouse once it was immunosuppressed. The BALB/c strain of inbred mice met these prerequisite conditions. When immunocompetent BALB/c mice are infected with CMV, virus replication persists for around 6 to 8 weeks pi in the salivary gland acinar epithelial cells. This is then followed by the establishment of latency. When the mice are immunodepleted, via exposure to $^{137}$Cs, reactivation of the virus occurs and disease ensues (Kosinowski et al., 1990). Experiments with these mice have provided important evidence unobtainable in a clinical situation.

1.7.2 Immune Responses to CMV

In humans, attempts to define protective, innate immune responses to CMV have generally been made in populations where there is some developmental or exogenous immunosuppression present. The effector functions of natural immunity to CMV have been most extensively studied in allograft recipients. Evidence suggests, following primary HCMV infection, a variety of cell mediated immune responses occur. In clinical studies, patients with decreased macrophage and NK activity (in vitro) have a poor prognosis on infection with HCMV (Rook, 1988). In line with this, detectable NK activity in renal transplant patients correlates with a more positive prognosis (Pasternack et al., 1990). Further, natural cytotoxicity in bone marrow transplant patients is an important correlate of survival. In newborn children, an increase in NK cell numbers and NK activated cells is often the only difference observed between asymptomatic patients and those with congenital CMV infection (Rasmussen, 1990). It would appear that, in humans, NK cell activity is crucial in responses to HCMV infection. However, it has been demonstrated that clinical isolates of HCMV can suppress the functions of large granular lymphocytes (including IFN-γ production). This is significant since it is these cells which are primarily responsible for NK activity (Rook, 1988).
In mice, a strong protective role for NK cells in anti-MCMV immunity has been demonstrated both by depletion methods (using anti-asialo GM1 or anti-NK1.1 antibody) and by adoptive transfer experiments (Bukowski et al., 1985). Indeed, evidence for the protective role of NK cells in certain strains of mice is substantial and convincing. For example, beige mutant mice lacking NK cell function have elevated susceptibility to MCMV infection (Koszinowski, 1990). In 1996, it was demonstrated that the host genotype of mice directly influences the importance of NK cell mediated immunity in the control of MCMV infection. Lathbury et al. (1996) showed that the replication of MCMV in the organs of CBA and C57BL/6 mice was controlled by NK cells and interferon α/β release before a T cell response was generated. In contrast, it was found that T cells were essential for the control of MCMV replication in BALB/c mice (see later). It was concluded from this study that the presence of an efficient NK cell response early in an infection is a major factor in determining whether T cells are required for the ultimate control of MCMV infection (Lathbury et al., 1996).

Very recent evidence has confirmed this conclusion and further demonstrated that NK cells often control infections in an IFN-γ dependent (and cytolytic independent) manner. Salazar-Mather et al. (1998) have shown that NK cells often migrate to MCMV infected organs such as the liver where they contribute to foci of inflammation. Migrations such as this seem to be dependent on the production of the chemokine MIP-1α by hepatocytes in response to MCMV infection.

Monocytes and macrophages are of key importance in the pathogenesis of HCMV and MCMV for a number of reasons. These cells are a site of MCMV replication and dissemination of MCMV (and probably HCMV) is mediated by monocytes within the blood. Further, monocytes and macrophages are a site of long term latency for HCMV and MCMV.
In relation to the host, macrophages are important in the presentation of antigen to CD4+ T cells and the production of cytokines. It should be noted, however, that some studies have suggested that the production of IFN-γ and TNF-α by macrophages in response to MCMV infection may elicit immunopathological lesion formation (Pasternack et al., 1990).

In conclusion, it would appear that the intrinsic difficulties of studying CMV infection have led to relatively little evidence being published in relation to natural immune defences to the virus.

1.7.3 CD4+ T cell responses to CMV infection

The function of CD4+ T cells which proliferate in response to HCMV infection is not understood. In CMV infection the most important cellular responses appear to be those of CTL which appear predominantly MHC class I molecule restricted (Merigan and Resta, 1990, Britt and Alford, 1996). Despite the importance of CD8+ CTL, evidence from HIV infected individuals points to some role for CD4+ T cells in anti-CMV immunity. Individuals with HIV suffer a progressive decline in the number and function of CD4+ T helper cells. This results in an inability to drive normal anti-viral and specifically anti-CMV responses most likely via an inability to produce IL-2 (Britt and Alford, 1996). Sufferers of AIDS regularly succumb to highly destructive HCMV led disease. A number of studies have demonstrated the ability of pp65 and gB to stimulate CD4+ T cells in vitro and in vivo. The exact functional outcome of such immune system stimulation remains unclear.

In the mouse, Kosinowski et al. (1990) demonstrated, using adoptive transfer experiments, that the CD4+ T cell subset had no anti-viral effect on its own. This finding
was contrary to those of Shanley (1987) who reported an anti-viral function for CD4+ T cells in the adrenal glands. This finding was confirmed by Lucin et al. (1992). They demonstrated that clearance of MCMV from the salivary glands depends entirely on CD4+ T cells and is mediated by IFN-γ production. It should be noted that in the mouse model of CMV pneumonitis, data suggests that DTH-type T cells (most likely CD4+) might be responsible for eliciting this pathological condition (Grundy, 1990).

1.7.4 CD8+ T cell responses to CMV infection

Clinical studies of immunosuppressed patients have underlined the importance of cell mediated immunity to recovery from HCMV infection. A universally common feature of individuals suffering severe CMV infections is a specific suppression or defect in cellular immune responses. The earliest evidence in humans establishing the importance of CTL in the recovery from HCMV infection came from studies of bone marrow recipients (Rook, 1988). The presence of virus-specific CTL in these individuals is correlated with a good prognosis in the post-transplant period.

It is now well recognised that anti-CMV cellular responses are predominantly by CD8+ CTL. In recent years, many attempts have been made to define which virus-encoded proteins are targets for the CD8+ immune response. Early studies in this area indicated that protein products of the IE genes were immunodominant (Borysiewicz et al., 1988). Further studies, however, have implicated virion structural proteins as more important antigens prior to de novo virion protein synthesis. Current evidence points to pp65 specific CD8+ T cells being dominant in CTL responses to HCMV infection. In some individuals 70% to 90% of circulating HCMV-specific CTL precursors are specific for epitopes derived from pp65. It should be noted, however, that these percentage values were obtained by limiting dilution assay analyses. In recent years it has become clear this form of analysis can heavily influence results which are obtained. When techniques
such as MHC-tetramer staining and flow cytometric analysis are applied to this problem it may become clear that a broader spectrum of anti-CMV specificities exists in infected individuals (Wills et al., 1998).

Very recently it has been demonstrated that HCMV infected endothelial cells can function as a direct activating stimulus for autologous T cells independent of accessory professional APC. This may be occurring as a result of CMV protein complex expression on the surface of these cells. Such molecules may mimic the HLA/peptide complex sufficiently to engage T cell receptors and elicit an activation response. It has been suggested this non-specific T cell activation may enhance dissemination of the virus throughout the host (Waldman et al., 1998).

The first observations of CMV-specific CTL in animals were in the spleens of sublethally infected mice. CTL activity increased in the spleens as virus titres decreased indicating that the cell mediated response was acting to lower the virus burden. In nude mice enhanced susceptibility to MCMV infections is observable, implicating T lymphocytes in MCMV resistance (Britt and Alford, 1996). More specifically it has been observed that MCMV replication is confined to the salivary glands of mice inoculated with the virus via an intraplantar route. This is accompanied by a strong CD8+ T cell response in the draining popliteal lymph node. This finding led to the conclusion that the CD8+ T cell subset is responsible for controlling virus replication in host tissues. This conclusion was confirmed by adoptive transfer studies demonstrating independent CD8+ anti-viral function which prevented virus mediated histopathology (Kosinowski et al., 1990).

Once CD8+ T cells were identified as critically important in the anti-CMV response in mice, interest turned to identifying which viral proteins yielded antigenic peptides recognisable by the host anti-viral effector cells. It was soon observed that the frequency
of immediate early (IE) antigen specific CTL in mice is very high indeed. It was concluded that IE proteins are immunodominant. This was confirmed when adoptively transferred cell populations lacking IE specific CTL failed to control MCMV infection (Kosinowski et al., 1990). In order to identify and characterise critical IE antigenic peptides a CTL clone was isolated. Following deletion mutation of the IE reading frame, a pentapeptide was identified as being the normal antigenic determinant required for CTL recognition and activation in BALB/c mice. It should be remembered, however, that BALB/c mice are an inbred strain. This peptide, therefore, may bear no resemblance to those which are important in the human immune response to CMV. Advances in molecular biology have generated new insights into immune responses to CMV infection. In recent years adoptive transfers of HCMV specific T lymphocytes have been undertaken. The results from these experimental therapies have proven promising but as yet not exceptional. In mice, however, T cell therapy has been shown to accelerate viral latency establishment, reduce viral tissue burdens and perhaps most significantly, reduce viral recurrence in immunosuppressed animals (Kurz et al., 1996).

1.7.5 Antibody responses to CMV infection

Many studies have investigated the antibody responses of individuals to CMV infection. Unfortunately, despite a great deal of knowledge concerning responses to specific viral proteins, little is known about the importance of antibody responses in host protection (Britt and Alford, 1996). Clinically, transplacentally derived antibody can protect premature infants from CMV infection via blood transfusion. Further, preconceptual antibody is known to prevent fetal CMV infection and in allograft recipients, passively transferred antibody may play an important protective role.

It is thought that because antibody is an important protective factor in primary infections, it is likely that its major role is to prevent generalised systemic spread of the virus.
However, it is abundantly clear that circulating antibodies can neither eliminate CMV or prevent infection with a second strain (Rasmussen, 1990). The glycoproteins of CMV have, on numerous occasions, been identified as important targets of antibody activity. Antibodies to gB, gH, the tegument and nonstructural proteins have been identified. Unfortunately, protective humoral immunity to CMV remains something of a mystery. With the exception of adoptive transfer studies, antibodies currently remain useful only as important indicators of prior CMV infection.

### 1.7.6 Subversion of the immune response by CMV

Over the last 2 years one of the most rapidly expanding areas of CMV research has been the study of immune system subversion by the virus. Reports have emerged which describe the ability of CMV to interfere with all 'arms' of the immune response from non-specific, innate effector mechanisms to subversion of specific T-lymphocyte led responses.

### 1.7.7 Subversion of the innate immune response

Acute infection with HCMV is often associated with a sustained, general immunosuppression of the host. The mechanisms underlying CMV mediated immunosuppression are unclear. In 1996, it was demonstrated that CMV infection of peripheral blood monocytes can induce the release of prostaglandin E$_2$ (PGE$_2$) via a TNF-α dependent pathway. PGE$_2$ produced in this way appears to contribute to CMV mediated immunosuppression and might be involved in the pathogenesis of CMV-led disease (Nokta et al., 1996).

Farrel et al. (1997) and Reyburn et al. (1997) showed that both murine and human CMV use MHC class I molecule homologues to escape surveillance by NK cells. Farrel et al.
constructed a recombinant MCMV with a functional deletion in its MHC class I molecule homologue. This deletion did not affect the replication of the virus in vitro but did severely attenuate the virus in vivo. This attenuation was due to a significantly increased sensitivity of infected cells to NK cell lysis.

In parallel with the above study, Reyburn et al. (1997) showed that if the UL18 MHC class I homologue of HCMV was cloned into cells which normally lack class I molecule expression the cells became resistant to NK cell lysis.

Thus, it appears that by encoding its own MHC class I molecule homologue, CMV provides itself with a mechanism which allows it to avoid NK cell attack.

Very recently, Miller et al. (1998) described the ability of HCMV to disrupt IFN-α stimulated immunoregulatory and direct antiviral effector mechanisms. It was concluded that this occurred as a result of HCMV interference with Jak/STAT signal transduction pathways in infected cells.

Over the last five years a number of G-coupled receptor (GCR) homologues have been found in both HCMV and MCMV. For example, the gene US28 of HCMV encodes a protein with 50% homology to a human β-chemokine receptor. Unfortunately, information concerning the biological function of Herpesvirus GCR homologues is limited. It is thought they may play a role in: disrupting inflammatory processes or altering the chemotactic properties of infected cells (Davis-Poynter and Farrell, 1996).

1.7.8 CMV subversion of specific immune responses

Both Human and murine CMV have the ability to produce more than 200 protein products. Amongst these proteins are a number which can directly interfere with MHC
class I molecule expression on the surface of infected cells. Interference with MHC class I enables CMV infected cells to avoid CD8+ T cell mediated immunosurveillance.

At very early times after infection, HCMV can produce the protein US3. US3 is an endoplasmic reticulum-localised glycoprotein which causes the accumulation of stable MHC class I molecule complexes in the ER. Such an accumulation may increase the efficiency of the proteins US11 and US2. Both of these transmembrane viral proteins transport MHC class I molecules from the ER back into the cytosol where they are degraded by the proteosome.

HCMV can further interfere with antigen presentation via the protein US6. US6 shuts down the translocation of cytosolic peptides into the ER lumen. It does this by regulating transporter associated with antigen presentation (TAP) function at the transporters ER luminal face.

As a functional group these proteins act to promote HCMV escape from immune surveillance during the crucial first stages of infection (Hengel and Koszinowski, 1997, Kleijen et al., 1997).

Murine CMV also encodes a number of protein products which enhance the ability of the virus to maintain its position in the infected host. In 1997, MCMV gp34 was characterised (Kleijen et al., 1997). This glycoprotein forms a tight complex with fully formed MHC class I molecules. Gp34-bound MHC class I molecules escape MCMV imposed ER retention and are expressed at the cell surface where they act as decoy antigen presenting complexes. In vivo, this may prevent NK cell mediated destruction of CMV infected cells (Kleijen et al., 1997). MCMV also encodes a 40Kd glycoprotein which has the ability to arrest the export of MHC class I complexes from the ER-Golgi
intermediate compartment. This protein is another example of a virally encoded molecule which acts to inhibit or prevent the presentation of viral peptides at the cell surface (Ziegler et al., 1997).

It has recently been demonstrated that MCMV can also affect MHC class I molecule presentation in infected cells at a transcriptional level. Slater and Campbell (1997) found that MCMV infection of immortalised fibroblasts induced a profound decrease in MHC class I mRNA production in these cells. This decrease in mRNA, however, was not observed in MCMV infected primary fibroblasts. This led to the suggestion that MCMV can affect the expression of host cell proteins at multiple levels and this may be dependent on a host cells differentiation state and transcriptional activity.

From the substantial number of studies published over the last 5 years, it is clear CMV has an impressive array of mechanisms for avoiding host immune responses. It is important to note, however, that effective immunity and in particular CD8+ CTL responses are invariably generated to this virus on infection of the host. It has been suggested that CMV may require an excess of immune avoidance mechanisms because viral genes are regulated very differently in differing cell types. Alternatively, different MHC class I alleles may have different susceptibilities to CMV protein binding.

It is clear from the recent work of many laboratories that if CMV (or any Herpesvirus) can gain even a tiny advantage over the host this may have an enormously beneficial effect on the survival of the virus (Johnson and Hill, 1998).
1.8 The Immune Response to Epstein Barr Virus

Epstein Barr virus (EBV), the prototypic member of the gammaherpesvirinae, has a genome of around 172Kbp and a capacity to encode at least 50 to 100 proteins. EBV is one of the more prevalent members of the human herpes virus family with around 95% of the world's human population being infected (Rickinson and Kieff, 1996). The existence of EBV was first noted in 1964 in Burkitt's lymphoma-derived cell lines. In common with all herpesviruses, EBV adopts a persistent infection and targets one cell type for replication, probably mucosal epithelium, and another for latency, the B lymphocyte (Rickinson and Kieff, 1996). The majority of the world's population are infected with EBV during the first three years of life. At this time primary infection is generally asymptomatic. An exception to this is in the Western world where primary infection is often postponed until the late teens/early twenties and is frequently accompanied by the symptoms of infectious mononucleosis (see later). It is generally agreed that EBV is predominantly an orally transmitted agent, since free virus is often detectable in throat washings or saliva. It should be noted, however, that EBV has been detected in the cervical epithelium and in genital ulcers. It is important, therefore, not to discount other routes of spread such as sexual transmission or blood transfusion (Straus, 1988). If EBV is transmitted orally it is likely that the oropharyngeal epithelium is the primary site of EBV infection and replication (Rickinson and Gregory, 1993). It is not confirmed, however, if epithelial infection occurs after an initial replicatory step in tonsillar crypt or epithelial-bound B cells (Rickinson and Kieff, 1996). During productive infection, the EBV genome is in a linear form and 3 groups of EBV 'lytic' antigens are expressed. These are termed the 'Early antigen (EA)', the 'Viral Capsid antigen (VCA)' and the 'Membrane antigen (MA)'. It is thought that progeny virus produced in the epithelium, enters a proportion of circulating B lymphocytes with high efficiency during primary infection. These cells act to generalise the infection throughout the body (Rickinson and Gregory, 1993, Rickinson and Kieff 1996,).
EBV entry into B cells occurs via an interaction between the viral envelope glycoprotein Gp340 and CD21 molecules on the cell surface. The virus is endocytically internalised and the nucleocapsid is delivered into the cell cytoplasm. This involves a membrane fusion which is probably mediated by Gp85. EBV can stimulate the host B cell population to proliferate and become polyclonally activated. This may serve to expand the infected B cell pool prior to an immune response developing. During a B cell infection the EBV genome is episomal and the virus usually expresses a restricted number of viral proteins. These are the latent cycle products and are termed Epstein Barr Nuclear antigens (EBNA) 1,2,3a,3b,3c and LP and the latent membrane proteins LMP1 and LMP2. Another latent cycle product termed BARFO is encoded by a rightward reading frame which was originally identified in a BamHI-A restriction fragment of the EBV genome. These proteins are responsible for activating the B cell or maintaining a latent infection (Rickinson and Gregory, 1993). In recent years, distinct forms of latent infection in which differing combinations of latent gene expression occur have been identified. These are summarised in Table 1.3. This is important to remember when antigenic challenges to the immune system are being considered.

The EBV/ B cell interaction is usually non-productive. However, a small proportion of infected cells (<1%) does allow viral replication and release. Primary infection with EBV, be it subclinical or associated with the symptoms of infectious mononucleosis (IM), always leads to a lifelong virus carrier state (Rickinson and Gregory, 1993). Often EBV can be found in epithelial and lymphoid habitats in a long-term virus carrier. Argument is underway at present as to how virus is maintained at a low level in the saliva. Some groups have evidence that low level replication is occurring in the oropharynx. Others have argued that B cells are responsible for reseeding this epithelial site or are even entirely responsible for the production of virus in the oropharynx (Rickinson and
Whichever argument is correct, EBV+ latently-infected B cells are present in infected individuals for life. A common feature of latency is the abundant transcription of untranslated EBV encoded small RNAs which have been used to detect latency in tissues via in situ hybridisation (Schmidt and Misko, 1995).

Investigations into a range of autoimmune diseases and lymphoproliferative syndromes have provided evidence of T lymphocyte infection by EBV. Much of the work in this area has exploited immature thymocytes or lymphoblastoid cells from patients suffering from acute lymphoblastoid leukaemias. In vitro work has shown that EBV infection of immature T lymphocytes results in the expression of some lytic gene products without progression to cell lysis or production of infectious virions. It has been suggested that this form of infection, if it occurs in vivo, may interfere with T cell selection and T cell repertoire development (Dreyfus et al., 1996). The suggestion has also been made that endothelial cells are targets for EBV infection and may be important in the life cycle and pathology of the virus (Jones et al., 1995).

1.8.1 The consequences of EBV infection

Around 50% of individuals who are infected with EBV in their late teens or early twenties suffer from the symptoms of the self limiting lymphoproliferative disease 'infectious mononucleosis' (IM). The symptoms of IM are fever, malaise, pharyngitis, lymphadenopathy and hepatosplenomegaly. These symptoms occur coincidentally with an unusually exaggerated T cell response to EBV activated B cells. This has been perceived as evidence of IM being an immunopathological condition. It has been argued that the greater frequency of symptomatic primary infection in adolescents may be due to the sufferers receiving a much higher dose of virus. This theory is still unproven. Almost all understanding of the immunological events occurring in primary EBV infection has been obtained from patients with IM. Some debate has arisen over the validity of
such information and whether it reflects events occurring during asymptomatic infections. Although it can be debilitating, primary EBV infection is rarely fatal. An exception to this occurs in sufferers of X-linked lymphoproliferative syndrome. This is characterised by the rather unique susceptibility of male members of affected families to EBV infection. Infection results in fatal infectious mononucleosis, hypogammaglobulinaemia and/or malignant lymphoma formation. 60% of males affected by the syndrome die by the age of 10. The disease ‘phenotype’ is at present unclear, however, it is known that NK and CD8+ CTL responses to EBV transformed B cells are often impaired in sufferers (Borysiewicz and Sissons, 1994).

EBV is also associated with the pathogenesis of a wide variety of malignancies. These include: immunoblastic lymphomas of T and B cell origin in immunosuppressed people, endemic Burkitt’s lymphoma, anaplastic large T cell lymphoma and midline granuloma, Hodgkin’s disease and lymphoepitheliomas of the nasopharynx, thymus and stomach (de Campos-Lima et al., 1996).

Severe impairment of T cell mediated immune responses renders an individual much more susceptible to lymphoproliferative disease (LPD). This points to an EBV+ B cell expansion occurring in vivo due to a lack of T cell control. This has been confirmed on numerous occasions when removal of immunosuppressive therapy results in the regression of EBV+ tumours (Rickinson and Gregory, 1993). In recent years the incidence of post-transplant lymphoproliferative disease has increased enormously with the use of more profoundly effective immunosuppressive therapy (Rickinson and Kieff, 1996). To counteract this, several groups around the world are now attempting to treat EBV-led LPD via immunotherapeutic reconstitution of patients with virus specific T lymphocyte populations. These attempts are discussed later.
EBV was originally discovered as a human cancer-associated virus in Burkitt’s lymphoma (BL) biopsy specimens. BL is a monoclonal B lymphoblastic tumour which is approximately 30x more common in areas of the world where malaria is endemic. Endemic BL is usually evident in children of age 6 to 10. All forms of BL are also associated with chromosomal translocation and deregulated expression of the c-myc oncogene. EBV, therefore, remains a co-factor rather than a cause of the malignancy (Schmidt and Misko, 1995). Nasopharyngeal carcinoma (NPC) is a tumour of the post-nasal space mucosal epithelium with a high incidence in East Asian populations. Again, EBV infection is a co-factor in the formation of these tumours alongside immunogenetic, dietary and environmental factors. However, 70% to 100% of all cases are positive for episomal EBV DNA (Schmidt and Misko, 1995).

The condition oral hairy leukoplakia (OHL) is of note as it has allowed a unique opportunity to follow the natural course of EBV infection in an epithelial environment. OHL predominantly develops in HIV infected individuals in which T cell responses are impaired. It occurs due to a focussed EBV replication in the differentiating outer layers of the stratified lingual epithelium of the tongue (Rickinson and Kieff, 1996).

1.8.2 The Study of EBV

*In vitro*, infection of resting B lymphocytes with EBV results in the highly efficient production of lymphoblastoid cell lines (LCL). Over the past two decades, LCL have provided a standard model for the study of latent EBV infection. Enormous advances have been made in the understanding of EBV latent gene expression and immunological control of latent infections using LCL (Borysiewicz, and Sissons, 1994). In contrast, it has been impossible to reproduce epithelial cell infections efficiently *in vitro* though a number of attempts have been made (Li *et al.*, 1992). Information on the lytic EBV
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replicative cycle and genes expressed during this phase comes from clinical examinations of OHL sufferers or from semi-permissive B cell line analysis.

Since the 1960s several attempts have been made to develop ‘accurate’ animal models of EBV infection. In recent years 3 species of New World monkey have been infected with EBV and both the Cotton Top Tamarin and the Owl Monkey are susceptible to B cell lymphoma formation. Unfortunately, the value of these animals for EBV study limited as they cannot be infected via a nasopharyngeal route and often do not display any virus replication or adoption of latency despite lymphoma development. From a practical viewpoint they are also protected species and are difficult to rear and experimentally manipulate. It is thought that endogenous primate lymphocryptovirus infection may ultimately provide a model for the study of aspects of EBV infection (Rickinson and Kieff, 1996, Wilson et al., 1996).

A rather novel model of EBV/ B cell interaction has been developed via the infection of SCID mice with EBV. In these animals, which lack T or B cells, it is possible to reconstitute certain facets of human immune function. This model has a number of drawbacks as it is a study of a human virus in a murine system and, therefore, lacks a number of key components which may be important in responses to the virus (Borysiewicz and Sissons, 1994). The most promising model of recent years is that of Nash et al. which is a murine infection with a gamma Herpesvirus, known as murine gammaherpesvirus 68 (MHV-68). This model shares many features with human EBV infection. The characterisation of this model will be described in a later section.

1.8.3 Immune responses to primary EBV infection

The majority of information currently held on the human immune response to primary EBV infection has been obtained from sufferers of infectious mononucleosis. It is not
known how closely this response resembles that which occurs in asymptomatic infection of young children. The study of immune responses during IM is not ideal since the majority of sufferers present their symptoms clinically some weeks after initial infection. This leaves a crucial period during which immune responses to EBV are something of a mystery (Rickinson and Gregory, 1993, Rickinson and Kieff 1996).

IM is one of the few conditions in humans in which the in vivo T cell response to a defined agent is strong enough and sufficiently generalised to allow fairly straightforward analysis (Strang and Rickinson, 1987). The antigenic challenge to the host in IM arises from the lytic antigens of EBV expressed in epithelial sites and latent antigens expressed on infected B lymphocytes. IM is characterised by the presence of elevated numbers of lymphocytes in the circulation and tissues. These are predominantly T lymphoblasts of the CD8+ subset plus some activated CD4+ T cells (Sheldon et al., 1973). Broadly, this was first recognised in 1975 when a study provided evidence of the presence of effector cells in the peripheral blood of patients with acute IM (Svedmyr and Jondal, 1975). It has been suggested that an IL-10 homologue encoded by EBV may dampen local cellular responses in epithelial sites of virus replication and augment viral-lead B cell activation. This would result in an increased antigenic challenge to the immune system from the B cell compartment and enhanced T cell activation (Rickinson and Kieff, 1996).

Over a decade ago, it was shown that the expanded lymphocyte populations present in IM, display a broad suppressive activity. This was demonstrated in vitro with the suppression of antigen or mitogen-led T or B cell responses (Reinherz et al., 1980). In 1975, it was demonstrated that the cell population expanded during IM may have a role in limiting proliferation of EBV-transformed lymphoblasts (Royston et al. 1975). It is now clear that the lymphocyte response to EBV during IM contains a major, virus-specific
HLA restricted component which is functionally analogous to that established in the CTL memory response to the infection (Strang and Rickinson, 1987, Rickinson and Moss, 1997).

The first reports that, some, or all, of the ‘alloreactive’ and ‘nonspecifically’ activated CTL seen in IM are EBV specific appeared around 1995. Hill et al. (1995) demonstrated that increasing antigenic density on target LCLs in their cytotoxicity assays (involving lymphocytes from patients with IM) led to target lysis by CTLs of previously undetectable specificity. Further, in 1996, in studies of acute human serum, White et al. (1996) identified a CD4+ T cell clone whose cytolytic response was directed against the BHRF-1 EBV IE protein.

Studies which have extended the work of White et al. and employed target sensitisation with antigen or peptide have revealed that primary infection induces numerous CD8+ CTL reactivities. These reactivities are broad and are to a range of IE and E lytic cycle proteins. For example, it is now clear that in the majority of IM patients, CTL responses to BZLF-1 IE protein are considerably higher than they are to any of the EBNA-3 latent proteins (Steven et al., 1997). Many of the IE reactivities generated during acute infection are maintained for several years. This continued high frequency of lytic-cycle specific memory cells in healthy carriers may arise due to a persistant immune stimulation by latently-infected B cells switching to lytic cycle gene expression (Elliot et al., 1997).

Over the past two years, some studies of the T lymphocytes, expanded during IM, have employed a novel method of identifying antigen-specific cells. Tetrameric MHC class I molecule complexes have been used to label cells of a particular specificity such that they can subsequently be analysed by flow cytometry. MHC tetramer ‘staining’ of
lymphocytes has revealed that up to 40% of CD8 T lymphocytes in some IM sufferers are specific for IE proteins such as BZLF-1. Further, it has been demonstrated that some EBV specific cells are detectable in individuals up to 3 years after primary infection. Unfortunately, a functional role for these cells has yet to be confirmed (Callan et al., 1998). In summary, CTL appear to be critically important in controlling foci of EBV replication either during primary acute infection or during the carrier state when reactivation of the virus occurs (Steven et al., 1997).

In the last 5 years, the enormity of the T cell response to primary EBV infection has led to speculation that EBV may encode or induce superantigen expression (Sutkowski, 1996). This theory, however, now appears to be out of favour. Callan et al. (1996) have demonstrated that the enlarged cell populations present during IM are driven to expand in an antigen specific manner.

The regulatory disturbance of the human immune system, induced by EBV infection, is not restricted to cellular responses. IM is also characterised by antibody responses to the lytic antigens EA, MA and VCA which begin just prior to the onset of symptoms. IgA responses may be detectable at this time although these are not yet defined fully (Rickinson and Kieff, 1996). Some days after the onset of IM the spectrum of immunoglobulin responses to EBV alters with antibodies to latent proteins such as EBNA 2, and eventually EBNA 1, becoming evident. The importance of antibody to the host in controlling primary EBV infection is unclear. Neutralising antibody may prevent systemic viral spread, although anti-Gp350 IgA has been implicated in the enhancement of viral movement between body compartments.
Alongside EBV-directed antibody responses, IM is characterised by a generalised Ig production with heterophile and auto-antibodies apparent. These may occur as a result of EBV-induced polyclonal B cell activation (Rickinson and Kieff, 1996).

1.8.4 Immune responses to persistent EBV infection

Primary infection with EBV always leads to a lifelong carrier state being established. The detection of cell-free virus in throat washes suggests that life-long persistence in B cells is accompanied by periodic low grade EBV replication in the oropharyngeal epithelium (Rickinson and Gregory, 1993). This means both lytic and latent antigen can maintain antibody and cell mediated immune responses for life. EBV latency in B cells of an infected individual remains an indistinct area, however, a range of cell mediated immune responses probably play a role in controlling persistent infections. This area has been made more difficult to understand through the lack of an accurate animal model of EBV infection. Evidence from in vitro studies, often involving LCL, points towards virus-specific CD8+ CTL being key in controlling EBV infections. Thorley-Lawson et al. (1977) demonstrated that events of transformation could be delayed at an early stage if autologous T cells were added back to an infected B cell population soon after exposure to the virus. Other early studies with infected in vitro cultures identified a number of activities capable of slowing the progression of infected B cells to proliferation. At this time, this effect was ascribed to the activity of NK cells and IFN-α and -γ production.

Subsequently, in the late 1970s and early ‘80s, it was demonstrated a number of times that the regression of EBV-led B cell outgrowth in vitro was being brought about by virus-specific CD8+ CTL (Rickinson, 1986). From these data it was concluded that CTL probably play an active surveillance role in controlling EBV infection in the host.

Until the mid to late 1980s when the class I MHC molecule + peptide/ T cell receptor interaction was defined, it was thought that EBV-specific CTL recognised virus-positive
in vitro-generated LCL and in vivo targets via a novel, latent viral antigen on the infected cell membrane. This was called the lymphocyte-dependent membrane antigen or LYDMA. In more recent years EBV-specific CD8+ CTL clones and latterly MHC tetrameric complexes have enabled work which has identified specific EBV epitopes to which immune responses are directed. Studies have also shown that CTL from a variety of HLA backgrounds are reactive to EBNA 3a, 3b and 3c in a high proportion of individuals (Rickinson and Kieff, 1996). In 1996, it was not known if this highly focussed specific response was generated during acute infection/IM or whether focussing of the memory response occurred over time. Recent findings have indicated that, in fact, latent CTL reactivities are highly skewed towards the EBNA-3 proteins and their epitopes even during the acute response (Steven et al., 1996). Other reactivities identified include those against EBNA-2, -LP, LMP-1 and LMP-2. In relation to LMP-2, although this is a subdominant antigen relative to EBNA-3, epitopes from this protein can elicit CTL responses on a number if different HLA backgrounds. Interestingly, at least some LMP-2 epitopes are presented in a TAP independent manner (Lee et al., 1996).

Significantly, CTL-reactive epitopes from EBNA-1 have only recently been detected. A great deal of evidence has been compiled in vitro which points to the glycine-alanine repeat of EBNA-1 protein being critical in the avoidance of CTL recognition by EBV (Blake et al., 1997). In 1997, a study set out to investigate the significance of the gly-alα repeat on EBNA-1 presentation in vivo. This study demonstrated that CD8+ MHC class 1 molecule-restricted responses to EBNA-1 are in fact detectable in seropositive individuals. The conclusion of this work was that professional antigen presenting cells probably pick up exogenous EBNA-1 protein released in vivo. Peptides from this protein are then presented in the context of MHC class 1 molecules. Thus CD8+ T lymphocyte priming can occur. It should be noted, however, that CTLs generated against peptides from EBNA-1 will not be able to recognise naturally infected cells (Blake et al., 1997).
This is important since Burkitt's lymphoma cells which express only EBNA-1 are, in most cases, resistant to CTL lysis (Borysiewicz and Sissons, 1994). It is obviously an advantage to the virus in establishing persistence if it can restrict gene expression to EBNA-1 and limit the ability of CTL to react to EBV-induced disease (Murray et al., 1992). Generally, only immunoblastic B cell lymphomas in immunocompromised patients express a full spectrum of viral latent proteins and would therefore be expected to remain sensitive to CTL control (Rooney et al., 1997).

The identification of EBV target antigens recognised by CTL is proceeding. The suggestion has been made that the relative immunogenicity of EBV-derived epitopes may be strongly influenced by peptide-driven persistence of antigenic complexes at the cells surface (Levitsky et al., 1996). In 1996, it was proposed that variation in HLA types and highly specific CTL responses may influence the long term evolution of EBV by selection of variants which lack immunodominant CTL epitopes i.e. escape mutants might be generated. However, studies in this field have concluded that immune pressure plays a minimal role in influencing which CTL epitopes are immunoreactive (Rickinson and Moss, 1997).

In relation to the evolution of immune responses to EBV, recent results show that the characteristic oligoclonality of EBV CTL responses is stably preserved in individuals over time. Despite the continuous presence of antigen, the avidity or affinity of interactions with specific antigens apparently is not a major driving force in the selection and perpetuation in memory of particular T cell specificities. Thus affinity maturation of the T cell response to EBV does not seem to occur (Levitsky et al., 1998).

In the last few years, evidence has appeared which suggests that immune responses to EBV in individuals of particular HLA types may actually be damaging to the host. Certain
HLA B8 individuals have anti-EBV specific CTL populations which are capable of cross-reacting with alloantigens. In the future, research in this field may explain clinical associations between EBV infections and auto-immune disease (Rickinson and Moss, 1997).

As in a number of other viral infections, EBV-specific CD4+ MHC class II restricted CTL have been identified. Some of these have a specificity for structural proteins such as Gp340. The function of these cells is unknown although it has been suggested that they are important in very early responses to EBV (Borysiewicz and Sissons, 1994).

1.8.5 Antibody responses to persistent EBV infection

Normal, healthy carriers of EBV in a persistent form have detectable titres of IgG to VCA, MA and most importantly Gp 340. Titres of these antibodies vary between individuals but do not seem to vary within an individual over time. It is thought these antibodies are probably of little functional use once a carrier state has been established. Anti-Gp340 antibodies may function to prevent super-infection with different strains of EBV (Rickinson and Kieff, 1996).

1.8.6 EBV escape from immune-surveillance

During a persistent infection a balance exists between EBV maintaining a non-pathogenic state and the virus avoiding immune responses and eliciting tumour formation or reactivation and transmission.

EBV can escape immune recognition via the adoption of a form of latency in which a highly restricted programme of gene expression allows the virus to stay in a resting, non proliferating B cell environment. Resting B cells have low adhesion molecule expression. This feature alongside a down-regulation of virus gene expression and the lack of
immunogenic epitopes in EBNA 1 means EBV can maintain an invisible state in the host (de Campos-Lima, 1996). As mentioned earlier, evidence has emerged in the literature which suggests that an internal repetitive amino acid sequence in EBNA1 containing glycine-alanine motifs can inhibit class I MHC cell surface expression in infected cells (Levitskaya et al., 1995).

The homologue of the human cytokine IL-10 encoded by EBV can inhibit CTL activation, suppress macrophage activation and downregulate Th1-type cytokine production. It is thought this may be of most significance in epithelial infection or in nasopharyngeal carcinomas in which local immune defences are suppressed (Ziedler et al., 1997, Yao et al., 1997).

EBV also has the ability to alter patterns of gene expression when it is present in neoplastic cells and their precursors. For example, only the latent gene EBNA1 is expressed in Burkitt’s lymphoma cells (de Campos-Lima, 1996).

In summary, it is evident that EBV has a number of highly evolved strategies for maintaining its position in the infected host.

1.8.7 The treatment of EBV-associated lymphoproliferative disease
As discussed earlier in this chapter, EBV has been linked with a number of malignant diseases, particularly in patients whose immune system is compromised. EBV associated cancers are becoming more common as the numbers of immunodeficient patients in society increases. In the ‘Western world’, for example, many more people are undergoing organ or bone marrow transplants today compared with a decade ago. In these patients it is the use of profoundly immunosuppressive regimes, designed to
prevent graft versus host disease (GvHD) and tissue rejection, which leaves them susceptible to EBV-related lymphomas (Smith et al., 1996).

The clinical presentation of EBV-associated lymphoproliferative disease (EBV-LPD) is highly variable. The condition may be first recognised when patients present with a mild infectious mononucleosis-like syndrome. This can then proceed to a disseminated disease of lymph nodes, the gastrointestinal tract, kidneys, CNS or bone marrow. For diagnostic purposes biopsy material is usually taken from patients. These tissues are used to assess B cell morphology and phenotype within tumours. The presence in tumours of EBV DNA is usually confirmed by in situ hybridisation or Southern blot analysis. Further, the clonality of the tumour is investigated, as is the HLA origin of the cells to determine whether they are of donor or host origin (Lucas et al., 1997).

The clinical presentation, origin, histology and clonality of EBV-LPD complicating solid organ allografts vary depending on the organ transplanted and the immunosuppressive regime employed. On average, around 20% of solid organ recipients present with LPD after transplantation. This percentage is somewhat higher in bone marrow recipients (30%) if they receive T cell depleted bone marrow from unrelated donors or HLA mismatched family members (O'Reilly et al., 1997, Rooney et al., 1997).

In solid organ recipients EBV-LPD can be treated by withdrawing immunosuppression. This often results in the complete resolution of tumours. Unfortunately, removal of immunosuppressive drug or antibody therapy significantly increases the risk of graft rejection. Invariably, chemoradiotherapeutic treatment of EBV-LPD results in problems of toxicity. Several transplant centres around the world have investigated immunological approaches to the treatment of EBV-LPD. These have included the administration to patients of IFN-α and monoclonal antibodies against B cell markers such as CD21 and
CD24. This approach has proven successful in the treatment of polyclonal tumours but it is inconsistent and is not an effective therapy for monoclonal malignancies.

In bone marrow recipients, EBV-LPD has been treated by administration of acyclovir, immunoglobulin, steroids, IFN-α and undepleted marrow. Despite these therapies, little success has been achieved in this field and around 90% of bone marrow recipients who develop LPD die (Rooney et al., 1997).

An immensely important feature of all EBV-LPD is that constituent B cells of these tumours all express a full array of latent EBV antigens including EBNA-1, 2, 3a, 3b, 3c, EBNA-LP and LMP-1. These cells are, therefore, highly susceptible to recognition and lysis by EBV-specific CTL. Because of this, over the last 5 years a great deal of effort has been directed towards the development of cellular immunotherapeutic regimes for the treatment of LPD. Such therapies involve the reconstitution of EBV-specific T lymphocyte populations in immunocompromised patients. This results in the restoration of a specific anti-tumour response against virus-infected and transformed B cells.

LPD is an ideal target for cellular immunotherapy for a number of reasons. Firstly, as mentioned above, the cells of lymphoproliferative lesions express a wide range of virus specific target antigens. Secondly, EBV antigen presenting cells can be readily prepared from any normal donor by infection of peripheral blood B cells with exogenous virus. This results in the production of EBV transformed B cell lines. Lymphoblastoid cell lines express all of the appropriate latent antigens for CTL induction. Lastly, most organ donors or recipients are immune to EBV and thus they carry a high frequency of EBV-specific CTL which can be activated and expanded in vitro prior to their infusion back into an immunosuppressed transplant recipient (Rooney et al., 1997).
The first reported use of T lymphocytes in the treatment of EBV-LPD was in 1994. Papadopoulos et al. (1994) gave bulk, unselected T cells to 5 allogeneic bone marrow recipients. After infusion of these cells, EBV lymphomas regressed clinically in the patients by 21 days post infusion. Unfortunately, several of the patients suffered from GvHD due to the presence of alloreactive T cells in the donor population. In order to examine and define the functional contributions of EBV specific T cells (and other effector cells) recruited to tumour sites, several groups have developed models of human adoptive cell therapy in mice with a severe combined immunodeficiency (SCID mice). In a model employed by Lacerda et al. (1996), SCID mice were depleted of NK cells by infusion of anti-asialo GM1 antibody and are then inoculated subcutaneously or peritoneally with EBV transformed LCL. This resulted in the development of EBV-induced immunoblastic B cell lymphomas with characteristics similar to lymphoproliferations arising in immunocompromised hosts.

Using this model Lacerda et al. demonstrated the preferential homing and infiltration of adoptively transferred EBV-specific T lymphocytes into lymphoproliferations. These cells were predominantly CD8+ T lymphocytes and had been infused into the animals by either a peritoneal or intravenous (IV) route. It was found when lymphocytes were delivered intravenously, successful tumour regression only occurred if a simultaneous dose of interleukin 2 (IL-2) was administered. It was concluded that this cytokine enhanced the survival and homing of the adoptively transferred lymphocytes. Unfortunately, this model did not provide any direct evidence of a mechanism for tumour regression.

In recent years, to avoid the problems of GvHD due to alloreactive T lymphocytes in adoptively transferred cell populations, EBV specific CTLs have been administered to bone marrow recipients (Smith et al. 1996). Immunotherapy using antigen specific CTLs
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was first attempted in 1992 when CMV-specific CD8+ T cell clones were given to recipients of HLA matched, sibling derived bone marrow (Riddell et al., 1992).

CTL, for the treatment of EBV-LPD in bone marrow transplant patients, are generated by repeatedly stimulating donor peripheral blood mononuclear cells with autologous LCL in medium containing IL-2. The expansion of virus-specific cells using this method takes between 3 and 4 weeks. At this time the EBV-specific lymphocytes are transduced with a retroviral vector containing a neomycin resistance gene. This is used to identify adoptively transferred cells at times after infusion. Cells are then tested for EBV specificity and are frozen until required by patients who are receiving immunosuppressive therapy.

Transferred cells are detectable for up to 23 months after their infusion into a patient. This is long after transplanted bone marrow has reconstituted an individual's T cell population. Encouragingly, these cells have been shown to have both antiviral and antilymphoproliferative effects in the vast majority of patients treated (Rooney et al., 1997).

Unfortunately, despite the success of these trials, when they are subject to scientific (and statistical) scrutiny it is evident that patients chosen for therapy are far from randomly selected. Further, no conclusive evidence has been provided to show whether it is CD8+ T cells in this system which are active in eliciting tumour regression. The possibility remains that it is the CD4+ T cells, which are invariably also infused into patients, which are functionally significant in this situation. The further development of an animal model of immunotherapy may help to advance understanding in this area.

In the future, it has been suggested that donor PBMC, genetically modified to express a selectable suicide gene, may be used to treat patients with EBV-LPD. These cells would
have a broad specificity, could be generated very quickly and could be rapidly destroyed \textit{in vivo} if GvHD were to appear (O'Reilly \textit{et al}., 1997).

It has been proposed that EBV-LPD in solid organ transplant recipients could be treated by infusion of host derived EBV specific CTL. The cells could be generated prior to the initiation of immunosuppressive therapy (Heslop \textit{et al}., 1997). In opposition to this, several groups have argued that transplant recipient CTLs could be difficult to obtain and use. Nalesnik \textit{et al}.
\textit{(1997)} have proposed that IL-2 activated lymphocytes may be more useful for the treatment of solid organ recipients in the future.

In relation to the treatment of EBV-associated malignancies other than LPD, Roskrow \textit{et al}.
\textit{(1998)} have recently reported some success in the immunotherapeutic treatment of EBV-associated Hodgkins lymphoma. It remains to be seen whether further progress can be made in this area.

\textbf{1.9 Human Herpesvirus 8}

Since its discovery in Kaposi's sarcoma (KS) lesions, HHV-8 has been the subject of intense study around the world. The condition KS was first characterised in 1872 by Moritz Kaposi. Kaposi described a rare and relatively benign skin tumour prevalent in ageing Mediterranean males. It is now clear that KS is not only found in the Mediterranean but is also endemic in areas of Central and Eastern Africa where it occurs in men and women at equivalent frequency. Significantly, until the late 1970s KS was considered a rare tumour in most of the world. However, during the AIDS epidemic of the early 1980s the incidence of KS rose dramatically. The phenotype of KS in individuals with AIDS (and also in immunosuppressed transplant patients) differs greatly from that originally described by Kaposi. KS in patients with compromised immunity is often highly aggressive with tumours disseminating to cutaneous sites, the lymph nodes
and spleen. In fact, KS is the most common malignancy found in HIV+ individuals in which it is regularly a fatal condition. Unfortunately, conventional cancer therapies have proven to be poor in the treatment of KS, many of them being associated with unacceptable side effects such as toxicity (Brooks et al., 1997, Greenblatt, 1998, Biberfeld et al., 1998).

Prior to 1994 an enormous amount of epidemiological evidence supported the theory that KS was associated with an infectious agent. This was confirmed when Chang et al. (1994) reported the identification of Herpes-like DNA sequences in KS tissues from patients with AIDS. Using PCR-based 'representational difference analysis' this group detected DNA sequences present in KS lesions which were absent in normal tissues. This study was rapidly followed by other reports of a novel Herpesvirus (now known as Human Herpesvirus 8) associated with KS lesions (Greenblatt, 1998).

To date, HHV-8 has been found in all forms of KS and has also been identified in AIDS related primary effusion or body-cavity associated lymphomas as well as in some cases of multicentric Castlemans’ syndrome (Brooks et al., 1997, Greenblatt, 1998, Biberfeld et al., 1998).

Studies of KS lesions have shown that most cells in these tumours are latently infected with HHV-8. A small proportion of the cells also support lytic viral replication. A wide range of epidemiological and serological studies support the theory that HHV-8 is transmitted sexually. It should be noted, however, that the virus has been found in a replicating form in the oropharynx suggesting it may be transmitted in the saliva.

There is much debate over the prevalence of HHV-8 in the general population. Some studies suggest that HHV-8 is, in general, limited to populations of individuals at risk of
developing KS. Others, however, have suggested that HHV-8 is prevalent throughout the world's population. Unfortunately, at present there is no standard serological or PCR based diagnostic test available for the irrefutable identification of HHV-8. It may be some time, therefore, before the true distribution of this virus within the population is known. At present it has been estimated that between 1 and 25% of the world's population is infected with this virus.

The complete sequence of HHV-8 has recently been published. These data demonstrate that HHV-8 is a gammaherpesvirus which can encode upwards of 81 ORFs. Most of the HHV-8 genome is highly conserved between different isolates, however, the ends of the genome do show some variability. To date, 3 classes of HHV-8 genes have been determined. Class 1 genes are expressed in endothelial tumour (spindle) cells of KS lesions and/or in primary effusion lymphoma (PEL) cell lines. Expression of these genes is not up-regulated in PEL cell lines after treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) or sodium butyrate. Class 2 genes are constitutively expressed in PEL cell lines at a low level. Expression of these genes can be enhanced by treatment of PEL cell lines with TPA or sodium butyrate. Class 3 'lytic' genes are only expressed in phorbol ester-stimulated PEL cell lines (Schulz, 1998).

Many of the genes encoded by HHV-8 are homologous to cellular proteins. It is thought these have been acquired from the infected host at some time during the evolution of the virus. These genes are predominantly expressed during the replication of the virus and include homologues of MIP-1 and MIP-2, IL-6, interferon regulatory factor and bcl-2. HHV-8 also encodes D-type cyclin and G-coupled receptor homologues.

At present it is thought that HHV-8 probably plays a primary role in the pathogenesis of KS. Unfortunately, however, a direct oncogenic role for the virus has not yet been
identified. It is important to note that KS can occur in individuals who are not immunosuppressed. It is likely, therefore, that additional cofactors such as HIV tat gene expression are also required for the development of malignant disease.

To date there is virtually no evidence documenting host immune responses to HHV-8 infection. It is known, however, that HHV-8 is very well equipped to modulate various arms of the immune response. For example, as already mentioned, HHV-8 encodes chemokine homologues such as MIP-1 and MIP-2 which may profoundly alter the migration of immune effector cells such as monocytes and eosinophils. Further, HHV-8 encodes an IFN-regulatory factor which, at least in vitro, can inhibit interferon mediated signalling.

It is hoped in the future that it may be possible to model the pathogenesis of HHV-8 infection in animals such as mice. An animal model of this infection may reveal how this virus contributes to the development of malignancies and also might provide more information on how the host responds (if it can) to HHV-8 infection.

1.10 Murine gammaherpesvirus 68

Studies of gammaherpesvirus infections have, over the years, been limited almost entirely to clinically apparent EBV infections of humans and experimental inoculations of non-human primates. An affordable, amenable model of gammaherpesvirus infection is therefore required for the study of all aspects of permissive natural infections and latency (Nash and Sunil-Chandra, 1994).

It is in response to this demand that murine gammaherpesvirus 68 (MHV-68) may prove invaluable as it allows the study of gammaherpesvirus infections in inbred laboratory mice. MHV-68 was originally isolated from Clethrionomys glareolus (a bank vole) in
Czechoslovakia in the late 1970s (Blaskovic et al., 1980). The complete sequence of MHV-68, however, has only recently been determined by two independent groups (Virgin et al., 1997, Davison, A., personal communication). The MHV-68 genome contains 118Kb of unique sequence flanked by multiple copies of a 1,213bp terminal repeat. The unique region of the genome is estimated to encode around 80 genes and is largely co-linear with the genomes of HHV-8, HVS and EBV. A number of the genes encoded by MHV-68 are homologous to mammalian or other gammaherpesvirus genes. For example, MHV-68 encodes a complement regulatory protein homologue which may enable the virus to interfere with C3 function. Further, the virus encodes an IL-8 receptor homologue which may function to bind chemokines and dampen inflammatory responses. As well as having open reading frames (ORF) which bear a resemblance to other known genes the MHV-68 genome encodes a number of unique ORFs termed M1 to M11.

At the left end of the genome are 8 genes with homology to mammalian tRNAs. These genes were first characterised by Bowden et al. (1997) and are similar to the EBV early RNA (EBER) genes in that they are expressed during viral latency. The tRNAs are detectable by in situ hybridisation and as such they are an important tool for identifying latently infected cells. The biological function of these uncharged tRNA-like sequences remains unclear. Suggestions for a functional role include, the idea that the tRNAs may, in some way, modulate MHV-68 translation.

Intranasal infection of 3 to 4 week old mice with MHV-68 results in the lungs of these animals becoming productively (and persistently) infected. Virus titres peak in the lung around 4 days post infection (Sunil-Chandra et al., 1992a). In the lung, virus can be found in alveolar epithelial cells and some mononuclear cells. MHV-68 is cleared from
the lungs of mice around day 10 post infection and the virus spreads to the spleen by some as yet unidentified route.

Analysis of B cell deficient (μMT) transgenic mice using a PCR based MHV-68-detection system has shown that MHV-68 can persist in the lungs of mice indefinitely in the absence of infection of the spleen or other organs. In this site of persistence, both linear and episomal forms of the viral genome are detectable. This demonstrates that both latent and productively infected cells are present. Further, in situ hybridisation analyses have confirmed the presence of latently infected alveolar cells in the lungs of infected mice. The conclusion drawn from this work is that the lungs are a true independent site of MHV-68 persistence and do not need to be constantly re-seeded by circulating B cells (Stewart et al., 1998).

Once MHV-68 has spread to the spleen some productive replication occurs. Most significantly, however, the virus adopts a latent form of infection at this stage in which it is associated predominantly with B lymphocytes. This is very similar to the situation in EBV infection (Sunil-Chandra et al., 1992b).

There is some debate as to whether a latent MHV-68 infection occurs or is detectable in mice with a genetically determined absence of mature B cells. Usherwood et al. (1996c) failed to show evidence of latent infection in the spleens of B cell KO (μMT) mice by infective centre assay and nested PCR of DNA from spleen cells. In contrast, evidence presented by Weck et al. (1996a,b) demonstrates recovery of latent virus from viable spleen cells 4 to 6 weeks after initial culture. No answer as to what cell types are latently infected in these mice has yet appeared. Suggestions such as T lymphocytes and IgM negative B cells have been made, however, evidence from 1993 argues against a
productive or latent MHV-68 tropism for cells of the T lymphocyte lineage (Sunil-Chandra, 1993).

Under normal circumstances, numbers of latently infected B lymphocytes peak around 15 days post infection, at which time a marked splenomegaly is evident. Infection of μMT mice results in an absence of splenomegaly suggesting this cellular expansion is initiated by B lymphocytes (Usherwood et al., 1996c). Splenomegaly results in an increase in splenic B and T lymphocytes. In particular, CD8+ T cell numbers can increase four-fold. This cellular expansion resembles infectious mononucleosis occurring in human EBV infection. Depletion of CD4+ T cells prevents splenomegaly and lowers latent virus load at this stage of infection. This is thought to occur as a result of the removal of cytokine production by these cells (Usherwood et al., 1996a).

From day 15 after infection, the number of latently infected B cells in the spleen drops over a few weeks to a steady state level on the limit of detection. There is currently no well defined evidence that explains why this decline in infected B lymphocytes occurs during the resolution of splenomegaly.

In contrast to the situation described above, infection of immunologically immature or immunocompromised mice results in a systemic spread of virus to the liver, kidneys, heart and adrenal glands. The mice succumb rapidly to this generalised infection.

Latently infected B lymphocytes can be detected by in vitro reactivation assay and in situ hybridisation of viral tRNAs. Spleen cells from latently infected animals under normal circumstances do not undergo long term and sustained spontaneous transformation in vitro. In fact, there is currently little evidence to suggest that MHV-68 drives B cell proliferation by transformation. All attempts to 'transform' or 'immortalise' B cells, using
techniques commonplace in the study of EBV, have been unsuccessful. In vivo, however, around 10% of infected laboratory mice develop some form of lymphoproliferative disease. This percentage rises when mice are subjected to an immunosuppressive regime with cyclosporin A (Sunil-Chandra et al., 1994).

In 1996, 5 cell lines were derived from long term MHV-68 infected BALB/c mice with LPD. When the phenotype of these cells was investigated it was found all the lines expressed MHC class I and ICAM-1 molecules and none expressed CD3. Further, one of the lines, termed S11, was also found to express surface IgM, intracellular Ig, MHC class II molecules and low levels of B220. It was concluded the line ‘S11’ was of B cell lineage, whilst the other lines were of ambiguous phenotype (Usherwood et al., 1996b).

Further analysis of S11 revealed; each cell carries around 40 copies of the MHV-68 genome (in either episomal and linear forms), a proportion of the cells produce virus particles and the longer the line is cultured for, the more lytic virus replication it supports. When S11 cells were cultured in the presence of the anti-Herpes drug 2'-deoxy-5-ethyl-beta-4'-thiouridine (4'-S-EtdU) free virus became undetectable. Latently infected cells, however, were detectable at all times.

When S11 cells were injected subcutaneously into the flanks of nude mice, solid tumours developed. Tumours did not, however, develop following similar injections of S11 into normal immunocompetent mice. It was concluded that S11 is more reminiscent of a Burkitt's lymphoma cell line than a LCL derived by in vitro infection of B cells (Usherwood et al., 1996b).

Detailed analyses of activated T cells in the peripheral blood of mice at late times after infection have suggested that MHV-68 may drive cellular proliferation via a super
antigen. In 1997, it was reported that there was a disproportionate number of activated CD8+ T lymphocytes expressing a Vβ4 T cell receptor in mice 21 days after infection. It was suggested that MHV-68 encodes a novel super antigen which can preferentially activate CD8+ T lymphocytes (Tripp et al., 1997).

1.10.1 Immune responses to MHV-68 infection

Acute infection of mice with MHV-68 leads to an inflammatory infiltrate in the lungs of these animals at days 0 to 4pi. This infiltrate consists of mononuclear phagocytes and lymphocytes (Sunil-Chandra, 1992a). From days 4 to 10pi. the monocyte infiltrate decreases making way for CD8+ T cells which peak in numbers at days 8 to 10pi. CD4+ T cells and B cells have also been detected in the lungs of mice at this time by monoclonal antibody staining of lung sections (Sunil-Chandra, 1992a). The presence of CD4+ and CD8+ T cells in the lung during acute infection warranted further investigation into the roles of these cells.

1.10.2 T lymphocyte responses to MHV-68

BALB/c mice were depleted of CD4+ and CD8+ T cells by monoclonal antibody treatment and were then infected with MHV-68. It was found, in the absence of CD8+ T cells, MHV-68 infection proceeded in an uncontrolled manner with lung and spleen infections being dramatically exacerbated. Increased levels of infectious virus were measurable in the lungs of CD8+ T cell depleted mice at 12 days post infection. Ultimately this group failed to clear the virus and suffered from severe clinical symptoms of disease despite the presence of CD4+ T cells and antibody. In contrast, only a slight delay in virus clearance was noted when CD4+ T cells were depleted from mice prior to infection (Ehtisham et al., 1993). Thus, it appeared from this study that CD8+ T cells were critical in the control of the acute infection and CD4+ T lymphocytes only played a minor role in this process. The importance of CD8+ T lymphocytes in the control of
MHV-68 infection was further highlighted by Weck et al. in 1996. This group showed that β2-microglobulin KO mice, which lack functional CD8+ T lymphocytes, fail to clear infectious virus from their spleens at late times post infection (Weck et al., 1996).

To investigate the role of CD4+ T lymphocytes in MHV-68 infection, H-2b mice with a constitutive CD4+ deficiency (due to a disruption in the H-2IAb MHC class 2 glycoprotein) were infected with MHV-68. These animals displayed an apparently normal response to the viral infection up until day 22 after infection. At this time, infectious virus was detected in the respiratory tracts and adrenal glands of the mice. Viral titres in these sites grew progressively larger until the majority of the animals died around day 125 pi. During this experiment, some of the CD4+ T lymphocyte deficient mice were depleted of CD8+ T cells at day 100 pi. After this depletion, high titres of virus appeared in the lungs of these animals and they rapidly died. It was concluded from these studies that CD8+ T cell mediated control of MHV-68 infection had progressively diminished due to a lack of CD4+ T lymphocyte cytokine help (Cardin et al., 1996). Very recently, Stevenson et al. (1998) have confirmed this finding by demonstrating the long term maintenance of MHV-68 specific CD4+ T lymphocytes in infected animals. They suggest these CD4+ cells not only maintain anti-viral CD8 lymphocyte responses but probably also provide help for the long term production of virus-specific IgG.

The first description of MHV-68 specific CD8+ cytotoxic T cell (CTL) responses was published in 1998. Using a standard chromium release assay, virus specific CTL were demonstrated in bronchialveolar lavage fluid from mice infected for 10 to 25 days. CTL were also detected in cells of the draining mediastinal lymph nodes and in the spleen during this period. By day 67, however, a dramatic decline in the numbers of CTL precursors detectable in lymph nodes and the spleen had occurred. It was suggested that this decline occurs as result of progressive immune exhaustion. MHV-68 CTL
precursors may be maintained in a partial state of activation by persistent antigen presentation which results in lymphocytes ultimately undergoing apoptosis (Stevenson, 1998).

In summary, this study seemed to confirm previous findings that CD8+ T lymphocytes are most important during the acute phase of MHV-68 infection. The mechanism or mechanisms by which these cells exert their effects in this model have not been fully characterised. In 1996, Usherwood et al. demonstrated that CD8+ T cells are not dependent on perforin for the control of MHV-68 infection. The possibility remains that these lymphocytes employ a fas-ligand/ fas interaction to kill infected cells. Alternatively, they may release cytokines which have antiviral effects (Usherwood et al., 1996).

There is no available evidence suggesting that numbers of MHV-68 latently infected B cells are directly regulated by virus-specific T lymphocytes either during the resolution of splenomegaly or at late times post infection.

1.10.3 Humoral responses to MHV-68 infection

Analyses of virus specific immunoglobulin and neutralising serum antibody profiles have demonstrated a low, delayed IgG response to MHV-68 which increases in magnitude over several months. Detectable antibody levels do not appear until around 16 days after infection. It is thought this may be due to MHV-68 subverting B lymphocyte function by infecting these cells (Stevenson et al., 1998).

Kulkarni et al. (1997) have provided two further observations which indicate that antibody does not play a major role in virus clearance from the lung. Firstly, in their hands, μMT mice with no mature B cells have no detectable virus in their lungs 28 days after infection with MHV-68. Secondly, Kulkarni et al. have demonstrated that CD8+ T
cell deficient mice with high levels of anti-viral serum antibody, have elevated levels of virus in their lungs (again 28 days after infection). It appears, therefore, that antibody is not important in the clearance of acute MHV-68 infection. It may play a role, however, in neutralising reactivating virus at late times post infection.

1.10.4 Cytokine responses to MHV-68 infection

Studies on cytokine responses to MHV-68 infection have revealed that infected mice produce high levels of IFN-\(\gamma\) and IL-6. Lower levels of IL-2 and IL-10 are also produced, however, little or no IL-4 or IL-5 has been detected (Sarawar et al., 1996).

To investigate the role of IFN-\(\gamma\) in murine responses to MHV-68, IFN-\(\gamma\) and IFN-\(\gamma\) receptor knockout (KO) mice have been experimentally infected with the virus. In IFN-\(\gamma\) KO mice, clearance of MHV-68 from the lungs was slightly delayed and no reactivation of the virus was observed at late times post infection. It is clear that a lack of IFN-\(\gamma\) did not limit cellular proliferation or accumulation in the spleen or lungs in response to MHV-68 infection.

It was concluded from this study that there is a redundancy of IFN-\(\gamma\) function in the responses of mice to MHV-68 infection and other cytokines can compensate for the absence of this immunomodulator (Sarawar et al., 1997).

Contradictory evidence on the necessity for IFN-\(\gamma\) in immune responses of mice to MHV-68 has been published recently by Kulkarni et al. (1997). They found that infection of IFN-\(\gamma\) KO mice resulted in at least a third of all animals dying by 17 days pi. It was concluded that IFN-\(\gamma\) probably exerts its effects in this system by inducing the expression of the enzyme nitric oxide synthase in macrophages. This would result in the production
of NO which could directly exert an anti-viral effect. This hypothesis is reinforced by evidence from the same group that mice lacking the enzyme 'inducible nitric oxide synthase' all die when infected with MHV-68 (Kulkarni et al., 1997).

Work in this laboratory (Dutia et al., 1997) utilised IFN-γ receptor (IFN-γr) KO mice to investigate the role of IFN-γ in immune responses to MHV-68. These results indicated that IFN-γ is not important in the clearance of the initial viral infection or in long term surveillance in the lungs. However, infection of IFN-γr KO mice did point to a major role for this cytokine in control of MHV-68 spleen infections. Further, results from this group indicated that IFN-γ is critical in the maintenance of splenic architecture during MHV-68 infection of mice.

Experiments involving the depletion of CD4 and/ or CD8 T lymphocytes in MHV-68 infected IFN-γr KO mice demonstrated that it was CD8+ T cells which were responsible for observed splenic destruction in these infected animals. To date, no evidence of a mechanism for this destructive process has been published (Dutia et al., 1997).

A recent publication by Weck et al. (1997) has described MHV-68 induced large vessel arteritis in IFN-γr KO mice. This work has demonstrated that MHV-68 has a tropism for smooth muscle cells. Further, it shows that IFN-γ is essential in protective responses against an MHV-68-induced chronic pathology. The implication of this finding is that MHV-68 infection of mice may provide an animal model for the evaluation of acute and chronic human vascular pathological conditions.

Gene KO mice have also been used to investigate the role of type 1 interferons in murine responses to MHV-68 infection. Infection of mice with a lesion in the α/β
interferon receptor gene results in around 90% animal mortality. Death in these mice occurs around 6 to 8 days post infection when virus lung titres in these animals have reached approximately 100 to 1000 times those found in wild type mice. It appears therefore that type 1 interferons play a major role in controlling the initial replication of MHV-68 in the lung (Dutia et al., 1999).

It is hoped that the murine gammaherpesvirus model will enable the further study of, amongst other things, critical early in vivo events in gammaherpesvirus infection and how these are controlled by the immune system. It is also hoped new insights into the tropism of latency and how this is immunoregulated can be obtained. Finally, the model may prove invaluable in the study and development of immunotherapeutic strategies such as vaccination and cellular adoptive transfers. These may ultimately have a bearing on the treatment of human gammaherpesvirus infections.

1.11 To conclude...

Over the past 2 years, one of the most controversial and competitive areas in herpesvirus study has been the characterisation, isolation, sequencing and association with Kaposi's Sarcoma of HHV8. Despite these potentially exciting studies of this gammaherpesvirus a great deal of research has continued on what are arguably the prototypic herpesvirus representatives HSV, CMV and EBV.

More is known about HSV than any other herpesvirus. Several models of infection exist and the virus has been extensively characterised and manipulated over the years. Treatment of HSV infection has become commonplace with antiviral preparations now available 'over the counter'. HSV, however, remains a severe clinical problem particularly in immunosuppressed individuals in which antiviral resistance is now
occuring. In recent years the ability of HSV to modulate immune responses has been demonstrated a number of times. Further, it is now recognised that HSV can induce apoptosis of infected cells. In the future it will be of interest to discover how and why this occurs and whether it is of some advantage to the host or perhaps even the virus.

Cytomegalovirus remains a major problem in immunocompromised patients. In recent times detailed information has appeared concerning persistent CMV infection and immunodominant viral peptides to which cellular responses are directed. In the future, the promise of successful adoptive transfer therapy in patients is exciting as are further studies into the ability of CMV to interfere with or manipulate anti-viral immune responses.

EBV remains a significant pathogen in immunocompromised patients, areas of endemic malaria and other immunocompromised hosts. Currently, there is a great deal of discussion concerning the range of latent or lytic infections EBV can adopt in infected individuals. There is, perhaps, no definitive answer, however, as to which viral genes are expressed in all situations. It is not inconceivable that the virus can ‘mould’ it’s gene expression to or around prevailing immune responses in the infected host.

Increasingly, information gathered from healthy infected individuals is revealing how memory responses may control levels of virus during a latent infection. Questions have been asked as to whether memory immune responses are predominantly against latent antigens or are against lytic antigens expressed during reactivation episodes.

In the clinical situation, adoptive transfer therapies with EBV-specific CTL have been attempted with limited success. On a global scale these will only be available to
privileged transplant patients and this form of treatment is unlikely to help resolve viral-led disease in areas such as Africa.

In recent years an animal model of gammaherpesvirus infection has shown promise. Murine infection with MHV68 has perhaps, the broadest application possibilities. Other models, however, may prove useful for studies of selected aspects of infection.

Information concerning the immunomodulatory capabilities of EBV continues to appear, particularly in relation to EBNA-1 and the virus encoded IL-10 homologue. In fact some researchers have begun to consider the potential therapeutic application of virally encoded cytokines. It is felt these may differ in their functional capabilities from their host immunomodulatory counterparts.

In summary, an enormous amount of information concerning herpesvirus pathogenesis and immune responses is currently available. However, these viruses remain a clinical problem due to their complexity and the increasing numbers of immunocompromised patients appearing around the world and many questions remain to be answered, particularly with regard to application of novel therapeutic strategies.

1.12 Overall aims of this study

The overall aim of the work described in this thesis was to investigate the role of T lymphocytes in the immune responses of mice to the gammaherpesvirus MHV-68.

To date, studies investigating immune responses to gammaherpesviruses have focussed almost exclusively on EBV infection of humans. This is not surprising since EBV is clinically a major problem anywhere immunosuppressed individuals are encountered. The study of EBV has yielded an enormous amount of useful information
which may ultimately lead to the development of effective antiviral vaccination regimes or therapies. The most significant discovery of the last 20 years work on immune responses to EBV is that CD8+ T lymphocytes play a crucial role in protecting an infected individual from EBV-led disease. Current work in this field, reviewed earlier in this chapter, has now reached a staggering level of resolution. It is now recognised that CD8+ CTL are generated to both lytic cycle and latency associated EBV-derived peptide antigens and that a hierarchy of immunodominance exists amongst these peptides.

Despite this level of knowledge, however, a number of questions remain to be answered on immune responses to gammaherpesvirus infection. First of all, the vast majority of studies investigating immune responses to EBV have focussed on events occurring after the onset of infectious mononucleosis (IM). Very little is known about the responses of individuals to EBV prior to the onset of IM. Further, it is not known why the vast majority of individuals infected with EBV never develop IM.

Taking a wider perspective, it is unclear if immune responses to EBV bear any resemblance to those which are generated to gammaherpesviruses in other species.

Infection of inbred mice with the gammaherpesvirus MHV-68 has provided an opportunity to study gammaherpesvirus pathogenesis in a highly amenable model species. The aim of the work described in thesis was to assess, both in vitro and in vivo, the importance of T lymphocytes in immune responses to MHV-68 infection. It was hoped, any information obtained would:

- Give an insight into whether well characterised human immune responses to EBV reflect those which are generated to another gammaherpesvirus in a very different species.
and

- Enable the design of therapeutic strategies which could be used to alter the progress of MHV-68 infection and ultimately be applied to the treatment of human gammaherpesvirus infections.

In order to assess T lymphocyte responses to MHV-68, several approaches were adopted

- Attempts were made to develop an *in vitro* method of assessing cytotoxic T lymphocyte function

- To study the functional roles of CD4+ and CD8+ T lymphocytes *in vivo*, monoclonal antibodies were used to deplete animals of specific T lymphocyte subsets at a range of times after infection.

- Adoptive transfers of specific T lymphocyte subsets were made into immunocompromised nude mice to assess the ability of these cells to respond to a subcutaneous MHV-68 infected lymphoma.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Chemicals

Unless otherwise stated chemicals and general laboratory reagents were supplied by Sigma-Aldrich Chemical Co. or Merck-BDH (Both UK).

The radio-labelled compound sodium chromate (Na$_2$CrO$_4$) in normal saline (pH8) (5mCi/ml) was supplied by ICN pharmaceuticals.

Euthatal – used to kill mice when cervical dislocation was inappropriate (Rhone-Merieux, Canada).

Unless otherwise stated plastic culture vessels were supplied by Life Technologies Ltd.

2.1.2 Apparatus

Centrifuge – Centrifuging was carried out in a Beckman TJ-6 refrigerated centrifuge

Fluorescence Activated Cell Sorter (FACS) – Flow cytometry was carried out using a ‘FACStar’ fluorescence activated cell sorter (Becton Dickinson, Bedford, UK).

Pipettes – All automatic pipettes were manufactured by Gilson.

Scintillation Counter – Measurements of chromium release were carried out using a ‘Microbeta Plus’ Liquid Scintillation Counter (Wallac, Turku, Finland).
**Chapter 2 – Materials and Methods**

**Cell Homogeniser** – In the preparation of virus stocks a Dounce homogeniser was used (Wheaton, USA)

**Organ Homogeniser** – Glass homogenisers used in the preparation of samples for virus titration were obtained from Soham Scientific, UK.

**Microscope** - Plaque counting was carried using a dissecting microscope (Wild, Heerbrugg).

**Microscope** – All counting of slide mounted cells stained with fluorescent markers was carried using a Leitz fluorescent microscope (Leitz, Switzerland)

### 2.1.3 Tissue Culture Reagents, Disposable Plastics and Equipment

Dimethyl sulfoxide (DMSO) (Merck-BDH).

‘Low-Tox-H’ Rabbit complement (Cedar Lane laboratories Ltd., Westbury, New York)

Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Ltd).

Essential amino acids (50x) (Squibb).

Non-essential amino-acids (Squibb).

Foetal calf serum (FCS) (Globepharm).

Formal-saline (Surgipath-Europe Ltd) 10% neutral buffered formaldehyde solution.

Glasgow’s modified Eagle medium (GMEM) (Life Technologies Ltd).

L-Glutamine (100x) (Merck-BDH) 200mM in sterile distilled water (SDW).

β-mercapto-ethanol (100x) 5mM in STD and filter sterilised.

Metrizamide (Nycomed Pharma AS, Torshov, Norway)

New-born calf serum (NBCS) (Harlan Sera-Lab).

Fungizone (100x) (Squibb) 200μg/ml in sterile distilled water (SDW).

Penicillin (100x) (Merck-BDH) 7mg/ml dissolved in SDW and then filter sterilised.
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Phosphate buffered saline (PBS) 80mg/ml sodium chloride, 2mg/ml potassium chloride, 2mg/ml potassium dihydrogen phosphate (KH$_2$PO$_4$), 11.05mg/ml disodium hydrogen phosphate (Na$_2$HPO$_4$) (pH 7.2, sterilised by autoclaving: SPBS).

RPMI 1640 (Life Technologies Ltd).

Scrapers (Sterilin)

Filter sterilisation carried out using sterile syringes (Becton Dickinson) and 0.2μm sterilising filters (Sartorius).

Streptomycin (100x) (Sigma-Aldrich Chemical Company, UK) 1mg/ml dissolved in SDW and filter sterilised.

Toluidine blue (Merck-BDH) 1% (W/V) in distilled water.

Trypan blue (stock) 1% (W/V) trypan blue (Merck-BDH) in PBS and filter sterilised.

Trypan blue (working stocks) 1/10 dilution of stock with sterile PBS.

Trypsin/EDTA solution, tissue culture grade (Life Technologies, Paisley).

Tryptose phosphate broth (TPB) (Life Technologies, Paisley).

0.02% Versene - in 1 litre of SPBS: 200μg/ml ethylenediaminotetraacetic acid (disodium salt: EDTA), 1% phenol red, pH 7.1 – 7.3.

2.1.4 Antiviral Compound

The antiviral compound 2’deoxy-5’ethylβ-4’thiouridine (4’-S-EtdU) was a gift from GlaxoWellcome (GlaxoWellcome Laboratories, Kent) (Rahim et al., 1996). A stock solution of 2mg/ml was prepared using SDW at 60°C with constant agitation. The solution was then filter sterilised whilst hot and stored at room temperature.
2.1.5 Antibodies

Avidin-FITC conjugate was obtained from Sigma-Aldrich Company Ltd, UK.

Goat anti-rabbit biotinylated IgG was obtained from Vector Laboratories (Burlingame, California).

Hamster anti-mouse CD3 FITC-conjugated monoclonal antibody was obtained from Pharmingen (San Diego, California).

Mouse anti-mouse monoclonal I-A^d antibody (0.5 mg/ml) was obtained from Pharmingen (San Diego, California).

Mouse anti-mouse MHC H-2K^d biotinylated monoclonal antibody was obtained from Pharmingen (San Diego, California).

Polyclonal swine anti-rabbit FITC-conjugated secondary antibody was obtained from Dako Ltd, UK.

Rabbit polyclonal anti-MHV-68 hyperimmune serum, derived by NP Sunil-Chandra, was obtained from departmental stock (Sunil-Chandra, PhD Thesis).

Rabbit anti-rat FITC-conjugated 'STAR 49' antibody (0.5 mg/ml) was obtained from Serotec, UK.
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Rabbit anti-mouse CD4 phycoerithrin-conjugated antibody (50μg/ml), Becton Dickinson (Bedford, UK).

Rabbit anti-mouse CD8 FITC-conjugated antibody (250μg/ml) was obtained from Becton Dickinson, (Bedford, UK).

Rat monoclonal anti-H-2K<sup>d</sup> antibody (0.5mg/ml) was obtained from Pharmingen (San Diego, California).

Rat anti-mouse CD45R (B220) biotinylated monoclonal antibody, Pharmingen (San Diego, California).

Rat anti-mouse CD4 (anti-mouse L3T4) IgG2b antibody, clone YTS 191.1, derived from ascites by ammonium sulphate precipitation was a gift from S. Cobbold (Cobbold et al., 1984).

Rat anti-mouse CD8 (anti-mouse Lyt-2) IgG2b antibody, clone YTS 169.4, derived from ascites by ammonium sulphate precipitation was a gift from S. Cobbold (Cobbold et al., 1984).

Rat anti-mouse CD44 antibody (hybridoma I 4215.1), was obtained from departmental stock.

Rat anti-mouse F4/80 monoclonal antibody was obtained from Serotec, UK.
2.1.6 Tissue Culture Growth Media

**S11 growth medium (S11-RPMI)**

RPMI 1640 with 10% FCS (non-heat inactivated), penicillin/ streptomycin (70μg/ml / 10μg/ml respective final concentrations), L-glutamine (2mM final concentration) and 2 - Mercaptoethanol (50μM final concentration).

**Lymphoma cell line culture medium (RPMI)**

RPMI 1640 with 10% FCS, penicillin/ streptomycin (70μg/ml / 10μg/ml respective final concentrations), L-glutamine (2mM final concentration), Fungizone (2μg/ml final concentration) and 2 - Mercaptoethanol (50μM final concentration).

**Fibroblast cell line culture medium (DMEM)**

DMEM with 10% FCS, penicillin/ streptomycin (70μg/ml / 10μg/ml respective final concentrations), L-glutamine (2mM final concentration)

**BHK Growth Medium (ETC)**

GMEM with 10% NBCS, 10% TPB, penicillin/ streptomycin (70μg/ml / 10μg/ml respective final concentrations), L-glutamine (2mM final concentration) and Fungizone (2μg/ml final concentration).
2.1.7 Cell lines

**A20 cells**

A20 cells are a pre-B lymphoblastoid cell line, originally derived from a BALB/c mouse with a spontaneously growing reticulum tumour. Grown in RPMI. (Kim et al., 1979)

**Baby Hamster Kidney cells (BHK)**

BHK cells are an immortal fibroblastoid cell line. Clone 21 was originally derived from kidneys of one day old hamsters and was grown in ETC medium (previously described by Sunil-Chandra, 1992a).

**L-929 cells**

L-929 cells are a fibroblastoid cell line originally derived from normal subcutaneous areolar and adipose tissue of a 100 day old C34/An mouse. These cells were obtained from the European Collection of Animal Cell Cultures and were grown in DMEM.

**NS0 cells**

NSO cells are a transformed B-cell myeloma cell line and were grown in RPMI medium (previously described by Sunil-Chandra, 1993).

**S11**

S11 lymphoma cells were originally isolated from an MHV-68 infected mouse with lymphoproliferative disease. These cells have the phenotype of a mature B cell.
and are predominantly infected with a latent form of MHV-68. A small proportion do support productive viral replication and release infective virus (Usherwood et al., 1996). S11 lymphoma cells were grown in S11-RPMI.

3T3
3T3 cells (clone A31) are fibroblastoid and were derived from a BALB/c mouse embryo. These cells were obtained from the European Collection of Animal Cell Cultures and were grown in DMEM.

YAC-1
YAC-1 cells are murine lymphoma cells originally derived by inoculation of Moloney leukaemia virus into newborn A/Sn mice. These cells were obtained from the European Collection of Animal Cell Cultures and were grown in RPMI medium.

2.2 Methods
2.2.1 Tissue Culture Procedures
2.2.2 Cell counting
Cells were suspended at 1:10 in 0.1% trypan blue in PBS and counted in a haemocytometer. Viability was determined by the proportion of cells unstained after a 5 minute incubation with the trypan blue.
2.2.3 Culture

BHK, L-929 and 3T3 cells were grown in T175 tissue culture flasks containing 50ml of the appropriate medium at 37°C and 5%CO₂. Flasks were seeded with 5 x 10⁶ or 1 x 10⁷ cells and cultured for 2 to 3 days or until confluent. Once confluent, spent medium was removed from the flask and the monolayers were washed once with 5ml of the chelating agent, Versene. Monolayers were then incubated with 5ml of trypsin/EDTA until the cells had detached from the surface of the flask at which point the trypsin was inactivated with 5 ml of fresh medium at 37°C. The cells were dispersed into a single cell suspension by pipetting up and down and pelleted by centrifugation at 450 x g for 5 minutes. The supernatant was discarded and the cells re-suspended in 10ml of fresh medium.

A20, NS0, S11 and YAC-1 cells were grown in T75 or T175 tissue culture flasks (Nunc) at 37°C and 5%CO₂ with 25 or 50 ml, respectively, of the appropriate medium. Flasks were seeded at a concentration of 1 x 10⁵ cells/ ml and cultured for 3 to 4 days. All of these cell lines are semi-adherent and weakly attached cells were re-suspended by a gentle ‘tap’ of the flask. The cells were pelleted by centrifuging at 450 x g for 5 minutes and then re-suspended in 10ml of fresh medium (Sunil-Chandra et al, 1993).

2.2.4 Storage and retrieval of cell lines from liquid nitrogen

Cell lines were stored in liquid nitrogen. Cells were pelleted by centrifugation and re-suspended at a concentration of 1 x 10⁶ cells/ ml in an ice-cold mixture of 90% FCS/ 10% DMSO. 1ml aliquots were dispensed into labelled cryo-vials and frozen.
at \(-70^\circ\text{C}\) for 24 hours. The cryo-vials were transferred to liquid nitrogen for long term storage.

Cells were recovered from liquid nitrogen storage by rapid thawing at \(37^\circ\text{C}\). Ice-cold growth medium was then added drop-wise to the cells with constant swirling. In total, 10ml of cold medium was added to the cells which were subsequently pelleted by centrifugation at 450 x g for 5 minutes. The supernatant was discarded and the cells re-suspended in 10ml of fresh medium. This cycle of cell pelleting and supernatant removal was repeated 3 times to remove all traces of DMSO. Finally, the cells were re-suspended in 1ml of medium and counted on a haemocytometer to assess viability. If more than \(5 \times 10^5\) viable cells were present the cells were placed into a T25 tissue-culture flask in 10ml of medium. If less than \(5 \times 10^5\) viable cells were counted, a second aliquot of cells was recovered from liquid nitrogen storage, cell populations were pooled and the cells were cultured as above.

### 2.2.5 Preparation of splenocytes

Spleens were removed from mice immediately following death and were placed into RPMI medium on ice. Splenocytes were obtained by ‘teasing’ cells from the organ using curved forceps in a 60mm petri-dish (Sterilin) containing 5ml of RPMI medium (Sunil-Chandra et al., 1992a). Alternatively, whole spleens were disrupted by crushing them through a sterile stainless steel tea strainer (John Lewis Ltd.) using a 10ml syringe plunger with 5ml of RPMI medium in a 60 mm petri-dish. Cell suspensions were Pasteur pipetted up and down to break up any cell clumps and were stored on ice throughout the procedure. Red blood cells were removed by
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water-lysis as follows. Cell suspensions were pelleted by centrifuging at 500 x g for 5 minutes at 4°C and supernatants were discarded. Sterile distilled water (1ml/spleen) was added to the dissociated pellet for 10 seconds and then a 10 fold excess of SPBS was added to the cells which were pelleted as previously. The cell pellet was re-suspended in RPMI, red-cell 'ghosts' were allowed to settle-out and the splenocyte suspension was pipetted into a sterile universal and stored on ice.

2.2.6 Culture of splenocytes

Splenocytes were cultured in 24 well flat-bottomed tissue culture plates (2ml medium/well) or upright T75 tissue culture flasks (40ml medium/flask) at a concentration of 2 x 10⁶/ml in S11 RPMI or RPMI. In the majority of experiments, splenocytes from infected mice were cultured with irradiated splenocytes (infected with MHV-68 in vitro) or S11 cells. These irradiated and non-dividing cell populations were intended to be a source of antigen presenting cells (APCs) capable of presenting viral antigen to the splenocyte population and eliciting the expansion and activation of virus-specific effector cells.

2.2.7 Preparation of splenocyte APC populations

Normal, uninfected spleens were removed from animals and stored in RPMI on ice. Splenocyte suspensions were obtained and counted as previously described. An appropriate number of cells was pelleted by centrifugation at 450 x g for 5 minutes and the supernatant was discarded. The cells were infected at a multiplicity of infection (MOI) of 0.1 to 5 plaque forming units (PFU) of MHV-68 per cell in a final volume of 1ml of RPMI medium. The cell suspension was incubated
at 37°C for 1 hour, on a shaker, to allow the virus to adsorb to the cells. Post-incubation, infected cells were washed twice by re-suspending in 10ml of RPMI medium and centrifuging at 590 x g for 5 minutes. These cells were then γ-irradiated with 2000R from a 137Cs source to damage cellular DNA, preventing cell growth and division yet retaining the antigenic integrity of the cells. Following irradiation, the cells were washed twice, as above, and counted. The irradiated splenocytes were then re-suspended at 1 x 10^6/ ml if they were to be cultured with infected splenocytes or 2 x 10^5/ ml for culturing with cells from the cervical lymph nodes of ear-infected mice. After re-suspension the cells were aliquoted at 1ml/ well into 24 well plates containing potential effector/ responder lymphocytes.

2.2.8 Preparation of S11 ‘APC’ populations

S11 cells were cultured and harvested as previously described. The cells were then centrifuged at 450 x g for 5 minutes and re-suspended in 5ml of S11-RPMI in a bijou. S11 suspensions were γ-irradiated with 6000R from a 137Cs source. Post-irradiation, the S11 cells were washed in RPMI by centrifugation at 450 x g for 5 minutes and counted. The S11 cell concentration was adjusted to 2x10^5 cells/ ml or 1 x 10^6 cells/ ml in RPMI depending on the experiment and an appropriate volume was added to the tissue culture plate or flask. When 24 well plates were used, 1ml of the S11 cell suspension was added to each well whilst 20ml of the S11 cell suspension was added to T75 tissue culture flasks.
2.2.9 Culture of cells from the cervical lymph nodes of mice

Cervical lymph nodes were removed from mice immediately following death and were stored in RPMI medium on ice. Lymphocytes were obtained by squeezing the lymph nodes between the ends of forceps in cold medium until all material was disrupted and a cell suspension was obtained. The cells and tissue debris were then pelleted by centrifugation at 450 x g for 10 minutes at 4°C. The supernatant was discarded and replaced with 20ml of cold medium after which the cells were shaken and debris was allowed to settle out. Lymphocytes were obtained by drawing off the medium above the debris at the base of the tube. The cells were counted as previously described. Subsequently, the cells were either re-suspended at 1.5 x10⁶/ ml in RPMI and pipetted in 200µl aliquots into a round bottomed 96 well plate or the cells were re-suspended at 2 x 10⁶/ ml in RPMI and 1ml aliquots were pipetted into the wells of a 24 well tissue culture plate. When 24 well plates were used the lymphocytes were cultured with 6 x 10⁵ irradiated and infected splenocytes as previously described.

All tissue culture plates were incubated at 37°C and 5%CO₂ in a humidified incubator. The period of incubation was always 3 days for lymph node-derived cells and between 3 and 14 days for splenocytes depending on the experiment.

2.2.10 Harvesting of lymphocytes after culture

Lymphocytes were harvested from 24 or 96 well plates by pipetting 'up and down' using an automatic pipette (Gilson) to dislodge and remove cells from the plate wells. When T75 flasks were used to culture lymphocytes, the cells were harvested by using a 10ml sterile plastic pipette. Cells were then pelleted by
centrifuging at 590 x g for 10 minutes after which they were re-suspended in 10ml of RPMI medium and counted.

2.2.11 Measurement of lymphocyte cytolytic activity

A range of lymphocyte concentrations was prepared in RPMI medium. The concentrations were varied according to the number of viable cells available but were, in all experiments, at least three of the following: $1 \times 10^7$, $5 \times 10^6$, $2.5 \times 10^6$, $2 \times 10^6$, $1.25 \times 10^6$ and/or $5 \times 10^5$ cells/ml. These concentrations corresponded to lymphocyte:target ratios in assay wells of; 100:1, 50:1, 25:1, 20:1, 12.5:1 and 5:1 respectively. 100μl of each concentration was pipetted in triplicate into the wells of a 96 well round-bottomed tissue culture plate. Where antibody blocking was investigated, 50μl volumes containing the same numbers of cells were used.

2.2.12 Target cells

The cell types used as targets in the various cytotoxic T cell assays, were as follows: A20, L-929, NS0, S11, 3T3 and YAC-1. All cells were cultured as described previously.

2.2.13 S11 and YAC-1 cells as targets

Cells were harvested on the day of an assay as previously described. After counting, $5 \times 10^6$ cells were removed and pelleted by centrifugation at 590 x g for 5 minutes. The supernatant was discarded and the cells were re-suspended in the smallest volume of medium achievable prior to labelling. If the effects of the antiviral compound 4'-S-EtdU on S11 cells as targets were being investigated the
cells were cultured, as normal, in the presence of >1mg/ml of the drug for more than 7 days prior to the assay.

2.2.14 3T3 and L-929 cells as targets

Cells were harvested on the day of an assay as described previously. The cells were then infected with MHV-68. Briefly $1 \times 10^7$ cells were pelleted and then re-suspended in an appropriate volume of virus stock. The multiplicity of infection (MOI) used was varied between experiments but was in all cases between 3 and 9. Following addition of virus to the cells they were incubated for 1 hour at $37^\circ C$ with agitation to allow virus adsorption. 10ml of RPMI was subsequently added to the cells and they were centrifuged at $590 \times g$ for 5 minutes. The supernatant was discarded and the cells were re-suspended in the smallest volume achievable prior to labelling.

2.2.15 A20 and NS0 cells as targets

These cells were infected with MHV-68 for more than a week before their use as targets in a cytotoxicity assay. Briefly, the cells were harvested as previously described and infection was carried out as in section 2.2.33. After infection the cells were cultured for more than 7 days and were harvested for use as targets on the day of an assay. The cells were pelleted by centrifuging at $590 \times g$ for 5 minutes, supernatant was discarded and the cells were re-suspended in as small a volume as possible prior to labelling.
2.2.16 Labelling of target cells with $^{51}$Cr

Once target cells had been pelleted in a universal, a 100µCi aliquot of the radioactive isotope $^{51}$Cr was used to re-suspend them. Targets were then incubated in a pre-warmed lead box at 37°C for 1 hour. After incubation, excess extra-cellular $^{51}$Cr was washed from the cells by re-suspending them in 10ml of SPBS and centrifuging at 900 x g for 5mins. This washing step was repeated 3 times, a total of 4 washes. The target cells were then re-suspended in 1ml of RPMI medium and counted in 0.1% trypan blue dye. Following this count, labelled cells were re-suspended at $1 \times 10^5$/ ml in RPMI. 100µl aliquots were pipetted into the wells of a 96 well round-bottomed tissue culture plate containing the potentially cytolytic effector cells. To measure the minimum (spontaneous) and maximum release of $^{51}$Cr from the target cells, 100µl target aliquots were incubated in the 96 well plate with 100µl of RPMI only or 100µl of 5% Triton X-100 detergent in PBS respectively.

Anti-CD4, anti-CD8, anti-H-2K<sup>d</sup> and anti-IA<sup>d</sup> antibodies were used in attempts to interfere with cytotoxic cell/ target cell interactions during cytotoxic T-lymphocyte (CTL) assays. The antibodies were diluted to a concentration of 50µg/ ml in RPMI and were added to assay wells in 100µl aliquots.

Where antibody blocking was being investigated, identical numbers of target cells were placed into the assay wells in 50µl RPMI.
Following addition of effector, target and control cells to a 96 well plate, plates were centrifuged at 450 x g for 1 min to pellet cell types together. The plates were then incubated in a humidified incubator at 37°C in 5% CO₂ for 5 hours.

2.2.17 Assessing the extent of cytolytic activity in assay wells
After the 5 hour incubation, plates were centrifuged at 450 x g for 5 minutes to pellet effector and target cells together. Subsequently, 40μl supernatant from the assay wells was transferred to a duplicate 96 well plate (Falcon, Becton Dickinson Labware California, USA) containing 120μl of scintillation fluid (Wallac) per well. The extent of ⁵¹Cr release into the supernatants was assayed by measuring levels of scintillation in the duplicate plate wells using a liquid scintillation counter.

2.2.18 The allo-specific cytotoxic T-lymphocyte assay
In order to assess the suitability of S11 lymphoma cells as targets for MHC class 1 molecule directed killing an allo-specific CTL assay was undertaken.

2.2.19 Obtaining allo-responsive effector cells
Spleen cell preparations from normal, uninfected C57BL/6 and BALB/c mice were obtained as in section 2.8. The BALB/c splenocytes were then irradiated with 2000R from a ¹³⁷Cs source. Post-irradiation these cells were washed and 2.5 x 10⁷ were cultured in a 25cm³ flask with 5 x 10⁷ normal C57BL/6 splenocytes. This represented a stimulator:responder ratio of 2:1. After 5 days incubation at 37°C in 5% CO₂ the cells were harvested, counted and the following dilutions were prepared - 5 x 10⁵, 2 x 10⁵, 5 x 10⁵ and 1 x 10⁵ cells/ml. As before these
concentrations corresponded to effector:target ratios of 50:1, 20:1, 5:1 and 1:1 in the final assay.

2.2.20 Target cells
The cell types used as targets in this assay were as follows; (i) S11, (ii) BALB/c concanavalin A (Con A)-stimulated splenocyte blasts and (iii) C57BL/6 Con A-stimulated splenocyte blasts. (ii) and (iii) were obtained as follows. Spleens were removed from BALB/c and C57BL/6 mice and splenocytes were obtained as before. 5x10^6 of these cells were then cultured for 5 days in 10ml RPMI and 4μg/ml Con A. Two days prior to their use as targets, the Con A stimulated blasts were split to ensure all cells were in a log phase of growth.

2.2.21 Assessing the extent of C57BL/6 cytotoxic activity in the stimulated splenocyte population
100μl aliquots of effector cells at the dilutions previously stated were pipetted in triplicate into the wells of a 96 well round-bottomed, tissue culture plate as before. 100μl aliquots of 1x10^4 51Cr labelled target cells were also pipetted into the plate which was incubated as in section 2.2.17. The extent of anti-H-2d and thus anti-S11 activity post incubation was assessed as in section 2.2.17.

2.2.22 Data interpretation
In all CTL assays, triplicate wells of each dilution of effector cells were prepared. Scintillation values were expressed as counts per minute (CPM) for individual
wells by the plate reader. For each CPM value specific chromium release was calculated using the formula;

\[
\frac{\text{Experimental CPM} - \text{Minimum CPM}}{\text{Maximum CPM} - \text{Minimum CPM}} \times 100 = \% \text{ Specific Release}
\]

Mean specific release for each effector:target ratio was calculated as were the standard deviation and standard error of the mean. Results were expressed in graphical form by plotting effector:target ratio against % specific \(^{51}\)Cr release.

2.2.23 \textit{In vitro} expansion of T lymphocyte lines

Mice were infected intranasally with MHV-68 as described elsewhere for between 20 and 30 days. After spleens were removed from the mice, splenocyte suspensions were prepared as previously described and the cells were cultured with irradiated S11 cells in 24 well plates for 6 days as described in section 2.2.6. The cells were harvested, pelleted by centrifugation at 450 x g for 10 minutes and re-suspended in fresh RPMI medium. This was repeated 3 times to remove cell debris and the cells were counted.

Viable lymphocytes were ‘re-seeded’ into a 24 well plate at a concentration of 2 x 10^6 cells / well with irradiated S11 cells at the same concentration. Into half of the wells was added 40u of mouse recombinant IL-2 (rIL-2) (Sigma-Aldrich, UK.). The remaining wells received 100u/ ml of rIL-2 and the plate was incubated for 7 days at 37°C/ 5% CO\textsubscript{2}. It was found after this incubation that the yield of viable
lymphocytes from the wells receiving 100u of rIL-2 was twice that of the wells given the lower dose of the cytokine. Therefore, viable lymphocytes remaining were re-cultured at a 1:1 ratio with irradiated S11 cells and 100u of rIL2 for 7 days. The cells were then harvested, washed and returned to culture for a further 7 days under the same conditions.

Following this incubation, the cells were harvested, pelleted and re-suspended in 2ml of fresh RPMI medium. To remove cell debris, the cell suspension was pipetted gently onto the surface of 4ml of Metrizamide (35% w/v) in a 15ml v-bottomed, clear polypropylene tube and the tube was centrifuged at 500 x g for 15 minutes. After this spin, dead cells from the population had formed a pellet in the base of the tube whilst viable cells remained on the upper surface of the Metrizamide. Viable cells were removed using a Pasteur pipette and were placed into 10ml of fresh RPMI medium. The cells were then pelleted and re-suspended in medium 3 times to remove all traces of Metrizamide and were re-cultured 1:1 with irradiated S11 cells and 50u/ml of rIL-2, as above, for 3 weeks. At the end of each week the cells were harvested, counted and re-cultured in fresh medium with a new population of irradiated S11 cells and fresh rIL-2. During this period of culture the lymphocytes expanded almost 100-fold in number and at its conclusion 1 x 10^7 cells were frozen down as described elsewhere.

The remaining cells were either analysed by flow-cytometry in an attempt to characterise their phenotype or were tested for their ability to lyse a range of targets in a standard chromium release assay (described in section 2.2.16)
The cells taken for flow cytometric analysis were incubated with anti-CD4, anti-CD8, anti-CD3 and anti-B220 monoclonal antibodies as described in section 2.2.37 prior to analysis.

The cells being tested for cytolytic activity were incubated with S11, NSO, MHV-68 infected NS0 and YAC-1 target cells as in section 2.2.16.

This protocol for generating T cell lines was employed 3 times but only the first attempt gave sufficient cells to carry out the subsequent analyses. Attempts to resurrect the original lymphocytes from liquid nitrogen storage were unsuccessful.

### 2.2.24 Virus

Murine gammaherpesvirus 68 (MHV-68) was originally isolated in Czechoslovakia from Clethrionomys glareolus (a bank vole) (Blaskovic et al., 1980). The G2.4 infectious clone used in these experiments was originally derived from MHV-68 stock supplied to the Department of Pathology, Cambridge University by Professor Blaskovic.

Working stocks of MHV-68 were prepared by infection of BHK-21 cells, in suspension, at a multiplicity of infection (MOI) of 0.1 or 0.01 PFU of MHV-68. Suspensions were incubated at 37°C for 1 hour on a shaker. Infected cells were then transferred to T175 tissue culture flasks containing 50ml of medium and incubated for 5 to 6 days in a humidified incubator at 37°C. After incubation, BHK monolayers were removed from flasks by using a scraper (Sterilin) and the cells were pelleted by centrifugation at 275 x g for 10 minutes at 4°C. Low titre supernatant was discarded. To obtain cell associated virus a Dounce
homogeniser (Wheaton, USA) was used to disrupt the BHK cells. Virus was then clarified by centrifuging at 750 x g for 30 minutes at 4°C. The resultant supernatant was stored on ice whilst the pellet was homogenised a second time and re-centrifuged at 750 x g for 30 minutes at 4°C. Supernatants were pooled and dispensed into 200μl or 500μl aliquots for use as working stock. All aliquots were stored at -70°C until use.

2.2.25 Assay for infectious virus

If required, sample organs were allowed to thaw and were then homogenised individually in 1ml of ice cold ETC medium using glass homogenisers. Cell suspensions were transferred back to their cryovials and were refrozen at -70°C. Freeze/ thawing samples increases cell lysis and infectious virus release.

Tissue homogenates were thawed rapidly at 37°C and centrifuged at 1100 x g for 5 minutes at 4°C to pellet cell debris. Homogenates or virus samples were then diluted, in duplicate, in bijoux and 1x10^6 BHK-21 cells added in a 200μl volume. The samples, in a final volume of 2ml of ETC medium, were incubated at 37°C for one hour on a shaker, to allow virus adsorption. After incubation, the samples were vortexed then poured into 60mm petri dishes containing 4ml ETC medium. The suspensions were gently swirled to evenly mix and distribute the cells. All petri dishes were subsequently incubated at 37°C/ 5% CO₂ for 4 days. Following this incubation, the BHK cell monolayers were fixed by removing supernatant from the plates using a 25ml pipette and replacing it with around 3ml of 10% formal saline. The plates were then incubated for a minimum of 15 minutes at room temperature. In a fume hood, the formal saline was poured off and around 5ml of
0.1% toluidine blue was added to each plate to stain the monolayer. After around 20 minutes the stain was poured off and the plates were rinsed in water and allowed to air dry.

The number of plaques per plate was quantitated using a microscope and the virus titre per organ was calculated.

### 2.2.26 Assay for Infectious Centres

Splenocyte suspensions were obtained and counted as previously described. 1 x 10^7, 1 x 10^6 and 1 x 10^5 cells were pipetted in duplicate into 60mm petri dishes containing 6ml of RPMI medium and 1 x 10^6 BHK-21 cells. 1ml of each splenocyte suspension was refrozen for subsequent infectious virus titration. Plates were incubated for 5 days at 37°C/ 5%CO₂. After this period monolayers were fixed as described above. Numbers of infectious centres/ plate was quantitated using a microscope and the infectious centre titre was calculated per 10^7 splenocytes and per spleen.

### 2.2.27 Mice

1) BALB/c females, age 3 to 4 weeks on arrival from Banton and Kingman (Grimston, Aldborough, Hull, UK)

2) CBA females, age 3 to 4 weeks on arrival from Banton and Kingman (Grimston, Aldborough, Hull, UK)
(3) BALB/c01aHsd-nu mice (athymic), age 6-8 weeks of age on arrival (Harlan, UK)

2.2.28 Administration of virus to mice

2.2.29 Intra-nasal route

Mice were inoculated intranasally with $4 \times 10^5$ PFU of MHV-68 in a 40μl infusion. Prior to administration mice were lightly anaesthetised using halothane. Virus dilutions were made in sterile phosphate buffered saline immediately prior to use and were kept on ice until the inoculation.

2.2.30 Sub-dermal route

Mice were inoculated sub-dermally in the ear pinna with $4 \times 10^5$ PFU of MHV-68 in a 20μl volume using a 27 gauge needle. Prior to administration mice were lightly anaesthetised using halothane. Virus dilutions were made in sterile phosphate buffered saline immediately prior to use and were kept on ice until the inoculation.

2.2.31 Anti-viral administration

Sterile 4'-S-EtdU stock solution was diluted with tap water (1/6) to give a final concentration of 0.33mg/ml. The 4'-S-EtdU solution was then filter sterilised and administered as drinking water to the mice.
2.2.32 Sampling of Mice

Mice were killed by cervical dislocation and relevant organs removed by dissection. When cervical lymph nodes were required, mice were killed by intraperitoneal injection of 100μl of euthatal (Rhone-Merieux).

2.2.33 In vitro infection of potential target cells

1 x 10⁷ A20 or NS0 cells were pelleted in a universal and re-suspended in 3 x 10⁷ PFU of MHV-68 stock (an MOI of 3). The final volume in the tube was made up to 1ml with RPMI medium and the cells were incubated at 37°C with agitation for 1 hour to allow virus adsorption. The cells were re-suspended in 10ml RPMI, pelleted by centrifugation at 450 x g for 5 minutes and supernatant was discarded. This was repeated 4 times. After the infection and washing stages the cells were returned to a T75 tissue culture flask in 25ml medium and were cultured until required.

2.2.34 Immunocytochemistry of potential target cells

S11, A20 and NS0 cells were harvested and counted as previously described. 2 x 10⁶ cells were aliquoted into a sterile universal and pelleted by centrifugation at 450 x g for 5 minutes. The supernatants were discarded. Pellets were re-suspended ‘dry’ and 50μl of fresh medium was added. 2μl of this cell suspension was applied to each well of a ‘multispot’ slide (Erie Scientific, Portsmouth, NH, USA). Cell spots were allowed to air dry for 15 to 20 minutes. Once dry, the cells were fixed by placing the slides in pre-cooled acetone for 3 to 5 minutes at -70°C and allowed to air dry. To block non-specific antibody binding, 20μl of 5% normal
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swine serum in PBS was added to the cell spots and slides were incubated flat in a humid box for 30 minutes at 37°C. Post-incubation, blocking serum was shaken off the slides and the primary antibody was added. 20μl of a 1/250 dilution of rabbit anti-MHV 68 hyper-immune serum in PBS was placed onto each cell dot and slides were incubated at 37°C for 1 hour. Slides were then washed 3 times in PBS containing 0.1% Triton X-100 and 20μl of swine anti-rabbit FITC conjugated secondary antibody diluted to 1/30 in PBS was added to the cells. Slides were incubated at 37°C for 30 minutes and were subsequently washed 3 times in PBS. Finally the slides were immersed in 0.1% Evans Blue (in PBS) for 30 seconds and rinsed in water. Mounting was carried out using the organic, aqueous mount “Citiflour“ (Citiflour, UKC, Canterbury) and slides were stored in a humid box, in the dark at 4°C. Cells were viewed using a fluorescent microscope (Leitz, Switzerland). One thousand cells were counted on each slide and results were expressed as the percentage of cells which were MHV-68 positive in the population counted.

2.2.35 Tumour regression experiments

Splenocytes from BALB/ c mice, obtained 21 days post-infection with MHV-68, were prepared and counted as described previously. These cells were then cultured in T75 flasks with irradiated S11 cells for 7 days as described in section 2.2.6. After incubation, the cells were harvested and pelleted by centrifugation at 450 x g for 10 minutes. The supernatant was discarded and the cells were resuspended in 20ml of fresh RPMI medium, then counted.
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To remove CD4+ or CD8+ T lymphocytes from a re-stimulated mixed splenocyte population a ‘complement depletion’ method was employed as follows. 5x10^7 splenocytes were pelleted by centrifugation at 450 x g for 10 minutes. During this spin an aliquot of lyophilized rabbit complement was re-constituted with 1ml of ice-cold sterile distilled water. 500μl of the complement solution was then added to 1500μl of ice-cold RPMI medium and this was sterilised by passing through a 0.2μm sterilising filter. An appropriate volume of anti-CD4 and/ or anti-CD8 antibody was then added to the complement/ RPMI to give a final antibody concentration of 100μg/ ml. After the supernatant had been discarded from the pelleted splenocytes the cells were re-suspended in 1ml of the antibody/ complement mixture and were incubated at 37°C for 1 hour, on a shaker. Post-incubation, splenocytes were pelleted by centrifugation at 450 x g for 5 minutes, supernatant was discarded and the cells were re-suspended in 10ml of fresh RPMI medium. This was repeated 3 times to remove unused complement and cell debris from the cell suspension. The cells were then counted, pelleted and re-suspended at a concentration of 5x10^7 cells/ ml in sterile PBS. To confirm antibody depletions had been successful, an aliquot of 1x10^6 cells was taken at this stage for FACS analysis.

Splenocytes were administered IP to Nu/ Nu mice in 200μl aliquots. Each mouse received 1x10^7 cells. The mice were then partially anaesthetised using halothane and 2x10^7 S11 cells (cultured and harvested as described previously) were injected subcutaneously into the animals flank. Where the effects of the antiviral 4'-S-EtdU were being assessed, mice were administered 4'-S-EtdU in their
drinking water for more than 7 days as described in section 2.2.31. In addition, S11 cells were cultured in 2μg/ml 4′-S-EtdU for more than 7 days prior to subcutaneous implantation.

Tumour growth was assessed by measurement of the tumour diameter using a standard stationary ruler at appropriate times after implantation. Visual appearance of the tumours was also noted over time. In two experiments, photographs were taken of all mice and their tumours.

Mice were killed when they began to show signs of illness i.e. crouched posture, shivering or when tumours began to necrose. After death, tumours were removed and frozen in either liquid nitrogen or in iso-pentane on dry ice. Alternatively, in some experiments, tumours were removed, fixed in 10% formal-saline for two hours and then stored in 70% ethanol. In one experiment the lungs, spleen and liver were taken from mice and frozen at -70°C for virus titration at a later date.

**2.2.36 Monoclonal antibody depletion of T lymphocytes *in vivo***

CD4+ and CD8+ T lymphocytes were depleted from mice by intravenous injection of 1mg of YTS-191 or YTS 169 rat anti-mouse CD4 or CD8 monoclonal antibody in 100μl of SPBS using a 27 gauge needle. T lymphocyte depletion was maintained by intraperitoneal injection of 1 mg of the antibody in 100μl of SPBS 2 days after the initial i.v. injection and then weekly for as long as required. In all experiments, to confirm successful depletions, splenocytes from control and depleted animals were taken for flow cytometric analyses.
At appropriate times after infection lungs and spleens were removed from the mice and assayed for infectious virus or infectious centres respectively. In one experiment, half of each spleen taken was fixed in 10% formal-saline for 4 hours and then transferred to 70% ethanol for subsequent processing, wax embedding and sectioning.

2.2.37 Flow cytometric analysis of cells

Reagents:

- 1% formal-saline - 10% formal-saline solution diluted 1/10 v/v with sterile PBS
- FACS buffer - PBS with 1% BSA and 0.1% Sodium Azide (w/v)

All antibody working stocks diluted in FACS buffer

2.2.38 Immunostaining and flow cytometry

Flow cytometric analyses were carried out on splenocytes prepared or cultured as previously described. $1 \times 10^6$ cells were pipetted into micro-test tubes and the cells were pelleted at $370 \times g$ for 5 minutes at $4^\circ C$. The cells were re-suspended by agitation and 30μl of primary antibody was added to the appropriate tubes which were then incubated for 20 minutes on ice. Following incubation the cells were pelleted by centrifugation as above and the supernatants were removed by aspiration. Each aliquot of cells was washed 3 times with ice-cold FACS buffer. 200μl of the buffer was added to each tube containing a re-suspended cell pellet and the cells were re-pelleted by centrifugation. 30μl of secondary antibody was added to the cells if required. Following addition of the secondary antibody, the cells were incubated on ice for 20 minutes and then washed as before. Finally, the
cells were fixed by addition of 200μl of 1% formal-saline to all tubes and analysed using a FACStar (Becton Dickinson) FACS scanner.

2.2.39 In situ hybridisation detection of MHV-68 tRNAs

2.2.40 Preparation of slides

Tissue sections were mounted on slides coated with the adhesive 'Biobond' (British Biocell International, UK). Prior to coating, slides were cleaned by immersion in a 2% solution of Decon in SDW for 1 hour, then rinsed thoroughly in distilled water and air-dried. Slides were immersed in a 2% solution of Biobond in acetone for 4 minutes, rinsed 6 times with distilled water and air dried at room temperature.

2.2.41 In situ hybridisation

2.2.42 Materials

T3 & T7 mRNA probes for the detection of MHV-68 tRNA-like molecule expression were obtained from Dr. Stacey Efstathiou, Cambridge University, UK. Details of the probes were published in 1997 (Bowden et al., 1997).

Diethylpyrocarbonate (DEPC) (inhibits RNases) was obtained from Sigma-Aldrich Company Ltd, UK.

Digoxigenin detection kit was obtained from Boehringer-Mannheim, Germany

Micromount mounting medium was obtained from Surgipath, Grayslake, IL. USA.

Proteinase K was obtained from Sigma-Aldrich Company Ltd, UK.

RNase A was obtained from Sigma-Aldrich Company Ltd, UK.

Salmon sperm DNA was obtained from Sigma-Aldrich Company Ltd, UK.
Tris-buffered saline (TBS) (50mM Tris (hydroxymethyl)methylamine, 0.15M NaCl, pH 7.6).

All solutions were prepared in DEPC-treated deionised water

2.2.43 Pretreatments

_in situ_ hybridisation studies were carried out on paraffin-wax embedded sections only. Tissue sections were dewaxed by immersion in fresh xylene for 10 minutes, in a fume hood. Sections were then hydrated through a series of alcohols (100%, 95%, 70%, 50%, 30%) containing 0.33M ammonium acetate (NH₄Ac). At the end of this series, sections were immersed twice in deionised water for one minute. This was followed by a 10 minute incubation in 0.2M hydrochloric acid (HCl) after which slides were washed twice in water as above, then immersed in 1% Triton X-100 in distilled water for 1.5 minutes. Sections were washed twice for 5 minutes in deionised water and once for 5 minutes in PBS, rinsed briefly in water and subjected to a proteinase K digestion. Digestion was carried out by incubating sections in the following solution: 10µg/ml fresh proteinase K in 20mM TBS/2mM calcium chloride, pH7.0 for 20 minutes at 37°C. Following this digestion, sections were immersed for 5 minutes in PBS containing 2g/L glycine/5mM diaminoethanetetra-acetic acid (EDTA) and then for 3 minutes in PBS. The slides were subsequently immersed twice in DEPC-treated water for 2 minutes and for 10 minutes in 0.1M triethanolamine HCl (pH8.0). Acetic anhydride was added to give a 0.25% solution. All sections were then dehydrated through a series of alcohols (30%, 50%, 70%, 95%, 100%) all of which (except the 100% alcohol) contained 0.33M ammonium acetate (NH₄Ac) and air dried.
2.2.44 Pre-hybridisation

The constituents of the pre-hybridisation (prehyb) and hybridisation mixtures are
detailed in table 2.1 (for 25 slides). Constituents were added together in the order
shown. Salmon sperm DNA was boiled and then immediately stored on ice (to
maintain strand separation) before its addition to the solution.

150µl of pre-hybridisation solution was added to each section. To increase contact
between the prehyb mixture and the sections and to prevent evaporation, small
‘Parafilm’ strips were placed on top of the sections. Sections were incubated with
the prehyb mixture for 1 hour at 55°C.

During this incubation the hybridisation mixture was prepared.

2.2.45 Hybridisation

8µl of digoxigenin-labelled T3 or T7 probe was added to 800µl of hybridisation
mixture. The mixture was then boiled for 2 minutes and immediately stored on ice.
Following the prehybridisation step, the prehyb mixture was decanted from the
sections and replaced with 25µl of hybridisation mixture containing the appropriate
probe. Sections were then covered with a small ‘Parafilm’ strip as before, slides
were placed in a moist slide tray and incubated at 55°C overnight.

After the overnight incubation, slides were immersed in 4x sodium chloride/
sodium citrate (0.6M NaCl, 0.06M Na3C6H5O7·2H2O in distilled water, pH 7.0: 4x
SSC), ‘Parafilm’ strips were removed and the sections were washed in a solution
of 0.5M sodium chloride/ TE (10mM Tris, 1mM EDTA in DEPC-treated water, pH
7.6) at room temperature for 20 minutes. 600µl of RNase A (Sigma-Aldrich Company Ltd, UK.) (stock 10mg/ml) was then added to the 300ml of NaCl/TE solution to give a final concentration of 20µg/ml of the enzyme. The sections were incubated in this solution for a further 30 minutes at 37°C and the slides were then washed 8 times. The first 4 washes were 15 minutes each in 0.5M NaCl/TE, two 15 minute washes were then carried out in 2 x SSC. Finally two 15 minute washes were undertaken in 0.2 x SSC. All washes were at 37°C.

2.2.46 Probe visualisation

Probe visualisation was carried out using a Boehringer-Mannheim digoxigenin detection kit. Prior to blocking and incubation with the anti-digoxigenin antibody sections were washed 3 times in 0.1M Tris, 0.15M NaCl (pH7.5, known as 'buffer#1': 5 minutes each wash). Sections were then blocked by incubation with 2% normal goat serum in 0.3% Triton X-100 in buffer#1 for 30 minutes at room temperature. Blocking serum was decanted and replaced with 100µl of sheep anti-digoxigenin alkaline phosphatase-conjugated F'ab2 fragments (Boehringer-Mannheim, Germany) diluted 1/1000 in buffer#1. Sections were incubated for 2 hours at room temperature and washed 3 times in PBS (15 minutes per wash) at room temperature. All sections were then immersed in 0.1M Tris, 0.1M NaCl, 0.05M MgCl2 (pH9.5, known as buffer#3) for 5 minutes. 100µl of chromagen/substrate solution [92µl buffer#3, 4.5µl nitroblue tetrazolium chloride (NBT) solution, 3.5µl X-phosphate (5-Bromo-4-chloro-3 indolylphosphate: BCIP)] was then added to each slide followed by a strip of 'Parafilm'. Slides were incubated in a sealed humid chamber in the dark until a colour reaction was observed on
inspection. The colour reaction was stopped by immersing the slides in water and the sections were counterstained with 1% Jensen’s neutral red (BDH laboratory supplies, Poole, Dorset, UK). Finally, sections were rinsed in water, allowed to air dry and mounted in Surgipath ‘micromount’.

2.2.47 MHV-68 immunostaining of paraffin-embedded spleen sections

2.2.48 Materials

Biobond (British Biocell International, UK)

Micromount mounting medium was obtained from Surgipath, Grayslake, IL, USA.

Hydrogen Peroxide, 100 volumes (30%), diluted to 0.3% in methanol for use.

Proteinase K was obtained from Sigma-Aldrich Company Ltd, UK.

Sigma FastTM 3,3'-Diaminobenzidine tablet set (DAB peroxidase chromagen) (Sigma-Aldrich Company Ltd, UK).

Tris-buffered saline (TBS) (50mM Tris (hydroxymethyl)methylamine, 0.15M NaCl, pH 7.6).

Triton X-100 was obtained from Sigma-Aldrich Company Ltd, UK.

Vectastain ABC POD reagent was obtained from Vector Laboratories (Burlingame, California)

2.2.49 Immunostaining

Tissue sections, mounted on ‘Biobond’ coated slides (see section 2.2.40), were dewaxed by immersion in fresh xylene for 10 minutes in a fume hood. Slides were then immersed in 100% ethanol for 5 minutes, followed by 30 minutes in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections
Chapter 2 – Materials and Methods

were hydrated through a series of alcohols (100%, 95%, 70%, 50%, 30%) followed by two 1 minute immersions in distilled water. The sections were then immersed in 1% Triton X-100 in water for exactly 1.5 minutes, followed by two washes in distilled water (5 minutes each) before being subjected to a proteinase K digestion. The digestion was carried out by incubating sections in the following solution: 10μg/ ml of freshly prepared proteinase K in 20mM TBS, 2mM calcium chloride, pH7.0 for 20 minutes at 37°C. Following this digestion step, sections were immersed for 5 minutes in PBS containing 2g/ L glycine/ 5mM EDTA and then washed for 3 minutes in PBS.

Slides were laid out in a moist incubation tray and 200μl of 2% normal goat serum in PBS was added to each slide followed by a small section of ‘Parafilm’ to prevent evaporation. Slides were incubated overnight at room temperature. This step was included to minimise non-specific binding of secondary immune reagents to the tissue.

Prior to the addition of primary antibody, ‘blocking’ serum was decanted from the slides. 50μl of rabbit polyclonal anti-MHV-68 antibody (diluted 1/500 in 2% normal goat serum) was added to each tissue section and slides were incubated in a humid chamber at room temperature for 2 hours. After this incubation, the slides were washed 3 times in PBS (5 minutes per wash).

50μl of goat anti-rabbit biotinylated IgG (diluted 1/500 in 2% normal goat serum) (Vector Laboratories, Burlingame, California, USA) was then added to each section and the slides were again incubated for 2 hours at room temperature in a
humid chamber. Following this incubation, slides were washed 3 times in PBS as above.

For visualisation, the Vectastain ABC POD (avidin/ biotin/ peroxidase complex) reagent was used (Vector Laboratories, Burlingame, California, USA). This was made up 30 minutes prior to use by adding 1 drop of reagent A to 5ml of PBS and then 1 drop of reagent B to the same mixture. The solution was mixed by inversion (as outlined in the supplied instruction sheet). 100μl of this reagent was added to the sections which were incubated for 30 minutes at room temperature in a humid chamber. The sections were washed 3 times in PBS (as above). Finally, a 0.7mg/ ml solution of 3,3'-diaminobenzidine tetrahydrochloride / 2mg/ ml urea hydrogen peroxide, 0.06M Tris (DAB/H₂O₂: chromagen/substrate) was added to the sections and they were monitored closely for any colour changes. On appearance of a colour change, slides were washed in PBS as before and then immersed in water for 5 minutes. Sections were counter-stained lightly with Harris's haematoxylin (BDH laboratory supplies, Poole, Dorset, UK), dehydrated through a series of alcohols (30%, 50%, 70%, 95%, 100%) and mounted in Surgipath 'micromount'.
2.2.50 Immunohistochemistry on paraffin-embedded S11 tumour sections

2.2.51 Antibodies

Rat anti-mouse CD4 monoclonal antibody (clone YTA 3.1.2), tissue culture supernatant (diluted 1/2), was obtained from departmental stock.

Rat anti-mouse macrophage monoclonal antibody, F4/80 (IgG2b), was obtained from Serotec (UK) and was used at a dilution of 1/100.

Rat anti-mouse B220 monoclonal antibody (clone RA3.24) (neat tissue culture supernatant) was obtained from departmental stock.

Rat anti-mouse CD8 monoclonal antibody (clone YTS 105) (neat tissue culture supernatant) was obtained from departmental stock.

2.2.52 Immunostaining

All paraffin sections were mounted on Biobond coated slides. Tissue sections were dewaxed by immersion in fresh xylene for 15 minutes in a fume hood, immersed in 100% ethanol for 5 minutes then hydrated through a series of alcohols (95%, 70%, 50%, 30%) followed by two 1 minute immersions in water. Sections were incubated in 0.85% NaCl in distilled water for 5 minutes and then subjected to a microwave (antigen retrieval) treatment. Briefly, sections were immersed in 600ml of citrate buffer (0.1M citric acid, 0.1M tri-sodium citrate in H₂O) and were microwaved for 7 minutes in a 950 watt microwave at 70% full power. The slides were then allowed to cool for 20 minutes in the same buffer and rinsed in PBS for 5 minutes, following which, sections were incubated in 2% paraformaldehyde, lysine, periodate fixative (PLP) for 20 minutes and twice in
PBS for 5 minutes each time before being subjected to a trypsin digestion step. This digestion was carried out by incubating sections in: 1mg/ml of fresh trypsin (Difco) in TBS, 0.1% calcium chloride, pH7.0 for 10 minutes at 37°C.

Sections were washed in PBS for 5 minutes, immersed in fresh PLP for 5 minutes, washed in PBS for a further 5 minutes and then rinsed in 0.85% NaCl twice for 5 minutes each rinse.

Slides were laid out in a moist incubation tray and 200μl of 5% normal mouse ‘blocking’ serum in PBS was added to each section followed by a small piece of ‘Parafilm’ to prevent evaporation and slides were incubated for 1 hour at room temperature.

Prior to primary antibody addition, blocking serum was decanted from the slides. 50μl of antibody (diluted in 5% normal mouse serum) was added to each tissue section and slides were incubated in a humid chamber at room temperature, overnight. After this incubation, the slides were washed 3 times in PBS (5 minutes per wash).

50μl of mouse anti-rat biotinylated IgG (diluted 1/500 in 5% normal mouse serum) (Sigma-Aldrich Chemical Co, UK) was added to each section and the slides were again incubated for 2 hours at room temperature, in a humid chamber. Following this incubation, slides were washed 3 times in PBS, as above.
50μl of streptavidin-alkaline phosphatase conjugate (Boehringer-Mannheim, Germany), diluted 1/1500 in PBS, was then added to each section and the slides were incubated for 1 hour at room temperature. Following this incubation the slides were washed 3 times in PBS, as before. To visualise staining a 5-bromo-4-chloro-3-indolylphosphate / nitroblue tetrazolium (BCIP/ NBT) alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, California) was used. The BCIP/ NBT chromagen/ substrate working solution was prepared just prior to use. Briefly, 2 drops of ‘reagent 1’ were added to 5ml of 100mM Tris-HCL (pH 9.5) buffer and the solution was thoroughly mixed. Two drops of reagents 2 and 3 were then added sequentially to the Tris-HCL buffer and the solution was again mixed thoroughly. 50μl of this substrate solution was added to each section and slides were incubated at room temperature for 10 to 30 minutes until the development of colour. Sections were washed in PBS as before and counterstained with Jensen’s neutral red (BDH laboratory supplies, Poole, Dorset) for 2 minutes. Finally, sections were rinsed in water, allowed to air dry and mounted in Surgipath ‘micromount’.
<table>
<thead>
<tr>
<th>Material</th>
<th>Pre-hybridisation</th>
<th>Hybridisation</th>
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<tbody>
<tr>
<td>Formamide</td>
<td>2.5ml</td>
<td>400μl</td>
</tr>
<tr>
<td>20x Salts</td>
<td>1.25ml</td>
<td>200μl</td>
</tr>
<tr>
<td>50x Denhardts</td>
<td>500μl</td>
<td>80μl</td>
</tr>
<tr>
<td>Dextran Sulphate</td>
<td>-</td>
<td>80μl</td>
</tr>
<tr>
<td>Salmon sperm DNA</td>
<td>125μl (10mg/ ml stock)</td>
<td>20μl</td>
</tr>
<tr>
<td>Yeast tRNA</td>
<td>125μl (10mg/ ml stock)</td>
<td>20μl</td>
</tr>
<tr>
<td>Heparin</td>
<td>50μl (2000u/ ml stock)</td>
<td>8μl</td>
</tr>
<tr>
<td>SDS (10% in SDW)</td>
<td>50μl</td>
<td>8μl</td>
</tr>
<tr>
<td>SDW</td>
<td>400μl</td>
<td>-</td>
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Table 2.1 Constituents of the pre-hybridisation and hybridisation buffers used during *in situ* hybridisation detection of MHV-68 tRNA-like molecule expression.
Chapter 3

The *in vitro* assessment of cytotoxic T lymphocyte responses to MHV-68
3.1 Introduction

Studies in both human and animal systems have demonstrated that an effective CD8+ cytotoxic T lymphocyte (CTL) response is often key to the survival of a virally infected host (Doherty and Ahmed, 1997). Viral infection often leads to the efficient generation of a CTL response which can completely clear the infectious organism. A proportion of these CTL, which expand in response to the primary infection, survive long after initial exposure to the virus. These are memory cells which prevent re-infection with the same virus and/or prevent the potentially harmful emergence of viruses from latency.

There is now a wealth of information, reviewed in the introduction of this thesis, on the importance of CTL in host responses to Herpesvirus infection. Perhaps the most significant observation from the last 20 years research in this field, is that individuals who have impaired T lymphocyte responses are highly susceptible to Herpesvirus reactivation and virus-led disease (Martin et al., 1984, Zutter et al., 1988).

Studies investigating CD8+ CTL responses to Epstein-Barr virus infection are very advanced. It is clear that EBV-specific CD8+ CTL are critical in the resolution of infectious mononucleosis and in the maintenance of the virus in a 'harmless' latent form. Many of the viral peptide epitopes to which these CD8+ CTL respond have now been identified (Rickinson and Moss, 1997). Despite this detailed knowledge of T lymphocyte responses to EBV, a number of questions remain unanswered. For example, it is unclear whether the
immune responses of humans to EBV reflect those of other species to gammaherpesvirus infection.

To date, a number of studies have suggested that CD8+ T lymphocytes are important in the protection of mice from MHV-68 infection. Firstly, monoclonal antibody depletion of CD8+ T cells from BALB/c mice, prior to infection, results in the mice rapidly succumbing to a generalised viral infection (Ehtisham et al., 1993). Secondly, infection of MHC class II knockout mice with MHV-68 results in an uncontrolled reactivation of the virus in the lungs and lymph nodes of infected animals. This is apparently due to an impaired CD8+ memory T cell response in the absence of CD4+ T helper cell cytokine release (Cardin et al., 1996). Lastly, mice which are deficient in β2 microglobulin and therefore MHC class I, have great difficulty in clearing infectious virus from their spleens (Weck et al., 1996b). Therefore, in vivo evidence suggests that CD8+ T lymphocytes contribute to the control of MHV-68 at all stages of infection.

The overall objective of the work described in this chapter was to develop an in vitro assay system for the measurement and analysis of CTL responses to MHV-68 infection of mice. Such an assay would enable the characterisation of CTL responses during acute infection, in the resolution of splenomegaly and in long-term latent or persistent MHV-68 infection. Further, such an assay would also contribute to the identification of antigenic viral peptides and the development of therapeutic or vaccination strategies.
In order to assess cytotoxic cellular responses to MHV-68 in mice, a ‘standard’ chromium release assay was adopted. Alternatives to the use of chromium do exist, for example the release of the enzyme lactate dehydrogenase (LDH) or tritiated thymidine. The chromium method was selected as it is widely employed and has consistently proven to be of use in assessing cytotoxic cellular responses to viral infections.

3.2 Preliminary work

The aim of preliminary work undertaken was to characterise and assess a range of potential target cell lines for use in the study of MHV-68 specific CTL.

At the outset of these studies, the decision was made to assess CTL responses to MHV-68 in BALB/c mice which are H-2<sup>d</sup> haplotype. This strain of mice was selected in response to the availability of the cell line S11. S11 is an MHV-68 infected B cell lymphoma line which was originally derived from an infected BALB/c mouse. It was felt, since this line expresses both lytic and latent viral antigens, as well as MHC class I and II molecules, that it held great potential as either a stimulator of anti-MHV-68 CTL in vitro or as a target for virus-specific lymphocytes. Unfortunately, ‘uninfected’ S11 cells are not available. Usherwood et al. (Usherwood, E., personal communication) demonstrated, using PCR based techniques, that cloned S11 lymphocytes always contain MHV-68 genome. For the purposes of the CTL assay, it was necessary to identify a virus-negative B cell line which resembled S11. Such a cell line, when employed as a target, would enable the confirmation of virus-specific cytolytic responses.
Candidate cell lines, on the H-2d background, that were investigated were as follows:

1) NSO cells: a virus-negative B cell myeloma derived lymphocyte line.
2) A20 cells: virus-negative pre-B lymphoblastoid cells.
3) 3T3 cells: virus-negative mouse embryo fibroblast cells. 3T3 cells can be infected with MHV-68 and were selected as representative of the cells infected by the virus during its replication in the mouse lung.

A number of uninfected cell lines, originally isolated from MHV-68-infected mice, were also considered for use in this study. However, these cells were of indeterminate phenotype and were not used.

3.2.1 Analysis of potential CTL assay targets

Once potential targets for virus-specific CTL had been identified, it was necessary to determine (a) how spontaneous chromium (51Cr) release from target cells would contribute to levels of release elicited by effector cytolysis of targets, (b) whether these cells could be infected by the virus and (c) whether MHV-68 infection alters the expression of MHC class I molecules on the surface of these cells.

3.2.2 Radioactive chromium uptake and release by candidate cell lines

Potential target cells were incubated with 51Cr for 1, 2 or 3 hours at 370C. Cells were then washed and levels of spontaneous 51Cr release after a 5 hour incubation at 370C were measured. The results of this experiment are shown in Figure 3.1.
Figure 3.1  The spontaneous release of Chromium from potential target cell lines. Cell lines were cultured and harvested as described in sections 2.2.13 to 2.2.15. Potential target cells were incubated with 100μCi of $^{51}$Cr at 37°C for 1 (■), 2 (□) or 3 (▲) hours. Cells were then washed and incubated for 5 hours at 37°C. Spontaneous and maximum possible $^{51}$Cr release was measured and values for spontaneous release as a percentage of the maximum were calculated. Each bar represents spontaneous release of $^{51}$Cr as a percentage of the maximum released from the cells. Spontaneous and maximum release values were assessed in triplicate, mean values taken and these were used to calculate the relative percentage figures presented. Error bars represent the standard error from the mean of three spontaneous release values.
Overall, spontaneous release of $^{51}$Cr was lowest from target cells incubated with sodium chromate for 1 hour. In these experiments, the lymphocyte cell lines retained the radioactive chromium more efficiently than the fibroblastoid cell line. The spontaneous release of chromium from the 3T3 cells was always above 35% of the maximum achievable value. Therefore, the H-2d restricted lymphocyte targets retained $^{51}$Cr sufficiently well to justify their use as targets in a CTL assay. In contrast, the fibroblast cell line, 3T3, exhibited an unacceptably poor ability to retain the radioactive compound.

The decision was made to proceed in the development of a cytotoxicity assay using lymphocyte targets. This was deemed a logical approach since infected lymphocyte targets have been used successfully for many years in the study of CTL responses to EBV (Svedmyr and Jondal, 1975, Strang and Rickinson, 1987).

### 3.2.3 MHV-68 infection of target cells

To confirm that candidate B lymphocyte targets could be infected with MHV-68, cells were infected in vitro with the virus and were cultured overnight or for 7 days. The cells were then harvested, smeared onto slides and stained with anti-MHV-68 hyperimmune serum and an FITC-conjugated secondary antibody. Numbers of MHV-68 infected cells were then quantified. The results of this experiment are summarised in Table 3.1. When the lymphocyte cell lines were infected with MHV-68 and then cultured for 17 hours a small percentage of cells appeared to be infected with the virus. At this time, 1% of
<table>
<thead>
<tr>
<th></th>
<th>A20 cells</th>
<th>NSO cells</th>
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<tr>
<td>Mock infection</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17 hours post infection</td>
<td>1%</td>
<td>3%</td>
</tr>
<tr>
<td>7 days post infection</td>
<td>2%</td>
<td>5.9%</td>
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</table>

Table 3.1 Infection of potential cytotoxic T cell assay targets with murine gammaherpesvirus-68. A20 and NSO cells were cultured and harvested as described in section 2.2.3. 1 x 10^7 cells were then pelleted and re-suspended in an appropriate volume of medium or virus stock such that the multiplicity of infection (MOI) was 3. Following addition of the medium or virus, the cells were incubated at 37°C on a shaker for 1 hour to allow virus adsorption. Post-incubation the cells were pelleted by centrifuging at 450 x g for 5 minutes, supernatant was discarded and replaced with fresh medium. This washing procedure was repeated a further 3 times. Mock infected or infected cells were returned to culture overnight or for 7 days after which they were harvested and stained with 'Rabbit A' polyclonal anti-MHV68 serum as described in section 2.2.33. Cells were viewed using a fluorescence microscope. One thousand cells were counted on each slide and the results were expressed as the percentage of cells which were positive for MHV-68 in the population counted.
the A20 cells and 3% of the NS0 cells showed positive staining for MHV-68. These percentages increased after 7 days in culture to 2% of A20 cells and 5.9% of NS0 cells lytically-infected with the virus. In contrast, neither of the mock infected B lymphocyte populations showed any positive staining with the hyperimmune serum.

In parallel with this study, S11 cells were also stained with anti-MHV-68 hyperimmune serum. The results of this experiment (not shown) were identical to those of Usherwood et al. in 1996 who demonstrated that around 5% of S11 cells are positive for lytic cycle antigen expression at any one time (Usherwood et al., 1996).

It should be noted that although 2 to 6% of the lymphocytes investigated stained positive for lytic antigen expression after 7 days in culture, it is likely that this figure increased as the lymphocytes were cultured for longer periods of time. In 1993, Sunil-Chandra et al. demonstrated that 16% of NS0 cells, cultured for 28 days after infection were positive for anti-MHV-68 hyperimmune serum staining (Sunil-Chandra et al., 1993). Further, by infectious centre assay, it was shown that around 70% of NS0 cells infected with MHV-68 in vitro become latently infected with the virus (results not shown). As previously mentioned, although only 5% of S11 cells stained positive with MHV-68 hyperimmune serum, all S11 cells carry the viral genome (Usherwood, E., personal communication). To date, no systematic study of viral gene transcription and protein expression in S11 cells has been undertaken. Therefore, it is difficult to draw conclusions on which viral gene
products were being recognised by the hyperimmune serum used in this experiment.

3.2.4 MHC class I molecule expression on MHV-68 infected cells

To determine whether MHV-68 affects the level of MHC class I molecule expression on the surface of infected cells, NS0 and A20 cells were infected \textit{in vitro}. These cells were then cultured and at regular intervals mock infected or infected cells were harvested and stained with anti-mouse H-2K\textsuperscript{d} antibody. MHC class I molecule expression on the surface of these cells was then analysed by flow-cytometry. A summary of results, showing the expression of MHC class I molecules on the surface of NS0 and A20 cells is presented in Figure 3.2a. Representative data obtained from the analysis of the NSO cells used in this experiment is presented in figure 3.2b.

Between 90% and 100% of all NS0 cells express MHC class I molecules at their cell surface. This level of expression is not notably affected by MHV-68 infection of these cells. A very similar profile of MHC class I molecule expression was observed on infected A20 cells. Usherwood \textit{et al.} (1996b) demonstrated that all S11 cells express MHC class I molecules at their cell surface. This finding was confirmed in this study by flow cytometric analysis of S11 (results not shown).

These results demonstrate that both A20 and NS0 cells can be infected with MHV-68. Further, infection does not seem to affect MHC class I molecule expression on the surface of NS0, A20 or S11 cells.
Figure 3.2a The expression of MHC class I molecules on the surface of cells at times after infection. A20 or NSO cells were cultured as described in section 2.2.3 of materials and methods. On day 0 of the experiment cells were harvested. $1 \times 10^7$ cells were then resuspended in an appropriate volume of medium or virus stock such that the multiplicity of infection (MOI) was 3. Following addition of the medium or virus, the cells were incubated at $37^\circ C$ on a shaker for 1 hour to allow virus adsorption. After incubation, the cells were pelleted by centrifuging at 450 x g for 5 minutes, supernatant was then discarded and replaced with fresh medium. This washing procedure was repeated a further 3 times. The cells were then returned to culture for 4, 24 or 48 hours. At each of these times $1 \times 10^6$ mock infected (□) or infected (▲) cells were harvested and stained with a biotinylated anti-mouse MHC class-I molecule H-2K$^d$ antibody and with a streptavidin/ FITC conjugate. After staining, the cells were analysed by flow cytometry. In each analysis 10000 cells were assessed. The number of lymphocytes expressing MHC class I is expressed above as a percentage of the total cells assessed. Error bars represent the standard error of the mean of three analyses.
Figure 3.2b

MHV-68 Infected

Mock Infected
Figure 3.2b  The expression of MHC class I molecules on the surface of NS0 cells at times after infection. NS0 cells were cultured as described in section 2.2.3. On day 0 of the experiment, cells were harvested as normal. $1 \times 10^7$ cells were then pelleted and re-suspended in an appropriate volume of medium or virus stock such that the multiplicity of infection (MOI) was 3. Following addition of the medium or virus, the cells were incubated at $37^\circ C$ on a shaker for 1 hour to allow virus adsorption. Post incubation the cells were pelleted by centrifugation, supernatant was discarded and replaced with medium. This washing procedure was repeated a further 3 times. The mock infected or infected cells were then returned to culture for 4, 24 or 48 hours. At each of these times $1 \times 10^6$ mock infected or infected cells were harvested and stained with a biotinylated rat anti-mouse H-2K$^d$ antibody and then a streptavidin/ FITC conjugate. After staining the cells were analysed by flow cytometry. In each analysis 10,000 cells were assessed. Analyses were carried out in triplicate. The histograms presented in this figure are typical of those obtained during this analysis.
3.3 The use of S11 lymphoma cells in an analysis of CTL responses in MHV-68 infected mice

3.3.1 MHC class I molecule-directed allogeneic responses to S11 target cells

Since S11 cells are infected with MHV-68 and express both class I and class II MHC molecules, a study was required into the susceptibility of these cells to MHC class I molecule-targeted killing (Usherwood et al., 1996b).

To assess the potential of S11 cells as targets of specific CTL, the ability of C57BL/6 mouse derived CTL to lyse MHC class I mismatched S11 and BALB/c blast cells was investigated. As a control, C57BL/6 blast cells were included as targets in this assay. It was hoped this assay would confirm the usefulness of S11 cells as targets of MHC class I-directed killing. Further, it was hoped this experiment would reveal if the protocol adopted for measuring chromium release from these target cells required modification. The results of the experiment are presented in Figure 3.3.

Cytolytic activity against the MHC mismatched BALB/c blast cell targets was evident. This lytic response peaked around 35% at an effector:target (E:T) ratio of 20:1. In contrast, no lysis of the MHC matched C57BL/6 blast cell targets was observed.

The specific killing profile of the BALB/c derived S11 cells closely resembled that of the BALB/c blasts with no significant difference in the peak value of
Figure 3.3  Cytotoxic activity of alloreactive C57 BL/ 6 lymphocytes

Splenocytes from C57BL/ 6 mice were cultured for 5 days with irradiated BALB/ c splenocytes. After 5 days the C57BL/ 6 splenocytes were assayed for their ability to lyse $^{51}$Cr loaded S11 ( ) cells or BALB/ c ( ) or C57BL/ 6 ( ) blast cells targets in a standard 5 hour chromium release assay (as described in sections 2.2.18 to 2.2.20).

All assays were carried out in triplicate. Mean maximum and minimum release values were obtained for each target cell type and specific $^{51}$Cr release values were calculated for each assay. Each point represents the mean specific value for three wells at each effector to target ratio. Error bars represent the standard error from the mean of the three specific release values calculated.
killing at an E:T ratio of 20:1. This result demonstrated that S11 cells were susceptible to MHC class I molecule-directed cytolysis. Further, it confirmed that cytolytic responses could be assessed using the protocol described in sections 2.2.11 to 2.2.17.

3.3.2 Sources of MHV-68-specific CTL

Over the last 20 to 30 years, EBV-specific CTL have been obtained from the peripheral blood of infected individuals. This is the only anatomical site from which these cells are available, without surgery being undertaken. In contrast, cells can be obtained from a wide range of murine tissues following viral infection of the animal.

For the purposes of the experiments described in this chapter, attempts were made to measure MHV-68-specific CTL activity in the splenocytes of mice 15 to 30 days after infection. It was thought, if the immune responses of mice to the gammaherpesvirus MHV-68 resembled those of humans to EBV, the spleen would be a likely source of virus-specific CTL. Later in the course of this study, attempts were made to measure MHV-68 specific CTL activity in lymphocyte populations obtained from lymph nodes shortly after infection. The results and implications of these lymph node studies are considered in the discussion section of this chapter.

3.3.3 Ex vivo anti-MHV-68 CTL responses

Murine infection with lymphocytic choriomeningitis virus (LCMV) induces such a profound CD8+ CTL response that anti-viral cytolytic activity is measurable
in lymphocytes taken directly from the infected animal (Doherty and Ahmed, 1997). Typically, however, Herpesviruses do not induce such enormous responses. Herpesvirus-specific CTL usually require a period of culture in the presence of irradiated antigen presenting cells before significant cytolytic activity can be measured.

To investigate whether T lymphocytes, which have expanded in response to MHV-68 infection, showed anti-viral activity _ex vivo_, the ability of these cells to lyse S11 targets or infected and uninfected NS0 cells was investigated. Results presented in Figure 3.4 demonstrate that no significant anti-S11 response was obtained when lymphocytes were assayed directly from infected animals. Further, no lysis of either infected or uninfected NS0 cells was detectable (results not shown). In contrast, when splenocytes from MHV-68 infected mice were cultured with irradiated S11 cells for 7 days, these cells lysed S11 targets. In this experiment, specific lysis of S11 targets peaked at 30% at an E:T ratio of 50:1.

Thus, MHV-68 resembles most other Herpesviruses in that it does not induce a CTL response which is measurable _ex vivo_.

### 3.3.4 In vitro culture and re-stimulation of lymphocytes

#### 3.3.5 S11 cells as inducers of anti-lymphoma cell responses

Since S11 is an MHV-68 infected B cell line which expresses MHC class I molecules on its surface, it was hoped it could act as an efficient presenter of
Figure 3.4 Splenocytes from MHV-68-infected mice require a period of *in vitro* re-stimulation before they will kill S11 target cells. Splenocytes from a group of infected BALB/c mice were cultured for 7 days with irradiated S11 cells. The ability of these re-stimulated cells (■■■) to lyse chromium loaded S11 cells was compared with that of splenocytes obtained directly from mice (□□□) in standard 5 hour chromium release assay (as described in sections 2.2.11 to 2.2.17).

All assays were carried out in triplicate. Mean maximum and minimum release values were obtained for each target cell type and specific $^{51}$Cr release values were calculated for each assay. Each point represents the mean specific value for three wells at each effector to target ratio. Error bars represent the standard error from the mean of the three specific release values calculated.
antigenic viral peptides. As an irradiated target cell, S11 had the potential to be a useful tool for stimulating MHV-68 specific CTL to proliferate in \textit{in vitro} culture. However, S11 is a lymphoma cell line and at the outset of these studies there remained the possibility that S11 could elicit an anti-lymphoma rather than anti-viral response on co-culture with murine splenocytes. Such a phenomenon could lead to the misinterpretation of cytotoxic responses where cytolysis attributed to a virus-specific activity is in reality of an anti-lymphoma nature.

To investigate whether cells cultured with S11 display anti-viral or anti-lymphoma cell activity, splenocytes from normal, uninfected mice were cultured for 7 days with irradiated S11 cells at a splenocyte:S11 ratio of 10:1. Subsequently, the ability of these cells (and re-stimulated cells from an infected animal) to lyse S11 target cells was assessed. No anti-lymphoma cell response in the normal, uninfected splenocyte population was detected as specific chromium release values were zero at all E:T ratios. Specific lysis of S11 targets by the 'infected' lymphocytes peaked at around 28\% at an E:T ratio of 50:1 (results not shown). Throughout this study, measurable cytolytic activity against S11 targets was only observed in cells taken from MHV-68 infected animals.

\textbf{3.3.6 Re-stimulation of lymphocytes with infected splenocytes or S11 cells}

As discussed earlier, CTL responses to virally-infected targets are typically assessed after lymphocytes, from an infected animal, have been cultured with
irradiated, virally-infected target cells. This period of culture serves to expand and activate virus-specific CTL whose lytic activity is more readily detectable. In this study, splenocytes from infected mice were cultured with either irradiated splenocytes, infected with MHV-68 \textit{in vitro}, or irradiated S11 cells.

3.3.7 Re-stimulation of lymphocytes with infected splenocyte populations
Spleen cells from infected mice were cultured with infected and irradiated splenocytes at a range of effector:stimulator (E:S) ratios for 5, 7 or 14 days. Cells were then harvested and the viability of the lymphocytes was assessed. Despite numerous attempts, re-stimulation of spleen cells with infected splenocytes invariably produced too few viable lymphocytes (less than 5% of the total cells cultured) after 5 or 7 days in culture to warrant assaying these cells for cytolytic activity.

3.3.8 Re-stimulation of lymphocytes with irradiated S11 cells
Spleen cells from infected mice were cultured with irradiated S11 cells for 5, 7 or 14 days. Typically, lymphocytes were cultured with irradiated S11 cells at an E:S ratio of 10:1. Over the course of this study, a range of E:S ratios was investigated. It was found a ratio of 10:1 gave an optimal yield of viable lymphocytes. Typically, after a period of 5 or 7 days in culture with S11 (at an E:S ratio of 10:1) around 25% of lymphocytes recovered were viable. In contrast, only around 6% of lymphocytes were viable after a 14 day re-stimulation.
3.3.9 The phenotypes of S11 re-stimulated cells

To investigate which cell types were prevalent within S11-restimulated lymphocyte populations, cells were stained with anti-CD4, anti-CD8 and anti-CD44 monoclonal antibodies. The cells were then analysed by flow cytometry. The results of this analysis are presented in Figure 3.5.

Bulk re-stimulation of splenocytes with S11 induced the expansion of both CD4+ and CD8+ T lymphocytes. A significant proportion of these expanded cells were of the CD44lo phenotype. CD4+ T lymphocytes, expressing low levels of CD44, expanded by around 18% after 5 days and 23% after 7 days in culture with S11. CD8+ T lymphocytes, expressing low levels of CD44, also expanded when re-stimulated with S11 for 5 or 7 days (11%).

A similar profile of expansion was observed in the CD4+/ CD44hi population of T cells. Somewhat surprisingly, very little expansion of the CD44hi CD8+ T lymphocyte population was ever observed.

3.3.10 The cytolytic activity of S11 re-stimulated lymphocytes

To investigate the cytolytic function of S11 re-stimulated lymphocytes, splenocytes from MHV-68-infected mice were cultured for 5, 7 or 14 days with irradiated S11 cells. The ability of these lymphocytes to lyse a range of targets was then assessed. The targets employed in these experiments were S11 cells, infected and uninfected NS0 or A20 B lymphocytes and YAC-1 cells.
Figure 3.5 Analysis of T cell phenotypes following re-stimulation of splenocytes in bulk culture with irradiated S11 cells. BALB/c mice were infected intranasally with murine gammaherpesvirus 68. After 25 or 27 days, splenocytes were obtained from the infected animals, pooled and cultured with irradiated S11 cells as described in section 2.2.6. After 5 or 7 days in culture, lymphocytes were harvested and their phenotypes analysed by flow cytometry. $10^6$ normal (□), 5 ( ■ ) or 7 ( □ ) day re-stimulated lymphocytes were stained with rabbit anti-mouse CD4 monoclonal antibody or rabbit anti-mouse CD8 monoclonal antibody and rat anti-mouse CD44 monoclonal antibody. 10000 cells of each type were analysed.
Numerous attempts were made to develop a protocol which gave a consistent readout of CTL function. Unfortunately, despite many attempts to optimise the CTL assay, many results obtained were often uninterpretable. The results presented in Figures 3.6 to 3.9 were gathered from the few experiments which gave consistent data for analysis.

Table 3.2 summarises attempts which were made to modify or investigate a range of conventional infection strategies, lymphocyte sources and re-stimulation protocols in attempts to obtain consistent, meaningful results.

Data, presented in Figure 3.6, confirmed a 7 or 14 day re-stimulation with S11 cells induced functionally active lymphocytes which could kill S11 targets. An increasing level of target lysis was evident as the E:T ratio increased. For convenience, in subsequent experiments, lymphocytes were re-stimulated for 5 days. Cells cultured for this period were functionally identical to 7 day re-stimulated lymphocytes.

Overall, anti-S11 cytotoxic activity was consistently detected in re-stimulated splenocytes from MHV-68-infected mice.

Over the course of this study, many attempts were made to measure MHV-68 specific lysis of infected A20 or NS0 cells. Data presented in Figures 3.7, 3.8 and 3.9 shows that while the lysis of S11 targets was consistently obtained, no profile of specific chromium release from NS0 or A20 cells was ever observed. In all 3 of these experiments, peak specific chromium release from
Figure 3.6  Cytotoxic activity of lymphocytes against S11 targets following a 7 or 14 day in vitro re-stimulation with irradiated S11 cells. Splenocytes from MHV-68 infected mice were cultured with irradiated S11 cells for 7 (---) or 14 (- - ) days. After re-stimulation the cells were assayed for their ability to lyse chromium loaded S11 cells in a standard 5 hour chromium release assay (as described in sections 2.2.11 to 2.2.17).

All assays were carried out in triplicate. Mean maximum and minimum release values were obtained for each target cell type and specific $^{51}$Cr release values were calculated for each assay. Each point represents the mean specific value for three wells at each effector to target ratio. Error bars represent the standard error from the mean of the three specific release values calculated.
Cytotoxic activity of lymphocytes against S11 or A20 targets following a 5 day in vitro re-stimulation with irradiated S11 cells. Splenocytes from MHV-68 infected mice were cultured with irradiated S11 cells for 14 days. After incubation, these cells were assayed for their ability to lyse chromium loaded S11 cells (—♦—), uninfected A20 cells ( ) or A20 cells infected with MHV-68 (—a—) (described in sections 2.2.11 to 2.2.17) in a standard 5 hour chromium release assay.

All assays were carried out in triplicate. Mean maximum and minimum release values were obtained for each target cell type and specific $^{51}$Cr release values were calculated for each assay. Each point represents the mean specific value for three wells at each effector to target ratio. Error bars represent the standard error from the mean of the three specific release values calculated.
Figure 3.8 Cytotoxic activity of re-stimulated splenocytes against differing numbers of S11 targets and infected or uninfected NS0 cells. Splenocytes from MHV-68 infected mice were cultured for 5 days with irradiated S11 cells (as described in section 2.2.6). After incubation, the cells were assayed for their ability to lyse $1 \times 10^4$ (●) or $2 \times 10^4$ (●) S11 cells or uninfected (●) or infected (●) NS0 cells in a standard 5 hour chromium release assay (as outlined in sections 2.2.11 to 2.2.17).

All assays were carried out in triplicate. Mean maximum and minimum release values were obtained for each target cell type and specific $^{51}$Cr release values were calculated for each assay. Each point represents the mean specific value for three wells at each effector to target ratio. Error bars represent the standard error from the mean of the three specific release values calculated.
Figure 3.9  **Cytotoxic activity of lymphocytes from MHV-68 infected mice against a range of targets.** Splenocytes from MHV-68 infected mice were cultured for 5 days with irradiated S11 cells (as described in section 2.2.6). After incubation, cells were assayed for their ability to lyse normal S11 cells (—), S11 cells cultured with the antiviral C9 for 7 days (—), YAC-1 cells (—), A20 cells (—) and MHV-68 infected A20 cells (—) (see section 2.2.15 for infection protocol) in a standard 5 hour chromium release assay (sections 2.2.11 to 2.2.17).

All assays were carried out in triplicate. Mean maximum and minimum release values were obtained for each target cell type and specific $^{51}$Cr release values were calculated for each assay. Each point represents the mean specific value for three wells at each effector to target ratio. Error bars represent the standard error from the mean of the three specific release values calculated.
<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Route of inoculation</th>
<th>Period of infection (days)</th>
<th>Source of lymphocytes</th>
<th>Re-stimulator</th>
<th>Splenocyte infection (MOI)</th>
<th>Period of re-stimulation (days)</th>
<th>E:S ratio</th>
<th>Cells viable after re-stimulation?</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>IN</td>
<td>15 - 30</td>
<td>spleen</td>
<td>S11</td>
<td>n/a</td>
<td>5/7/14</td>
<td>10:1</td>
<td>Yes (~25%)</td>
<td>S11/ NSO/A20/ 3T3/ YAC-1</td>
</tr>
<tr>
<td>BALB/c</td>
<td>IN</td>
<td>15 - 30</td>
<td>spleen</td>
<td>splenocytes</td>
<td>0.1 - 5</td>
<td>5/7</td>
<td>3:1</td>
<td>Very few (&lt;5%)</td>
<td>S11/ 3T3</td>
</tr>
<tr>
<td>BALB/c</td>
<td>IN</td>
<td>15 - 30</td>
<td>spleen</td>
<td>S11</td>
<td>n/a</td>
<td>5</td>
<td>10:1</td>
<td>Yes (~25%)</td>
<td>S11 cells in culture</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Sub-dermal</td>
<td>9</td>
<td>Cervical lymph node</td>
<td>splenocytes</td>
<td>5</td>
<td>5</td>
<td>3:1</td>
<td>Yes (~25%)</td>
<td>3T3</td>
</tr>
<tr>
<td>CBA</td>
<td>Sub-dermal</td>
<td>9</td>
<td>cervical lymph node</td>
<td>splenocytes</td>
<td>5</td>
<td>5</td>
<td>3:1</td>
<td>Yes (~25%)</td>
<td>L-929</td>
</tr>
</tbody>
</table>

Table 3.2 A summary of strategies investigated in the development of an assay for MHV-68 specific CTL.
S11 targets occurred at an E:T ratio of 50:1. Chromium release from A20 or NS0 cells, whether infected or not, never rose above background levels.

In summary, no specific cytotoxic activity against infected lymphoma cell lines, other than S11, was ever observed.

3.3.11 S11 re-stimulation of lymphocytes did not induce or enhance lymphokine-activated killer cell or NK cell activity

To address the possibility that re-stimulation with S11 cells in culture was inducing a population of non-specific killer lymphocytes, YAC-1 cells were included as targets in a number of CTL assays undertaken. YAC-1 cells are highly susceptible to natural killer cell or lymphokine-activated killer (LAK) cell lysis.

As a positive control, lymphocytes from uninfected mice were cultured in the presence of the cytokine IL-2. IL-2 is known to non-specifically enhance the proliferation and function of NK and LAK cells. In this system, IL-2-activated lymphocytes lysed YAC-1 target cells efficiently. At an E:T ratio of 100:1 ‘specific’ chromium release from YAC-1 targets was 39% (results not shown).

In contrast, no measurable NK or LAK activity was found in any of the S11-restimulated lymphocyte populations investigated. This is illustrated in figure 3.9.
3.3.12 Effects of the anti-viral drug 4'-S-EtdU on S11 target cell lysis

The antiviral drug 4'-S-EtdU is known to inhibit the replication of MHV-68 DNA (Smee et al., 1997). The long term culture of MHV-68 infected cells in the presence of this drug results in a progressive decline in viral lytic-cycle protein expression (Stewart, J. personal communication).

To investigate whether 4'-S-EtdU could alter the susceptibility of S11 targets to effector cell lysis, S11 cells were cultured with the compound for more than 7 days.

Immunocytochemistry of normal and 4'-S-EtdU treated S11 cells was carried out to confirm differences in expression of MHV-68 lytic proteins on these cells. Under normal culture conditions, 4.8% of S11 cells were positive for the lytic cycle antigens to which MHV-68 hyperimmune serum binds. When S11 cells were cultured with the antiviral drug, however, replicative cycle antigen expression was eliminated. No positive immunostaining of these cells could be detected.

When S11 cells, cultured in the antiviral 4'-S-EtdU, were included as targets in assays of S11-restimulated lymphocyte cytotoxic activity, no significant difference in the lysis of normal or antiviral-treated targets was ever observed (see Figure 3.9).
3.3.13 Generation of a polyclonal S11-specific CD8+ T lymphocyte line

Long term culture and re-stimulation of lymphocytes with irradiated and infected targets expressing viral epitopes is often used to obtain virus-specific T lymphocyte lines. Since S11 cells were capable of eliciting the activation (and proliferation) of lymphocytes from MHV-68 infected mice, this cell line was used to generate a T lymphocyte line as described in section 2.2.23.

To confirm the identity of the lymphocyte line which had expanded in response to S11, cells were stained with a range of monoclonal antibodies against cellular markers and then analysed by flow-cytometry. The results of this analysis are presented in Figure 3.10a and 3.10b. This experiment confirmed that 96% of cells that had expanded in response to long term S11 stimulation were CD8+, although only 70.5% expressed CD3. No significant expansion of any other lymphocyte subset was observed.

To assess whether the CD8+ T lymphocyte line was functionally active, its ability to lyse a range of lymphocyte targets was investigated. The results of this experiment are presented in Figure 3.11. The CD8+ T lymphocyte line efficiently lysed S11 target cells. At an E:T ratio of 12.5:1 a specific chromium release value of 38% was obtained. This rose to a peak of 44% at an E:T ratio of 50:1. No specific lysis of any of the other lymphocyte targets in this assay was observed.

All attempts to recover this cell line from frozen stocks were unsuccessful. Further, despite repeated attempts to generate a new line, using an identical
Figure 3.10a  Phenotype of T lymphocyte line generated against S11 lymphoma cells. BALB/c mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68. Splenocytes were removed from the animals 25 days after infection and were cultured with irradiated S11 cells for 7 weeks as detailed in section 2.2.6. After this period the lymphocytes were harvested and analysed by flow cytometry. Normal splenocytes (□) or the re-stimulated cells (□) were stained with either PE conjugated anti-mouse CD4 monoclonal antibody, FITC conjugated anti-mouse CD8 monoclonal antibody, FITC conjugated anti-mouse B220 monoclonal antibody, anti-mouse CD3 monoclonal antibody or anti-mouse F4/80 monoclonal antibody. 10,000 cells were counted in each analysis. The percentage of each cell type present in this population is presented above. Error bars represent the standard error from the mean of three analyses.
Figure 3.10b

Normal splenocytes
Anti-S11 line

Figure 3.10b
Figure 3.10b Representative data from the flow cytometric analysis of a T lymphocyte line generated against S11 lymphoma cells. BALB/c mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68. Splenocytes were removed from the animals 25 days after infection and were cultured with irradiated S11 cells for 7 weeks as detailed in section 2.2.23. After this period the lymphocytes were harvested and analysed by flow cytometry (as described in section 2.2.36). Normal splenocytes or S11 restimulated cells were stained with either phycoerithrin conjugated anti-mouse CD4 monoclonal antibody, FITC-conjugated anti-mouse CD8 monoclonal antibody, FITC-conjugated anti-mouse B220 monoclonal antibody, anti-mouse CD3 monoclonal antibody or anti-mouse F4/80 monoclonal antibody. Analyses were carried out in triplicate. The histograms presented in this figure are typical of those obtained during this analysis.
Figure 3.11 Cytolytic activity of a CD8 T lymphocyte line against a range of targets. BALB/ c mice were infected intranasally with 4 x 10^6 PFU of murine gammaherpesvirus 68. Splenocytes were obtained from the animal 25 days after infection and were cultured with irradiated S11 cells for 6 to 7 weeks as detailed in section 2.2.23. After this period the lymphocytes were harvested and their ability to lyse S11 (---), NS0 (--), MHV-68 infected NS0 (---) and YAC-1 (---) targets cells was assessed by a standard 5 hour chromium release assay (outlined in sections 2.2.11 to 2.2.17).

All assays were carried out in triplicate. Mean maximum and minimum release values were obtained for each target cell type and specific ^{51}Cr release values were calculated for each assay. Each point represents the mean specific value for three wells at each effector: target ratio. Error bars represent the standard error from the mean of the three specific release values calculated.
re-stimulation protocol, this was never possible within the time constraints of this project.
3.4 Discussion

The experiments described in this chapter demonstrate the lysis of an MHV-68-infected B cell lymphoma line (S11) by re-stimulated splenocytes from MHV-68 infected mice. At present, it is unclear whether the cytolytic responses measured to S11 were MHC class I or II restricted. Long term re-stimulation of splenocytes with S11, however, induced the expansion and activation of CD8+ CTL capable of efficiently killing S11 cells. The killing of B lymphocyte cell lines, infected *in vitro* with MHV-68, was never observed. It is, therefore, difficult to confirm that measurable CTL responses were virus specific.

Characterisation of CTL responses to EBV infection began in the mid 1970s. In 1975, Svedmyr and Jondal (1975) demonstrated killing of EBV infected B cell lines by peripheral blood lymphocytes which had been cultured with EBV positive lymphoblastoid cell lines (LCL). Over the next decade, this strategy for assessing EBV-specific CTL responses was optimised. In 1987, Strang and Rickinson (1987) employed gamma-irradiated LCL to re-stimulate unfractionated mononuclear cells from patients with infectious mononucleosis (IM). These re-stimulated cells were used to lyse EBV positive LCL obtained by spontaneous transformation or exogenous infection of B cells in culture. In the early 1990s, the emphasis of studies shifted to the identification of individual immunoreactive proteins of EBV. Vaccinia virus recombinants, expressing specific EBV proteins, were used to infect fibroblast target cells. The ability of LCL re-stimulated CTL to lyse these fibroblast targets was then investigated (Murray *et al*, 1992). Several groups have analysed specific EBV-
derived peptide epitopes. To do this, peripheral blood lymphocytes from IM patients were re-stimulated with LCL and then included in cytotoxicity assays involving phytohaemoglutinin-stimulated, autologous T cell lymphoblasts as targets. T cell lymphoblasts were incubated with putative immunoreactive peptides prior to their inclusion in the assay (Steven et al., 1997).

Over the past two decades, studies such as those outlined above, have generated a wealth of information and CTL responses to EBV are now well characterised. This area is reviewed in section 1.8.4 of this thesis. Prior to 1997, T lymphocyte responses to MHV-68 infection of mice were studied by monoclonal antibody depletion of T cell subsets in animals or by the use of genetically disrupted mouse strains. In 1998, Stevenson and Doherty (1998) published the first detailed analysis of CD8+ CTL responses to MHV-68. Much of this chapter has been devoted to describing the difficulties encountered in developing an assay for the assessment of MHV-68-specific CTL responses. In the following discussion, the CTL assay protocol developed by Stevenson and Doherty (1998) will be analysed in detail and differences in approach will be considered.

Stevenson and Doherty (1998) elected to assess MHV-68 specific CTL responses in BALB/c mice. These animals were intranasally infected with 400PFU of MHV-68. Throughout the course of the experiment the mice showed typical characteristics of infection, which suggests that differences in infectious dose of the virus are probably not significant.
Lymphocytes were obtained at short intervals after infection from the broncho-alveolar region, the mediastinal lymph nodes (MLN) and the spleens of infected animals. These cells were then cultured for 5 days with autologous spleen cells, infected with MHV-68 in vitro at an MOI of 0.1. The final effector:stimulator (E:S) ratio was 3:1. After 5 days in culture, putative effector cells were characterised by flow cytometry and their ability to lyse infected 3T3 cells was assessed. The target cells used in these experiments had been infected with MHV-68 in vitro at a MOI of 10.

Stevenson and Doherty (1998) found that MHV-68-specific cytotoxic activity in broncho-alveolar lavage (BAL) lymphocytes peaked at around 30% at 10 days post-infection. The response remained at this level for another 10 days. After 25 days, cytotoxic activity in BAL cells had declined to a negligible level. Peak cytotoxic activity in the MLN and spleens of MHV-68 infected mice peaked at around day 13 pi. This activity decreased to a background level at day 45 pi. CD8+ T lymphocytes were responsible for all cytotoxic activity measured in these experiments.

Stevenson and Doherty (1998) did not identify any of the MHV-68 epitopes to which CD8+ CTL were responding and assumed these were probably derived from lytic cycle proteins. When the authors assessed overall cytotoxic activity in lymphocytes from MHV-68 infected mice, they found a significant level of activity against anti-CD3 antibody coated target cells. They suggested the CTL responsible for lysis of these targets were probably specific for MHV-68
Chapter 3 – Discussion

antigens not expressed in 3T3 cells. It is possible these cells recognised MHV-68 latent cycle derived antigens.

In the experiments described in this chapter, the spleen was used as a major source of putative MHV-68-specific CTL. The demonstration of S11 target cell killing, by re-stimulated splenocytes, led to further investigation of the spleen as a useful source of effector lymphocytes. Unfortunately, further progress using this strategy was limited.

Stevenson and Doherty’s study demonstrated that a major CTL response to MHV-68 occurs during the acute phase of infection. This response is primarily in cells of the MLN and broncho-alveolar infiltrate.

In this study, no systematic investigation of MLN responses was undertaken. Periodically, attempts were made to obtain MLN effector cells at early times after infection (days 2 to 8). However, some difficulty was encountered in identifying and obtaining MLN at this time. Recent work has shown that the extent and timing of lymphocyte population expansions, in response to MHV-68 (resulting in lymph node enlargement), is affected by the genetic background of mice being investigated. Evidence suggests that lymph node expansion is much greater in C57BL/6 mice than in BALB/c mice at times immediately after infection (Dutia, B. personal communication). This may explain why MLN lymphocytes were difficult to obtain early after intranasal inoculation. In hindsight, however, a systematic investigation of MLN
responses would have been highly informative as Stevenson and Doherty’s publication demonstrates.

In the latter stages of this study, in an attempt to ‘focus’ lymphocyte responses to MHV-68, CBA and BALB/c mice were infected subdermally in the ear pinna. This resulted in a marked increase in cell numbers in the local, draining cervical lymph node. When the ability of these cells to lyse MHV-68-infected L-929 or 3T3 cells was investigated (following a period of re-stimulation with infected splenocytes) no specific target killing was ever observed.

To expand and activate lymphocytes in in vitro culture, Stevenson and Doherty employed naïve spleen cells, infected with MHV-68 in vitro, as stimulators of CTL. MHV-68 infection of splenocytes in vitro has not been characterised. It is likely that the virus enters several different cell types such as B lymphocytes and macrophages which are capable of presenting antigens to CTL.

Stevenson and Doherty found that, if they re-stimulated MLN or spleen cells, using irradiated and infected splenocytes, they obtained expansion of both CD4+ and CD8+ T lymphocyte sub-populations in culture. A significant proportion of these expanded cells expressed a naïve CD62Lhi phenotype. In contrast, in this study, infected splenocytes were found to be very poor stimulators of lymphocyte expansion in culture. To address this problem, numerous attempts were made to improve the splenocyte re-stimulation
protocol adopted. For example, naïve splenocytes were infected with MHV-68 at a range of MOI values. Further, a variety of E:S ratios were investigated. Despite these attempts, very few viable lymphocytes were ever obtained from splenocyte-re-stimulated cultures. It is difficult to speculate why the above discrepancy in findings has occurred. However, it is probable that the efficiency of a re-stimulation is determined by where and when lymphocytes were originally obtained and the activation state of the cells when they are first cultured. Difficulties in the re-stimulation of lymphocytes in this study were probably encountered because cells were not obtained from sites such as the MLN when anti-viral responses were at their peak.

Almost all of the CTL response data presented in this chapter was obtained following a re-stimulation of infected splenocytes with S11 cells. S11 cells display many of the phenotypic characteristics of B-lymphocytes. Further, all S11 lymphocytes carry numerous copies of episomal MHV-68 DNA and around 5% support lytic viral replication. At the beginning of this study, it seemed that the S11 line could be an equivalent to lymphoblastoid cell lines used for many years in the study of EBV-specific CTL responses. Unfortunately, very little data is available on the protein products of MHV-68 which are produced during latent or lytic infections. Gene expression in S11 cells, therefore, has not been fully characterised. It is probable that these cells present a variety of viral antigens in the context of MHC class I molecules.

Re-stimulation of infected (but not uninfected) splenocytes with S11 resulted in the expansion of both CD4+ and CD8+ T lymphocytes. Interestingly, in line
with the work of Stevenson and Doherty, the majority of these cells did not display an activated phenotype and expressed low levels of CD44. It would perhaps have been useful to further characterise the activation status of these cells by looking for increased expression of molecules such as I-A^d, the IL-2 receptor and the down regulation of CD5.

It is not surprising that S11 cells were capable of eliciting the proliferation of both T lymphocyte subsets since the cell line expresses both MHC class I and II molecules at its surface. It is not clear, however, why proliferating T cells expressed a naïve phenotype.

Stevenson and Doherty (1998) demonstrated that re-stimulated lymphocytes from the broncho-alveolar infiltrate, spleen and MLN of MHV-68 infected mice, lysed virally infected and not uninfected 3T3 target cells.

Preliminary experiments in this study indicated that 3T3 cells were very poor in their ability to retain radioactive chromium. Spontaneous release of the radioactive label from these cells was always above 35% of the maximum achievable value. As a consequence of this finding, attempts were made to develop a method of measuring CTL responses to MHV-68 using lymphocyte targets. This seemed a logical approach since B cell lymphoblastoid lines have been used for many years as targets of EBV-specific CTL.

Overall, some difficulty was encountered in developing a consistent and reliable assay for cytolytic T lymphocyte function. As discussed in section
3.3.10 of this chapter, only lysis of S11 targets could be reliably obtained and no detectable responses against infected NS0, A20, or latterly 3T3 cells were ever observed.

There is currently no evidence relating to why only S11 cells were lysed by splenocytes from MHV-68 infected animals. Data suggests that the lysis of S11 targets was not due to the expression of a non-viral, immunogenic lymphoma-related antigen. Further, non-specific NK or LAK cells did not seem to be responsible for target killing.

It is possible that S11 cells express an immunoreactive viral antigen not found in other cell lines used in these experiments. Antiviral treatment of S11 targets did not affect their susceptibility to lysis. This demonstrated that killing of these cells was not due to the presentation lytic cycle antigen. It is feasible that S11 cells may support a different pattern of latent MHV-68 protein expression to infected NSO, A20 or 3T3 cells. This is a common feature of EBV infections. A full range of latent-protein expression is found in LCL whilst restricted patterns of expression are found in cell lines derived from Burkitt's lymphoma or nasopharyngeal carcinomas.

Unfortunately, efforts to determine whether anti-S11 responses were MHC class I or II-restricted were hampered by the inconsistency of the assay. On several occasions, attempts were made to interfere with cytotoxic responses using MHC class I molecule H-2K^d-specific monoclonal antibody. Latterly, attempts were also made to deplete effector cell populations of CD4+ or
CD8+ T lymphocytes. Neither of these strategies ever yielded interpretable results.

It is interesting to note that long term re-stimulation of lymphocytes with S11 resulted in the outgrowth of a CD8+ T lymphocyte line. This cell line efficiently killed S11 targets. Thus, it can perhaps be argued that at least a proportion of the anti-S11 cytolytic response consistently measured was due to functionally active, MHC class I restricted, CD8+ CTL.

In the past year, Stevenson and Doherty's study has revealed that cytolytic T cell responses to MHV-68 differ in many respects from the CTL responses of humans to EBV infection. The design of the experiments described in this chapter was strongly influenced by successful studies of CTL responses to EBV. For example, it was felt that the mouse spleen would be a key source of effector cells with specificity for antigens expressed on B lymphocytes. To date, no evidence of MHV-68-specific CTL lysis of infected B lymphocyte targets has been published.

It appears that CTL responses to MHV-68, detectable by conventional means, are against lytic cycle antigens of the virus. Peak cytolytic activity, against lytically infected target cells, occurs around day 13 in lymphocytes from the MLN of infected mice. The work of Stevenson and Doherty focussed on lytic cycle antigen-specific CTL responses. It is probable that less immunodominant CTL responses, to latent epitopes of MHV-68, will be
characterised in the future now that the virus has been completely sequenced and candidate proteins of latent infection are being identified.

A recent sequence analysis of putative lytic cycle MHV-68 early proteins, revealed 7 peptide epitopes which are capable of binding to $H-2^b$ MHC class I molecules. When $H-2^b$-restricted target cells were incubated with these epitopes, it was found that two of the peptides sensitised the targets to lysis by lymphocytes obtained from MHV-68 infected animals. Interestingly, it was found that peptide-coated targets were not recognised by the vast majority of expanded CD8+ lymphocytes in spleens of infected mice, nor were they killed by cells taken from animals at late times post-infection (Stevenson, P., personal communication). Evidence suggests, therefore, that a significant CD8+ CTL response to MHV-68 lytic cycle antigens is generated during acute infection. However, it would seem that detectable lytic cycle specific CTL are not maintained into memory. The specificity of the majority of the CD8+ T cells in the spleen during splenomegaly remains unclear.

Preliminary evidence, relating to the specificity of CD8+ T cells raised against S11, suggests that at least some of these lymphocytes are specific for epitopes within a putative latent cycle protein, M2. This suggests that CTL, specific for MHV-68 latent antigens, are generated during a normal infection. No functional role has yet been attributed to these cells (Usherwood, E., personal communication).
Current evidence suggests that immune responses to EBV in humans may not reflect how other species respond to gammaherpesvirus infection. A key difference between responses to EBV and MHV-68 is that CTL do not appear to be critical in the maintenance of the murine virus in a harmless form during long-term latent or persistent infection. There may, however, be some similarity in the CD8+ CTL responses of mice and humans to acute MHV-68 or EBV infection respectively. Over the last 5 years, it has been revealed that a significant proportion of CD8+ CTL, which expand during IM, are specific for lytic cycle EBV antigens (Steven et al, 1996). Stevenson and Doherty have demonstrated that CD8+ CTL, with specificity for lytic cycle antigens of MHV-68, play a key role in the resolution of acute MHV-68 infection in the lung. Once again there is a distinction between the human and rodent viruses. Detectable EBV lytic-antigen-specific CD8+ CTL responses are maintained in memory for long periods of time. CD8+ T cell responses to MHV-68, however, are undetectable by conventional means by day 45 post-infection. It seems likely that other mechanisms, such as antibody neutralisation of MHV-68, play a significant role in dampening reactivation of the murine virus at late times post-infection.

It is difficult to draw firm conclusions on whether CD8+ CTL responses to MHV-68 resemble T lymphocyte responses to subclinical EBV infection. The majority of the world's population are seropositive for EBV and carry functional EBV-specific CTL which have been generated in the absence of infectious mononucleosis. It is impossible to say whether these cells were responsible
for clearing the acute viral infection or whether other immune mechanisms were more significant on primary exposure to the virus.

The importance of CD8+ T cell responses, during subclinical EBV infections, may be determined by the dose of virus to which an individual is exposed and their genetic background. In BALB/c mice, lowering MHV-68 inoculation dose decreases the dependence of these animals on CD8+ T cells for survival (Stevenson, P. personal Communication). Further, other strains of mice such as C57 BL/ 6 animals, which are H-2K^b and H-2D^b restricted rely less upon CD8+ T lymphocytes for clearance of MHV-68.

In the future, the identification of epitopes, to which CTL respond in different inbred strains of mice, will allow MHC class I tetramer construction. Tetramer staining of cells (in conjunction with cytotoxicity assays) will allow the characterisation of immunodominant and subdominant T cell epitope-specificities. Identification of immunoreactive MHV-68 epitopes may help to explain why CD8+ T cells are functionally significant in some strains of mice and not others. Further, as significant immunoreactive epitopes are identified these will prove to be useful tools in the development of vaccines.
Chapter 4

Adoptive immunotherapy of MHV-68 infected tumours in immunocompromised nude mice
4.1 Introduction

The experiments described in Chapter 3 of this thesis demonstrated the killing of S11 lymphoma cells by lymphocytes obtained from MHV-68 infected mice. Unfortunately, due to problems encountered in the development of the \textit{in vitro} cytotoxicity assay, these experiments did not confirm if this cytolysis response was MHC restricted or virus specific. Further, it was unclear which lymphocyte subset was active in killing the S11 cells. In an attempt to discover more about lymphocyte responses to S11 cells a change in strategy was required. Therefore, the decision was made to consider functional lymphocyte responses to S11 cells \textit{in vivo}. This approach was appealing for two reasons. Firstly, it was hoped the analysis of lymphocyte responses to S11 cells \textit{in vivo} would reveal more about the functional characteristics of S11-specific cytotoxic effector cells. Secondly, the killing of S11 cells \textit{in vivo} allowed us to model the therapeutic treatment of a virus-associated tumour in a severely immunocompromised host.

The immunotherapeutic treatment of tumours has been widely studied for many years in laboratories around the world. If tumour development in a patient is associated with a viral infection, lymphocytes specific for viral antigens expressed on tumour cells, can be exploited to target, exclusively, transformed or malignant tissues. Persistent EBV infection is usually clinically unnoticeable in immunocompetent individuals. It is thought that under normal circumstances, CD8+ CTL are responsible for the control of EBV infected B lymphocytes which spontaneously transform in infected individuals. However, patients with severe congenital or acquired deficiencies of cell mediated
immunity are at a significantly increased risk of developing EBV-induced B cell lymphoproliferative disorders (Lacerda et al., 1996b, Rooney et al., 1997). Patients suffering from EBV-induced lymphoproliferative disorders are ideal candidates for cellular immunotherapy. The reasons for this are outlined in section 1.8.7 of this thesis. Over the last 6 years, several attempts have been made to develop strategies for the treatment of EBV-induced lymphoma (Smith et al., 1996). Initially, attempts were made to transfer ‘bulk’, LCL-re-stimulated lymphocytes into immunosuppressed, bone-marrow transplant recipients. Unfortunately, these studies were hampered by patients developing graft versus host disease (Papadopoulos et al., 1994). To overcome this problem, several groups are currently transferring EBV-specific CD8+ CTL lines into immunocompromised patients (Rooney et al., 1997). To date, transfer of virus-specific, polyclonal CD8+ T cells lines into bone-marrow recipients has been very successful in preventing EBV-induced lymphoma development in these patients. CD8+ T lymphocytes survive in patients for up to 2 years after their transfer. Further, these cells retain their cytotoxic activity for this period. In patients who have received virus-specific lymphocyte infusions, EBV DNA levels in the blood invariably drop and immunoblastic lymphomas either do not appear or regress. Therefore, this strategy has proven to be successful. Despite this success, a number of limitations hamper current bone marrow transplant tumour therapy trials. Firstly, fully randomised therapeutic trials are currently not being undertaken. Patients are eligible for adoptive-transfer therapy only if they are receiving bone-marrow from a mismatched family member or an unrelated donor. Secondly, only historical controls are available for these studies. The success of a trial is determined
by how many patients survive relative to the number of individuals who would have developed fatal lymphoproliferative disease (LPD) in the past. Thirdly, the majority of patients receive prophylactic lymphocyte infusions. Very few studies have investigated adoptive transfer therapy of established lymphomas. In the few studies that have examined the treatment of established tumours, inflammation due to tumour infiltrating lymphocytes has proven to be a significant problem. Finally, it is unclear at present whether adoptive transfer of lymphocytes will be a suitable therapy for the treatment of lymphomas in solid organ recipients.

In view of these problems, there is currently scope for an animal model of EBV-induced lymphoma development and therapy. To date, several groups have developed murine models of EBV-associated LPD. These models have all involved the inoculation of SCID mice with PBL from EBV seropositive donors or with EBV-transformed LCL. This results in the development of EBV-induced immunoblastic lymphoma with characteristics similar to EBV-LPD which arise in immunocompromised human hosts. If HLA-matched, in vitro-expanded EBV-specific T cells are also transferred into these SCID mice, regression of autologous tumours occurs (Lacerda et al., 1996). The principal drawback of this model system is that it involves the transfer of lymphocytes between species. Differences in key components of the immune system undoubtedly exist between species and these differences may fundamentally alter the initiation and subsequent development of immune responses.
Infection of inbred laboratory mice with MHV-68 results in approximately 9% of these animals developing tumours. Several tumour cell lines have been derived from afflicted mice, the most significant of which is termed 'S11'. S11 cells display all the phenotypic characteristics of B lymphocytes and are all infected with MHV-68. Significantly, if S11 lymphocytes are implanted subcutaneously into nude mice the cells proliferate in an uncontrolled manner. This results in large tumours forming which can grow to around 15mm in diameter before animal welfare is affected. Subcutaneous proliferation of S11 lymphocytes does not occur if the cells are implanted into normal, immunocompetent mice. This suggests that T lymphocytes play a role in the immune responses of mice to this cell line (Usherwood et al., 1996b).

To confirm this hypothesis, a number of experiments were undertaken to investigate whether immune responses to S11 cells could be restored in nude mice by the adoptive transfer of T lymphocyte subsets into these animals.

4.2 The restoration of an immune response to S11 cells in nude mice
To investigate whether immune responses to S11 cells could be restored in athymic BALB/c nude mice, groups of animals received adoptive transfers of either uninfected splenocytes, MHV-68 'immune' splenocytes or S11-restimulated 'immune' splenocytes from syngeneic donor animals. 1 x 10^7 splenocytes were delivered via an intra-peritoneal route and then all of the nude mice received a subcutaneous injection of S11 cells on their flank. Tumour development was assessed by measurement of graft diameter at a range of times after implantation and the experiment was terminated when
tumours began to affect the welfare of the nude mice. The results of this experiment are presented in Figure 4.2.

In agreement with the previous study of Usherwood et al. (1996b), S11 cells rapidly proliferated in untreated nude mice. Subcutaneous tumours in these animals grew from around 6mm (+/- 1mm) in diameter at 6 days post implantation, to an average of 12.75mm (+/- 1.75mm) in diameter after 19 days.

In contrast, animals which received an adoptive transfer of S11 re-stimulated lymphocytes were able to mount an effective immune response to the lymphoma cell line. S11 tumours in this group of mice decreased in size from 5.5mm in diameter at 6 days post-implantation to less than 1mm in diameter 19 days after implantation. The photographs presented in Figure 4.1 show S11 tumours on the flanks of untreated mice or mice which had received an adoptive transfer of re-stimulated lymphocytes.

The nude mice which received either uninfected or infected splenocytes from mice displayed an intermediate ability to control S11 tumour growth. S11 tumours did not notably increase or decrease in size from day 6 onwards in either of these groups. Throughout the experiment, subcutaneous tumours in these animals were between 5 and 7mm in diameter.

On termination of the experiments, tumours were removed for immuno-histological analysis. The results of these analyses are described later.
Figure 4.1 Subcutaneous S11 tumours in nu/nu mice. 10 BALB/c mice were infected intranasally with murine gammaherpesvirus 68. After 25 days, spleens were removed from these animals and splenocytes were cultured as described in section 2.2.6 of materials and methods. Following this bulk re-stimulation in culture, splenocytes were harvested, washed and counted. $1 \times 10^7$ re-stimulated cells were then adoptively transferred into 4 nude mice via an intraperitoneal route. $2 \times 10^7$ S11 cells were then implanted subcutaneously into the flanks of the animals. Tumour growth was assessed by measurement of the tumours at various times after implantation. Photograph 1 above shows S11 tumours in control animals which received no adoptive transfer of cells. Photograph 2 above shows a group of animals which received an adoptive transfer of complete re-stimulated lymphocytes at time of tumour implantation. Both photographs were taken at 19 days post tumour implantation. White squares highlight the subcutaneous tumours.
Figure 4.2  Diameter of sub-cutaneous S11 tumours in nude mice following the adoptive transfer of splenocyte populations. 3 to 4 week old BALB/c mice were infected intranasally with murine gammaherpesvirus 68. After 25 days, splenocytes from the mice were cultured with irradiated S11 cells at a ratio of 10:1 for 7 days. 1x10^7 spleen cells from uninfected or infected mice or 10^7 re-stimulated splenocytes were injected IP into nude mice. 1x10^7 S11 cells were then injected subcutaneously into the left flank of the nude mice and lymphoma development was assessed by measurement of the tumour diameter at the times indicated. (—) Tumour only, (—) Tumour + Normal splenocytes, (—) Tumour + Infected splenocytes, (—) Tumour + S11 re-stimulated splenocytes. Each point represents mean diameter of 4 tumours. Error bars represent standard error of the mean.
Further, in this experiment, the lungs and spleens of all animals were removed for a subsequent investigation of whether MHV-68 had spread from the subcutaneous tumours to other anatomical sites in the nude mice. When infectious virus and infectious centre assays were carried out on these tissues, no evidence of MHV-68 infection was ever obtained.

The results of this experiment demonstrate that adoptively transferred lymphocytes can restore the ability of nude mice to respond to subcutaneous S11 tumours.

4.3 The role of CD8+ T lymphocytes in S11 tumour regression

Current trials, involving the transfer of EBV-specific lymphocytes into immunocompromised bone-marrow recipients, indicate that it is CD8+ CTL which are active in preventing EBV-induced lymphoma development in these patients (Rooney et al., 1997, O’Reilly et al., 1997).

To investigate whether CD8+ T lymphocytes played a significant role in S11 tumour regression, groups of nude mice received injections of either complete S11 re-stimulated ‘immune’ splenocytes or re-stimulated cells depleted of CD8+ T lymphocytes. All of these mice then received a subcutaneous injection of S11 cells on their flank.

In this experiment, one group of mice received the antiviral drug 4’-S-EtdU in drinking water for more than a week prior to S11 implantation. The S11 cells implanted into these animals had been cultured with the anti-viral drug for
more than 7 days. This group of animals was included to investigate whether the down-regulation of MHV-68 lytic cycle protein expression in S11 cells altered the growth characteristics of these lymphocytes in mice receiving immunotherapy. In this experiment, tumour development was assessed as before and results are presented in Figure 4.3.

S11 cells rapidly proliferated in the untreated nude mice. The mean tumour diameter in these animals increased from 6mm at 5 days after implantation to 14mm after 2 weeks. In mice which received S11 re-stimulated splenocytes, tumours decreased in size from 5mm in diameter 5 days after implantation to 2mm in diameter after 12 days of the experiment. 4'-S-EtdU treatment of S11 cells and recipient animals had no notable effect on the regression of S11 tumours in the nude mice.

Significantly, when cells expressing CD8 were removed from adoptively transferred lymphocyte populations, S11 tumours in recipient mice did not increase in size beyond a mean diameter of 5mm. Flow cytometric analysis of re-stimulated splenocytes showed that prior to depletion, around 18% of these cells were positive for CD8 expression. After depletion, negligible levels of CD8+ T lymphocytes remained. Data from this analysis are presented in Figure 4.5(1).

This experiment demonstrated that CD8+ T lymphocytes were not primarily responsible for eliciting the regression of subcutaneous S11 tumours in this
Figure 4.3 CD8+ T cells are not primarily responsible for the regression of S11 tumours in nude mice. 3 to 4 week old BALB/c mice were infected intranasally with murine gammaherpesvirus 68. After 25 days, splenocytes from the mice were cultured with irradiated S11 cells at a ratio of 10:1 for 7 days. The re-stimulated cells were divided into two populations. CD8+ T lymphocytes were complement depleted from one of these populations. Nude mice were then injected IP with $10^7$ CD8 depleted or complete re-stimulated splenocytes. All mice were then injected subcutaneously with $10^7$ S11 cells on their left flank. One group of mice received 4'-S-EtdU in their drinking water and a subcutaneous injection of $10^7$ S11 cells which had previously been cultured with 4'-S-EtdU for 7 days. Lymphoma development was assessed by measurement of the tumour diameter at the times indicated. (-O-) Tumour only, (-■- ) Complete splenocytes, (-△-) No CD8 splenocytes, (-×-) 4'-S-EtdU treated tumour + complete splenocytes. Each point represents the mean diameter of 4 tumours. Error bars represent standard error of the mean.
model. Further, it showed that the regression of tumours was not affected by a lack of MHV-68 lytic gene expression in implanted S11 cells.

4.4 The role of CD4+ T lymphocytes in S11 tumour regression

Studies have demonstrated that in vitro re-stimulation of PBL with LCL often results in the production of CD4+ T lymphocyte lines which can lyse LCL targets. These CD4+ T cell lines are, however, generally ineffective in inducing the regression of EBV positive lymphomas in SCID mice (Rencher et al., 1994). To assess the role of CD4+ T lymphocytes in the regression of S11 tumours in nude mice, groups of animals received complete S11-restimulated ‘immune’ splenocytes or ‘immune’ re-stimulated splenocytes depleted of CD4+ and/or CD8+ T lymphocytes. All mice were then given a subcutaneous injection of S11 cells. To ensure that depletions were successful, lymphocyte populations were analysed by flow cytometry. Representative data from this analysis is presented in figure 4.4 and a summary of the results is presented in Figure 4.5 (2). In this experiment, around 50% of lymphocytes in the complete re-stimulated population expressed CD4 whilst approximately 8% expressed CD8. Depletion resulted in a greater than 99% reduction in the number of CD4+ and/or CD8+ lymphocytes present in transferred populations of cells. Tumour growth profiles for this experiment are presented in Figure 4.6.

The result was consistent with those of previous experiments, in that S11 cells rapidly proliferated in untreated nude mice. The average tumour diameter in this group of animals increased from around 6mm in diameter 4 days after
Figure 4.4
Figure 4.4 Flow cytometric analysis of complement depleted lymphocyte populations used in the therapy of S11 tumours. BALB/c mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68. After 25 days, splenocytes were removed from the animals and cultured with irradiated S11 cells at a ratio of 10:1 for 7 days. At the end of this period, lymphocytes were harvested and depleted of CD4+ or CD8+ T lymphocytes using monoclonal anti-CD4 or anti-CD8 antibody and complement (as detailed in section 2.2.34). After depletion, $1 \times 10^6$ lymphocytes from each group were analysed by flow cytometry (as described in section 2.2.37). Both CD4+ (1) and CD8+ (2) T lymphocytes were present in the re-stimulated splenocyte population prior to depletion. Depletion resulted in a greater than 95% reduction in numbers of CD4+ (3) and CD8+ (4) T cells in the lymphocyte populations transferred into nude mice.
Figure 4.5
Figure 4.5  Flow cytometric analysis of CD4 or CD8 depleted re-stimulated lymphocyte populations used in therapy of S11 tumours. BALB/c mice were infected intranasally with 4 x 10^5 PFU of murine gammaherpesvirus 68. After 25 days splenocytes were removed from the animals and cultured with irradiated S11 cells at ratio of 10:1 for 7 days. At the end of this period lymphocytes were harvested and depleted of either CD4+ or CD8+ T lymphocytes using a combination of the monoclonal anti-CD4 antibody YTS-191 and/or the monoclonal antibody YTS-169 and complement (as detailed in section 2.2.35). After depletion, 10^6 lymphocytes from each group were analysed by flow cytometry for the presence of CD4 and/or CD8 lymphocytes as described in section 2.2.37. Panel 1 shows that depletion resulted in a greater than 99% reduction in the number of CD8+ lymphocytes transferred into nude mice (experiment described in section 4.3). Panels 2 and 3 also illustrate that antibody depletion resulted in a greater than 99% reduction in the number of CD4+ and CD8+ lymphocytes transferred into nude mice. Panel 2 refers to results presented in Figure 4.6. Panel 3 refers to results presented in Figure 4.7.
Figure 4.6 CD4+ T cells elicit S11 tumour regression in nude mice. 3 to 4 week old BALB/c mice were infected intranasally with murine gammaherpesvirus 68. After 21 days, splenocytes from the mice were cultured with irradiated S11 cells at a ratio of 10:1 for 7 days. The re-stimulated cells were then divided into 4 populations. Populations 2, 3 and 4 were complement depleted of CD4 or CD8 or CD4 and CD8 lymphocytes respectively. Nude mice were injected IP with $10^7$ complete, CD4 depleted, CD8 depleted or CD4 and CD8 depleted splenocytes. All mice were then injected sub-cutaneously with $10^7$ S11 cells on the left flank and tumour development was assessed by measurement of the tumour diameter at the times indicated. (—young) Tumour only (each point represents mean diameter of 3 tumours), (—■—) complete splenocytes (each point represents mean diameter of 3 tumours), ( —△— ) CD4-depleted splenocytes (each point represents mean diameter of 4 tumours), ( —○— ) CD8-depleted splenocytes (each point represents mean diameter of 4 tumours), ( —□— ) No CD4 or CD8 lymphocytes (each point represents mean diameter of 4 tumours). Error bars represent the standard error of the mean.
implantation, to 15.5mm (+/-0.5mm) in diameter after 11 days. At this point, the mice were sacrificed as tumours were beginning to affect the welfare of the animals. Depleting either CD4+ or both CD4+ and CD8+ T lymphocytes from transferred cells resulted in significant S11 proliferation in nude mice. Tumours in these animals grew to around 13mm in diameter before animals were sacrificed.

In this experiment, complete re-stimulated splenocytes were less effective than previously in eliciting tumour regression. S11 tumours grew in diameter from 3mm at 4 days post-implantation to around 8mm (+/-2mm) in diameter after 12 days.

Significantly, no tumour expansion occurred in nude mice which had received CD4 splenocytes. This suggests that CD4+ T lymphocytes play a significant role in eliciting tumour regression in this model system. To confirm that this was a legitimate result, this experiment was repeated.

Nude mice were again injected with S11 re-stimulated 'immune' splenocytes or re-stimulated 'immune' splenocytes depleted of CD4+ or CD8+ T lymphocytes. As before, a flow cytometric analysis was undertaken to confirm lymphocyte subset depletion. The results of this analysis are summarised in Figure 4.5(3). The data presented in Figure 4.6 confirm that CD4+ T lymphocytes were at least as effective as complete splenocytes in eliciting S11 tumour regression.
Figure 4.7 CD4+ T cells elicit S11 tumour regression in nude mice. 3 to 4 week old BALB/c mice were infected intranasally with murine gammaherpesvirus 68. After 25 days, splenocytes from the mice were cultured with normal, irradiated S11 cells at a ratio of 10:1 for 7 days. The re-stimulated cells were then divided into 4 populations. Populations 3 and 4 were complement depleted of CD4 or CD8 lymphocytes respectively. Nude mice were then injected IP with $10^7$ complete, CD4- or CD8- depleted re-stimulated splenocytes. All mice were injected sub-cutaneously with $10^7$ S11 cells on the left flank and tumour development was assessed by measurement of the tumour diameter at the times indicated. (---) Tumour only (each point represents the mean of 2 animals), (--•--) complete splenocytes (each point represents the mean of 2 animals), (△--) CD4-depleted splenocytes (each point represents the mean of 4 animals), (×--) CD8-depleted splenocytes (each point represents the mean of 4 animals). Error bars represent the standard error of the mean.
Once again, S11 cells rapidly proliferated in the untreated nude mice with tumours reaching a maximum diameter of 15mm after 10 days. In mice which received CD4-depleted lymphocytes S11 tumours expanded rapidly in diameter from 4.5mm at day 3 after implantation to 12mm (+/- 3mm) after 13 days.

From day 3 to day 10 of this experiment, S11 tumours did not noticeably increase in size in mice which had received either complete splenocytes or CD4 lymphocytes. From day 10 to day 13, however, S11 proliferation did occur in mice which had received CD8 depleted lymphocytes. By day 13 post implantation, tumours in these animals had reached 7mm (+/-3mm) in diameter.

These data confirmed that CD4+ T lymphocytes play a significant role in eliciting S11 tumour regression in this model.

**4.5 Immunohistochemical analysis of S11 tumours**

To investigate cellular infiltration into subcutaneous S11 grafts, tumour sections were stained with a range of antibodies as described in section 2.2.50. Photographs of stained sections are presented in Figure 4.8. In agreement with Usherwood et al. (1996b), when tumour sections were stained with rabbit polyclonal anti-MHV-68 serum, positively stained cells appeared in large numbers throughout the tumour sections analysed. Further, large numbers of positive cells were also observed when sections were stained with an anti-mouse B220 monoclonal antibody. This was expected since S11 is a
Figure 4.8
Figure 4.8 Immunohistochemical analysis of S11 tumours. Subcutaneous S11 tumours were removed from nude mice two weeks after implantation, fixed in formal saline for 4 hours and then transferred to 70% ethanol. Tumours were then embedded in paraffin wax and 6μm sections were obtained. A and B were stained with haematoxylin and eosin to illustrate pathological features of the tumour. C was immunostained with polyclonal anti-MHV68 anti-serum. Virus positive cells were visualised using a DAB/ H2O2 chromagen/ substrate combination (brown). Sections were counter-stained with haematoxylin. D represents a negative control section which received non-immune serum in place of primary antibody and was counterstained with neutral red. E was immunostained with a rat anti-mouse B220 monoclonal antibody. B220 positive cells (B lymphocytes) were found, widely distributed, throughout all sections analysed. F and G were immunostained with a rat anti-mouse macrophage monoclonal antibody (F4/80). Macrophages were found in all sections analysed but were most abundant in tumours from mice which had received an infusion of CD4+ T lymphocytes (G). H was immunostained with a rat anti-mouse CD4 monoclonal antibody. CD4+ T cells (X) were mainly observed in the vicinity of blood vessels (Y) with sporadic staining in other areas of the tumours analysed. Positive cells in E to H were visualised by the addition of a mouse anti-rat biotinylated secondary antibody, a streptavidin/ alkaline phosphatase conjugate followed by NBT/ BCIP (blue/ black) as described in section 2.2.50. E-H were counter-stained with neutral red. Original magnification x200. White bars represent 0.2mm. Arrows show positive staining with relevant antibody.
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B lymphocyte cell line. Tumour sections were also stained with the macrophage-specific antibody F4/80. Macrophages were found in all tumour sections analysed. Greater numbers of these cells were evident in tumours from mice which had received an infusion of lymphocytes. Broadly, these cells were evenly distributed throughout the tumour sections analysed. CD4+ T lymphocytes were observed in tumours from nude mice which had received an infusion of either complete or CD8-depleted splenocytes. CD4+ T cells were not evenly distributed throughout tissue sections and were found in the vicinity of blood vessels. Unfortunately, CD8+ T lymphocyte staining was never achieved using the protocol outlined in section 2.2.50.

4.6 The *in vitro* culture of S11 cells with re-stimulated lymphocyte populations

In an attempt to mimic the lymphocyte-induced regression of S11 tumours *in vivo*, S11 cells were cultured *in vitro* in 96 well round-bottomed plates with re-stimulated lymphocyte populations. A range of S11:lymphocyte ratios were investigated, however, re-stimulated lymphocytes were, in all instances, unable to prevent S11 proliferation.
4.7 Discussion

The experiments described in this chapter demonstrate that T lymphocyte responses to S11 lymphoma cells can be 'restored' in athymic nude mice. In this model, CD4+ T lymphocytes efficiently controlled subcutaneous S11 proliferation. In contrast, CD8+ T lymphocytes were ineffective in preventing tumour development.

To date, only a few groups have investigated the transfer of EBV-specific T lymphocytes into solid-organ transplant recipients. However, the adoptive transfer of such cells, into immunocompromised bone-marrow recipients, has proven to be a useful means of preventing virus-induced lymphoma development. Both CD4+ and CD8+ T lymphocytes are routinely transferred into patients who have received bone-marrow grafts. In vitro studies suggest that the CD8+ T lymphocytes are functionally active in preventing tumour development. These cells retain an EBV-specific cytolytic function for up to 2 years after their transfer (Rooney et al., 1997). CD4+ T lymphocytes are included in transferred lymphocyte populations to provide cytokine 'help' for the maintenance or expansion of EBV-specific CD8+ T cells in vivo. No direct anti-tumour activity has been attributed to these cells although they have been found in histological studies of tumour biopsy material (Thomas, J.A., personal communication). Therefore, current evidence suggests that, in vivo, adoptively transferred CD8+ T cell lines directly lyse transformed B lymphocytes which are presenting EBV-derived peptide sequences (Rooney et al., 1997).
Several attempts have been made to model EBV-induced lymphoma development using animals. The best recognised of these models employs the transfer of human EBV-infected peripheral blood lymphocytes (PBL) or LCL into mice with a severe combined immunodeficiency (SCID). These mice lack functional T or B lymphocytes and on receipt of EBV+ PBL or LCL develop tumours which closely resemble those found in humans suffering from EBV+ LPD (Rowe et al., 1991).

In 1996, Lacerda et al. (1996) demonstrated that an intra-peritoneal infusion of EBV-specific, CD8+ HLA-restricted T lymphocytes, into SCID mice, could elicit the regression of peritoneal EBV+ tumours. CD8+ T cells were found infiltrating these autologous B lymphocyte grafts. In contrast, when EBV-specific T lymphocytes were administered intravenously (IV) (a more clinically relevant route), SCID mice bearing peritoneal tumours died. To overcome this problem, the cytokine IL-2 was administered i.v. at regular intervals after the lymphocyte infusion. This resulted in the increased survival of transferred lymphocyte populations in vivo. As a consequence of this enhanced survival, adoptively transferred lymphocytes preferentially migrated to, and infiltrated peritoneal tumours in these animals. The animals also survived. The SCID mice used in this study were depleted of NK cells by i.v. injection of anti-asialo GM1 antibody at the outset of the experiments (Lacerda et al., 1996a). Unfortunately, the mechanism employed by the infused cells to induce the regression of the B cell lymphomas remains unconfirmed. The CD8+ T lymphocyte lines used in this study lysed EBV-infected targets in vitro. However, the in vivo evidence published in 1996 does not exclude the
possibility that tumour regression was occurring due to the release of cytokines by graft-infiltrating lymphocytes (Lacerda et al., 1996).

Wolf et al., (1995) investigated the responses of athymic nude mice to a range of EBV-infected B cell lymphoma lines. When permanent Burkitt's lymphoma (BL) lines are implanted subcutaneously into nude mice they form fast-growing tumours which do not become necrotic or regress. In contrast, EBV-positive LCL do not proliferate efficiently in nude mice and tumours regress 7 to 14 days after implantation. This observation suggests that immune responses are generated to LCL in nude mice. These immune responses may be induced by LCL antigen expression or cytokine production by these cells. Wolf et al. (1995) demonstrated that co-inoculation of EBV-positive BL cell lines and LCL into nude mice resulted in the regression of BL tumours in these animals. Successful co-culture of the two cell lines together indicated that this was not due to the production of cytokines by the LCL directly inhibiting the growth of the BL line. In a previous study of LCL-induced EBV-negative BL line regression in nude mice, no infiltration of mouse cells was observed on analysis of immunostained BL graft sections (Tosato et al., 1994). However, Wolf et al. (1995) found both macrophage and natural killer cell infiltrates in regressing BL line tumours. These cells were absent in non-regressing lymphoma. This evidence suggested that the LCL was inducing the activation and migration of non-specific inflammatory cells into BL grafts.

How does the evidence obtained in these studies of cellular immunotherapy relate to the present investigation of S11 tumour regression in
immunocompromised nude mice? Overall, the results outlined in this chapter indicate that CD4+ T lymphocytes are functionally significant in the suppression of subcutaneous S11 proliferation in nude mice. Initially, a combination of re-stimulated CD4+ and CD8+ T lymphocytes was shown to elicit regression of S11 tumours. Both naïve and unstimulated-'immune' lymphocytes were also partially effective in preventing uncontrolled S11 proliferation. In this instance, the naïve splenocytes were probably mounting a primary response to the S11 graft and it seems likely that the primed splenocytes were responding to viral antigens previously encountered in MHV-68 infected donor mice. In contrast to this result, unprimed or non-specifically activated lymphocytes do not inhibit EBV-induced LPD development in xenografted SCID mice. In the SCID-Hu-PBL model, the transfer of LCL-re-stimulated CD8+ T lymphocytes into mice is required for tumour regression.

Also in the present study, depletion of CD8+ T lymphocytes from transferred cell populations had little effect on tumour regression. Once again, there is sharp contrast between this observation and data obtained from experiments involving the treatment of EBV-LPD in SCID mice. In these animals, EBV-specific CD4+ T lymphocytes are generally unable to prevent the development of EBV-LPD.

Immunohistological analyses of tumour sections revealed that both macrophages and CD4+ T cells had migrated into S11 grafts in these experiments. Macrophages were observed in tumours from all groups of nude
mice. However, greater numbers of these cells were observed in tumours from mice which had received a lymphocyte infusion. This suggests that cytokine release by T lymphocytes enhanced monocyte/macrophage migration into this site.

In the nude mouse model, CD4+ T lymphocytes might be instigating an anti-tumour response in several ways. CD4+ T cells may be migrating to tumours and directly lysing S11 lymphoma cells via an MHC class II molecule/T cell receptor interaction. Against this idea, there is currently no evidence which suggests that CD4+ CTL are generated during the immune responses of mice to MHV-68. Therefore, CD4+ T cells could be eliciting an anti-S11 response directly or indirectly via the production of cytokines. Local production of cytokines such as IFN-γ or GM-CSF by re-stimulated CD4+ T lymphocytes may have a direct negative effect on S11 proliferation. However, it seems more likely that these cytokines are initiating an anti-tumour delayed-type hypersensitivity (DTH) response in the nude mice. Nude mice have significant, functional populations of macrophages and NK cells (Wolf et al., 1995). The secretion of cytokines such as IFN-γ by tumour infiltrating lymphocytes may serve as a signal for monocyte or NK cell recruitment and activation.

It has been suggested that cytokine production by LCL may be responsible for the initiation of non-specific immune responses to BL lines in nude mice (Wolf et al., 1995). The fact that S11 cells proliferate in untreated nude mice argues against the possibility of cytokine production by these lymphoma cells being
important in initiating non-specific immune responses. The identification of a role for macrophages in anti-tumour DTH responses might be significant. If tumour-infiltrating lymphocytes initiate effective DTH responses then differences in levels of viral antigen expression on tumour cells may be less significant (Barth et al., 1991). S11 tumour infiltrating lymphocytes may be exerting their effects via the production of cytokines such as TNF-α.

Evidence on the inability of re-stimulated lymphocytes to prevent S11 growth in vitro (section 4.6), supports the theory that macrophages are involved in preventing in vivo S11 proliferation. It seems reasonable to conclude that lymphocytes were unable to exert any effects on the proliferation of S11 cells in vitro due to an absence of macrophages in this system.

It is unclear why CD8+ T lymphocytes alone were incapable of eliciting tumour regression in this model. Evidence suggests that these cells play a significant role in the acute immune responses of mice to MHV-68 infection (Stevenson and Doherty, 1998). Further, several MHV-68 derived epitopes, presented in the context of S11 MHC class I molecules, have now been identified (Usherwood, E. personal communication). It is possible that CD8+ T lymphocytes when transferred into nude mice are functionally active and yet are unable to suppress the rapid proliferation of S11 cells in this environment.

It is difficult to speculate why immune responses to S11 can be 'restored' in nude mice when these cells originally proliferated in an apparently normal animal. It is possible that the mouse from which S11 was first isolated did not
have a fully functional immune system. The animal was over 18 months old and at this age it is probable that some form of decline in immune function had occurred. Such a decline, coupled with the potential resistance of S11 cells to certain immune effector mechanisms, may have led to the development of a lymphoma in this animal. Other factors, such as the anatomical site of S11 implantation and possible changes in the phenotypic characteristics of S11 in culture, may also have played a role in rendering this cell line sensitive to lymphocyte-induced responses in recipient nude mice.

To learn more about lymphocyte responses to S11 in nude mice, some basic characterisation studies are required. For example, it is not known whether S11-restimulated lymphocytes can inhibit the subcutaneous proliferation of virus negative cell lines in nude mice. Further, nothing is known about the growth characteristics of S11 if the cell line is infused into immunocompromised mice via an intravenous route. Studies on lymphocyte responses to systemic S11 lymphoma development may be very revealing. To analyse the mechanisms by which subcutaneous S11 lymphoma development is curtailed, it may be possible to recover and re-culture graft infiltrating lymphocytes. This would enable the study of the cytotoxic capabilities of these cells or their cytokine production profiles. In a recent publication, Hung et al. (1998) demonstrated that IFN-γ and IL-4 production by CD4+ T cells resulted in the rejection of subcutaneous B16 melanomas in mice. In this model of tumour therapy, CD4+ T cells orchestrated macrophage nitric oxide (NO) production and eosinophil degranulation both of which contributed to tumour cell death. Interestingly, Hung et al. (1998) also showed
that B16 melanoma cell-specific CD8+ CTL which exhibited notable anti-tumour cell cytotoxicity \textit{in vitro} were ineffective in eliciting melanoma rejection \textit{in vivo}.

In a murine model of cellular immunotherapy for the treatment of chemically-induced sarcoma, \textit{in vivo} antibody depletion of IFN-\(\gamma\) has been used to inhibit DTH-like anti-tumour responses (Barth \textit{et al.}, 1991). This type of study, in conjunction with an analysis of cytokine production by S11 cells, macrophages and re-stimulated lymphocytes, may provide some important clues to the mechanism of S11 tumour regression.

Nude mice have a partially functional B lymphocyte system and antibody responses to human antigens have been demonstrated in these animals (Sprent, 1974). It is possible that some form of anti-S11 antibody response might be mediating the rejection of S11 tumours. This could be operating via an antibody-dependent cellular cytotoxicity-type mechanism.

In future experiments, variations in the timing of therapeutic lymphocyte infusions could be investigated since it is not known if lymphocyte transfers into nude mice will elicit the regression of established tumours.

The work discussed in this chapter has demonstrated that CD4+ T lymphocytes can inhibit the proliferation of S11 \textit{in vivo}. Unfortunately, it is unclear whether these cells exert their effects directly or indirectly. This model of S11 tumour regression seems to share a number of features with murine
models of BL development and immunotherapy. This is in agreement with the findings of Usherwood et al. (1996b) who proposed that phenotypically, S11 more closely resembles BL lines than LCL. However, in vitro studies have shown that S11 is more ‘immunogenic’ than BL lines which express a tightly restricted EBV latent protein profile. In the future, characterisation of MHV-68 infection of S11 cells may reveal that this cell line displays characteristics of both LCL and BL lines. It is hoped that further study in this field will produce information which is applicable to the treatment of human diseases such as EBV-LPD, BL or even Kaposi’s sarcoma.
Chapter 5

In vivo CD4+ and CD8+ T lymphocyte responses to murine gammaherpesvirus 68
5.1 Introduction

Human CD8+ CTL responses to EBV during infectious mononucleosis (IM) and long-term infection are now well characterised from *in vitro* investigations (Rickinson and Moss, 1997). Both *in vitro* and *in vivo*, the biology of the gammaherpesvirus MHV-68 seems to differ in certain key aspects from its human counterpart. One obvious example is that MHV-68 does not transform B lymphocytes in culture. As reported in the previous chapter, the *in vitro* assay for the measurement of anti-viral CTL responses proved to be unreliable. An alternative approach was to investigate T lymphocyte responses to MHV-68 infection *in vivo*.

As previously discussed, most individuals 'acquire' EBV early in life and at this time infection with the virus is not usually associated with any signs of illness. As a result, immune responses during primary, asymptomatic infection of humans are poorly characterised. In the 'Western' world, infection with EBV is occasionally delayed until the late teens or early twenties. Approximately 50% of people infected with EBV at this time develop infectious mononucleosis (IM). It is this group of individuals which has served as the principal source of information on 'primary' immune responses to EBV infection. Immune responses to EBV during IM and long-term infection are reviewed in detail in sections 1.8.3 and 1.8.4 of this thesis. It is clear from the current literature that a great deal of information has been gathered on T lymphocyte response to EBV during IM. However, it is currently not known if the well characterised immune responses, which develop during IM, reflect those which develop in young children following primary EBV infection. Further, it is unclear whether
immune responses of humans to EBV resemble the immune responses of other species to gammaherpesvirus infection. The infection of inbred laboratory mice with MHV-68 provides an amenable model for the study of acute and latent gammaherpesvirus infection and may help to clarify some of the uncertainties mentioned above.

The characteristics of MHV-68 infection of inbred mouse strains are described in section 1.10.1. However, it seems pertinent to summarise the major features of the process at this stage. Briefly, intranasal inoculation of inbred mice with MHV-68 results in the lungs of these animals becoming productively infected with the virus. Virus titres peak in the lungs around 4 days after infection and MHV-68 is ‘cleared’ from this site approximately 10 days after infection. By this time the virus has spread to the spleen where it adopts a latent form of infection in B lymphocytes (Sunil-Chandra et al., 1992b). Numbers of MHV-68-infected B lymphocytes peak around 15 days after infection. This is accompanied by a notable splenomegaly in which all lymphocyte subsets are expanded. Three to 4 weeks after infection, a marked decline in the number of MHV-68-infected B cells occurs and the splenomegaly is no longer apparent. In the long term, latently-infected B lymphocytes are detectable for the life of the animal.

Several studies have highlighted the importance of CD8+ T lymphocytes in the immune responses of mice to MHV-68 infection. Ehtisham et al. (1993) demonstrated that the depletion of CD8+ T cells from mice, prior to intranasal infection, resulted in mice dying due to an overwhelming viral replication in the
lungs. Further, Weck et al. (1996) showed that intraperitoneal infection of \( \beta_2 \) microglobulin knockout mice with MHV-68, results in these animals being incapable of clearing infectious virus from the spleen. Finally, in the past year, Stevenson et al. (1998) have characterised CD8+ CTL responses to acute MHV-68 infection using an in vitro cytotoxicity assay.

The aim of the work described in this chapter was to analyse in vivo the functional roles of CD8+ (and latterly CD4+) T lymphocytes during acute and persistent MHV-68 infection of mice. It was hoped that results from this study might lead to an understanding of cellular responses to MHV-68 and, in turn, give some insight into whether in vivo immune responses of mice to the virus resemble those of humans to EBV infection. To investigate T cell responses to MHV-68 in vivo, monoclonal antibodies were used to deplete specific T lymphocyte subsets in BALB/c mice. This approach has been used successfully on a number of occasions to study T cell responses to HSV and CMV (Nash et al., 1987, Koszinowski et al., 1990). To ensure that injections of antibody were effectively depleting specific T cell subsets from mice, flow cytometric analyses of splenocytes were undertaken. The results from these analyses are presented alongside the appropriate experiment and are discussed later in this chapter. All of the results described in this chapter were statistically analysed using Student’s T test with Bessel’s correction for small group sizes (as described in Moroney, 1970). Data described as significant had a 'p' value of less than 0.05. This value represents a confidence interval of 95%.
5.2 CD8+ T lymphocyte responses to MHV-68 infected B lymphocytes during the resolution of splenomegaly

It is well documented that CD8+ T lymphocytes are critical in preventing EBV reactivation and virus-induced lymphomagenesis in humans. As discussed earlier, Ehtisham et al. (1993) showed that CD8+ T cells are also important in the control of acute MHV-68 infection of mice. Sunil-Chandra et al. (1992a) demonstrated that numbers of splenic MHV-68-infected B lymphocytes peak approximately 2 weeks after infection. Following this peak, there is a decline in the number of latently-infected B cells detectable by infectious centre assay. At the outset of this study it was felt that this decline might be occurring for a number of reasons. Firstly, from day 15 post-infection onwards, MHV-68 may be adopting some form of non-reactivatable, latent infection in a proportion of B cells. Such a restricted infection would mean the virus would be undetectable by infectious centre assay. Alternatively, or in conjunction with this, if murine immune responses to MHV-68 resembled those of humans to EBV, the reduction in latently-infected B cell numbers might be occurring due to CTL killing of a proportion of infected B lymphocytes. If this were true, MHV-68-infected B cells, which are detectable for the life of the animal might represent a sub-population of infected lymphocytes which are immunologically unreactive. Finally, the decline in B cell numbers might represent the apoptosis of activated B lymphocytes. This event may be triggered directly by the virus or by other cells during the resolution of splenomegaly.

To begin this series of experiments, a pilot study was undertaken. The aim of this study was to investigate whether CD8+ T cells were responsible for the
decline in MHV-68 infected B lymphocytes observed in mice 2 to 3 weeks after infection. Two groups of mice were infected intranasally with MHV-68. From day 10 after infection onwards, one group of mice was depleted of CD8+ T lymphocytes as described in section 2.2.36 of this thesis. Spleens were removed from both groups of mice at regular intervals and the numbers of latently infected B cells were quantified by infectious centre assay. The results from this experiment are presented in Figure 5.1. To ensure that antibody depletions were successful, splenocytes were analysed by flow cytometry at days 15 and 25 after infection. The results of this analysis are summarised in Figure 5.2.

In this pilot study, numbers of latently-infected B lymphocytes on day 15 post-infection were significantly higher in depleted mice than in control animals. Twenty one days after inoculation with MHV-68, numbers of infected B lymphocytes in depleted and control animals were not significantly different. Figure 5.2 shows that negligible levels of CD8+ T lymphocytes were present in depleted mice 15 days after infection. However, on day 25 a potentially important population of CD8+ T lymphocytes had returned to the spleens of these animals. The implications of this result are discussed later in this chapter.

This pilot study suggested that CD8+ T lymphocytes were not responsible for the decline in numbers of latently infected B cells from day 15 onwards in MHV-68 infected mice. However, CD8+ T lymphocytes did appear to play a key role in determining the extent of B lymphocyte infection up to 2 weeks
Figure 5.1 Infectious centres in spleens of mice depleted of CD8+ T lymphocytes from day 10 after infection onwards. BALB/c mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68. On day 10 after infection half of the mice were injected intravenously with 1mg of YTS-169 rat anti-mouse CD8 antibody in a 100μl volume. Two days later the same group of mice were injected IP with 1mg of YTS-169 antibody. Further IP injections of the antibody were then given weekly until the end of the experiment. To ensure depletion of the CD8+ T lymphocytes, splenocytes were analysed by flow-cytometry. Mice were sampled at the times indicated when spleens were removed and infectious centre assays undertaken. (•) Control, (■) day 10 depleted mice.
Figure 5.2 Flow cytometric analysis of splenocytes from mice depleted of CD8+ T lymphocytes from day 10 after infection with murine gammaherpesvirus 68. BALB/c mice were infected with $4 \times 10^5$ PFU murine gammaherpesvirus 68. On day 10 after infection half of the mice were injected intravenously with 1mg of YTS-169 rat anti-mouse CD8 antibody in a 100μl volume. Two days later the same group of mice were injected IP with 1mg of YTS-169 antibody. Further IP injections of the antibody were then given weekly until the end of the experiment. To ensure depletion of the CD8+ T lymphocyte subset, splenocytes were analysed by flow-cytometry on day 15 and day 25 after infection. Briefly, $10^6$ control and depleted splenocytes from each animal were stained with FITC-conjugated anti-mouse CD8 antibody and were then analysed as described in section 2.2.37. The percentage of CD8+ lymphocytes in each animal was assessed. The graph above shows the mean percentage of CD8+ lymphocytes/ mouse for each group. Error bars represent the standard error of the mean.
after virus inoculation. Average numbers of latently-infected B cells were approximately 6-fold greater in depleted animals compared to the control group of mice at this time point.

To discover more about CD8+ T lymphocyte responses to MHV-68, a more systematic *in vivo* analysis was required. Therefore, T lymphocytes were depleted from groups of BALB/c mice at various times before or after infection with MHV-68. At regular intervals after infection, lungs were removed from mice and viral titres were assessed. Further, spleens were obtained from mice and numbers of infected B cells were quantitated by infectious centre assay. As before, to confirm the success of CD8+ T lymphocyte depletions, splenocyte populations were analysed by flow-cytometry.

Figure 5.3 shows that viral titres in the lungs of control animals peaked on day 4 in this experiment. However, by day 8 no detectable virus was present in the lungs of these animals. In contrast, mice depleted of CD8+ T lymphocytes prior to infection failed to control MHV-68 replication in their lungs and titres rose from day 4 onwards. By day 11 all of these mice had died. Such results are consistent with those of Ehtisham *et al.* (1993).

In mice depleted of CD8+ T lymphocytes from day 3 after infection, viral titres rose to a peak on day 15. At this time, these mice exhibited many signs of illness including ruffled fur and crouched posture. However, all of these animals survived and over the next two weeks viral titres fell. On day 29, a mean of 214 PFU of virus was detected in the lungs of these animals. Mice
Figure 5.3 Infectious virus titres in the lungs of mice depleted of CD8+ T lymphocytes prior to or after infection with murine gammaherpesvirus 68. 85 BALB/c mice were divided into 5 groups. All of the mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 4 out of the five groups of mice being investigated were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 (○) were control mice which did not receive any anti-CD8 antibody. Group 2 mice (■) were depleted of CD8+ T cells 2 days prior to infection and then every 7 days for the course of the experiment. All group 2 mice died on day 12 of this experiment. Group 3 mice (▲) were depleted of CD8+ T lymphocytes from day 3 after infection. Group 4 (◆) mice were depleted of CD8+ T cells from day 7 onwards and group 5 which contained 5 mice was depleted from day 10 after infection until completion of the experiment (results not shown as data not significantly different from data presented in Figure 5.1). As before, to ensure depletion of the CD8+ T lymphocyte subset, splenocytes were analysed by flow cytometry. Mice were sampled at the times indicated when lungs were removed for subsequent disruption and titration. The limit of viral detection (10PFU) is represented by a dotted line.
depleted of CD8+ T lymphocytes from 7 days after infection onwards displayed a similar lung infection profile to the control animals. On day 21, a very low and statistically insignificant level of virus reappeared in the lungs of these mice.

Figure 5.4 shows how the number of latently infected B cells in the spleens of CD8+ T cell depleted mice varied over the course of the experiment. Very few latently-infected B lymphocytes were detected in control mice 8 days after infection with MHV-68. However, in agreement with previous experiments, numbers of infected B lymphocytes peaked in these animals on day 15 and then decreased to a level at the limit of detection for the course of this experiment. Mice depleted of CD8+ T cells prior to infection had a significantly greater number of latently-infected B cells in their spleens (relative to other groups in this study) on day 8 and, as stated above, by day 11 all of these mice had died. The depletion of CD8+ T cells from mice 3 days after infection resulted in a small but significant increase in numbers of latently-infected B cells in these animals on day 8 of this experiment. By day 15, this number of infected B lymphocytes had increased dramatically. Two weeks after infection, this group of mice had approximately 5 fold more infected B lymphocytes in their spleens than control animals at the same time. Notably, this number of latently-infected B cells rapidly declined over the next 7 days and on day 21 of this experiment, the number of infected B lymphocytes in these animals was on the limit of detection (10 IC/ spleen). A slight increase in numbers of infected B cells in these mice on day 29 of this experiment was found to be statistically insignificant.
Figure 5.4 Infectious centres in spleens from mice depleted of CD8+ T lymphocytes prior to or after infection with murine gammaherpesvirus 68. 85 BALB/c mice were divided into 5 groups of animals. All of the mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 4 out of the five groups of mice being investigated were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 (●) were control mice. Group 2 mice (■) were depleted of CD8+ T cells 2 days prior to infection and then for the course of the experiment. All mice in group 2 died on day 12 of this experiment. Group 3 mice (▲) were depleted of CD8+ T lymphocytes from day 3 after infection. Group 4 mice (X) were depleted of CD8+ T cells from day 7 onwards and group 5 which contained 5 mice was depleted from day 10 after infection until completion of the experiment (data not shown). To ensure depletion of the CD8+ T lymphocyte subset, splenocytes were analysed by flow cytometry. Mice were sampled at the times indicated when spleens were removed and infectious centre assays undertaken.
A very similar profile of B lymphocyte infection was observed in mice depleted of CD8+ T lymphocytes 7 days after infection. Notably, a greater variability in the number of infected B lymphocytes was observed in this group of mice on day 15 of the experiment. By day 21, numbers of IC/ spleen in this group of animals had declined to a level which was not significantly different to other groups in this study.

Flow cytometry of splenocytes from depleted mice demonstrated that several days after the initial infusion of anti-CD8 antibody, negligible levels of CD8+ T lymphocytes were present in mice. However, data presented in Figure 5.5 shows that around 3 weeks after the first injections were given, CD8+ T lymphocytes had begun to re-populate the mice used in these experiments. The implications of this are discussed later in this chapter.

This study confirmed that mice lacking CD8+ T lymphocytes cannot effectively control acute MHV-68 infection. Significantly, it also demonstrated that if CD8+ T cells are present for the first three days after infection, then mice can eventually overcome MHV-68 lung infection. Clearance of MHV-68 occurs despite greatly increased levels of virus in the lungs of mice around 2 weeks after infection. The depletion of CD8+ T lymphocytes 3 or 7 days after MHV-68 infection had a dramatic effect on numbers of latently-infected B lymphocytes in mice 15 days after infection. Removal of this lymphocyte subset resulted in a 5 fold increase in numbers of such B cells at this time. Significantly, an absence of CD8+ T lymphocytes had no effect on the decline
Figure 5.5 Flow cytometric analysis of splenocytes from mice depleted of CD8+ T lymphocytes prior to or after infection with murine gammaherpesvirus 68. 85 BALB/c mice were divided into 4 groups of 20 animals and 1 group of 5 animals. All of the mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 4 out of the five groups of mice being investigated were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 were control mice. Group 2 mice were depleted of CD8+ T cells 2 days prior to infection and then for the course of the experiment. Group 3 mice were depleted of CD8+ T lymphocytes from day 3 after infection. Group 4 mice were depleted of CD8+ T cells from day 7 onwards and group 5 which contained 5 mice was depleted from day 10 after infection until completion of the experiment (data not shown). To ensure depletion of the CD8+ T lymphocyte subset, splenocytes were analysed by flow-cytometry on day 6 and day 29 after infection. Briefly, $10^6$ control and depleted splenocytes from each animal were stained with FITC conjugated anti-mouse CD8 antibody and were then analysed as described in section 2.2.37. The percentage of CD8+ lymphocytes in each animal was assessed. The graph above shows the mean percentage of CD8+ lymphocytes/ mouse for each group. Error bars represent the standard error of the mean.
of infectious centre numbers in these animals between 15 and 21 days after infection.

5.3 How does the depletion of CD8+ T lymphocytes from day 3 after inoculation alter long-term MHV-68 infection?

The depletion of CD8+ T lymphocytes from mice 3 days after MHV-68 infection, resulted in several intriguing observations which warranted further study.

BALB/c mice were again infected with MHV-68 and depleted of CD8+ T lymphocytes 3 days after infection. One group of mice received injections of depleting antibody on days 3 and 5 after infection and then weekly for the course of the study. In addition, a further group of mice were injected with antibody on days 3 and 5 after infection and received no further infusions for the course of the investigation. This group of mice were included in an attempt to investigate how the re-population of depleted mice with CD8+ T lymphocytes would affect long-term MHV-68 infection. Lungs and spleens were removed from mice at regular intervals until 60 days after infection. In this experiment, half spleens were fixed, embedded in paraffin wax and 6µm thick tissue sections were obtained. Spleen sections were then analysed for either MHV-68 tRNA expression by in situ hybridisation or MHV-68 lytic antigen expression by immunocytochemistry, the results of which are discussed later.
Figure 5.6 shows that mice depleted of CD8+ T cells failed to clear MHV-68 from their lungs until around 2 to 3 weeks after infection. Viral titre in the lungs of these animals peaked between 9 and 13 days after infection. In contrast, MHV-68 was almost undetectable in the lungs of control animals by day 8 of this experiment. A peak in the number of MHV-68 infected B lymphocytes was observed 14 days after infection in all mice. Once again, numbers of infected B cells at this time point were notably higher in mice depleted of CD8+ T cells from day 3 after infection onwards (Figure 5.7). By day 26 of this experiment, numbers of infectious centres in CD8+ T lymphocyte-depleted mice were no different to those found in control animals.

It has been suggested that the expression of MHV-68 tRNA-like sequences within cells may be a useful marker of MHV-68 latent infection (Bowden et al., 1997). To investigate how numbers of tRNA+ve cells varied in spleens of normal mice and mice depleted CD8+ T cells from day 3 onwards, spleen sections were subject to an in situ hybridisation analysis as described in section 2.2.40 of this thesis. In parallel with this in situ analysis, spleen sections were also stained with anti-MHV-68 hyperimmune serum. It was hoped these analyses would enable a comparison to be made of latently- and productively- infected cell numbers in the spleens of normal and CD8+ T cell depleted mice. Data from the in situ hybridisation analysis are presented in Figure 5.8. In all groups of mice, numbers of tRNA positive cells peaked on day 14 of this experiment. However, numbers of these cells were notably greater at this time in groups of CD8+ T lymphocyte-depleted mice. tRNA positive cell numbers rapidly dropped to zero in control animals by day 26.
Figure 5.6 Infectious virus titres in the lungs of mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 45 BALB/c mice were divided into 3 groups of animals. All of the mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 2 out of the 3 groups of mice being investigated were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 (○) were control mice. Group 2 mice (■) were depleted of CD8+ T cells 3 days after infection and then for the course of the experiment. Group 3 mice (△) were injected with depleting antibody on days 3 and 5 after infection and then received no further injections for the course of the experiment. To ensure depletion of the CD8+ T lymphocyte subset, splenocytes were analysed by flow cytometry at times after depletion. Mice were sampled at the times indicated when lungs were removed for subsequent disruption and titration. The limit of viral detection (10PFU) is represented by a dotted line.
Figure 5.7 Infectious centres in spleens from mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 45 BALB/c mice were divided into 3 groups of 15 animals. All of the mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 2 out of the 3 groups of mice being investigated were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 (●) were control mice. Group 2 mice (■) were depleted of CD8+ T cells 3 days after infection and then for the course of the experiment. Group 3 mice (▲) were injected with depleting antibody on days 3 and 5 after infection and then received no further injections for the course of the experiment. To ensure depletion of the CD8+ T lymphocyte subset, splenocytes were analysed by flow cytometry at times after depletion. Mice were sampled at the times indicated when spleens were removed and infectious centre assays undertaken.
Figure 5.8 tRNA positive cells in spleen sections from mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 45 BALB/c mice were divided into 3 groups of 15 animals. All of the mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 2 out of the 3 groups of mice being investigated were depleted of CD8 T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 ( ) were control mice. Group 2 mice ( ) were depleted of CD8+ T cells 3 days after infection and then for the course of the experiment. Group 3 mice ( ) were injected with depleting antibody on days 3 and 5 after infection and then received no further injections for the course of the experiment. Mice were sampled at regular intervals and spleen sections were analysed by in situ hybridisation as detailed in section 2.2.41 of materials and methods. Four spleen sections per group were analysed for tRNA expression at each time point and the mean number of positive cells per section was calculated. Error bars represent the standard error of the mean value.
post infection. The decline in detectable tRNA staining in depleted mice was less dramatic, taking between 40 and 60 days. Figure 5.9 shows that productively-infected cell numbers in control spleens were negligible throughout this experiment. In contrast, a peak in MHV-68 viral antigen staining was observed 14 days after infection in CD8+ T lymphocyte depleted mice. By day 26 of this experiment, no detectable viral antigen staining was observed by immunohistochemistry in either control or CD8 depleted animals. The photographs presented in Figure 5.10 and 5.11 show representative spleen sections on which tRNA and anti-MHV-68 serum staining was observed. In general, tRNA-positive cells were only observed within B lymphocyte follicles of the spleen. In contrast, MHV-68 positively stained cells were found to be distributed throughout the sections analysed.

Flow cytometric analysis of splenocytes on day 7 of this experiment confirmed that initial infusions of anti-CD8 antibody had reduced numbers of CD8+ T lymphocytes to a negligible level. Data from this analysis are presented in Figures 5.12 and 5.13. In contrast, by day 41 of this experiment, CD8+ T lymphocytes had re-populated both groups of 'depleted' animals. On day 41, around 5% of splenocytes were CD8+ in mice receiving antibody infusions for the course of the experiment. In mice which only received antibody infusions at the outset of this study, around 8% of splenocytes were CD8+ on day 41 of the experiment. The implications of this CD8+ T lymphocyte re-population are discussed later in this chapter.
Figure 5.9 Viral antigen-positive cells in spleen sections from mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 45 BALB/c mice were divided into 3 groups of 15 animals. All of the mice were infected intranasally with $4 \times 10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 2 out of the 3 groups of mice being investigated were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 (–♦–) were control mice. Group 2 mice (–––) were depleted of CD8 T cells 3 days after infection and then for the course of the experiment. Group 3 mice (––△–) were injected with depleting antibody on days 3 and 5 after infection and then received no further injections for the course of the experiment. Mice were sampled and tissues analysed by immunohistochemistry as detailed in section 2.2.47 of materials and methods. Four spleen sections per group were analysed for MHV-68 lytic cycle antigen expression at each time point and the mean number of positive cells per section was calculated. Error bars represent the standard error of the mean value.
Figure 5.10  tRNA positive cells in spleen sections from mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 6μM thick sections were cut from formalin fixed paraffin-embedded spleen tissue and an in situ hybridisation analysis was undertaken as outlined in section 2.2.41. Sections A and B were hybridised with a sense ‘T3’ probe and are negative for staining. Sections C and D were hybridised with an antisense ‘T7’ probe and positive staining can be observed. The probes used in this experiment were specific for the latency-associated tRNA-like molecules of MHV-68. Sections were counter-stained with 1% neutral red. Arrows show positively stained cells. Panels A and C, x 4 (bar represents 0.5mm), panels B and D, x 40 (bar represents 0.05mm).
Figure 5.11 Infectious virus positive cells in spleen sections from mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 6μM thick sections were cut from formalin fixed paraffin-embedded spleen tissue and immunostaining was undertaken as outlined in section 2.2.47. Sections A and B are negative for anti-MHV-68 hyperimmune serum staining. Sections C and D show positive staining with anti-MHV-68 hyperimmune serum. Sections were counterstained with haematoxylin. Arrows show positively stained cells. Panels A and C, x 4 (bar represents 0.5mm), panels B and D, x 40 (bar represents 0.05mm).
Figure 5.12 Flow cytometric analysis of splenocytes from mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 45 BALB/c mice were divided into 3 groups of 15 animals. All of the mice were infected intranasally with 4 x 10^5 PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 2 out of the 3 groups of mice being investigated were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 were control mice. Group 2 mice were depleted of CD8+ T cells 3 days after infection and then for the course of the experiment. Group 3 mice were injected with depleting antibody on days 3 and 5 after infection and then received no further injections for the course of the experiment. To ensure depletion of the CD8+ T lymphocyte subset, splenocytes were analysed by flow-cytometry on day 7 and day 41 after infection. Briefly, 3 aliquots of 10^6 control and depleted splenocytes from each animal were stained with FITC conjugated anti-mouse CD8 antibody and were then analysed as described in section 2.2.37. The percentage of CD8+ lymphocytes in each animal was assessed. The graph above shows the mean percentage of CD8+ lymphocytes/ mouse for each group. Error bars represent the standard error of the mean.
Figure 5.13 Representative data from the flow cytometric analysis of splenocytes from mice depleted of CD8+ T lymphocytes 3 days after infection with murine gammaherpesvirus 68. 3 groups of mice were infected intranasally with murine gammaherpesvirus 68. 2 out of the 3 groups of mice were depleted of CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-169 rat anti-mouse CD8 monoclonal antibody. Group 1 were control mice. Group 2 mice were depleted of CD8+ T cells 3 days after infection and received antibody injections for the course of the experiment. Group 3 mice were injected with depleting antibody on days 3 and 5 after infection and then received no further injections. To ensure depletion of the CD8+ T cell subset, splenocytes were from each animal were analysed by flow cytometry on day 7 and 41 after infection (as described in section 2.2.37). Histograms represent CD8+ T lymphocyte staining in a representative animal from each group.
This experiment confirmed that mice depleted of CD8+ T lymphocytes from day 3 after infection with MHV-68 can ultimately clear the virus from their lungs. Further, it demonstrated again that CD8+ T lymphocytes do not play a role in the decline of latently-infected B lymphocyte numbers from day 15 after infection onwards. In this study, the analysis of CD8+ T cell numbers at late times after infection further highlighted an inherent problem in long term antibody depletions of this lymphocyte subset. By day 41 of this experiment, antibody infusions were ineffective in preventing the re-population of mice by CD8+ T lymphocytes.

5.4 Do CD4+ T lymphocytes play a role in immune responses to acute MHV-68 infection?

Over the course of the experiments described in this chapter, several important questions arose in relation to the immune responses of mice to acute MHV-68 infection. One of these questions was: how do mice ‘control’ acute MHV-68 lung infection in the absence of CD8+ T lymphocytes from day 3 after infection onwards? The most obvious answer to this question was that CD4+ T lymphocytes were playing a role, either directly or indirectly, in protecting mice from an overwhelming MHV-68 infection in the absence of CD8+ T cells. To investigate this theory, CD4+ and/ or CD8+ T lymphocytes were depleted from mice at a range of times before or after infection. Again, to ensure that antibody infusions were successfully depleting animals of T lymphocyte subsets, flow cytometric analyses of mediastinal and/ or spleen cell populations were undertaken.
Figure 5.14 shows how the MHV-68 titre varied in the lungs of mice in this experiment. Virus titre in the lungs of control animals peaked between 4 and 6 days after MHV-68 infection. By day 11, infectious virus was undetectable in the lungs of the control mice used in this experiment. In agreement with previous studies, when CD4+ or CD8+ T lymphocytes were depleted from mice prior to or 3 days after infection respectively, clearance of MHV-68 from the lungs was delayed. In mice depleted of CD4+ T lymphocytes prior to infection, viral titre in the lungs peaked on day 6 and fell to an undetectable level by day 13. Viral titre in the lungs of CD8+ T cell-depleted mice peaked 11 days after MHV-68 infection and fell to an undetectable level by day 25. Significantly, when both T lymphocyte subsets were depleted from mice, the animals were unable to control MHV-68 replication in the lungs and ultimately died. When mice lacking CD4+ T cells were administered with anti-CD8 antibody from day 3 after infection onwards, lung virus titres peaked in these animals on day 11. All members of this group died on day 12. Similarly, when mice lacking CD4+ T cells were administered with anti-CD8 antibody from day 7 onwards, lung virus titres peaked in these animals on day 19 of this experiment. By this time all but two of the mice in this group were dead.

During the first 11 days of this experiment, flow cytometric analyses of spleen and mediastinal lymph node cell populations confirmed that CD4+ or CD8+ T lymphocytes had been effectively depleted. The results of these analyses are summarised in Figures 5.15 and 5.16. Figure 5.15 shows that numbers of CD4+ T lymphocytes were reduced to negligible levels after animals had received monoclonal anti-CD4 antibody infusions. Mediastinal lymph nodes
Figure 5.14 Infectious virus titres in the lungs of mice depleted of CD4+ or CD8+ T lymphocytes prior to or after infection with murine gammaherpesvirus 68. 75 mice were divided into 5 groups of animals. All of the mice were infected intranasally with 4 x $10^5$ PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 4 out of the 5 groups of mice being investigated were depleted of CD4+ and/ or CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-191 and/ or YTS-169 rat anti-mouse CD4 or CD8 monoclonal antibody respectively. Group 1 (●) were control mice. Group 2 mice (■) were depleted of CD4+ T lymphocytes 2 days prior to infection and then for the course of the experiment. Group 3 mice (▲) were depleted of CD8+ T lymphocytes from day 3 after infection. Group 4 mice (★) were depleted of CD4+ T lymphocytes prior to infection and CD8+ T lymphocytes 3 days after infection. All mice in group 4 died around day 12 after infection. Group 5 mice (▲) were depleted of CD4+ T lymphocytes prior to infection and CD8+ T lymphocytes 7 days after infection. All group 5 mice died around day 19 post infection. To ensure depletion of the T lymphocyte subsets was successful, splenocytes were analysed by flow-cytometry at times after depletion. Mice were sampled at days 4, 6, 11, 13 and 25 post infection when lung tissues were removed and frozen for subsequent virus titration.
Figure 5.15 Flow cytometric analysis of CD4+ T lymphocytes from mice depleted of CD4+ and CD8+ T lymphocytes prior to or after infection with murine gammaherpesvirus 68. 5 groups of mice were infected with 4 x10^5 PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 4 of the 5 five groups of mice were depleted of CD4+ and/ or CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-191 or YTS-169 rat anti-mouse CD4 or CD8 antibody respectively. Group1 were control mice. Group 2 mice were depleted of CD4+ T lymphocytes 2 days prior to infection and then for the course of the experiment. Group 3 mice were depleted of CD8+ T lymphocytes from day 3 after infection. Group 4 mice were depleted of CD4+ T lymphocytes prior to infection and CD8+ T lymphocytes 3 days after infection. All mice in group 4 died around day 12 after infection. Group 5 mice were depleted of CD4+ T lymphocytes prior to infection and CD8+ T lymphocytes 7 days after infection. All group 5 mice died around day 19 after infection. To ensure that depletion of T lymphocyte subsets was successful, splenocytes or mediastinal lymph node (MLN) cells from representative animals were analysed by flow-cytometry on day 4 (■), day 6 (■) and day 11 (□) after infection. Briefly, 10^6 control and depleted lymphocytes were stained with either phycoerythrin-conjugated anti-CD4 antibody or FITC-conjugated anti-CD8 antibody and were then analysed as in section 2.2.37. The percentage of CD8+ lymphocytes in each animal was assessed. The graph above shows the mean percentage of CD4+ lymphocytes/ mouse for each group. Error bars represent the standard error of the mean.
Figure 5.16 Flow cytometric analysis of CD8+ T lymphocytes from mice depleted of CD4+ and CD8+ T lymphocytes prior to or after infection with murine gammaherpesvirus 68. 5 groups of mice were infected with 4 x10^5 PFU of murine gammaherpesvirus 68 on day 0 of the experiment. 4 of the 5 five groups of mice were depleted of CD4+ and/ or CD8+ T lymphocytes by intravenous and subsequently intraperitoneal injection of YTS-191 or YTS-169 rat anti-mouse CD4 or CD8 antibody respectively. Group 1 were control mice. Group 2 mice were depleted of CD4+ T lymphocytes 2 days prior to infection and then for the course of the experiment. Group 3 mice were depleted of CD8+ T lymphocytes from day 3 after infection. Group 4 mice were depleted of CD4+ T lymphocytes prior to infection and CD8+ T lymphocytes 3 days after infection. All mice in group 4 died around day 12 after infection. Group 5 mice were depleted of CD4+ T lymphocytes prior to infection and CD8+ T lymphocytes 7 days after infection. All group 5 mice died around day 19 after infection. To ensure that depletion of T lymphocyte subsets was successful, splenocytes or mediastinal lymph node (MLN) cells from representative animals were analysed by flow-cytometry on day 4 (■), day 6 (■) and day 11 (□) after infection. Briefly, 10^5 control and depleted lymphocytes were stained with either phycoerythrin-conjugated anti-CD4 antibody or FITC-conjugated anti-CD8 antibody and were then analysed as in section 2.2.37. The percentage of CD8+ lymphocytes in each animal was assessed. The graph above shows the mean percentage of CD8+ lymphocytes/ mouse for each group. Error bars represent the standard error of the mean.
were never successfully recovered from animals which had received infusions of T lymphocyte-depleting antibody. Figure 5.16 shows that antibody infusions were successful in depleting CD8+ T lymphocytes from mice until at least day 11 of this experiment. Unfortunately, after day 11 of this study, facilities for the flow cytometric analysis of lymphocytes became unavailable.

This experiment demonstrated that both CD4+ and CD8+ T lymphocytes almost certainly play a significant role in the control of acute MHV-68 infection of mice.
5.5 Discussion

The experiments described demonstrate that both CD4+ and CD8+ T lymphocytes contribute to the clearance of MHV-68 from the lungs of mice during the acute phase of infection. Further, the results of this study indicate that CD8+ T lymphocytes are not responsible for reducing numbers of virally-infected B lymphocytes in mice 3 to 4 weeks after infection.

A significant number of studies have demonstrated that CD8+ T lymphocytes are often critical in the immune responses of humans and mice to acute and persistent viral infections. For example, CD8+ CTL are required for the termination of primary influenza virus infection (Karzon, 1996). Further, CTL also prevent virus-induced B cell lymphoma development and viral reactivation in individuals infected with EBV (Rickinson and Moss, 1997). The aim of the work described was to characterise in vivo CD8+ T lymphocyte responses to the gammaherpesvirus MHV-68. It was hoped this study might reveal whether the immune responses of mice to MHV-68 resemble those of humans to EBV.

As discussed previously, little is known about how most humans respond to EBV on primary exposure to the virus. Current data available on immune responses to primary EBV infection has been gathered from the relatively small group of patients who have acquired the virus relatively late in life and who have developed infectious mononucleosis. However, the majority of humans 'acquire' EBV during the first few years of life and in these individuals the infection is usually 'silent'. The infection of inbred laboratory mice with
MHV-68 provides a model system for the study of immune responses to acute and persistent gammaherpesvirus infection. To investigate T lymphocyte responses to MHV-68 in vivo, monoclonal antibodies were used to deplete specific T cell subsets from BALB/c mice. This proved to be a useful technique for studying the importance of T lymphocyte responses during the acute phase of infection. To simplify this discussion, T lymphocyte responses during acute and long-term MHV-68 infection will be considered separately. A summary later in this chapter will provide an overview of immune responses to both phases of infection.

5.5.1 T lymphocyte responses to acute MHV-68 infection

The depletion of CD8+ T lymphocytes from mice prior to MHV-68 infection resulted in all animals dying due to uncontrolled viral replication in the lungs. This result was in agreement with the findings of Ehtisham et al. (1993). In a more recent study, Weck et al. (1996) demonstrated that β2-microglobulin knockout mice, which lack functional CD8+ CTL, do not die following intraperitoneal infection with MHV-68. However, these mice do have an impaired ability to clear infectious virus from the spleen. These studies emphasise that immune responses to MHV-68 differ depending on the route and dose of virus that is administered to mice.

In this study, when CD8+ T lymphocytes were depleted from mice 3 days after infection, clearance of MHV-68 from the lungs was delayed. However, these mice eventually overcame the viral infection and survived. Viral titres in the lungs peaked between 12 and 15 days after infection. In contrast, when CD8+
T lymphocytes were depleted from mice 7 days after infection, a normal profile of viral clearance from the lungs was observed. These data suggest that infusion of antibody on day 3 after infection resulted in the depletion of a functionally significant population of lymphocytes which express CD8. Unfortunately, the lineage of these functional cells has not been confirmed. Stevenson and Doherty (1998) demonstrated that MHV-68-specific CD8+ CTL appear in bronchoalveolar lavage fluid of mice 6 or 7 days after intranasal MHV-68 infection. At this time cytolytic responses are at the limit of detection. Therefore, it is unlikely that conventional MHV-68-specific T lymphocyte responses were generated 2 to 3 days after infection in the experiments described here. However, it is possible that virus-specific CD8+ T lymphocytes release cytokines whilst they were being primed at early times after infection. Sarawar et al. (1996) demonstrated that significant quantities of IFN-γ and IL-6 are produced by lymphocytes from the mediastinal lymph nodes of mice 5 days after MHV-68 infection. Very early IFN-γ production might serve to activate macrophage populations in the lungs of infected mice. However, current evidence suggests that IFN-γ does not play a significant role in the clearance of MHV-68 during the acute phase of infection (Sarawar et al., 1997). Further, evidence on the importance of macrophages in the immune response of mice to MHV-68 is conflicting. Kulkami et al. (1997) recently demonstrated that mice lacking inducible nitric oxide synthase (iNOS), a key functional enzyme of macrophages, all die when infected with MHV-68. In contrast, in this laboratory it was found that iNOS knockout mice all survived MHV-68 infection and displayed a normal pattern of lung and B lymphocyte infection (Dutia, B.M., Personal communication). Interestingly, IFN-α/β
knockout mice all die when infected with MHV-68 due to an overwhelming lung infection. Macrophages are a major source of type 1 interferons and the inability of mice to survive MHV-68 infection in the absence of these cytokines is perhaps an indication of a role for macrophages in immune responses to acute MHV-68 infection.

In addition to macrophages, natural killer (NK) cells may play a role in protecting mice from MHV-68. In the present study, intravenous infusions of anti-CD8 antibody may have depleted NK or lymphokine-activated killer (LAK) cells which express CD8 at their surface. Evidence is accumulating which suggests that a range of NK/T lymphocyte subsets can contribute to innate immune responses in humans and animals. For example, in certain strains of mice, NK-T cells have been identified which express molecules such as CD4 (Di Santo et al., 1998). These cells have been associated with the recognition of β2-microglobulin-associated CD1 molecules and the production of cytokines. However, cells of this phenotype have not been identified in BALB/c mice, which were used throughout the experiments described in this chapter. In humans, several groups have characterised CD8+ T lymphocytes within EBV-associated nasopharyngeal carcinoma lesions which display NK or LAK cell activity (Ferradini et al., 1991). Unfortunately, no systematic study of NK or LAK cell responses to MHV-68 infection of mice has been undertaken. However, several lines of evidence suggest that NK cells probably do not play a significant role in protecting mice from the virus. Usherwood et al. (1997) demonstrated that immunological control of MHV-68 was unaffected in perforin knockout mice. Under normal circumstances, NK
cells exert their cytolytic effects via perforin. Further, the treatment of mice with anti-IL-12 antibody (in an attempt to limit NK cell activation) makes little difference to the normal clearance of MHV-68 from mice (Usherwood, E., personal communication). However, there is much redundancy of function in the immune system and NK cell activation can be elicited by cytokines such as IL-2. Therefore, these latter experiments do not rule out the possibility that NK or LAK cells play a functional role at early times after MHV-68 infection although available evidence suggests that this is unlikely.

It has been suggested that infusions of monoclonal antibody may not be effective in depleting T cells from mice (Doherty, P. Personal communication). There is a possibility that a down-regulation of CD8 expression occurs at the surface of activated lymphocytes in some circumstances. However, there is little data available which indicates that the activation of CD8+ T cells during viral infections results in a reduction in the expression of CD8 at the cell surface (Cobbold, S., personal communication). Flow-cytometric analyses of splenocytes throughout this study demonstrated that initial infusions of anti-CD8 antibody effectively depleted CD8+ T lymphocytes from the spleen. Further, mediastinal lymph nodes were undetectable in mice which had received antibody infusions. This suggested that lymphocyte populations had been effectively removed from this peripheral site.

The experiments described in this chapter indicate that CD4+ and CD8+ T lymphocytes can act together to effect the clearance of MHV-68 from the lungs of infected mice. When CD8+ T lymphocytes were depleted from mice 3
days after infection, clearance of MHV-68 was delayed. However, when CD8+ T cells were depleted from day 7 after infection a normal profile of lung clearance was observed. Significantly, when both T lymphocyte subsets were depleted from mice, uncontrolled MHV-68 replication was observed in the lungs of mice.

It is likely that CD4+ T lymphocytes exert their anti-viral effects in this system via the production of cytokines. There is currently no evidence that CD4+ CTL play a significant role in immune responses to MHV-68. However, Stevenson and Doherty (1998) have shown that CD8+ CTL are critical in immune responses to the virus. In the present study, mice which received anti-CD4 antibody prior to infection and anti-CD8 antibody from day 7 after infection all died. This suggests that CD4+ T lymphocyte cytokine production is important in the development of effective CTL responses. It is unlikely that antibody plays a role in the clearance of MHV-68 during the acute phase of infection since virus neutralising antibody production is only detectable 2 to 3 weeks after infection. It is thought that this delay in antibody production occurs as a result of MHV-68 infection of B lymphocytes (Stewart et al., 1998c).

It is possible that CD4+ T lymphocytes limited MHV-68 replication in the lungs directly in these experiments. As discussed previously, Sarawar et al. (1996) demonstrated that IFN-γ production in MHV-68-infected mice peaks on day 5 after infection and remains high for the next 20 to 30 days. However, this group also demonstrated that mice, which are unable to produce IFN-γ due to a disruption of the gene encoding this cytokine, clear MHV-68 in a normal
fashion. Therefore, current evidence suggests that this cytokine does not play a significant role in the clearance of MHV-68 despite its potential to inhibit viral replication directly and influence the function of numerous innate and specific immune pathways (Sarawar et al., 1997).

Overall, immune responses which develop during acute MHV-68 infection of the lungs appear to be complex. Conflicting data has been obtained from studies involving differing strains of mice infected via different routes. Evidence obtained in this study suggests that lymphocytes that express CD8 play a role in limiting the very early stages of MHV-68 lung infection. However, IFNα/β production by infected cells or even macrophages is probably of equal importance in limiting MHV-68 replication at this time. Several days after infection, it seems that more conventional CD4+ and CD8+ T lymphocytes are active in containing MHV-68 replication and preventing overwhelming lung infection.

5.5.2 T lymphocyte responses during long-term MHV-68 infection
The purpose of this part of the study was to provide some insight into the long-term immune responses of mice to MHV-68. CD8+ T lymphocyte responses to the human gammaherpesvirus EBV are complex and essential in preventing virus-induced disease in infected individuals. It is thought that EBV-specific CTL act to suppress viral reactivation and prevent the expansion of EBV-transformed B Lymphocytes in the host (Rickinson and Moss, 1997). The experiments described in this chapter demonstrate that monoclonal antibody depletion of CD8+ T lymphocytes is a useful technique for studying
immune responses during the first 2 to 3 weeks after viral infection. However, this technique is of limited use in the analysis of long-term immune responses to MHV-68. Several weeks after antibody infusions began, populations of CD8+ T lymphocytes reappeared in the BALB/c mice used in these experiments. It is likely that mice receiving the rat anti-mouse monoclonal antibody developed a 'neutralising' antibody response to this protein. Such a response would render the antibody ineffective. In the past, in an attempt to alleviate this problem, groups have thymectomised mice prior to the administration of depleting monoclonal antibody (Nash et al., 1987). However, CD8+ T lymphocyte maturation can occur in peripheral lymphoid sites in mature mice. Therefore, even when the thymus is removed, CD8+ T lymphocyte re-population of mice is a significant limitation. In spite of this drawback, antibody infusions can effectively remove CD8+ T lymphocytes from mice for periods of up to 3 weeks. During this time it is possible to analyse CD8+ T lymphocyte responses to viral infection and draw conclusions from these analyses.

The data presented in this chapter show that numbers of latently infected B cells were greatly elevated in the spleens of CD8 depleted mice 2 weeks after infection. Significantly, numbers of infected B cells in CD8 depleted mice returned to a 'normal' level 7 to 14 days later. Evidence suggests that the peak in B lymphocyte infection, observed in CD8 depleted mice, is due to a lack of immunological control of MHV-68 in the spleen. When CD8+ T lymphocytes were depleted from mice 3 days after infection, a peak in lung virus titre was observed 10 or 11 days later. This elevated level of virus in the
lungs was accompanied by an increase in the number of lytic antigen-positive cells observed in the spleen. Interestingly, only a slight increase in infectious virus titre (assessed by plaque assay) was observed in the spleen at this time. In isolation, these data might suggest that sustained viral replication in the lungs of depleted mice led to a greater number of B lymphocytes becoming infected as a result of increased levels of virus reaching the spleen. However, an elevated peak in infected B lymphocyte numbers was also observed in mice depleted of CD8+ T lymphocytes from day 7 after infection. The lung infection profiles of day 7-depleted mice were identical to those of the control animals in these experiments. Therefore, CD8+ T lymphocytes appear to play a role in limiting the extent of MHV-68 infection of B lymphocytes in the spleen. Unfortunately, it is unclear how these CD8+ T cells exert their effects. Stevenson and Doherty (1998) demonstrated that CD8+ T cell responses to lytic MHV-68 antigens peak in the spleen 13 days after infection. These CTL may be responsible for the control of MHV-68-infected B cells in which lytic gene expression occurs. In control mice, little or no evidence of infectious virus or lytic gene expression was found in the spleen 2 weeks after infection. In contrast, lytic antigen expression peaked in the spleen 15 days after infection in mice depleted of CD8+ T cells from day 3 of the experiment onwards. No systematic double-staining analysis of MHV-68 infected spleen sections has ever been undertaken. Therefore, it is impossible to say whether lytic antigen-positive cells observed in these experiments were B lymphocytes. Interestingly, numbers of MHV-68 tRNA-positive cells were also elevated in the spleens of CD8 depleted mice. It has been suggested that the tRNA-like sequences of MHV-68 may be used as markers of latent
infection in the absence of viral lytic gene expression (Bowden et al., 1997). However, in this study widespread lytic cycle antigen staining accompanied tRNA expression until day 26 after infection in spleen sections from depleted mice. Therefore, although antigen and tRNA positive cells were observed in different areas of the spleen, it would be unwise to assume that tRNA-positive cells in these sections represented lymphocytes in which only a 'true' MHV-68 latent infection existed. After day 26, MHV-68 lytic antigen staining disappeared in spleen sections from CD8-depleted animals whilst tRNA positive cells could still be detected. Over the next 30 days tRNA staining also declined until positive cells were no longer detectable. 2 to 3 months after infection it is likely that MHV-68 tRNA expression occurs in so few cells that only a systematic analysis of the whole spleen would reveal any positive staining.

At present there is no satisfactory strategy for accurately quantifying the total number of tRNA-positive cells in an infected spleen. Therefore, it would be unwise to compare directly the numerical data obtained via in situ hybridisation analyses with infectious centre data. It is of interest that both of these techniques show a similar pattern of MHV-68 infection in the spleens of CD8+ T lymphocyte depleted mice.

The possibility remains that CTL specific for latent antigens of MHV-68 determine the extent of B lymphocyte infection in the spleen. However, CD8+ T cell responses to MHV-68 latent antigens have yet to be convincingly
demonstrated although some evidence is available which suggests that they do exist. This evidence is reviewed in section 3.4 of this thesis.

The elevated numbers of infected B cells observed in these experiments may be a consequence of enhanced B cell proliferation occurring in the spleen due to an absence of IFN-γ production by CD8+ T cells in this system. IFN-γ is known to suppress lymphocyte proliferation and CD8+ CTL can be a significant source of this cytokine (Boehm et al., 1997). In the future, the infection of IFN-γ knockout mice with MHV-68 may help to clarify the functions of IFN-γ in the spleen during MHV-68 infection.

5.5.3 T lymphocyte responses during the resolution of splenomegaly

A significant observation made during the course of this study was that CD8+ T lymphocytes are not responsible for the decline in the number of MHV-68 infected B cells which occurs in mice from day 15 after infection onwards. This decline in infected cell numbers was observed using both infectious centre assays and in situ hybridisation analyses of MHV-68 tRNA expression in the spleen.

In EBV-infected humans, the number of B lymphocytes that are latently infected with the virus varies between individuals, but within individuals, numbers of latently infected cells remain remarkably constant for life (Rickinson and Kieff, 1996). It is not known if the immune response which develops during primary EBV infection ultimately determines the number of B cells infected in an individual. It is known that CD8+ CTL strictly regulate the
out-growth of EBV transformed B lymphocytes and suppress viral reactivation (Rickinson and Moss, 1997). Initially, it was anticipated that CD8+ T lymphocytes could be responsible for the decline in numbers of virally infected B lymphocytes observed in mice 2 to 3 weeks after infection. However, throughout this study, the decline consistently occurred in the absence of CD8+ T cells in the spleens of mice. Usherwood et al. (1996a) demonstrated that CD4+ T cells and MHV-68-infected B lymphocytes are required for the development of splenomegaly in infected mice. It is unclear what elicits the resolution of splenomegaly and the decline in MHV-68 infected-B cells which accompanies this event. Clearly it is in the interests of the host to suppress lymphocyte proliferation and activation before this leads to immune exhaustion or tissue damage. Therefore, host regulatory mechanisms may be critical in initiating the suppression or death of activated lymphocytes in the spleen. To date, no systematic analysis of the mechanisms involved in the resolution of splenomegaly has been undertaken. However, recent work in this laboratory has demonstrated that widespread apoptosis is observed in MHV-68 infected spleens 2 to 3 weeks after MHV-68 infection (Ebrahimi, B., personal communication).

Although data obtained by in situ hybridisation and infectious centre assay suggest that a significant proportion of MHV-68 infected B cells are lost during the resolution of splenomegaly, it is not possible to confirm this beyond doubt. In the future, the identification of an MHV-68 gene which is expressed exclusively during latent infection will reveal if the virus adopts a non-reactivating form of infection in a significant proportion of B cells. Further,
such a marker will help to reveal what proportion of infected B cells die or migrate from the spleen during the resolution of splenomegaly.

It would be unwise to draw firm conclusions from data presented in this chapter on the importance of T lymphocytes in the long-term surveillance of MHV-68. It is possible that memory CTL, specific for replicative cycle antigens of MHV-68 are responsible for killing B cells in which viral reactivation is occurring. However, Stevenson and Doherty (1998) demonstrated that significant CTL responses to MHV-68-infected fibroblast targets are not maintained beyond 25 days after infection. As discussed earlier, it is currently unknown whether CTL specific for latent antigens of MHV-68 play a significant role in the long-term immune responses of mice to MHV-68. A recent study by Stewart et al. (1998a) highlights the importance of antibody in the control of MHV-68 after reactivation. Therefore, long-term immuno-surveillance of MHV-68 in the host may not require CTL. Jonjic et al. (1990) demonstrated that MCMV can be controlled by mice which lack CD8+ T cells. Further, in this system, antibody played a major role in limiting recurrent MCMV infection.

In summary, following intranasal inoculation MHV-68 replicates within the lung epithelium of mice. Recent work in this laboratory suggests that the virus is carried to the local draining mediastinal lymph nodes by dendritic cells (Selvarajah, S. personal communication). It is probable that in this site MHV-68 infection of B lymphocytes first occurs and the priming of CD4+ and CD8+ T lymphocytes is initiated. Numerous components of the immune system seem to play functional roles in the clearance of MHV-68 from the lungs of
mice. It is clear from published data and results presented in this thesis that CD8+ CTL play a major role in this process. However, IFN α/β production appears to be equally important and CD4+ T cells can contribute to some stages of viral clearance. Interestingly, IFN-γ production does not seem to play a significant role in the clearance of MHV-68. However, it is unclear at present whether responses induced by this cytokine are insignificant or whether other functional molecules have rendered this cytokine redundant. To date, no evidence had been published which supports a role for conventional NK cell responses in the clearance of MHV-68 from murine lungs. Further, little is known about the importance of macrophages in this process.

In the spleen, CD8+ CTL appear to play a role in limiting numbers of B lymphocytes which are infected with MHV-68. However, it seems that only a proportion of infected cells in this site are susceptible to CD8+ T cell mediated control. Further, it is unclear whether responding CD8+ T lymphocytes are specific for lytic and/or latent antigens of MHV-68.

Significantly, CD8+ T lymphocytes do not seem to be responsible for the decline in MHV-68 infected B cells which occurs in mice 2 to 3 weeks after infection. This may be occurring as a result of host or virus-induced cell death and the mechanisms responsible for triggering this event remain a mystery. Long-term administration of anti-CD8 antibody was ineffective in preventing CD8 T cell re-population of mice. Therefore, it is difficult to draw conclusions on the importance of T lymphocytes in the surveillance of MHV-68 at late
times after infection. However, it is possible that virus-specific antibody plays a significant role in preventing viral dissemination following reactivation.

It seems that a variety of immune system components co-operate at a range of times after MHV-68 infection to prevent the virus overwhelming the host. Despite the attentions of the immune system, MHV-68 seems to be capable of consistently establishing a latent infection within the mouse. It is difficult to draw conclusions on whether immune responses to MHV-68 infection of the mouse resemble the immune responses of humans to EBV infection. Overall, it seems that CD8+ CTL play a less significant role in the control of the murine gammaherpesvirus. Ultimately, it may not be possible to understand the immune responses of mice to MHV-68 until detailed studies of viral gene expression have been completed. Such analyses will reveal how MHV-68 regulates the expression of immunoreactive peptides and avoid immune responses which would otherwise rid the host of the virus.

In the short term, infection of CD4 and CD8 knockout mice with MHV-68 may allow us to overcome problems encountered with the long-term administration of antibody to mice. Elegant studies by Stewart et al. (1998a) demonstrated that the transfer of lymphocytes into knock-out mice can reveal much about viral tropism and long-term sites of latency. Similar studies with T lymphocyte knock-out mice may eventually lead to an understanding of how innate and adaptive immune responses operate together during MHV-68 infection. In conclusion, several arms of the immune system appear to limit acute MHV-68 replication in mice. However, it is apparent that the virus is well adapted and
can efficiently maintain a latent infection within the host despite the attentions of the immune system.
Chapter 6

Overview and Future Directions
6.0 An overview of current and future work

Since 1990 many significant advances have been made in the study of MHV-68 and its interaction with inbred laboratory mouse strains. A limited sequence analysis of MHV-68 by Efstathiou et al. (1990) demonstrated that the virus is closely related to the gammaherpesviruses of primates EBV and Herpesvirus saimiri. In 1992, Sunil-Chandra et al. (1992) showed that following intranasal infection, MHV-68 replicates in the lungs of mice and then adopts a latent infection in B lymphocytes. Recently, interest has increased in MHV-68 and this has led to differences of opinion over several features of the model. For example, Weck et al. (1996) described a latent MHV-68 infection of the spleen in μMT (B cell knockout) mice. In contrast, Usherwood et al. (1996c) failed to demonstrate any evidence of a latent spleen infection in the same strain of mice. Many of the discrepancies in data which have been published in the last 3 years have arisen as a result of differing infection strategies being adopted by groups around the world.

Perhaps the most significant recent advance in this field has been the release of the complete DNA sequence of MHV-68 (Virgin et al., 1997). To date, two independent laboratories have sequenced MHV-68 and the sequences obtained by these two groups differ by only one base pair. However, differences of opinion do exist between the two groups in relation to the number and orientation of predicted open reading frames (ORF) encoded within the genome (Virgin et al. 1997, Davison, A. Personal communication). Availability of the MHV-68 DNA sequence has led to a great deal of interest in gene products of the virus. For example, in this laboratory, work is underway
to characterise the novel ORF M2, M3 and M4 and an IL-8 receptor homologue encoded by MHV-68. In the future, it will be possible to study and characterise patterns of viral gene expression in differing cell types and anatomical sites within the host. For example, at present it is not known if MHV-68 infects the oropharyngeal tract of mice. It is possible that the virus may induce some form of nasopharyngeal carcinoma (NPC) during infection which resembles the NPC commonly observed in EBV sero-positive individuals in China and the Far-East.

In 1993, it was demonstrated that CD8+ T lymphocytes are critical in protecting mice from an overwhelming MHV-68 infection (Ehtisham et al., 1993). Antibody depletion of this lymphocyte subset in mice prior to infection results in animals dying due to uncontrolled viral replication in the lungs. In the last year, CD8+ T cell responses to MHV-68, during the acute phase of infection, have been partially characterised using an *in vitro* cytotoxicity assay (Stevenson and Doherty, 1998). Stevenson and Doherty (1998) demonstrated that CD8+ cytotoxic T lymphocytes (CTL) are detectable in the mediastinal lymph nodes and spleens of mice up to 1 month after infection. However, significant virus-specific CTL activity is not detectable in mice approximately 6 weeks after infection. Therefore, it appears that CD8+ CTL, with specificity for the lytic cycle proteins of MHV-68, are probably not involved in the elimination of reactivating virus at late times post-infection. Experiments described in this thesis confirm that CD8+ T cells are important in clearing replicating virus from the lungs of mice and, perhaps, play a significant role at very early times after infection. Unfortunately, CD8+ CTL functional activity proved to be
difficult to demonstrate *in vitro* by conventional means. To date, only one group in the world has managed to demonstrate MHV-68-specific CTL activity using a standard chromium release assay. Significantly, experiments described in chapter 5 demonstrate that CD4+ T cells can also play a major role in the clearance of MHV-68 from the lungs of infected mice. It seems, from this study and from recent results obtained in this laboratory demonstrating the importance of type I interferons in murine immune responses to MHV-68, that a variety of immunological components contribute to the clearance of the virus during the acute phase of infection.

In the spleen, it appears that CD8+ T cells play a role in limiting the extent of B lymphocyte infection during splenomegaly. In this situation, CD8+ T lymphocytes probably reduce infected cell numbers by lysis of virus-positive cells and suppress B cell proliferation via the release of cytokines such as IFN-γ. Significantly, CD8+ T cells do not appear to be important in eliciting the decrease in infected B cell numbers which occurs during the resolution of splenomegaly. Recent evidence from this laboratory suggests that there is widespread apoptosis of infected B cells at this time. However, what initiates this apoptosis of B lymphocytes is unclear.

Evidence suggests that the B lymphocyte is the principal site of MHV-68 latent infection. However, in the last year Stewart *et al.* (1998) have demonstrated that the virus is also capable of adopting a latent infection in the lung. It is, at present, unclear which components of the immune system play a significant role in preventing MHV-68 reactivation at late times post-infection. There is
currently no published data describing CTL which are specific for latent antigens of MHV-68. However, limited evidence is beginning to appear which suggests that these T lymphocytes may be important in anti-viral immune responses.

In summary, current data suggests that several components of the immune system function to control MHV-68 during the acute phase of infection and prevent death of the animal. However, it is not known if the immune system plays a significant role in preventing the reactivation of MHV-68 at late times post-infection. Therefore, there are many aspects of the immune response to MHV-68 which require further study. To date, no systematic investigations of macrophage or natural killer cell responses to MHV-68 have been undertaken. It is likely that these cells produce immunomodulatory or antiviral cytokines during the acute phase of viral replication in the lung. Further, no evidence has yet been published on the involvement of dendritic cells during MHV-68 infection. As mentioned previously in chapter 5, preliminary data from this laboratory suggests that dendritic cells may play a pivotal role in transporting MHV-68 from the lungs, through the lymphatic system to the mediastinal lymph nodes. It seems likely that these cells are also responsible for the priming of CD4+ and CD8+ T cell responses to the virus.

The functions of CD4+ T lymphocytes in this model system appear to be diverse. It seems that CD4+ T cells are involved in the clearance of MHV-68 from the lung and the development of splenomegaly in addition to being required for the efficient priming of anti-viral CD8+ T cell and antibody
responses. Therefore, there is scope for experiments investigating the functional roles of this T lymphocyte subset in the model.

As discussed previously, CD8+ T cell responses to MHV-68 have been partially characterised. It is now clear that CD8+ CTL, with specificity for lytic-cycle antigens of MHV-68, proliferate and are functionally active in the mediastinal lymph nodes and spleens of mice at early times after infection (Stevenson and Doherty, 1998). More recently, at least one group has progressed in the identification and characterisation of some of the peptide epitopes to which MHV-68-specific CD8+ CTL respond (Stevenson, P. Personal communication). Further, MHC class I tetramers are now available which bind MHV-68-specific CD8+ T lymphocytes. These molecules will be useful in the future identification and characterisation of MHV-68-specific CD8+ T lymphocytes which are undetectable by conventional cytotoxicity assays. As discussed in chapter 3, CD8+ T lymphocyte lines which are specific for an epitope from the putative MHV-68 latent protein M2 have been generated (Usherwood, E., personal communication). When these cells are adoptively transferred into mice, prior to intranasal inoculation with MHV-68, generalised proliferation of lymphocytes in the spleen does not occur. Therefore, it appears that CD8+ CTL with specificity for latent antigens of MHV-68 can play a functional role in this model system. To date, no systematic investigation of cytokine production by CD8+ T cells in MHV-68-infected mice has been undertaken. This is perhaps an oversight since evidence has emerged over the last 3 years which has shown that cytokine production by CD8+ T cells can efficiently clear viral infections in the absence
of a detectable cytotoxic response (Guidotti et al., 1996). In general, available data on cytokine production in MHV-68-infected mice is somewhat confusing and a clear picture of which cytokines and/or chemokines play significant roles in this system has yet to appear.

As the number of groups studying MHV-68 increases, an important consideration is the diversity of infection strategies being adopted by different researchers. For example, it has recently emerged that decreasing the dose of MHV-68 given to mice can dramatically decrease the reliance of the animals on certain immune system components such as CD8+ CTL (Stevenson, P., personal communication). Therefore, in the future, interpretation of results from different research groups should be undertaken very carefully indeed.

In vivo and in vitro studies suggest that, unlike EBV, MHV-68 is poorly tumorigenic. Despite this general characteristic, some progress has been made in the development of a model of MHV-68-positive lymphoma therapy in an immunocompromised host. Subcutaneous injection of S11 lymphoma cells into athymic nude mice results in the development of tumours within 1 to 2 weeks of cellular implantation. Experiments described in chapter 4 demonstrate that it is possible to 'restore' anti-tumour immune responses in recipient mice by means of an adoptive transfer of lymphocytes into tumour-bearing animals. Data suggests that CD4+ T cells are most effective in eliciting tumour regression in this model. Unfortunately, it is not known how these CD4+ T cells exert their anti-tumour effects. In the future, a study
investigating the functional properties of donor lymphocytes might reveal whether CD4+ T cells exert their effects directly or indirectly (via the induction of a DTH-like response) in this system.

6.1 To conclude

Now that many features of MHV-68 infection of inbred mice have been characterised, it is clear that caution must be taken when attempting to extrapolate results obtained with this model to other systems such as EBV infection of humans. Although MHV-68 is closely related to Herpesvirus saimiri, Human Herpesvirus 8 and Epstein-Barr virus, the virus has undoubtedly been subject to a variety of evolutionary pressures not encountered by its close relatives. As a result it appears that MHV-68 has developed a range of subtly different features and strategies for long-term survival in the murid rodent. Therefore, in the future, careful selection of appropriate and relevant features of the virus for investigation and study is essential.
Chapter 7

Bibliography
7.0 Bibliography


Appendix

Names and Addresses of Principal Suppliers

Bantin and Kingman — The Field Station, Grimston, Aldborough, Hull, UK.

Beckman Instruments (UK) — Progress Rd., Sands Industrial Estate, High Wycombe, Buckinghamshire, UK.


Coulter — Northwell Drive, Luton, Bedfordshire, UK.

European Collection of Animal Cell Cultures — Porton Down, Salisbury, UK.

Globepharm — Esher, Surrey, UK.

Harlan Sera-Lab — Hill Crest, Dodgeford Lane, Belton, Loughborough, UK.

ICN pharmaceuticals, Inc. — 1, Elmwood Chineham Business Park, Basingstoke, UK.

Life Technologies Ltd. — Inchinnan Business Park, Paisley, UK.

Merck-BDH — Merck House, Poole, Dorset, UK.

Pharmingen — San Diego, California, USA.

Sartorius — Longmead Business Centre, Blenheim Rd., Epsom, Surrey, UK.

Serotec — 22, Bankside, Station Approach, Kidlington, Oxford, UK.

Sigma-Aldrich Company — Fancy Rd., Poole, Dorset, UK.

Squibb — PR Squibb and Sons, Reeds Lane, Moretun, Wirral, Cheshire, UK.