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Investigating innate and adaptive immune responses against sexually transmitted viral infections

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B.Sc, M.Sc

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

Discipline of Pathology, Sydney Medical School, The University of Sydney, Australia

April 2014


Declaration

I hereby declare that all work presented in this thesis describes original research work undertaken in the Discipline of Pathology, Sydney Medical School, The University of Sydney. To the best of my knowledge, these results have not been previously submitted for any degree, and will not be submitted for any other degree or qualification. All studies reported within this thesis were performed by the author, except where specifically stated.
Dedication

This dissertation is dedicated to my brilliant and outrageously loving and supportive wife, Mahsa. Without her help, encouragement and also scientific ideas it simply never would have been.
Acknowledgment

First and foremost, I would like to express my gratitude to my supervisor Professor Nicholas King, whose expertise, understanding and patience added considerably to my graduate experience. I appreciate his vast knowledge and skill and his continual confidence in me throughout these years. This thesis would not have been possible without his help, support and patience. I must also express my profound gratitude to Dr. Shane Thomas for his brilliant ideas, insightful discussions and for also giving me the opportunity to use HPLC in the UNSW.

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Abstract

Viral sexually transmitted pathogens, including herpes simplex viruses (HSV), human immunodeficiency virus (HIV) and human papillomaviruses (HPV) are a major global health problem. These incurable viruses evade the genital immune system and generate life-long infections in the reproductive tract with attendant social repercussions. In particular, infection with HSV-2 is associated with increased risk of HIV acquisition. Despite the threat posed by these infections, there is a relative dearth of data about the genital mucosal immune response against viral sexually transmitted infections (STI), particularly in the early phase of the immune response.

Currently the most commonly employed STI model uses HSV-2, which is lethal in mice. Vaginal infection with this virus in a mouse model results in epithelial layer breakdown and further uncontrolled spread of the virus into the CNS. Therefore, it does not accurately reflect HSV-2 infection in humans. As such, our laboratory has developed a murine model of non-lethal STI using the flavivirus West Nile virus (WNV), which is characterised by mild and self-limiting viral infection. Similar to HSV-2 infection in the murine vagina, WNV productively infects vaginal epithelial cells and is cleared from the epithelium within seven days. However, unlike HSV-2 infection, the vaginal epithelial layer remains intact during WNV infection, enabling the investigation of the early vaginal immune responses with local defence mechanisms largely intact.

In this study, comprehensive analysis of leukocyte infiltration into the vagina and the draining iliac lymph nodes (ILN) during infection with both WNV and HSV-2 resulted in acute recruitment of macrophages, dendritic cells (DC), neutrophils and lymphocyte subsets. Interestingly, CD11c⁺, CD11b⁺ and F4/80⁺
macrophages and DCs infiltrated the vagina in large numbers, however, only CD11c+ DCs formed sub-mucosal clusters in close association with foci of epithelial cell infection. These subsets, as shown by adoptive transfer experiments, originated in the bone marrow and migrated to the site of infection. Studies showed that the cytokine and chemokine milieu in the vagina of C57BL/6 mice during WNV infection was dominated by Th1-type response characterised by high expression of IFN-γ. In contrast, intravaginal (i.vag) WNV infection in BALB/c mice elicited a Th2-type response, specifically, tumor necrosis factor (TNF) and nitric oxide synthase 2 (NOS2) were highly upregulated but not IFN-γ or IDO.

Although the numbers and relative percentages of infiltrating leukocytes in the vagina and ILN of C57BL/6 and BALB/c mice were comparable, the chemokines regulating DC and macrophage migration were differentially expressed in the two strains. In particular, CCL20 was only expressed in BALB/c mice, while CCL2 was expressed in the vagina of WNV-infected C57BL/6 mice. Although systemic anti-CCL20 antibody (Ab) neutralisation had no statistically significant effect on the number of recruited DCs and macrophages in the vagina and the ILN, increased WNV infection was observed in the vaginal epithelium of these BALB/c mice. On the other hand, systemic anti-CCL2 Ab blockade in WNV-infected C57BL/6 mice resulted in the reduction of several leukocyte subsets, including CCR2+ macrophages, B cells, CD4+ T cells and neutrophils in the vagina. Within the macrophage populations in the ILN, only the Ly6C+ CCR2+ subset was reduced following CCL2 neutralisation, while DC subsets and lymphocytes were reduced in the ILN of WNV-infected C57BL/6 mice. In an attempt to track macrophage migration, intravenous administration of 0.05 μm
polystyrene beads to i.vag WNV-infected C57BL/6 mice specifically reduced the infiltration of CCR2+ macrophages and neutrophils into the vagina. In the ILN however, lymphocytes and several DC subsets but not macrophages were reduced. Interestingly, bead+ cells were found to accumulate in the spleen, suggesting that monocytes and perhaps other cells enter the blood from the bone marrow, take up beads and become trapped in the spleen and are thus prevented from infiltrating infected tissues. However, whether this treatment has an effect on virus growth in the vaginal epithelium was not investigated.

Finally, one outcome of IFN-γ production in the vagina of C57BL/6 mice was the dramatic upregulation of the enzyme, indoleamine 2-3-dioxygenase (IDO). Investigating the production and activity of IDO at the site of HSV-2 infection and in the draining LN revealed that enzymatically active IDO was present in both tissues, however, IDO was only expressed by endothelial cells in the ILN. Attempts to delineate a possible anti-viral or immunosuppressive role of IDO in this model were unsuccessful, as the infection time course, pathology, disease severity and cytokine/chemokine expression milieu in response to the HSV-2 infection in the vagina, as well as the dynamics of leukocyte infiltration in the ILN, were broadly similar in both WT and IDO−/− mice.

Collectively, these results expand previous findings showing the similarities and differences in the innate and adaptive immune responses to lytic and non-lytic viruses that are elicited in C57BL/6 mice, i.e., generate Th1-type immune responses, and the Th2-prototypical BALB/c mouse strain, as two distinct STI models of viral infection.

Findings from this project throw light on the multiple ways in which the immune response may be initiated at the mucosal surface in response to viral
infection and in time may inform approaches to the development of more effective vaccine.
Abbreviations

1-MT 1-methyl tryptophan
Ab Antibody
Ag Antigen
AhR Aryl hydrocarbon receptor
APC Antigen presenting cells
BCR B cell receptor
BDC Blood-derived DCs
BHK Baby hamster kidney
BLIMP-1 B-Lymphocyte-Induced Maturation Protein
BM Bone marrow
CCL2/3/5/19/20/21 Chemokine (C-C motif) ligand 2/3/5/19/20/21
CCR2/5/6/7 C-C chemokine receptor type 2
CNS Central nervous system
CPE Cytopathic effect
CTL Cytotoxic T cells
CTLA-4 Cytotoxic T-Lymphocyte Antigen 4
CX3CR1 CX3C chemokine receptor 1
CXCL1/9/10/12 Chemokine (C-X-C motif) ligand 1/9/10/12
CXCR4 C-X-C chemokine receptor type 4
DAP12 DNAX activation protein of 12
DCs Dendritic cells
DDT Dithiothreitol
DMEM Dulbecco's modified Eagle's medium
ELISA Enzyme-linked immunosorbent assay
FACS Fluorescence-activated cell sorting
FCS Foetal calf serum
FMO Fluorescence minus one
FoxP3 Forkhead box P3
FRT Female reproductive tract
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GAS IFN-γ-activated sites
GM-CSF Granulocyte macrophage colony-stimulating factor
Gr-1 complex Glucocorticoid receptor
HBV Hepatitis B virus
HCV Hepatitis C virus
HIV Human immunodeficiency virus
HPLC High-performance liquid chromatography
HPV Human papillomaviruses
HSV-2 Human Simplex Virus type 2
i.c Intracranial
ICAM-1 Intercellular Adhesion Molecule 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.d</td>
<td>Intradermal</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFNα/β/γ</td>
<td>Interferon alpha/beta/gamma</td>
</tr>
<tr>
<td>IL1/4/12/13/15/17/18/23</td>
<td>Interleukin 1/4/12/13/15/17/18/23</td>
</tr>
<tr>
<td>IL-28RA</td>
<td>Interleukin-28 receptor</td>
</tr>
<tr>
<td>ILN</td>
<td>Iliac lymph node</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>i.p</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ISGs</td>
<td>Interferon stimulated genes</td>
</tr>
<tr>
<td>ISREs</td>
<td>Interferon-stimulated response elements</td>
</tr>
<tr>
<td>IRF</td>
<td>IFN regulatory factor</td>
</tr>
<tr>
<td>i.v</td>
<td>Intravascular</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi's sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>Kyn</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>L-Trp</td>
<td>L-tryptophan</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophages</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified Eagle's medium</td>
</tr>
<tr>
<td>MHC-II</td>
<td>Major histocompatibility molecule class II</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney Murine Leukemia Virus</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>(NF)-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum Cutting Temperature</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotides</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>pDCs</td>
<td>Plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming units</td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>prM</td>
<td>Premembrane</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SA-HRP</td>
<td>Streptavidin-horseradish peroxidase</td>
</tr>
<tr>
<td>s.c</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCC</td>
<td>Single colour-stained controls</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>STAT1/2/4</td>
<td>Signal transducer and activator of transcription1/2/4</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmissible infections</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDCs</td>
<td>Tissue-derived DCs</td>
</tr>
<tr>
<td>TDO</td>
<td>Tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>T helper</td>
</tr>
<tr>
<td>TIP-DCs</td>
<td>TNF/inducible nitric oxide synthase producing DCs</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNB</td>
<td>2,4,6-trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tregs</td>
<td>T cell responses by inducing regulatory T cells</td>
</tr>
<tr>
<td>TSA</td>
<td>Tyramide Signal Amplification</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon</td>
</tr>
<tr>
<td>vDCs</td>
<td>Vaginal dendritic cells</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>WNIV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
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General Introduction

1.1 The female genital tract

The female reproductive tract (FRT) is a mucosal-fibrous tube that can be divided into upper and lower main sections: the upper FRT includes the endocervix, uterus and fallopian tubes which are lined by columnar epithelium and maintain a sterile environment, while the vagina and ectocervix forming the lower FRT are lined with stratified squamous epithelium (Last, 1973). The vagina is populated by commensal microflora, predominantly lactobacilli. These bacteria metabolise glycogen released by vaginal epithelial cells, thus accounting for the low vaginal pH. This acidic environment plays a critical role not only in preserving the integrity of the vaginal epithelium but also in maintaining sexual and reproductive health (Quayle, 2002).

As a part of the female reproductive tract, the vagina is precisely controlled and regulated by the ovarian steroid hormones, oestrogen and progesterone. The hormones undergo periodic changes in the fertile female. Thus, fluctuation in hormonal levels results in cyclic changes in the vaginal epithelium, which, in turn, influences the physiology and immune function of the vagina.
1.2 The vaginal immune system

The immune system in the FRT is similar in some ways to the other mucosal environments, such as the gastrointestinal and respiratory tracts. In contrast, there are several critical aspects that are unique to the FRT, including particular microflora, an abundance of IgA and IgG immunoglobulins and the initiation of an immune response that targets a diverse array of sexually transmitted pathogens while being tolerant towards allogeneic spermatozoa and the immunologically distinct developing conceptus (Chentoufi and Benmohamed, 2012, MasCasullo et al., 2005, Quayle, 2002). Despite the threat posed by the major genital tract pathogens, very little is known about the role of the immune system in the protection against infection in this region.

1.2.1 Natural barriers

1.2.1.1 Vaginal mucus

The vaginal mucus is the first line of defence against pathogen invasion. It contains several immune components such as antimicrobial substances, cytokines (Crowley-Nowick et al., 2000, Hector and Brenna, 2011), defensins and cationic peptides (Fan et al., 2008), complement factors (Carroll, 2004, Burgener et al., 2013) and immunoglobulins (Parr and Parr, 1985, Wang et al., 2002). Among the antibody isotypes, IgA and IgG are the major antibodies in genital secretions and have been demonstrated to play an important role in protection against infection (Eriksson et al., 1998, Parr and Parr, 1998, Hunter et al., 2011), although the IgG
and IgA levels are dependent on various hormonal and inflammatory factors (Beagley and Gockel, 2003, Hel et al., 2010, Wira and Sandoe, 1989).

It has been reported that IgA is the predominant immunoglobulin in the other external mucosal secretions, including those of the intestinal tract, milk, tears and saliva. In contrast, however, cervico-vaginal secretions are dominated by IgG (Mestecky, 2006, Mestecky and Fultz, 1999, Mestecky et al., 2010, Mestecky et al., 2005). Moreover, nearly all of the specific viral antibody in immune mice is IgG (Milligan and Bernstein, 1995b, Tengvall et al., 2008).

Interestingly, the commensal microbiota that colonise the vaginal mucosa produce lactic acid and hydrogen peroxide (Boskey et al., 2001, Atassi and Servin, 2010, Hector and Brenna, 2011). The acidic environment in healthy females (pH 3.5–4.5) contributes to the protection against infections; for example, exposure of herpes simplex virus type 2 (HSV-2) to a pH 4.5 or lower, irreversibly inactivates the virus and reduces viral yields by some 90% (Keller and Herold, 2006, MasCasullo et al., 2005).

1.2.1.2 Vaginal epithelium

Vaginal epithelial cells are the first physical barrier encountered by various pathogens and must therefore effectively protect the FRT from, and respond to, a diverse array of sexually transmitted pathogens (Blaskewicz et al., 2011, Beagley and Gockel, 2003, Wira et al., 2010). Epithelial cell proliferation and maturation is under strict regulation by female sex hormones, which affects the thickness of the epithelial layer during the menstrual cycle. As a result, these
hormones influence susceptibility and disease predisposition towards FRT infections (Brabin, 2002, Kaushic et al., 2011, Wira et al., 2010, Kaushic et al., 2003). For instance, in a macaque model of vaginal infection, subcutaneous implants of progesterone rendered the monkeys more susceptible to simian immunodeficiency virus (SIV) vaginal transmission, while oestrogen was able to protect against SIV infection (Smith et al., 2000). Studies in mouse models of HSV-2 infection also showed similar effects of hormones on sexually transmitted infections. With an inoculation dose of $10^5$ plaque-forming units (PFU) of HSV-2, all progesterone-treated mice succumbed by day 4 post infection (p.i.) whereas mice treated with oestradiol did not display any vaginal pathology or viral shedding when infected with an even higher dose of HSV-2 ($10^7$ PFU) (Gillgrass et al., 2005a, Kaushic et al., 2003). The mechanism of increased susceptibility to vaginal HSV-2 infection by progesterone is not clear but may be partly attributed to structural differences in the vaginal epithelium. The progesterone-dominated vagina in the mouse is composed of a thin stratified epithelium containing a single layer of columnar, mucus-secreting cells, while the oestrogen-dominated epithelium is comprised of a thick, keratinised stratified epithelium (Parr and Parr, 1997b).

1.2.2 Innate immune responses in the vagina

As with other mucosal surfaces, the FRT and associated tissues are exposed to the outside environment. It is also the receptive organ during sexual intercourse making it further susceptible to the transmission of pathogens.
Therefore, the mucosal immune system must maintain innate and adaptive immune barriers against invading pathogens while avoiding overactive inflammatory responses that impair mucosal tissue function.

1.2.2.1 Pattern recognition receptors

Recognition of pathogenic intruders by specific pattern-recognition receptors, such as toll-like receptors (TLRs), induces a rapid innate immune response. The conserved pathogen-associated molecular patterns (PAMPs) from infectious microorganisms (bacteria, fungi, parasites and viruses) as well as endogenous ligands from damaged cells are recognised by membrane-bound TLR receptors. Viral infection is mainly detected by TLR3, TLR7, TLR8 and TLR9, which are designed to detect single- and double-stranded nucleic acids (Nasu and Narahara, 2010, Alexopoulou et al., 2001, Diebold et al., 2004, Heil et al., 2004, Bowie and Haga, 2005, Matsumoto et al., 2002). More recently, TLR2 and TLR4 have also been implicated in sensing viruses (Kumar et al., 2009, Kurt-Jones et al., 2004, Gill et al., 2006).

Ligand recognition by TLRs leads to the recruitment of various Toll/IL-1 receptor (TIR) domain-containing adaptors such as MyD88 (myeloid differentiation primary response gene 88) and TRIF (TIR-domain-containing adapter-inducing interferon (IFN)-β), which trigger different signalling pathways (Vogel et al., 2003). Signalling through MyD88 activates nuclear factor (NF)-κB and induces the expression of many cytokines including tumour necrosis factor (TNF) and IL-6 (Alexopoulou et al., 2001, Diebold et al., 2004). Stimulation of
the TRIF signalling pathway activates the IFN regulatory factor (IRF) family to stimulate the production of type I IFNs (Kaisho and Akira, 2006).

Among these TLRs, TLR2 and TLR9 are known to detect the HSV-2 virion and the genomic DNA of HSV-2, respectively (Sato and Iwasaki, 2004, Lund et al., 2003, Sato et al., 2006). Thus, TLR9 signalling induces the production of IFN-α by plasmacytoid dendritic cells (pDCs) in response to HSV-2 infection. In addition, TLR9-deficient mice suffer from more severe HSV-2 infection, which was accompanied by higher viral titres, increased vaginal pathology and accelerated mortality, even though a robust Th1 response was still generated (Lund et al., 2006, Sato and Iwasaki, 2004). TLR3 is also important in sensing HSV-2 infection after viral entry into the central nervous system (CNS), as TLR3 deficiency facilitated the infection of astrocytes and the establishment of viral infection in the CNS (Reinert et al., 2012). Based on these observations, it has been suggested that TLR agonists can provide host protection against vaginal HSV-2 infection. Correspondingly, inoculation of mice with CpG oligodeoxynucleotides (CpG) or polyinosinic:polycytidylic acid (poly I:C), TLR9 and TLR3 ligands, respectively, induces robust IFN-α/β production by pDCs, rapid thickening of the vaginal epithelium and migration of leukocytes to the vagina (Gill et al., 2006, Sajic et al., 2005, Shen and Iwasaki, 2006).

1.2.2.2 Interferons

IFNs are one of the first lines of defence against viruses because they are induced early after virus recognition by TLRs before many other defence mechanisms appear (Sen, 2001). IFN-α/β produced by pDCs, macrophages,
fibroblasts and epithelial cells limit viral replication in infected cells and also inhibit virus spread to neighbouring cells by activating IFN-stimulated genes (ISG) (Der et al., 1998, Sen, 2001, Stetson and Medzhitov, 2006). IFN-α/β also shape the extent and quality of the immune system in response to virus infection by promoting neutrophil survival (Sakamoto et al., 2005) and the activation of macrophages (Kropp et al., 2011), natural killer (NK) cells (Martinez et al., 2008), DCs (Longhi et al., 2009), B cells (Swanson et al., 2010) and CD8+ T cells (Stetson and Medzhitov, 2006), as well as inducing T helper (Th) 1 polarisation of effector CD4+ T cells (Longhi et al., 2009, Wang and Fish, 2012). Even though HSV-2 vaginal infection in mice is associated with high upregulation of both type I IFNs (Lund et al., 2003, Lund et al., 2006, Svensson et al., 2007), studies have demonstrated that IFN-α/β expression alone does not provide protection, as Peng and colleagues found that IFN-α and -β were poorly expressed in human vaginal lesions (Peng et al., 2009). On the other hand, IFN-α/β expression has found to be protective against West Nile virus (WNV) spread to the CNS. Mice with gene deficiency for IFN-α/β receptor (IFN-α/βR−) were acutely susceptible to WNV infection, thus, succumb earlier with greater numbers to infection (Samuel and Diamond, 2005).

Type II IFN (IFN-γ) is produced following the IFN-α/β response. IFN-γ is produced only by cells of the immune system (Sen, 2001). Natural killer (NK) cells and T cells are the primary producers of IFN-γ during the innate and adaptive phases of immune response, respectively, to viral infection (Randall and Goodbourn, 2008). In general, agents that promote T cell activation induce IFN-γ synthesis. Moreover, viruses trigger IL-12 production by monocytes and macrophages, which, in turn, act upon NK cells and T cells to promote IFN-γ
synthesis (Ye et al., 1995, Stubblefield Park et al., 2011, Schroder et al., 2004). The inhibitory role of host IFN-γ production in vaginal HSV-2 infection has long been investigated. This will be discussed further in the context of the adaptive cellular response to HSV-2 infection.

The more recently discovered type III IFNs (also known as IFN-λ or IL-28A/B and IL-29) are similar to type I IFNs, most notably in antiviral activity (Kotenko et al., 2003, Sheppard et al., 2003). Although virus infection or double-stranded RNA stimulation induces the expression of type I and III IFNs by most types of cells, the IFN-λ-specific receptor, IL-28RA, is only expressed by few subset of cells including epithelial cells and plasmacytoid DCs (Ank et al., 2008, Sommereyns et al., 2008).

Further investigation with a HSV-2 vaginal model of infection has found that IFN-λ, in contrast to type I IFNs, completely inhibits viral replication in the genital mucosa and halts progression of disease (Ank et al., 2006). Also, treatment of mice with IFN-λ before HSV-2 infection enhances the levels of IFN-γ in serum. Although type III IFN protects the animal from HSV-2 infection, it is more likely that IFN-λ exerts a significant portion of its antiviral activity via the stimulation of other aspects of the immune system rather than direct induction of the antiviral state (Ank et al., 2008, Ank et al., 2006).

Although IFNs are major innate antiviral immune factors, many viruses possess mechanisms to circumvent IFN-mediated effects in the host. For instance, WNV prevents activation of STAT1 (signal transducer and activator of transcription 1) and STAT2 (Guo et al., 2005). Further, Peng and colleagues showed that HSV-2 infection in the mucosal surface of the vagina is associated with low levels of IFN-α/β expression in humans. This blockade of the production
of type I IFN may be the important factor to allow HSV-2 to overcome host mucosal defences (Peng et al., 2009).

1.2.2.3 Cell-mediated innate immunity

1.2.2.3.1 NK and NKT cells

NK cells are large granular lymphocytes that have a major role in the elimination of virally infected cells without the need for antigen sensitisation (Andoniou et al., 2006, Emanuela et al., 2011). Upon activation, NK cells can secrete a number of cytokines and chemokines, particularly IFN-γ, that can control the growth and spread of viral infection as well as affect the initiation and maintenance of adaptive immune responses (Andoniou et al., 2006, Ishido et al., 2000).

In addition, a population of NK-like cells, known as NKT cells, has been recently identified that express TCR molecules and are found to share both NK and T cell markers, including NK1.1 and CD3, respectively (Biron and Brossay, 2001). NK and NKT cells increase in the vaginal mucosa and its draining lymph node (LN) following infection with HSV-2 (Kwant-Mitchell et al., 2009) and are required for protection against genital HSV-2 infection in mice (Ashkar and Rosenthal, 2003). In humans, NK cells accumulate at sites of HSV-2 infection and can lyse infected cells (McClelland et al., 2002, Koelle et al., 1998). Although these innate immune cells are critical for virus clearance, it has been shown that members of the herpesvirus (e.g., HSV-1 and 2) and flavivirus (e.g., WNV) families have developed mechanisms to evade NK cell responses (Orange et al.,
by methods such as interference with the ligands on the surfaces of infected cells that bind to the NK cell activating or inhibitory receptors. For example, Kaposi's sarcoma-associated herpesvirus (KSHV) K5 protein downregulates ICAM-1 and B7-2, which are ligands for the NK cell receptor mediating cytotoxicity. As a result, NK cell-mediated cytotoxicity reduces dramatically by the expression of K5 in vitro (Ishido et al., 2000, Coscoy and Ganem, 2001).

1.2.3.2 Neutrophils

Neutrophils are polymorphonuclear leukocytes (PMNs) that have long been recognised as one of the first lines of cellular defence at mucosal sites and their role in the resolution of intracellular viral infections has been increasingly investigated (Tate et al., 2008, Tate et al., 2011, Tumpey et al., 1996). Neutrophils represent the predominant leukocyte population in the vaginal epithelium (Nandi and Allison, 1993) and have been suggested to play a role in the protection against genital infection with sexually transmitted pathogens (Milligan, 1999, Milligan et al., 2001). Infection at mucosal sites such as the genital or respiratory tracts elicits rapid recruitment of Ly6Ghi PMNs (primarily neutrophils) in large numbers by 24 h p.i. (Milligan et al., 2001, Tate et al., 2011). Activated neutrophils phagocytose virions and damaged epithelial cells and secrete antiviral mediators, including TNF and defensins (Duerst and Morrison, 2003). Even though depletion of neutrophils prior to primary HSV-2 inoculation results in significantly higher virus titres, they have a minor role in the final resolution of the infection from the
vagina, indicating that the influx of neutrophils contributes to the suppression of initial HSV-2 replication (Chan et al., 2011, Milligan, 1999, Milligan et al., 2001).

1.2.2.3.3 Macrophages and dendritic cells

Although both macrophages and DCs are members of the mononuclear phagocyte family, these cells are highly heterogeneous (Iwasaki, 2007). Macrophages and DCs are terminally differentiated, non-dividing cells derived from circulating monocytes (Gordon and Taylor, 2005, Fogg et al., 2006). Macrophages and DCs play an important role in the innate and adaptive immune response. They phagocytose cellular debris and pathogens, but also act as professional antigen presenting cells (APC) (Hume, 2008), triggering cytokine and chemokine secretion by the presentation of pathogen-derived peptides via the major histocompatibility molecule (MHC)-II pathway to CD4+ T cells (Itano et al., 2003), and activate CD8+ cytotoxic T cells (CTL) by cross-presentation of viral antigens on MHC-I molecules (Fonteneau et al., 2003, Ackerman and Cresswell, 2004). In mice, macrophages and DCs are often distinguished by differential expression of surface markers such as CD11b, F4/80 and CD11c. However, there is a major problem in defining these subsets separately, as these cells are derived from a common myeloid progenitor (Murray and Wynn, 2011, Geissmann et al., 2010a). A distinct subtype of DC, defined as CD11c+ B220+, has been identified and appears to be the murine counterpart of human type I IFN-producing pDC (Ferrero et al., 2002, Villadangos and Young, 2008). pDCs are also capable of presenting foreign antigens on the cell surface to initiate a proper adaptive immune response against STI in the vaginal mucosa, although to a lesser
extent than macrophages and other DC (Schlecht et al., 2004, Shen and Iwasaki, 2006, Schuster et al., 2011).

Several studies have investigated the origin of macrophages and DCs in inflamed and infected tissues and have found at least two functionally distinct monocyte subsets that give rise to these macrophages and DCs (Auffray et al., 2009, Gordon and Taylor, 2005). CD11b+ Gr-1" monocytes are inflammatory and migrate to bacterial and viral infected sites (Dunay et al., 2008, Serbina and Pamer, 2006, Iijima et al., 2007), whereas CD11b" Gr-1" monocytes are associated with patrolling resting vessels, populating normal and inflammatory sites as well as participating in the resolution of inflammation (Auffray et al., 2009, Geissmann et al., 2003, Nahrendorf et al., 2007).

Further analysis of the Gr-1 monocyte surface marker led to the identification of two small, linked proteins, now known as Ly6C and Ly6G, that are a part of the Gr-1 complex. Ly6G is highly expressed on neutrophils and blocking this marker using neutralising Ab has no functional effect on the recruitment of leukocytes in mice (Yipp and Kubes, 2013). Ly6C, on the other hand, is more specifically expressed on monocytes and monocyte-derived subsets (Dunay et al., 2008, Geissmann et al., 2010a, Geissmann et al., 2003, Geissmann et al., 2010b, Gordon and Taylor, 2005, Swirski et al., 2009, Tacke and Randolph, 2006). More recently, phenotypic characterisation in the mouse revealed two distinct monocyte subsets based on their expression of Ly6C and the fractalkine receptor CX3CR1 (Geissmann et al., 2010b). Ly6C<sup>hi</sup> monocytes are characterised by the expression of CCR2 and low expression of CX3CR1. These cells have been termed “inflammatory” as they are selectively recruited to sites of inflammation and infection in a CCR2-dependent manner and constitute the
primary inflammatory subset. In contrast, Ly6C^{low} monocytes are smaller in size than their Ly6C^{hi} counterparts and express very low levels of CCR2 and high levels of CX3CR1 (Auffray et al., 2009, Geissmann et al., 2010a, Iijima et al., 2007, Iijima et al., 2011, Swirski et al., 2009).

The functions of Ly6C^{low} monocytes during steady state and inflammation are less well characterised than inflammatory monocytes, although interest in this subset has increased within the past few years (Auffray et al., 2009, Davison and King, 2011). Ly6C^{low} monocytes were initially termed “resident” as they have a longer half-life \textit{in vivo} and are found in both inflamed/infected and steady state tissue after adoptive transfer (Geissmann et al., 2003). Recent studies have demonstrated that Ly6C^{low} monocytes are in lower abundance than Ly6C^{hi} and adhere to and migrate along the luminal surface of endothelial cells that line small blood vessels in a process referred to as “patrolling”. However, under inflammatory conditions, this subset is also recruited to the site of infection, but to lesser extent than Ly6C^{hi} monocytes and are able to differentiate into DC (Auffray et al., 2009, Dunay et al., 2008, Geissmann et al., 2003, Iijima et al., 2007, Randolph et al., 1999, Serbina and Pamer, 2006, Sunderkötter et al., 2004, Swirski et al., 2009). Although some studies using either adoptive transfer of monocytes or latex bead-‘labelled’ monocytes, have proposed that circulating Ly6C^{low} monocytes originate from Ly6C^{hi} precursors (Sunderkötter et al., 2004, Tacke and Randolph, 2006, Davison and King, 2011, Varol et al., 2007), it is also clear that Ly6C^{low} monocytes can arise independently of their Ly6C^{hi} counterparts (Geissmann et al., 2008), thus highlighting the plasticity of monocyte differentiation. Notwithstanding, when monocytes extravasate through the endothelium, they differentiate into macrophages or DCs. Therefore, the primary
role of monocytes is to replenish the pool of tissue-resident macrophages and DCs in steady state and in response to infection.

Despite the extensive investigation to distinguish the unique functions of macrophages and DCs, researchers have been unable to directly identify the subset responsible for specific Ag presentation or CD4$^+$ and CD8$^+$ T cell priming (Fogg et al., 2006, Hume, 2008, Murray and Wynn, 2011, Geissmann et al., 2010a). This problem arises from the fact that most, if not all, macrophages express low amounts of CD11c (Santhakumar et al., 2010, Murphy, 2011). Moreover, although F4/80 is commonly known as a macrophage marker in mice, there are likely some CD11c$^+$ cells that also express F4/80 and some macrophages that lack F4/80 expression (Murray and Wynn, 2011). Moreover, macrophages and DCs that are isolated from the same organ can have similar stimulatory effects on naive T cells (Denning et al., 2011, Hume, 2008).

Following infection, Ly6C$^{hi}$ monocytes, and to a lesser extent, Ly6C$^{low}$ monocytes, egress from the bone marrow (BM) and infiltrate the site of infection in response to CCL2, a chemokine that is secreted by infected cells (Ansari et al., 2011, Davison and King, 2011, Dunay et al., 2008, Geissmann et al., 2003, Getts et al., 2008, Iijima et al., 2011, Lim and Murphy, 2011, Lim et al., 2011, Nakano et al., 2009, Satish et al., 2009, Sato et al., 2000, Serbina et al., 2003, Tsou et al., 2007, Tsui et al., 2007). These monocytes upregulate the expression of CD11c and MHC-II molecules in situ, indicating they have differentiated into macrophages and DCs (León and Ardavín, 2008, Heath and Carbone, 2001, Villadangos et al., 2001). Macrophages and DCs exert their effects by three different mechanisms, including secretion of microbicidal factors, stimulation of naive CD4$^+$ and CD8$^+$ T cells within the lymphoid organs and regulation of
immunoglobulin production by B cells after secondary challenge (León and Ardavín, 2008, Shi and Pamer, 2011).

It has been shown that after infection with *Listeria monocytogenes* and *Toxoplasma gondii* in the murine models, Ly6C\(^{hi}\) monocytes differentiate into specialised inflammatory cells called TIP-DCs (TNF/inducible nitric oxide synthase (iNOS)-producing DCs) that produce large amounts of TNF and NO (Dunay et al., 2008, Serbina et al., 2003). Furthermore, in a process known as “maturation”, DCs and macrophages upregulate MHC-II and other co-stimulatory markers such as ICAM-1, CD80 and CD86, and migrate from the site of inflammation/infection to the draining LN. At the LN, these DCs and macrophages present Ag to CD4\(^{+}\) and CD8\(^{+}\) T cells (Heath and Carbone, 2001, Nakano et al., 2009, Villadangos et al., 2001, Zhao et al., 2003) and stimulate B cells to produce Ag-specific immunoglobulins (Deal et al., 2013, León and Ardavín, 2008, Pulendran and Ahmed, 2006).

### 1.2.2.4 Adaptive immune responses in the vagina

#### 1.2.2.4.1 B cells

After leaving the BM, naïve B cells enter the circulation and act as APC and internalise Ag; however, they are not able to stimulate T cell responses (Zhao et al., 2003). B cells are activated when they recognise non-self Ag via the B cell Ag-receptor (BCR) and other secondary signals (Dal Porto et al., 2004, Geisberger et al., 2006).
B cells infiltrate the mucosal tissue in response to the chemokine CXCL12 (Mei et al., 2009). B cells recognise peptide Ags as well as lipids and bacterial polysaccharides via membrane bound IgM or IgD. Activated B cells clonally expand and differentiate into plasma or memory B cells (Geisberger et al., 2006). Plasma cells are responsible for the secretion of large quantities of specific IgA and IgG as part of the humoral immune response. Humoral immunity has several roles in combination with other elements of the immune response, including activation of the complement cascade (Blue et al., 2004), opsinisation and neutralisation of viral Ags (Traggiai et al., 2004, Doepper et al., 2000, Lindorfer et al., 2003). These processes neutralise the Ags or enhance immunogenicity to facilitate phagocytosis by macrophages and DCs (Abbas and Lichtman, 2004). Memory B cells are similar to memory T cells and rapidly differentiate and secrete large amounts of Ab following reinfection with the same pathogen (Abbas and Lichtman, 2004). Interestingly, direct contact with viral Ags in the vaginal tract is not required for B cell differentiation and Ab secretion. In other words, to induce relevant immune responses in the vaginal mucosa, alternative immunisation routes such as systemic immunisation with a subsequent mucosal (intranasal) immunisation proved to be effective in the induction of humoral immune responses in genital tract secretions (Mestecky, 2006, Mestecky and Fultz, 1999, Mestecky et al., 2005, Wang et al., 2002).

1.2.2.4.2 T cells

The cell-mediated branch of the adaptive immune response is primarily driven by T cells which have been shown to be crucial in resolving viral infection
in the vagina (Parr and Parr, 1997b, Milligan and Bernstein, 1995a). Extensive studies have found that T cell deficiency results in greater disease severity (Zhao et al., 2010, Milligan and Bernstein, 1995a, Parr and Parr, 1998). Traditionally, T cells are divided into two major subsets, specifically, CD4⁺ T cells (helper T cells) and CD8⁺ T cells (known as cytolytic T cells) (Lichtman et al., 2010). Following T cell receptor (TCR)-mediated recognition of foreign Ag in the context of MHC-II on the surface of APC, together with costimulatory molecules such as CD80 and CD86, which bind to CD28 on T cells, naïve CD4⁺ T cells differentiate and clonally expand into effector or memory CD4⁺ T cells (Lim et al., 2012).

Naïve CD8⁺ T cells undergo a similar activation process after recognising Ag that is displayed on MHC-I molecules (Wakim and Bevan, 2011). Traditionally, the antiviral activity of CD8⁺ T cells has been considered a key factor for viral clearance in the genital mucosa, although a greater emphasis on CD4⁺ T cells has recently emerged (Chan et al., 2011, Iijima et al., 2011). Originally, CD4⁺ T cells were viewed at least as having three distinct subsets: T helper 1 (Th1) cells, which express the transcription factor T-bet, and produce high levels of IFN-γ, and Th2 cells, which express the Gata3 transcription factor and produce interleukin (IL)-4 and IL-10 (Murphy and Reiner, 2002, Grogan and Locksley, 2002). The third subset of T helper cells, which are developmentally distinct from the first two subsets, produce IL-17 family cytokines and thus called Th17 cells (Aggarwal et al., 2003). These cells are mainly involved in inflammatory immune responses including autoimmune tissue injuries, allergic disorders and microbial infections (Lawrence, 2007, Aggarwal et al., 2003, Harrington et al., 2005), however, it has been reported that Th17 cells can also
exert regulatory effects specially those expressing CCR6, IL-6 and TGF-β on the surface (Yamazaki et al., 2008, Lim et al., 2008).

Regulatory T (Treg) cells are a CD4+ T cell subset that promotes immunosuppression (Sakaguchi, 2005, Sakaguchi et al., 1995). The primary function of Treg cells is to maintain a balance between the immunity (non-self Ag) and tolerance (self Ag) (Fontenot et al., 2003, Van Parijs and Abbas, 1998). Effective immune responses against viruses are sometimes accompanied by strong inflammatory reactions (Suvas et al., 2004). Thus, to prevent excessive and destructive immune activation, the immune system also initiates anti-inflammatory responses by promoting the differentiation of naïve CD4+ T cells into Treg cells, which are defined by expression of the forkhead box p3 (Foxp3) transcription factor (Jiang and Kelly, 2011, O’Garra et al., 2004). Treg cells secrete IL-10 or transforming growth factor (TGF)-β (Jiang and Kelly, 2011) which limit T cell expansion and suppress immune responses after mucosal infection (Oswald-Richter et al., 2004, Boussiotis et al., 2000).

1.3 Sexually transmitted infections

Sexually transmitted pathogens represent a diverse collection of microorganisms that are transmitted by intimate or sexual contact. Their impact on global health is substantial. The most recent World Health Organization (WHO) estimates suggest that there are approximately 490 million cases of curable STI that occur annually. Syphilis, gonorrhea and chlamydia were the major sexually transmitted infections (STI) 50 years ago. However, attention has now focused on viral pathogens, with the emergence of human immunodeficiency
virus (HIV), HSV-1 and -2, human papillomaviruses (HPV) and hepatitis B virus (HBV) (WHO, 2012). Even though there are commercially available vaccines for HPV and HBV, both HIV and HSV viruses, at present, are incurable (Frazer et al., 2011, Makidon et al., 2008, Schiffer et al., 2012). Moreover, HSV-2 infection has been shown to increase the risk of HIV acquisition among both men and women (Johnston et al., 2011, Freeman et al., 2006). Due to the higher rate of STI in women than in men (Annan, 2003, Jonsson and Wahren, 2004), viral STIs are a major threat to the reproductive health of women. Despite this, the mechanisms involved in the initiation of the immune response in the FRT are poorly understood. Thus, fundamental factors such as the interaction of innate and adaptive immune responses in the vaginal mucosa in response to viral infection or the precise determinants of FRT susceptibility to viruses, still need to be addressed.

1.3.1 Herpes simplex virus type 2

Herpes simplex viruses including HSV-1 and HSV-2 are prominent members of the Herpesviridae family and neurotropic alphaherpesviridae subfamily (Duerst and Morrison, 2003, Davison, 2010). Other members of this family such as varicella zoster virus, Epstein-Bar virus and cytomegalovirus, as well as HSV-1 and HSV-2, are extremely widespread among humans (Arvin, 2007). More than 90% of adults have been infected with at least one of these viruses (CDC, 2006, Cohen et al., 2011) and a latent form of the virus remains in most people (Chayavichitsilp et al., 2009).
HSV-2 has a relatively large double-stranded, linear DNA molecule that is encased in an icosahedral capsid and lipid bilayer envelope (Duerst and Morrison, 2003). The HSV-2 genome contains up to 94 open reading frames (ORF), of which seven are doubled. These ORFs encode for between 74 to 84 genes (Rajcáni et al., 2004). There is extensive homology in the genetic material and clinical manifestation between HSV-1 and HSV-2. Although HSV-1 and HSV-2 were initially considered as pathogens associated with cutaneous and genital infections, respectively, both viruses are now known to contribute to vaginal infection, and thus, this distinction between the two virus subtypes is no longer as straightforward (Cowan et al., 2002, Ryder et al., 2009).

HSV establishes lifelong disease with clinical manifestations including cold sores, genital ulcerations and encephalitis (Simmons, 2002). Primary genital infection with HSV-2 involves productive replication within vaginal epithelial cells. Thereafter, the virus invades the ganglionic sensory neurons in the peripheral nervous system (PNS) to establish a latent infection. During reactivation, the virus travels back to the site of primary infection and causes viral shedding, which accounts for a significant proportion of symptoms and morbidity among sufferers (Chentoufi and Benmohamed, 2012, Simmons, 2002).

Herpes simplex viruses are arguably the most common viral sexually transmitted pathogens throughout the world (WHO, 2012). One of the more serious consequences of STI, particularly HSV-2, is the increased risk of HIV transmission (Da Ros and Schmitt, 2008, Fleming and Wasserheit, 1999) as HSV-2 infection in the vagina can facilitate HIV virus entry by modulating innate and adaptive immune responses. More specifically, a study has demonstrated that HSV-2 infection is associated with a 3-fold increased risk of HIV acquisition.
among men and women (Freeman et al., 2006), suggesting that, in areas of high HSV-2 prevalence, a high proportion of HIV infection is attributable to HSV-2 (Celum, 2004, Freeman et al., 2006, Barbour et al., 2007). Ulcerative lesions in the genital mucosa may account for this increased risk of HSV-HIV co-infection (Strick et al., 2006). Acute lesions damage epithelial integrity and, as a result, allow HIV to enter and infect the vaginal stroma. In addition, several studies in murine models have indicated that HSV-2 infection leads to the recruitment of activated macrophages, DCs and CD4+ T lymphocytes to the vaginal mucosa (Chan et al., 2011, Harandi et al., 2001a, Milligan and Bernstein, 1995a, Parr and Parr, 1997b, Iwasaki, 2003). DCs and macrophages, as well as vaginal resident cells that express CCR5 or CXCR4 receptors on their surface, serve as the first potential target cells for HIV transmission and infection (Miller, 2007, Miller and Shattock, 2003, Shen et al., 2011). Following initial infection, CD4+ T cells infiltrate the vaginal mucosa and support robust viral replication of HIV (Barbour et al., 2007, Zariffard et al., 2009, Nazari and Joshi, 2008, Shen et al., 2011, Wilkin and Gulick, 2012). Conversely, the immunosuppression associated with HIV acquisition increases the risk of infection with other sexually transmitted pathogens and correlates with increased recurrent HSV shedding and reactivation (Strick et al., 2006, Da Ros and Schmitt, 2008).

1.3.2 Common experimental models of HSV-2 infection

At present, murine models of lethal and non-lethal HSV-2 infection are the most commonly used models to investigate the host response against HSV-2
Female mice are first injected subcutaneously (s.c.) with medroxyprogesterone acetate to synchronise the animals in the dioestrous phase of the hormonal cycle to ensure consistent levels of vaginal infection. In the lethal model, i.vag inoculation of mice results in extensive local epithelial infection that spreads to the sensory ganglionic neurons in the PNS and further to the spinal neuronal ganglia of the CNS, causing lethal neurological illness (Parr et al., 1994, Parr and Parr, 2003, Zhao et al., 2003, Chentoufi and Benmohamed, 2012). In this context, the murine model does not accurately recapitulate human HSV-2 infection, because HSV-2 infection in humans is associated with neurological symptoms that are generally, but not always, mild and self-limiting (Berger and Houff, 2008). Therefore, to overcome the limitations of this lethal model, an attenuated strain of HSV-2 was engineered by partial deletion of the thymidine kinase (TK) gene (McDermott et al., 1984). The TK gene is critical for the virus to replicate in the ganglionic neurons. Thus, inoculation of mice with HSV-2TK- induces the typical symptoms of wild type (WT) virus including vaginal epithelial infection, although the virus is unable to travel to the neurons to establish latency and mediate chronic infection. More importantly, mice infected intravaginally (i.vag) with HSV-2TK- successfully resolve the epithelial infection and exhibit strong immunity to rechallenge with WT HSV-2 even at higher titres (i.e., 10-fold LD₅₀) (McDermott et al., 1984, Parr et al., 1994).
1.3.3 Immune response to vaginal HSV-2 infection

A complex interaction between the normal vaginal flora, different innate and adaptive immune cells and various peptides create conditions that protect the vagina and thus inhibit infection. Each of these components contribute in such a way that homeostasis prevails and any changes in these compartments lead to the progression of disease. As mentioned earlier, the intact vaginal epithelium provides the first physical barrier to the invasion of sexually transmitted pathogens and thus plays an important role in protecting the underlying tissue against viral invasion. This protection is further enhanced by the vaginal mucus, which coats the vaginal epithelium and contains several components including soluble antimicrobial peptides (Hector and Brenna, 2011), cytokines (Crowley-Nowick et al., 2000), defensins or cationic peptides (Fan et al., 2008), complement factors (Carroll, 2004, Burgener et al., 2013) and immunoglobulins (Parr and Parr, 1985, Wang et al., 2002).

Within the mucus, complement factors play an important role in recognising and limiting HSV-2 infection. C1q, MBL and C3 molecules initiate the classical, lectin and alternative complement pathways, respectively. These molecules are present in vaginal secretions and are capable of binding to HSV-2, leading to neutralisation of the virus and decreased susceptibility to HSV-2 infection (Gadjeva et al., 2004, MasCasullo et al., 2005, Pellis et al., 2005, Seppänen et al., 2009).

Furthermore, defensins, a group of endogenous antimicrobial peptides, are secreted by epithelial cells and leukocytes in the FRT (John et al., 2005). They comprise 18–45 amino acids, including 6 to 8 conserved cysteine residues, and
are divided into three subfamilies including $\alpha$, $\beta$ and $\theta$ defensins (Selsted and Ouellette, 2005). Defensins protect against common sexually transmitted organisms, particularly HSV-2, by binding to the viral envelope and causing pore-like membrane defects as well as by directly inhibiting viral binding and replication (Hazrati et al., 2006, John et al., 2005, Wang et al., 2013, Yasin et al., 2004).

In addition, the normal vaginal flora consists of a rich network of organisms, dominated by hydrogen peroxide-producing lactobacilli (Boris and Barbés, 2000). These bacteria maintain the acidic pH within the vagina that makes it inhospitable for sexually transmitted pathogens. Disruption of this flora leads to an increase in the vaginal pH and is presumably associated with increased risk of HSV-2 infection (MasCasullo et al., 2005).

1.3.3.1 Innate immune response to HSV-2 infection

1.3.3.1.1 NK and NKT cells

NK cells were originally identified as an innate subset of lymphocytes that mediated the cytotoxicity of target cells expressing abnormal or reduced levels of MHC-I molecules. NK cells are also responsible for the early production of IFN-$\gamma$ following HSV-2 infection, although depletion of this subset showed that NK cells are dispensable for ultimate viral clearance from the vagina (Milligan and Bernstein, 1997). On the other hand, IL-15, which is essential for the development, maturation and function of NK and NKT cells, has been shown to contribute to defence against HSV-2. Studies using IL-15-deficient mice which
lack NK and NKT cells, or NK1.1 depletion in WT mice, resulted in a higher rate of morbidity and viral loads in the vaginal mucosa, suggesting a role for IL-15 in the early innate antiviral response, independently (Gill et al., 2005) or via NK/NKT cell-dependent mechanism(s) (Ashkar and Rosenthal, 2003). Furthermore, IL-12 and IL-18, which synergically enhance CCR5+ NK/NKT cell activity and induce the production of IFN-γ by T cells and NK cells, are critical in suppressing HSV-2 vaginal infection (Harandi et al., 2001b), since NK/NKT cell trafficking in CCR5− mice is impaired and, as a result, these mice display decreased resistance to viral infection (Thapa et al., 2007).

1.3.3.1.2 Neutrophils

Neutrophils represent a critical component of the innate immune response and are found throughout the female genital tract under healthy conditions (MasCasullo et al., 2005). The number of neutrophils does not fluctuate during the course of the menstrual cycle (Cauci et al., 2003), although they are rapidly recruited to the site of vaginal HSV-2 infection in response to the epithelial secretion of IL-8 or its functional homolog, CXCL1 (Rubio and Sanz-Rodriguez, 2007, Thapa and Carr, 2008). Neutrophils can phagocytose virions and release different types of antiviral products including TNF, defensins and proteases (Duerst and Morrison, 2003). The role played by neutrophils in the innate immune response against genital herpes in humans is yet to be identified. In murine models, neutrophils may not be critical for primary HSV infection, although HSV-2-immune mice are dependent on neutrophil-mediated HSV-2 clearance during the first few days after rechallenge, highlighting the interactions of
neutrophils with other innate and adaptive cell types in immune protection of the genital tract against viral pathogens (Milligan, 1999, Milligan et al., 2001).

1.3.3.1.3 Macrophages and dendritic cells

Recruitment of leukocytes is essential for eventual control of HSV-2 infection in the vagina. Macrophages and DCs represent leukocyte populations involved in the early immune defence against HSV-2. These subsets, present in small numbers in the vaginal mucosa, sample the vaginal tissue environment for Ag, such as those derived from dying/damaged cells, pathogens and molecules, through phagocytosis and the use of pattern recognition receptors (Fogg et al., 2006). Macrophages and DCs also constitute an important link between the innate and adaptive immune response in HSV-2 infection, through the production of cytokines such as type I IFNs, TNF, IL-4, IL-12, IL-13, IL-17, IL-18, IL-23, and chemokines such as CCL2, CCL3, CCL5, CXCL1 and CXCL10. The enhanced production of these cytokines and chemokines, in turn, recruits more effector cells to the site of HSV-2 infection (Melchjorsen et al., 2002, Ellermann-Eriksen, 2005, Malmgaard and Paludan, 2003, Malmgaard et al., 2000, Thapa and Carr, 2008). Moreover, in vivo and in vitro HSV-2 infection is associated with the production of antiviral compounds by macrophages and DC (e.g., reactive oxygen species (ROS) and NO), which can inhibit HSV-2 replication (Ellermann-Eriksen, 2005, Gonzalez-Dosal et al., 2011, Mogensen et al., 1989, Duerst and Morrison, 2003).

Even though HSV-2 does not appear to replicate efficiently in macrophages (Duerst and Morrison, 2003), in vitro studies have shown that DCs can be infected by this virus, which causes bystander DCs to phagocytose infected
DCs and mount an efficient CD8⁺ T cell response following Ag presentation via MHC-I (Bosnjak et al., 2005).

Upon vaginal HSV-2 infection, Ly6C<sup>hi</sup> inflammatory monocytes and Ly6C<sup>low</sup> monocytes migrate to the genital mucosa to give rise to CD11c⁺ MHC-II⁺ APCs (Iijima et al., 2007). Although several studies have investigated the role of chemokines in the recruitment of monocytes to the HSV-2-infected vagina (Thapa and Carr, 2008), it is likely that CCL2 plays the major role in this context as CCR2<sup>-/-</sup> mice have impaired immune responses and diminished numbers of CD11b<sup>-</sup> CD11c⁺ MHC-II⁺ and CD11b⁺ CD11c⁻ MHC-II⁻ subsets in the vagina (Iijima et al., 2011). pDCs are also important for the innate control of HSV-2, likely due to their ability to rapidly secrete high amounts of type I IFNs (Lund et al., 2006, Zhao et al., 2003). Depletion of pDC results in severe vaginal pathology and higher viral titres and morbidity rates, although, in contrast to CD11c⁺ MHC-II⁺ DCs, these cells appear to be dispensable for the generation of a Th1 response (Iijima et al., 2011, Lund et al., 2006, Zhao et al., 2003).

There is a long-held belief that only CD11c⁺ MHC-II⁺ DCs, but not macrophages or pDCs, can migrate to the draining LN, present viral Ag to naïve T cells and mount a Th1 type response against HSV-2 infection (Iijima et al., 2008a, Iwasaki, 2003, Iwasaki, 2007, King et al., 1998, Zhao et al., 2003). However, due to the recent findings indicating that inflammatory macrophages also upregulate CD11c and MHC-II, it is unclear whether, and to what extent, macrophages are also able to migrate to the vaginal draining LN and stimulate T cell responses (Geissmann et al., 2010a, Hume, 2008, Murray and Wynn, 2011).
1.3.3.2 Adaptive immune response to HSV-2 infection

1.3.3.2.1 B cells

Despite being classified as APCs, B cells are unable to present viral antigens and migrate to local lymph nodes to induce protective Th1 CD4+ T cell responses in HSV-2 infection (Zhao et al., 2003), but instead, play a role in limiting early infection and restricting latency (Milligan et al., 2005). In addition, HSV-2-specific memory B cells, referred to as “memory APCs”, can provide help to memory CD4+ T cells during secondary infection through BCR-specific uptake of Ag and re-stimulating IFN-γ production (Iijima et al., 2008a).

Intravaginal re-challenge of mice with HSV-2 or primary infection with the attenuated HSV-2 strain (HSV-2TK−) in mice elicit the accumulation of B cells and plasma cells in the vaginal mucosa (Milligan et al., 2005, Parr and Parr, 1997b). Many studies have shown that i.vag immunisation usually generates a weak local IgA response (Di Tommaso et al., 1996, Mestecky and Fultz, 1999, Mestecky et al., 2010, Parr and Parr, 1985). Interestingly, even though B cells and plasma cells of the IgA isotype accumulate in the secondary lymphoid organs, the spinal cord and the vaginal lamina propria after i.vag HSV-2 inoculation, specific IgG, rather than IgA, is the main protective antibody in vaginal secretions (Ashley et al., 1998, Di Tommaso et al., 1996, Naz, 2012). It has been shown that specific IgG blocks virus entry into epithelial cells and promotes phagocytosis by macrophages and DCs (Parr and Parr, 1998, Milligan and Bernstein, 1995b). Furthermore, only purified IgG, and not IgA, from the vagina of immune mice exhibits HSV-2-neutralising activity (Parr and Parr, 1997a, McDermott et al., 1984, Milligan and Bernstein, 1995b). However, despite the accumulation of B
cells and significant induction of antibody production in the vagina of mice immunised with HSV-2TK- or recombinant vaccinia virus expressing HSV-2 epitopes, infection of the vaginal epithelium still occurs when re-challenged with HSV-2, albeit with much lower viral titres than in primary infection mice (Milligan et al., 1998, Kuklin et al., 1998). Indeed, passive transfer of immune serum IgG to naïve mice is associated with reduced viral load following HSV-2 infection (Parr and Parr, 1997b). Moreover, i.vag re-challenge of B cell knockout mice that cannot produce antibodies generally results in complete HSV-2 clearance from the vaginal mucosa, although this response is delayed in comparison to normal mice (Dudley et al., 2000, Parr and Parr, 2000). These observations suggest that humoral responses are essential components for optimal immunity, although the presence of local HSV-2-specific antibody only limits infection and a specific T cell response is required to prevent vaginal infection upon rechallenge (Kwant-Mitchell et al., 2009, Naz, 2012, Parr and Parr, 2000).

1.3.3.2.2 T cells

It is well established that both CD4+ and CD8+ T cells infiltrate the vaginal mucosa after primary and secondary challenge with HSV-2 (Iijima et al., 2008a, Koelle et al., 1998). Traditionally, the antiviral activity of CD8+ T cells was considered to be the hallmark of viral clearance in the genital mucosa, as it has been shown in vivo that the depletion of all T lymphocytes or CD8+ T cells alone, resulted in reduced vaginal immunity against HSV-2 infection (Parr and Parr, 1998). However, CD8-deficient or CD8+ T cell-depleted mice are still protected against HSV-2 after immunisation, similar to in normal mice (Milligan and
Bernstein, 1997, Gill and Ashkar, 2009). In contrast, when CD4\(^+\) T cells are depleted, a remarkable decrease is observed in viral clearance and mice have reduced protection against HSV-2 vaginal infection (Gill and Ashkar, 2009, Milligan and Bernstein, 1997). Moreover, CD8\(^+\) T cells induce apoptosis in HSV-2-infected cells through perforin and/or Fas-mediated cytolytic mechanisms, which are regulated by IFN-\(\gamma\) production (Dobbs et al., 2005). Thus, the ability of primary CD8\(^+\) T cell to mediate cytolysis, enter infected tissue and generate protective CD8\(^+\) memory T cells against HSV-2 largely depends on the signal provided by CD4\(^+\) T cells (Tang and Rosenthal, 2010, Nakanishi et al., 2009). After vaginal HSV-2 infection, primed CD4\(^+\) T cells migrate to the vaginal mucosa earlier than effector CD8\(^+\) T cells and provide migratory assistance for CD8\(^+\) T cells by producing large quantities of IFN-\(\gamma\) (Milligan and Bernstein, 1995a). It has been found that the mobilisation of CD8\(^+\) T cells to the vaginal mucosa, as well as the ability of CD8\(^+\) T cells to resolve the HSV-2 infection, is impaired in mice that were either CD4-deficient, or depleted of CD4\(^+\) T cells (Dobbs et al., 2005, Harandi et al., 2001a, Milligan and Bernstein, 1995a). However, the WT phenotype can be restored in those animals by administration of IFN-\(\gamma\), suggesting a critical role for IFN-\(\gamma\) in the resolution of HSV-2 infection (Harandi et al., 2001a). Thus, both CD4\(^+\) and CD8\(^+\) T cells are necessary for robust adaptive immune responses against vaginal HSV-2 challenge and this response is orchestrated by IFN-\(\gamma\) produced by CD4\(^+\) T cells.

Furthermore, cytotoxic CD8\(^+\) T cell migration into HSV-2-infected sites requires CXCR3, whose ligands CXCL9 and CXCL10 are induced by IFN-\(\gamma\) produced by CD4\(^+\) T cells (Nakanishi et al., 2009). HSV-2-induced IFN-\(\gamma\) production itself is regulated by the transcription factor T-bet early during the
differentiation of naïve CD4+ T cells into Th1 cells (Sullivan et al., 2003, Svensson et al., 2005, Szabo et al., 2000) in an IL-12-independent manner (Mullen et al., 2001) or via STAT4 signalling (Svensson et al., 2012).

Surprisingly, Treg cells, which limit the late stages of pathogen-specific immunity as a means of minimising associated tissue damage, have also been implicated in facilitating the mobilisation of virus-specific CD8+ T cells from the draining iliac LN (ILN) into the infected vagina to control HSV-2 infection (Lund et al., 2008, Nakanishi et al., 2009), although this function of Treg cells can be overridden in the presence of effector CD4+ T cells (Nakanishi et al., 2009).

1.3.4 West Nile Virus (WNV): an emerging flavivirus

WNV is a neurotropic mosquito-borne virus of the genus, Flavivirus and the family, Flaviviridae. Originally, WNV was isolated from a woman in the northern West Nile district of Uganda in 1937 (Smithburn et al., 1940). WNV is currently endemic to the regions of the Africa, Asia, Europe and the Middle East, where it has caused sporadic outbreaks of various severities. Flaviviruses are named after the jaundiced appearance of patients infected with Yellow fever, the prototypic virus in this group, meaning ‘yellow’. The genus, Flavivirus is comprised of nearly 80 members, many of which are arthropod-borne, being transmitted by mosquitoes or ticks. Among the most important viruses to human health are Yellow fever and Dengue viruses and the neurotropic members of the Japanese encephalitis serogroup, St Louis encephalitis, Murray Valley encephalitis, Kunjin and WNV. Murray Valley encephalitis and Kunjin viruses
are endemic to Australia and can cause permanent neurological disease or death in humans (Scherret et al., 2001). Following the major outbreak in New York in 1999 (Lanciotti et al., 1999), WNV was identified as an important emerging pathogen, causing significant morbidity and mortality worldwide and is now regarded as the most widespread of all Flaviviruses (Bakonyi et al., 2005).

1.3.4.1 Common experimental models of WNV infection

WNV is an enveloped virus with a diameter of 45–50 nm that contains an 11-kilobase genome in an icosahedral-shaped capsid. The genetic material of WNV is positive-sense, single-stranded, RNA which is held within nucleocapsid proteins (Hayes et al., 2005, Gyure, 2009, Rossi et al., 2010). The RNA strand is translated as a single open reading frame that is proteolytically cleaved by viral and cellular proteases into 10 proteins. The 5’ end of the RNA encodes for 3 structural proteins including the capsid (C), premembrane (prM) and envelope (E) proteins and 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5), which likely contribute to virus replication and immune evasion (Li et al., 2010, Gyure, 2009, Rossi et al., 2010). Although, phylogenetic analysis of WNV genomic sequences has proposed eight distinct lineages of WNV strains, isolates grouped in lineage I and II are by far the most widespread (Savini et al., 2012). Strains of both lineages are able to cause severe disease in birds, horses and humans (Venter and Swanepoel, 2010). The majority of WNV infections in humans prior to the late 1990’s were asymptomatic and typically resulted in a mild, uncomplicated disease course termed WNV fever that was characterised by ‘flu-like’ symptoms such as a fever, headaches and general malaise (Prabhakar et
al., 2013, Solomon et al., 2003). However, the more recent epidemics of WNV have been associated with more severe symptoms including encephalitis, meningitis, ataxia, weakness, recumbency and muscle fasciculation (Papa et al., 2011, Vazquez et al., 2010, Venter and Swanepoel, 2010, Chowers et al., 2001).

Given the fact that animal models recapitulate many features of human disease, including the neuro-invasiveness and central nervous system complications of WNV infection, a number of robust models have been developed in mice to investigate various stages of WNV infection. Moreover, several routes of WNV inoculation have been employed, including subcutaneous (s.c), intraperitoneal (i.p), intravascular (i.v), intracranial (i.c), intranasal (i.nas) and intradermal (i.d), each of which mimic particular aspects of severe WNV infection (King et al., 2007).

Although it has not been specifically determined if WNV can be transmitted by sexual contact, the closely related Japanese encephalitis virus is known to cause abortion in swine populations following transmission via the reproductive tract (Imoto et al., 2010). More recently, Zika virus, a flavivirus that is related to WNV, has been shown to be sexually transmitted among humans (Foy et al., 2011). In addition, Japanese and St Louis encephalitis viruses can be excreted in the urine of infected humans and may thus increase the risk for genital tract infections (Luby et al., 1980).

More specifically, WNV has been employed as a tool to examine the murine response to vaginal infection in our laboratory (Burke et al., 2004). It has been established that vaginal WNV infection exhibits many similarities to the HSV-2 model (Burke et al., 2004; Wen, 1998), that is, it is characterised by productive infection of the vaginal epithelium followed by an acute inflammatory
infiltrate into the vaginal mucosa and draining ILN (Burke, 2005, Wen, 1998, Wu, 2006, Yeung, 2012). Interestingly, WNV is cleared in this model of murine vaginal infection within 7 days and infected mice are completely immune upon rechallenge 6–8 weeks later (Burke et al., 2004). In contrast to the common HSV-2 model of vaginal infection, which is associated with high mortality in mice, inoculation of WNV results in a mild and self-limiting disease scenario. Thus, comparative studies between WNV and HSV-2 models of infection may help identify effective innate and adaptive immune responses in the local vaginal mucosa and ILN. These immune responses are further examined in Chapters 3 and 4.

1.4 Indoleamine 2,3-dioxygenase (IDO)

IDO is an intracellular haem-containing enzyme that catalyses the first and rate-limiting step in the kynurenine (Kyn) pathway of L-tryptophan (L-Trp) metabolism. L-Trp, the least abundant essential amino acid, is degraded to N-formylkynurenine and then to Kyn, the stable end product. This results in the depletion of L-Trp from the local cellular environment with a concomitant increase in the downstream metabolites of Kyn pathway (Munn et al., 2004a, Thomas and Stocker, 1999). IDO was first discovered and isolated by Osamu Hayaishi’s group in 1967 from the rabbit intestine (Higuchi and Hayaishi, 1967, Yamamoto and Hayaishi, 1967).

The presence and activity of IDO has been examined in various tissues and as seen in the rabbit, IDO has been found to be expressed in many tissues from humans, rats and mice, with high IDO activity found in the lungs and
intestines during steady-state conditions (Yamazaki et al., 1985). In addition, the
highest expression of IDO is detected in the placenta (Takikawa, 2005, Takikawa
et al., 1988).

As IDO expression is able to deplete L-Trp and accumulate downstream
Kyn metabolites in sites of infection and inflammation, IDO has important roles
in many physiological and pathological situations, including fetal/maternal
tolerance, tumour resistance, chronic infection and autoimmune disease, as well as
host defence against infections (González et al., 2008, Munn et al., 2004a).

1.4.1 Structure and properties of IDO

cDNAs encoding for human and mouse IDO (Tone et al., 1990, Dai and
Gupta, 1990, Akemi et al., 1991) have been cloned and expressed in vitro and
then the structure deduced. The mouse IDO gene encodes a monomeric protein of
407 amino acids (Akemi et al., 1991), while the human IDO gene encodes a
protein of 403 amino acids with a molecular weight of approximately 45 kDa (Dai
and Gupta, 1990, Tone et al., 1990). The primary sequence of mouse IDO shows
approximately 61% identity to that of human IDO (Akemi et al., 1991). This
monomeric enzyme has a high affinity for D- and L-Trp and is able to use both O₂
and superoxide anion as a source of oxygen in the oxidative reaction. Even though
IDO is a haem-containing protein, there is no sequence identity or similarity to
other haemoproteins such as haemoglobin, myoglobin, or cytochrome P-450
(Takikawa, 2005, Hirata and Hayaishi, 1975). In contrast, tryptophan 2,3-
dioxygenase (TDO), another enzyme that catalyses the same reaction as IDO, is
only expressed in the liver and brain, is a homo-tetramer of 167 kDa (Stone and Darlington, 2002, Thackray et al., 2008, Haber et al., 1993). TDO was discovered earlier than IDO in 1937 (Kotake and Masayama, 1937) and differs notably in location, structure, substrate specificity and regulation (Yoshida and Hayashi, 1987, González et al., 2008). It shows substrate specificity for L-Trp, although with lower affinity than IDO, and uses only O₂ in the oxidative ring cleavage. (Dick et al., 2001, Mellor and Munn, 2004).

1.4.2 L-Trp metabolism through Kyn pathway

L-Trp is the rarest of the 22 standard amino acids (Peters, 1991) and is either obtained from the daily dietary intake or released by protein turnover (Brown, 1996). More than 95% of free L-Trp is metabolised along the Kyn pathway, thus leading to the biosynthesis of nicotinamide adenine dinucleotide (NAD) or the complete oxidation of the amino acid, whereas the remainder (about 1–5%) is converted to serotonin or used in protein synthesis (Figure 1.1) (Gál and Sherman, 1980, Oxenkrug, 2010).

IDO is the initial enzyme in the tightly regulated L-Trp degradation pathway. IDO catalyses the oxidative cleavage of the indole ring of L-Trp to produce N-formylkynurenine. N-formylkynurenine further loses the formyl group to form the stable product, Kyn, in a spontaneous reaction or via arylformamidase. The aromatic ring of Kyn is then hydroxylated and converted to either quinolinic acid or picolinic acid or is oxidised to CO₂ and H₂O (González et al., 2008, Thomas and Stocker, 1999, Stone and Darlington, 2002, Grohmann et al., 2003).
Hepatocytes in the liver are the only cells that contain the cascade of enzymes for the Kyn pathway that lead to the complete oxidation of L-Trp and production of NAD$^+$ from it’s precursor, quinolinic acid, that, in turn, links the Kyn pathway to the intracellular redox status (Thomas and Stocker, 1999, Stone and Darlington, 2002). The importance of NAD$^+$ as a co-factor in many cellular reactions, as well as in DNA repair and maintaining cellular redox balance, has been clearly illustrated (Kirkland, 2012, Surjana et al., 2010). Thus, the activity of IDO and production of this co-factor through Kyn pathway is of critical importance for host cell survival (Khan et al., 2007, Grant et al., 2000a, Kirkland, 2012).

IDO is not the only enzyme that metabolises L-Trp to downstream by-products, as TDO and a more recently discovered isoform, IDO-2, also exhibit L-Trp-catalysing activities (Ball et al., 2007). IDO-2 is thought to have originated from gene duplication and catalyses the oxidation of the indole ring of indoleamines, although with a much lower affinity than IDO (Austin et al., 2010, Ball et al., 2009, Metz et al., 2007). In contrast to IDO, which is highly induced by several different inflammatory stimuli in many cell types and tissues, the activity of TDO is primarily restricted to the liver and brain and is not induced or regulated by immunological signals (Mellor and Munn, 2004, Mellor et al., 2003b). Rather, TDO seems to principally function as a homeostatic enzyme in the liver for the maintenance of physiological concentrations of L-Trp under normal conditions. Moreover, the differential induction and expression pattern of IDO2 also suggests a differential function for this enzyme within the tissues and during inflammatory conditions (Ball et al., 2009).
Figure 1.1: A summary of the various pathways of tryptophan metabolism (Stone and Darlington, 2002).
1.4.3 IDO induction and post-translational regulation

The IDO gene is comprised of 10 exons spread over ~15 kbp of DNA on chromosome 8 in humans and mice (Kadoya et al., 1992). IDO gene transcription is tightly controlled and only responds to specific inflammatory signals, depending on the cell type and tissue (Mellor and Munn, 2004). Although the IDO gene contains multiple sequence elements that confer responsiveness to IFN-α/β, the most potent inducer of IDO expression is IFN-γ (Hassanain et al., 1993, Mellor and Munn, 1999, Taylor and Feng, 1991). It has been shown that professional APCs, such as macrophages and DCs, as well as other cell types including fibroblasts, endothelial cells and some tumor cell lines, express IDO following IFN-γ exposure (Burke et al., 1995, Hassanain et al., 1993, Hwu et al., 2000). Moreover, CD4+ T cells are able to produce IDO upon stimulation with IFN-α and IFN-γ (Boasso et al., 2005b, Curreli et al., 2001). Even though there is strong correlation between IFN-γ production and IDO expression, this interferon is not the only inducer of IDO. Other signalling pathways and cytokines can modulate IDO expression in a cell-specific manner. For example, co-stimulatory surface molecules on DCs, CD80 and CD86, can bind to cytolytic T-lymphocyte antigen-4 (CTLA-4) on the surface of T cells. CTLA-4 is a negative regulator of T cell activation, which is expressed on immunosuppressive Treg cells. This ligation leads to the expression of IDO in DCs and subsequent suppression of T cell-mediated immune responses (Boasso et al., 2005a, Munn et al., 2004b, Fallarino et al., 2003, Grohmann et al., 2002). Although TNF does not have the capacity to induce IDO production alone, together with IFN-γ and IL-1, it synergistically stimulates IDO expression in several cell types (Babcock and Carlin, 2000,
Robinson et al., 2003). Furthermore, ligation of several TLRs (TLR 3, 4, 7, 8 and 9) can induce the expression of IDO in a type I and II IFN-dependent manner (Hayashi et al., 2001, Adams et al., 2004a, Fallarino and Puccetti, 2006, Mellor et al., 2005, Furset et al., 2008, Hissong et al., 1995, Suh et al., 2007).

As IDO depletes the least abundant essential amino acid from the microenvironment, it is not surprising that IDO activity is subject to stringent regulation. It is well known that IDO is highly induced during maturation of monocyte-derived macrophages and DCs, however, fully functional IDO activation requires further stimulation, mainly by IFN-γ, indicating post-translational regulation by some types of IDO activators (Mellor and Munn, 2004, Munn and Mellor, 2013). For instance, murine splenic DCs constitutively express IDO protein with no enzymatic activity, but only the CD8⁺ DC subset exhibits fully active IDO following exposure to IFN-γ (Fallarino et al., 2002a, Orabona et al., 2006).

Emerging evidence indicates that the supply and insertion of haem and iron as well as the changes in the intracellular redox status also significantly affects cellular IDO activity (Thomas et al., 2001). However, among the IDO regulatory pathways, perhaps the most thoroughly studied post-translational modulator of IDO activity is nitric oxide (NO), which is generated via the catabolism of L-arginine by NO synthase (NOS) (Alberati-Giani et al., 1997). Under inflammatory conditions, a variety of stimuli that induce IDO such as IFN-γ, LPS and TNF, also induce expression of NOS in multiple cells types, specifically, inducible iNOS (also known as NOS2) (López et al., 2006, Thomas and Stocker, 1999). Research suggests that NOS2 and IDO are reciprocally
controlled, as the expression of one enzyme can regulate the enzymatic activity of the other (King and Thomas, 2007).

### 1.4.4 IDO role in the immune response

Historically, depletion of necessary nutrients has been considered to be one of the simple methods used by the innate immune system against the invasion of microorganisms. Evidence from in vitro studies clearly confirms that IDO-dependent L-Trp deprivation can impair the growth of intracellular pathogens including *Chlamydia pneumonia* (Pantoja et al., 2000), *Toxoplasma gondii* (Pfefferkorn, 1984), cytomegalovirus (Bodaghi et al., 1999), WNV (Yeung et al., 2012), hepatitis C (HCV) (Larrea et al., 2007) and HSV (Adams et al., 2004a, Adams et al., 2004b). This effect is mediated by increased L-Trp degradation, as adding high doses of exogenous L-Trp can reconstitute pathogen or viral replication. Although IDO has an effect on pathogen replication in vitro, the biological relevance of IDO in controlling infections in vivo remains unclear.

Interestingly, recent reports suggest that IDO has more than one role in the immune responses and that these roles are sometimes paradoxical. This enzyme modifies immune responses in two ways: by producing Kyn and other downstream metabolites, some of which are the natural immunologically active ligands for the aryl hydrocarbon receptor (AhR), and by local L-Trp withdrawal to trigger amino acid-sensing signal transduction pathways (González et al., 2008, Munn and Mellor, 2013). The AhR is a ligand-activated nuclear transcription factor that induces complex immunological effects on T cell subsets depending on
the characteristics of the ligands as well as their affinity for the AhR, the duration of signalling and other factors (Stockinger et al., 2011, Nguyen et al., 2013).

Generation of Kyn pathway metabolites or L-Trp deprivation is associated with promoting differentiation of FoxP3+ Treg cells (Mezrich et al., 2010, Chen et al., 2008, Jenabian et al., 2013), suppression of anti-tumour immune responses (Pilotte et al., 2012) and a decrease in the immunogenicity of professional APCs (Nguyen et al., 2013), clearly demonstrating the immunosuppressive role of IDO activity.

1.4.4.1 IDO as an immunosuppressive molecule

The fundamental function of the immune system is to establish tolerance for self while mounting appropriate immune responses against foreign or non-self Ags. The notion of IDO-mediated immune tolerance was first discovered in a mouse model of pregnancy, where pregnant mice rejected allogeneic foetuses after exposure to the pharmacological IDO inhibitor, 1-methyl tryptophan (1-MT; a competitive substrate inhibitor) (David et al., 1998) suggesting that placental IDO acts to inhibit the specific T cells response against the immunologically foreign foetus by L-Trp degradation. Since then, many subsequent reports have found that IDO-expressing APCs suppress the T cell responses by depriving them of L-Trp in vivo and in vitro (Munn et al., 2004a, Munn et al., 2004b, Xu et al., 2008). IFN-γ-stimulated macrophages and DCs that express IDO suppress the proliferation of activated CD4+ and CD8+ T cells, but not resting T cells, by halting T cells in the G1 phase (Munn et al., 1999). In addition to the indirect immunosuppressive effects of IDO via DCs, other studies have found that Kyn
pathway metabolites such as picolinic acid, quinolinic acid and 3-hydroxyanthranilic acid are toxic for Th1 cells, causing them to undergo cell cycle arrest and apoptosis (Fallarino et al., 2002b, Frumento et al., 2002).

On the other hand, Th2-type T cells are not sensitive to Kyn pathway byproducts, raising the possibility that IDO might alter the Th1/Th2 cell balance (Fallarino et al., 2002b). A more recent analysis of IDO-mediated T cell suppression in murine tumour-draining LNs shows that even though many macrophages and DCs express IDO protein, functional IDO expression is only confined to a small portion of DCs including B220⁺ pDCs and CD8⁺ DC subsets (Munn et al., 2004a, Fallarino et al., 2003, Mellor et al., 2004). These IDO⁺ subsets can activate resting CD4⁺ CD25⁺ FoxP3⁺ Treg cells for potent suppressor activity, not only in the tumour-bearing mouse, but also in the normal host, without the need for exogenous stimuli (Munn et al., 2004a). These data emphasise that the biologically relevant population of IDO-expressing DCs may only be a minor subset of cells. However, while murine CD8⁻ DCs do not have functional IDO activity, IFN-γ induction in combination with the presence of Kyn still stimulates these cells to acquire suppressive properties. This is because these CD8⁻ DC are still able to express other enzymes in the Kyn pathway, allowing them to take up Kyn from the microenvironment and generate immunosuppressive metabolites (Belladonna et al., 2006). This implies that DC subsets, which are exposed to enough Kyn, can be still induced to mediate certain tolerogenic effects independently of L-Trp concentration. Moreover, the proliferation of NK cells is effectively restricted in the presence of Kyn concentrations, possibly by inhibiting the responsiveness of NK cells to IL-2 and IL-12 (Della Chiesa et al., 2006). Indeed, Kyn prevents the cytokine-induced
upregulation of the NK cell activating cellular receptors (NKp46 and NKG2D) that are responsible for NK cell killing (Della Chiesa et al., 2006). Again, the suppressive effect of Kyn can be reversed by addition of 1-MT or when Kyn was removed from the microenvironment.

1.4.4.2 Immune response during viral infection

Conventionally, IDO activity is considered to be part of the innate immune response against infection. Deprivation of L-Trp and the subsequent rise in the toxic metabolites of the Kyn pathway both in the microenvironment and systemically, can affect a wide range of pathogens, particularly those closely reliant on the host cell for replication or survival (Mellor and Munn, 2004).

Given the fact that viruses must employ host cell machinery to replicate and proliferate, they are particularly susceptible to IDO-mediated L-Trp deprivation. Soon after the discovery of IDO, it was found that IDO activity is induced in murine lungs infected with influenza virus (Yoshida et al., 1979). Moreover, several other groups found that IDO activity is induced or upregulated in experimental animal models and human patients during viral infections with poliovirus (Heyes et al., 1992), LP-BM5 murine leukemia virus (Hoshi et al., 2010), dengue virus (Becerra et al., 2009) and Epstein-Barr virus (Bellmann-Weiler et al., 2008), as well as HSV-1 (Reinhard Jr, 1998).

In most cases, the induction of IDO is confined to the tissues infected with the viruses. For example, during pulmonary infection with influenza virus, the IDO-dependent L-Trp degradation occurs in the local infection site (Yoshida et al., 1979). L-Trp depletion was also suggested to be the mechanism by which IDO
induced by IFN-γ was able to suppress the growth of intracellular pathogens in vitro (Adams et al., 2004a, Larrea et al., 2007, Yeung et al., 2012). However, systemic induction of IDO is observed in systemic or chronic viral infections with dengue virus, Epstein Barr virus and HIV (Favre et al., 2010, Heyes et al., 1992, Becerra et al., 2009, Bellmann-Weiler et al., 2008). Therefore, these infections may cause a systemic reduction in L-Trp and a concomitant increase in Kyn levels in the blood, as well as increasing the urinary levels of xanthurenic acid (the major metabolite of blood Kyn in the urine of C57BL/6 mouse) (Yoshida et al., 1979, Takikawa et al., 1986, Takikawa, 2005), which in turn may have detrimental effects. Furthermore, these markers of IDO activity correlate with disease severity (Takikawa, 2005, Sardar et al., 1995, Sardar and Reynolds, 1995).

The antiviral activity of IDO has been widely documented in various viral infections in vitro. Several groups have examined the role of IDO induction in suppressing viral replication such as cytomegalovirus (Bodaghi et al., 1999), HSV-1 (Adams et al., 2004b) and -2 (Adams et al., 2004a), vaccinia (Terajima and Leporati, 2005), dengue (Becerra et al., 2009), measles (Obojes et al., 2005), hepatitis B (Mao et al., 2011) and WNV (Yeung et al., 2012). In these reports, IDO activity is highly upregulated by IFN-γ, but not IFN-α/β. Interestingly, similar to the results observed in other pathogens, the presence of excess amounts of L-Trp abrogates the antiviral effect induced by IFN-γ.

LP-BM5 murine leukaemia virus causes a fatal immunodeficiency syndrome in mice known as murine AIDS. Mice infected with this virus upregulate IDO expression and activity in several tissues. Remarkably, however, IDO-deficient or 1-MT-treated mice have enhanced type I IFN production and an
increased number of innate and adaptive immune effector cell subsets including NK cells, T cells and conventional and plasmacytoid DC compared to the WT control mice (Hoshi et al., 2010). This results in inhibition of viral replication. Neutralisation of type I IFN confirmed that the observed suppression of virus replication is mediated by IFN-α/β.

As HIV is a global threat and one the most important STI, several studies have investigated the role of IDO during HIV infection in vitro and in vivo. It has been suggested that HIV infection (or simian equivalent, SIV, in the rhesus macaques) induces high levels of functional IDO expression, mainly by APCs, in the brain and CNS (Favre et al., 2010, Heyes et al., 1992, Sardar et al., 1995, Sardar and Reynolds, 1995). This results in systemic reduction of L-Trp and increased levels of Kyn pathway metabolites, Kyn and quinolinic acid, which are neurotoxic and can therefore lead to neuronal dysfunction in these patients (Kandanearatchi and Brew, 2012).

In vitro studies with various HIV strains also indicate that infected human monocyte-derived macrophages (MDM) produce large amounts of IFN-γ that is responsible for the subsequent IDO expression and activity. Interestingly, MDM cells respond differently to the initial infection with different subtypes of HIV virus (Grant et al., 2000b, Grant et al., 2000c).

Moreover, a study from Favre and colleagues indicates that HIV and SIV infections are associated with increased Kyn pathway metabolite concentrations, specifically 3-hydroxyanthranilic acid (Favre et al., 2010). 3-hydroxyanthranilic acid enhances the differentiation and activation of Treg cells that changes the ratio between Th17 and Treg cells, further promoting a chronic inflammatory state in progressive HIV disease. In addition, neutrophils obtained from HIV-infected
patients as well as HIV-primed human pDCs express active IDO, inhibit Th1 cells and lead to the generation of CD4+ CD25+ FoxP3+ Treg cells (Boasso et al., 2007, Manches et al., 2008).

On the other hand, administration of the IDO inhibitor 1-MT suppresses IDO activity and enhances the trafficking of CD8+ T cells to the site of infection in a mouse model of HIV encephalitis, correlating with greater clearance of virus and infected macrophages. These mice, however, still fail to control the infection (Potula et al., 2005). This observation thus can be taken as evidence that viral-mediated IFN-γ induction and IDO expression can lead to the immune escape, helping HIV to evade the HIV-specific immune response in vivo.

Taken together, IDO activity has a significant role in establishing robust immunosuppression in many physiological microenvironments, directly and indirectly, in a manner that is dependent on both L-Trp degradation as well as the generation of Kyn metabolites. This activity could benefit the host in many situations such as in maternal tolerance to pregnancy, uncontrolled immune activation, autoimmune diseases, hypersensitivity and transplantation, although it is detrimental in cancer or infection.

1.5 Aims of this project

The primary aim of this research was to further understand the dynamics of the innate and adaptive immune response in the FRT in the early phase of murine models of HSV-2 and WNV infections. Specifically, we wanted to acquire data investigating the role of sub-mucosal DCs in the initiation of the vaginal response to infection. Also, we wished to define differential cytokine and
chemokine responses in the murine WNV model using two genetically and immunologically distinct mouse prototypes. In this early phase, the effects of the IFN-γ-induced tryptophan-depleting enzyme, IDO, in the regulation of the anti-HSV-2 immune response locally in the vagina and in secondary lymphoid tissue were also important to examine. Moreover, it was very interesting to delineate whether IDO induction in the vagina and ILN contributed to the anti-viral activity of the local immune system.
Chapter 2

Materials and Methods
Chapter 2:

Materials and methods

2.1 Materials

2.1.1 Viruses

The original WNV (Sarafend strain) (Poidinger et al., 1996) stock was acquired from Professor Alison Kesson (The Children’s Hospital at Westmead, Sydney, Australia), while HSV-2 (strain 186) (Rawls et al., 1968) was obtained from Dr Marian Fernandez (The Children’s Hospital at Westmead, Sydney, Australia).

2.1.2 Mice

Female WT C57BL/6 (H-2b), B6.SJL-Ptprc<sup>p</sup>Pep3<sup>b</sup>/BoyJ and BALB/c (H-2<sup>d</sup>) mice were acquired from the Animal Resources Centre (Canning Vale, WA, Australia); female IDO1<sup>−/−</sup> mice were obtained from a colony established by Dr. Shane Thomas and Professor Nicholas King at Australian BioResources (NSW, Australia). Construction methodology and genetic background of IDO knockout mouse has been described earlier (Mellor et al., 2003a). Briefly, DNA constructs containing complete IDO gene, were generated. Exons 3-5 of IDO gene was manipulated to be replaced by neomycin (selectable marker) and also, a
translational stop codon (TAG) inserted into exon 2. Then, constructs were electroporated into stem cells and clones showing targeted alleles of IDO were injected into blastocysts. Finally, IDO deficient mice generated by this method were mated with chimeric male C57BL/6 mice to produce a pool of IDO−/− mice (Mellor et al., 2003a). All mice were age-matched and used between 7–13 weeks of age. Upon arrival, mice were housed in specific-pathogen free conditions in Hepa cages in class II biohazard hoods. Mice were provided with autoclaved food and sterile tap water ad libitum and monitored daily according to The University of Sydney Animal Ethics Committee guidelines (Animal ethics protocol numbers: K20/11-2011/3/5660 and K20/8-2009/3/5127).

2.1.3 Avertin (anaesthetic)

Avertin was prepared as follows; 1 g of 2,2,2-tribromoethanol (Sigma-Aldrich, MO, USA) was dissolved in 1 ml of 2-methyl-2-butanol (Sigma-Aldrich). Hot tap water (50 ml) was added and the contents vortexed vigorously to mix. The solution was filter-sterilised and the aliquots were stored at -20°C until use. A dose of 0.012 ml/g of body weight was injected intraperitoneally to obtain optimal anaesthesia. This equated to approximately 250-300 μl per mouse.

2.1.4 Solutions and buffers

Sera

Foetal calf serum (FCS; In Vitro, VIC, Australia) was aliquoted and kept at -20°C until use. For in vitro studies (e. g., plaque assay), the FCS was heat inactivated at 56°C for 30 min before use.
Phosphate-buffered saline (PBS)

PBS was prepared by dissolving 9.6 g of PBS powder (Sigma-Aldrich) in 1 L MilliQ water and filter-sterilised. Tween-20 (0.05%) was added to PBS (TBST) for immunofluorescent staining.

Tris-buffered saline (TBS)

13.9 g Trizma base (Sigma- Aldrich), 60.6 g TRIS HCl and 87.66 g NaCl (Amresco, OH, USA) were dissolved in 900 ml of MilliQ water in order to make 1 L of 10x TBS. The solution was adjusted to a pH of 7.6–7.8. The 10x solution was diluted to 1x with MilliQ water and 5 ml Tween-20 was added for use.

Depo-Provera

Medroxyprogesterone acetate (Pfizer, NY, USA), a long-acting synthetic progesterone, was shaken thoroughly, diluted in sterile PBS and administered subcutaneously at 4 mg/100 μL per mouse.

TNB blocking buffer

A 1x stock solution of TNB was prepared based on the manufacturer’s instructions and then aliquoted and stored at -20°C until use. TNB blocking buffer contained 0.1 M Tris-HCl pH 7.5 and 0.15 M NaCl with 0.5% (w/v) blocking reagent from the Tyramide Signal Amplification kit (PerkinElmer, MA, USA).

Collagenase/DNase solution

A 1 mg/ml collagenase IV (Sigma-Aldrich) and 0.5 mg/ml DNase I (Sigma-Aldrich) were dissolved in PBS to prepare a 1x working solution of collagenase/DNase. This solution was made fresh prior to each flow cytometry experiment.
**FACS buffer**

FACS buffer was composed of PBS that was supplemented with 5% FCS and 5 mM ethylenediaminetetraacetic acid (EDTA; Lomb Scientific, NSW, Australia). FACS buffer was stored at 4°C until use.

**Cell lysis buffer**

Red blood cell (RBC) lysis buffer was supplied as a 10x solution (Biolegend, CA, USA) and stored at 4°C. The stock solution was diluted to 1x with distilled water, adjusted to a pH of 7.1–7.4 and allowed to warm to room temperature (RT) prior to use.

**Complete protease inhibitor cocktail**

One tablet of complete protease inhibitor cocktail (Roche Applied Science, Germany) was dissolved in 50 ml PBS. Then, the solution was aliquoted and stored at -20°C until required. Each tablet contained mixture of several proteases to inhibit protease activity including Pancreas extract, Pronase, Thermolysin, Chymotrypsin, Trypsin and Papain.

**HPLC mobile phase**

A 2 L of HPLC mobile phase was composed of 17.01 g chloroacetic acid (Sigma-Aldrich), which was dissolved in 1.8 L distilled water. 180 ml acetonitrile (Sigma-Aldrich) was also added. The mobile phase was adjusted to a pH of 2.4 using 10 M NaOH (Sigma-Aldrich). This resulted in a final concentration of 100 mM chloroacetic acid in 9% acetonitrile.

**Ear or tail tissue digestion buffer**

The stock tissue digestion buffer was composed of 1 M Tris (pH 8.0) (Sigma-Aldrich), 0.5 M EDTA (Lomb Scientific) and 10% (w/v) SDS (Amresco, OH, USA). To make 100 ml of the working digestion buffer, 5 ml Tris, 5 ml SDS
and 20 ml EDTA solutions were mixed and topped up to 100 ml with MiliQ water. The final concentration of reagents in the working digestion buffer were 50 mM Tris, 100 mM EDTA and 0.5% SDS. To start digestion process, 1 μL of 20 mg/ml protein kinase (New England Biolabs, MA, USA) was added to 100 μL of fresh tissue digestion buffer.

**TBE buffer**

A 10x stock solution of TBE was prepared. To make 500 ml, 54 g Trizma base (Sigma-Aldrich), 27.5g boric acid (Sigma-Aldrich) and 100 ml of 0.1 M EDTA were added to 400 ml MilliQ water. The solution was adjusted to a pH of 8.0. The 10x solution was diluted to 1x with MilliQ water.

### 2.1.5 Cell culture medium

All cell culture media were prepared fresh by dissolving media powder in MilliQ water. The solutions were sterile-filtered with a 0.22 μm filter and stored at 4°C. Medium was warmed to 37°C before use.

**MEM**

Lipopolysaccharide (LPS)-free Minimum Essential Medium containing L-glutamine and phenol red (Life Technologies, CA, USA) supplemented with 20 mM Hepes (Amresco), 2.2 g/L sodium bicarbonate (Amresco), 4 g/L penicillin G and 0.2 g/L streptomycin sulfate (Sigma-Aldrich). MEM was supplemented with 2% or 10% FCS as required. For the standard plaque assay overlay, 2x MEM with 4% FCS was used.
DMEM/10% FCS

LPS-free Dulbecco’s Modified Eagle’s Medium containing L-glutamine and phenol red (Life Technologies) was supplemented with 10% FCS, 20 mM Hepes (Amresco), 2.2 g/L sodium biocarbonate (Amresco), 4 g/L penicillin G and 0.2 g/L streptomycin sulfate (Sigma-Aldrich).

Medium 199V/10% FCS

Medium 199V was supplemented with 2 mM L-glutamine, 100 U/ml of penicillin G, 100 μg/ml streptomycin sulfate (all from Life Technologies) and 10% FCS (In Vitro).

2.1.6 Cell lines

All cells were maintained at 37°C in a HeraCell humidified incubator (Thermo Scientific, MA, USA) with 5% CO₂. BHK cells (baby hamster kidney cells) (Stoker and Macpherson, 1964) and Vero cells (African green monkey kidney epithelial cells) (Yasumura and Kawakita, 1963) were grown in DMEM/10% FCS. An overlay of MEM/4% FCS/1.5% agarose II was used in plaque assays.

2.1.7 TaqMan primer and probe sequences

The primers and probes for WNV and HSV-2 real-time PCR were custom-ordered (Applied Biosystems, CA, USA) based on the sequences that were previously used and published. The WNV primers and probe detecting a
conserved 92 bp of the WNV 3’ non-coding region (Tang et al., 2006) and the HSV-2 primers and probe detecting HSV-2 glycoprotein B region (Namvar et al., 2005) are listed in Table 2.1. Primers and probes were reconstituted or diluted with nuclease-free water (QIAGEN, Germany) to 18 and 5 μM, respectively, and aliquoted and stored at -20°C until use.

**Table 2.1.** TaqMan primer and probe sequences for WNV RNA and HSV-2 mRNA

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNV</td>
<td>Forward primer: AAG TTG AGT AGA CGG TGC TG</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer: AGA CGG TTC TGA GGG CTT AC</td>
</tr>
<tr>
<td></td>
<td>Probe: CTC AAC CCC AGG AGG ACT GG</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Forward primer: TGC AGT TTA CGT ATA ACC ACA</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer: AGC TTG CGG GCC TCG TT</td>
</tr>
<tr>
<td></td>
<td>Probe: CGC CCC AGC ATG TCG TTC ACG T</td>
</tr>
</tbody>
</table>

**2.1.8 Antibodies**

The antibodies listed in Table 2.2 and Table 2.3 were used for immunofluorescent staining and flow cytometry, respectively.
Table 2.2: Antigen specificity, isotype, clone, dilution and source for each antibody used for immunofluorescent staining. All are specific for mouse antigens unless specified.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Clone</th>
<th>Dilution</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1NS-1 FITC</td>
<td>Mouse IgG1</td>
<td>4G4</td>
<td>1:50</td>
<td>NA</td>
<td>Roy Hall, UQ</td>
</tr>
<tr>
<td>NS-1 biotinylated</td>
<td>Mouse IgG21</td>
<td>4G4</td>
<td>1:100</td>
<td>NA</td>
<td>Roy Hall, UQ</td>
</tr>
<tr>
<td>HSV-2 FITC</td>
<td>Sheep IgG</td>
<td>ab20971</td>
<td>1:400</td>
<td>4 mg/ml</td>
<td>Abcam</td>
</tr>
<tr>
<td>CD11c biotinylated</td>
<td>Armenian Hamster IgG</td>
<td>HL-3</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD11b biotinylated</td>
<td>Rat IgG2b</td>
<td>M1/70</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>F4/80 biotinylated</td>
<td>Rat IgG2a</td>
<td>BM8</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CCL2 biotinylated (human/rat/mouse)</td>
<td>Armenian Hamster IgG</td>
<td>2H5</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CCL20 FITC</td>
<td>Rat IgG1</td>
<td>114906</td>
<td>1:20</td>
<td>0.1 mg/ml</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>IDO (purified)</td>
<td>Rabbit IgG</td>
<td>---</td>
<td>1:5000</td>
<td>NA</td>
<td>Osamu Takikawa, Hokkaido University</td>
</tr>
<tr>
<td>IDO (purified)</td>
<td>Rat IgG2b</td>
<td>mIDO-48</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>

1 (Arnold et al., 2004)
Table 2.3: Antigen specificity, isotype, clone, dilution and source for each antibody used for flow cytometry analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Clone</th>
<th>Dilution</th>
<th>Concentration</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>CD3ε</td>
<td>Hamster IgG</td>
<td>145-2C11</td>
<td>1:300</td>
<td>0.2 mg/ml</td>
<td>BD</td>
</tr>
<tr>
<td>CD4</td>
<td>Rat IgG2a</td>
<td>RM4-5</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
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</tr>
<tr>
<td>CD8α</td>
<td>Rat IgG2b</td>
<td>53-6.7</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD11b</td>
<td>Hamster IgG</td>
<td>HL-3</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD11c</td>
<td>Rat IgG2b</td>
<td>M1/70</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
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<td>93</td>
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</tr>
<tr>
<td>CD25</td>
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<td>PC61</td>
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<td>Biolegend</td>
</tr>
<tr>
<td>CD45</td>
<td>Rat IgG2b</td>
<td>30-F11</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend, BD</td>
</tr>
<tr>
<td>CD45R (B220)</td>
<td>Rat IgG2a</td>
<td>RA3-6B2</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
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<td>CD45.1</td>
<td>Mouse IgG2a</td>
<td>A20</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend, BD</td>
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<td>CD45.2</td>
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<tr>
<td>I-A/I-E</td>
<td>Rat IgG2b</td>
<td>M5/114.15.2</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>I-A^4</td>
<td>Mouse (C3H.SW) IgG3</td>
<td>39-10-8</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Ly6C</td>
<td>Rat IgG2c</td>
<td>HK1.4</td>
<td>1:100</td>
<td>0.5 mg/ml</td>
<td>Biolegend</td>
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<tr>
<td>Ly6G</td>
<td>Rat IgG2a</td>
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<td>1:100</td>
<td>0.5 mg/ml</td>
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<td>Antibody</td>
<td>Species</td>
<td>Isotype</td>
<td>Dilution</td>
<td>Concentration/mL</td>
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<td>NK1.1</td>
<td>Mouse</td>
<td>IgG2a</td>
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<td>IgG2b</td>
<td>1:20</td>
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<td>F4/80</td>
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<td>IgG2a</td>
<td>1:100</td>
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<td>Biolegend</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Mouse</td>
<td>IgG1</td>
<td>1:5</td>
<td>0.1 mg/ml</td>
<td>Biolegend</td>
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</tbody>
</table>
2.2 Methods

2.2.1 Preparation of stock virus

2.2.1.1 WNV

Stock WNV (3 x 10⁵ PFU) was injected intracranially into newborn C57BL/6 suckling mouse pups. At the first signs of clinical illness (2-3 days), the pups were sacrificed and the brains aseptically removed (Kesson et al., 1987, Taylor and Marshall, 1975). The brains were homogenised in DMEM/10% FCS using a rotor/stator homogeniser (Tissue Tearor; BioSpec, OK, USA) and centrifuged to obtain the clarified supernatant and then aliquoted and stored at -80°C.

The resulting brain-derived WNV was used to propagate the stock virus. Semi-confluent BHK cells in 175 cm² flasks (Corning Incorporated, NY, USA) were infected for 1 h at 37°C in a Heracell incubator, rocking every 15 min to make sure the virus was evenly distributed. DMEM/10% FCS was added to the flask and was further incubated for approximately 2 days, until the cells first exhibited cytopathic effects (CPE). The flasks were freeze-thawed once to release the virus from intact cells. The supernatant was collected and centrifuged at 1000 g for 10 min at 4°C to sediment all cell debris. The clarified supernatant was aliquoted and stored at -80°C until further use.

All experiments used virus that had been freshly thawed. Viral titre was periodically determined by a plaque assay, as described in section 2.2.2, and calculated to be 1.7 x 10⁹ plaque-forming units (PFU) per ml.
2.2.1.2 HSV-2

Stock HSV-2 (10^7 PFU), strain 186 (Rawls et al., 1968) was used to infect semi-confluent 175 cm² flasks of Vero cells at 0.1 PFU/cell for 1 h at 37°C, rocking gently every 15 min. After 1 h, medium 199V/10% FCS was added to the flask and it was returned to the humidified incubator. At the first signs of CPE (2–3 days), the cells were detached by shaking and the cells and supernatant were freeze-thawed twice, sonicated with a Vibra-Cell sonicator (Sonics and Materials Inc., CT, USA) and centrifuged at 1000 g for 15 min at 4°C to remove the cell debris. The supernatant was aliquoted and stored at -80°C until use. The final viral stock concentrations were found to be 5 x 10^7 PFU/ml as determined by HSV-2 plaque assay (section 2.2.3).

2.2.2 Plaque assay for WNV

BHK cells (1 x 10^6) were added to each well in 6-well plates. To give a near-confluent monolayer (~90%), plates were incubated at 37°C overnight. The medium was then aspirated and the cells were washed with PBS to remove dead cells. Cells were infected with 150 μL serial 10-fold dilutions of stock virus for 1 h at 37°C, with gentle rocking every 15 min. At the end of the incubation time, the virus inoculum was removed and replaced with 2 ml 2x MEM/10% FCS mixed with 2 ml 3% (w/v) molten agarose II (Amresco) at ~37°C. Plates were left for approximately 10 min at RT and returned to the incubator for 3–5 days. After this incubation, cells were fixed and the overlay hardened with 10% formalin (Lomb Scientific) for 1-3 h. To visualise the plaques, the agarose plug was removed and
1 ml 3% (w/v) crystal violet (Sigma-Aldrich) in 20% (v/v) methanol added for 1 min. Excess dye was rinsed off with tap water and the plates left to dry. Plaques were counted and the titre calculated as PFU/ml.

2.2.3 Plaque assay for HSV-2

Vero cells (8 x 10^5) were seeded in 6-well plates in DMEM/10% FCS overnight to reach 90% confluence. Wells were rinsed once with PBS and infected with 150 μL serial 10-fold dilutions of virus stock for 1 h with rocking every 15 min. During the incubation, 0.5% (w/v) methylcellulose (Sigma-Aldrich) and 2x MEM/4% FCS were combined in a 1:1 ratio. The supernatant was removed from the wells and the cells were overlaid with the methylcellulose/MEM mix and incubated at 37°C for 3 days. At the end of this period, the overlay was removed and the cells fixed with absolute methanol for 15 min. To stain the plaques cells, 1 ml of 3% (w/v) crystal violet in 20% (v/v) methanol was added into each well for 1 min and the excess rinsed off with tap water. The plates were left to dry overnight and the plaques were counted.

2.2.4 Animal procedures

2.2.4.1 Intravaginal infection of mice

Mice for infection studies were treated with 4 mg of medroxyprogesterone acetate (Pfizer) by subcutaneous injection prior to infection to synchronised mice
in the same progesterone-dominant phase and make them uniformly susceptible to i.vag infection. After 5–7 days, mice were first anaesthetised via i.p injection with avertin and then assessed for their level of consciousness by squeezing the hind foot. Once the withdrawal reflex could not be detected, the vaginal lumen was washed three times with 50 μL of sterile PBS to remove the vaginal mucus using a flame-polished, bevelled-edge 200-μL plastic pipette tip. Mice were then infected with various doses of either WNV or HSV-2 via the i.vag route using a sterile 200 μl pipette tip in a volume of 20 μL. Mock-infected mice were treated with 20 μL sterile PBS. All mice were laid on their back with their pelvis raised for at least 30 min to make sure that there was no leakage before transfer into their cages. Mice infected with WNV did not exhibit any clinical signs of disease.

2.2.4.2 Disease monitoring in HSV-2 infection

HSV-2 infected mice were monitored every day for clinical signs of disease, including the changes in the external region of the vagina that indicated pathology. Clinical disease was scored on a scale of 0 to 5 as follows: 0, no signs of infection; 1, slight redness of genital area; 2, redness and swelling of external vagina; 3, severe swelling of the external vagina and hair loss in the surrounding area; 4, ulceration, severe swelling and redness of the vaginal tissue; and 5, continued ulceration, swelling and redness, occasionally accompanied by paralysis of the hind legs (mice were euthanised at this point) (Gill et al., 2006).
2.2.4.3 Collection of tissues

Mice were sacrificed by cervical dislocation. To collect the vagina, the lumen was first washed 3 times with 50 μL sterile PBS and an incision made in the lower abdominal cavity. The vagina was separated from the adjacent tissue and gently pulled out of the pelvic floor and cut free from the external part of the vaginal opening. The cervix was also removed. For PCR and immunostaining, the vagina was then cut in half longitudinally while for flow cytometry, plaque assay, ELISA or HPLC analysis, the vagina was kept intact. For immunofluorescence, the urethra was left attached to the vagina but the bladder was removed. The iliac lymph nodes (ILN) were also harvested for PCR, immunofluorescence, flow cytometry, ELISA and HPLC.

2.2.5 Real-time PCR

2.2.5.1 Preparation of cells and tissue

To avoid contamination and undue exposure to RNAses, after collection, vaginae and ILN were immediately placed into 2 ml microcentrifuge tubes containing 350 μL RLT buffer from the RNeasy Mini kit (QIAGEN, Germany) with 1% 2-mercaptoethanol (Sigma-Aldrich) and 250 μL of 1 mm zirconia homogenisation beads (Biospec, OK, USA) and frozen in liquid nitrogen. Samples were stored at -80°C until use. Tissues were thawed and then homogenised and disrupted with a FastPrep tissue homogeniser (ThermoElectron Corporation, MA, USA) for 20 s at a setting of 5.
2.2.5.2 RNA extraction

The RNeasy Mini kit (QIAGEN) was used to extract RNA from the vagina and ILN. Tissue lysates were thawed in a 37°C waterbath and centrifuged at 16000 g at RT for 3 min to sediment the debris. The supernatants were transferred to 1.5 ml microcentrifuge tubes where they were mixed with an equal volume of 70% molecular-biology grade ethanol (Sigma-Aldrich). The mixtures were transferred to RNeasy mini-columns in 2 ml collection tubes and centrifuged for 30 s at 8000 g at RT. The flow-through was discarded. Next, 700 μL RW1 buffer was added to the columns which were centrifuged at 8000 g for 30 s at RT. The flow-through was discarded again and the 500 μL RPE buffer was added to the column, which was washed by centrifugation for 30 s at RT at 8000 g before disposing of the flow-through. Another wash step was carried out using 500 μL of RPE buffer and the columns were centrifuged at 8000 g for 2 min to dry the membrane. The columns were carefully transferred to a fresh 2 ml collection tube and centrifuged at 16000 g for 1 min to make sure that there was no remaining contaminants. The columns were transferred to new 1.5 ml microcentrifuge tubes and 30–50 μL nuclease-free water was pipetted onto the column membrane and centrifuged for 1 min at 8000 g to elute the RNA. The RNA was then stored at -80°C until further processing.

The purity and yield of the RNA was analysed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The absorbance of each sample at 230, 260 and 280 nm was measured. Only RNA samples with an A260:A280 ratio between 1.8 and 2.0, and a A260:A230 ratio of ~2, were used for cDNA synthesis. A260:A280 ratios outside of the mentioned range were
indicative of protein contamination in RNA extracted sample and, A260:A230 ratio outside of 2 was indicative of ethanol contamination.

2.2.5.3 cDNA generation

cDNA was synthesised using Maloney Murine Leukaemia Virus reverse transcriptase (MMLV-RT). 1 μg of extracted RNA was diluted to a final volume of 13 μL with nuclease-free water (QIAGEN) and incubated at 70°C for 5 min in a Bio-Rad T100 gradient thermal cycler (CA, USA) to denature the RNA. A cocktail containing the following per sample was prepared: 2.5 μL 10x reaction buffer (Epicentre Biotechnologies, WI, USA), 0.01 M dithiothreitol (DTT) (Epicentre), 10 mM of dATP, dCTP, dGTP and dTTP (Bioline, NSW, Australia), 0.2 μg random hexamer primers (New England Biolabs) and 10 U MMLV-RT (Epicentre) made up to final reaction volume of 25 μL. The samples were centrifuged briefly and then returned to the thermal cycler and incubated at 37°C for 1 h. To denature the reverse transcriptase and terminate the reaction, the samples were further incubated at 85°C for 5 min. The final cDNA products stored at -20°C until further use.

2.2.5.4 TaqMan PCR

TaqMan gene expression assays were used to quantify vaginal mRNA levels and were purchased as pre-designed TaqMan assays from Applied Biosystems (Table 2.4). Each assay for the gene of interest was multiplexed with either 18S RNA (Applied Biosystems) or GAPDH (glyceraldehyde 3-phosphate
dehydrogenase) (Applied Biosystems) as the internal control genes (Table 2.4). A master mix was prepared for each assay as follows: 10 μL of 2x Universal PCR Master Mix (Bioline, NSW, Australia), 1 μL primer/probe mix, 1 μL eukaryotic 18S or mouse GAPDH endogenous control primer/probe mix, 7 μL of nuclease-free water and 1 μL cDNA.

Table 2.4. List of TaqMan gene expression assays

<table>
<thead>
<tr>
<th>TaqMan Primers/Probe</th>
<th>Catalogue #</th>
<th>Labelled Dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNV</td>
<td>Table 2.1</td>
<td>FAM</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Table 2.1</td>
<td>FAM</td>
</tr>
<tr>
<td>IFN-γ</td>
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<td>FAM</td>
</tr>
<tr>
<td>TNF</td>
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</tr>
<tr>
<td>NOS2</td>
<td>Mm 00440485_m1</td>
<td>FAM</td>
</tr>
<tr>
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<td>Mm 00441258_m1</td>
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</tr>
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</tr>
<tr>
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<td>FAM</td>
</tr>
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</tr>
<tr>
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<td>FAM</td>
</tr>
<tr>
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<td>FAM</td>
</tr>
<tr>
<td>CCR2</td>
<td>Mm 9999051_gH</td>
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</tr>
<tr>
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<td>VIC</td>
</tr>
<tr>
<td>18s RNA</td>
<td>4310893E</td>
<td>VIC</td>
</tr>
</tbody>
</table>
The master mix for WNV and HSV-2 TaqMan PCR consisted of the following: 10 μL Universal PCR Master Mix (Bioline), 1 μL forward primer, 1 μL reverse primer, 1 μL probe, 1 μL 18S or GAPDH endogenous control primer/probe mix, 5 μl nuclease-free water and 1 μl of cDNA.

To confirm the efficiency of the reactions, the gene of interest and the housekeeping gene were analysed in cDNA from tissue-extracted RNA in single- or multiplexed-format. Each reaction was carried out in duplicate or triplicate and amplification was performed using the Light Cycler 480 (Roche). The threshold cycles (Ct value) for the gene of interest and the endogenous internal control gene were obtained from the Light Cycler 480 and the relative expression levels were analysed using the ∆∆Ct method and compared to the mock-infected samples.

2.2.6 Immunofluorescence

2.2.6.1 Preparation of tissues

The vaginae and ILN were immersed in Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Fine Technical Co., Japan), slowly frozen in liquid nitrogen to avoid cracking and stored at -80°C until use. For cryosectioning, 5–7 μm sections of tissues were cut with a cryostat microtome (Thermo Fisher Scientific), mounted on Superfrost Plus slides (Menzel-Glaser, Germany), air-dried for 1 h and stored at -80°C.
2.2.6.2 Immunofluorescent staining

For immunofluorescent staining, slides were thawed, fixed with 4% paraformaldehyde for 10 min and washed 3 times with TBS washing buffer for 3 min. Endogenous peroxidase activity was then quenched with 0.3% hydrogen peroxide (Sigma-Aldrich) in methanol (Sigma-Aldrich) for 10 min. Slides were washed 3 times in TBS and blocked with avidin/biotin block (Vector Laboratories, CA, USA) for 10 min before blocking non-specific staining with TNB buffer (Perkin Elmer) containing 10% FCS for 30 min. Excess buffer was then tipped off and slides were incubated with the appropriate conjugated primary, biotinylated Ab or isotype control for 1 h or overnight, as shown in Table 2.2. If required, amplification was conducted using the Tyramide Signal Amplification (TSA) Kit (Perkin Elmer). Samples were incubated with streptavidin-horseradish peroxidase (SA-HRP) diluted 1:100 for 30 min, then washed 3 times in TBS. Samples were then incubated for 10 min with biotinyl tyramide (Perkin Elmer) diluted 1:50 in amplification diluent (Perkin Elmer) and washed 3 times in TBS. Slides were incubated with streptavidin-Alexa Fluor 488 or 594 (Life Technologies) diluted 1:500 for 30 min and washed 3 times in TBS. Prolong Gold mounting medium or Prolong Gold with DAPI (Life Technologies) was pipetted onto tissue samples and glass coverslips were lowered on top.

2.2.6.3 Image analysis

Immunofluorescent images were captured using an Olympus BX-51 microscope (Olympus, Japan) with a DP-70 camera and DP manager 2.2.1
software (Olympus). Image analysis was performed using ImagePro Plus (Media Cybernetics) or ImageJ (NIH, MD, USA) software on random fields-of-view using the 20x and 40x objectives. Colour channels representing different markers were split automatically by the channel splitter option of the software and the total area occupied by positive staining for each colour was expressed as a percentage of the total area of tissue. Results are expressed as the mean and standard error per time point.

2.2.7 Adoptive transfer experiments

For adoptive transfer studies, the femurs and tibias from both legs of B6.SJL-Ptprc<sup>−/−</sup>Pep3<sup>−/−</sup>/BoyJ (CD45.1) mice were dissected. The ends were cut using a scalpel and bone marrow cells were flushed with 10 ml cold PBS using a 1 ml syringe and 30 ½ gauge needle. Cells were collected into a 15 ml centrifuge tube and centrifuged at 460 g for 5 min. RBC were then lysed by suspension in 2 ml of RBC lysis buffer for 5 min at RT and the reaction stopped by the addition of 10 ml FACS buffer. Cells were centrifuged at 460 g for 5 min and resuspended in 10 ml of PBS.

To determine cell numbers, 10 μL aliquots of bone marrow cells were counted using a haemocytometer. Samples were then centrifuged for 5 min at 460 g and resuspended in 1 ml PBS containing 4 μM CellTracker Violet BMQC (Life Technologies) for 1 h at 37°C. The reaction was stopped by adding 1 ml of FCS and cells were resuspended in 10 ml of PBS. Cells were then centrifuged for 5 min at 460 g and washed twice with PBS. Samples were then filtered through a 70
µm nylon sieve and 1 x 10^7 cells in 250 µL of PBS were injected into each recipient [C57BL/6 (CD45.2)] mice.

**2.2.8 Administration of immune-modulating agents**

**2.2.8.1 Intravenous injection of blocking antibodies**

CCL2 neutralisation was conducted by intravenously (i.v.) injecting WNV-infected mice with 100 µg of LEAF purified anti-MCP-1 (Biolegend) in 250 µL of PBS. Control animals received 100 µg of the isotype control Ab (Hamster IgG; Biolegend). Mice were given one dose of Ab daily starting from D1 p.i. for 4 consecutive days. To block CCL20, purified anti-CCL20/MIP-3α (R&D Systems) or goat IgG isotype control (R&D Systems) were used. 100 µg of blocking and isotype Ab were diluted in 250 µL of PBS and injected i.v. into WNV-infected mice 6 and 48 h after infection.

**2.2.8.2 Intravenous injection of beads**

FITC-conjugated carboxylate polystyrene beads that were 0.05 µm in size were obtained from Polysciences (NY, USA). As described before (Terry, 2012), beads were diluted to the concentration of 4.41 x 10^{12} FITC beads in 250 µL of sterile PBS and was injected i.v. into mock- or WNV-infected mice on a daily basis starting from D1 p.i. until D4 p.i (4 injections). In some experiments, mice
were treated with both beads and anti-MCP-1 Ab or the isotype control in a total volume of 250 μL with the concentrations described above.

**2.2.9 Flow cytometry**

**2.2.9.1 Preparation of tissue**

To obtain single cell suspensions for flow cytometry, vaginas were harvested and immediately placed in a 5 ml vial containing 500 μL collagenase/DNase solution. Vaginas were mechanically digested using small scissors in 1.5 ml collagenase/DNase solution and alternately incubated at 37°C for 30 min (shaking every 15 min intervals) and on a shaker at RT for 30 min for 2 h. Samples were then gently passed through a 70 μm cell strainer (BD Biosciences) which was washed with 10 ml FACS buffer. The cells were centrifuged at 460 g for 5 min and the supernatant discarded. Cells were resuspended in 200 μL FACS buffer containing a 1:100 dilution of purified anti-mouse CD16/32 Ab and incubated for 30 min at 4°C. This blocks non-specific binding to the Fc portion of the staining Abs (Fc block). Cells were counted using a haemocytometer and 1 x 10⁶ cells were collected for staining.

ILN were placed into a 70 μm cell strainer (BD Biosciences) with 3 ml of cold FACS buffer in a 6-well plate and then forced through the strainer with the plunger of a 1 ml syringe. The single cell suspension was topped up to 10 ml with cold FACS buffer and then collected into a 15 ml centrifuge tube. The pellet, obtained after 5 min centrifugation and at 460 g, was resuspended in 250 μL Fc
block and incubated at 4°C for 30 min. Cells were counted using a haemocytometer and 1 x 10^6 cells were collected for staining.

For some experiments, the spleen was also isolated and placed into a 70 μm cell strainer (BD Biosciences) with 3 ml of cold FACS buffer in a 6-well plate. A single cell suspension obtained by passing the spleen through the sieves before centrifugation for 5 min at 460 g. To lyse the RBC, the pellet was resuspended in 5 ml RBC lysis buffer for 10 min at RT. 10 ml FACS buffer was added to the samples to stop the reaction. Cells were then centrifuged at 460 g for 5 min, resuspended in 600 μl Fc block and incubated at 4°C for 30 min. Cells were counted with haemocytometer and 1 x 10^6 cells collected for staining.

2.2.9.2 Antibody labelling of single cell suspensions

The single cell suspensions that were collected from isolated tissues were transferred into a 96-well round-bottom plate and was centrifuged to remove the Fc block. Samples were then resuspended in a 100 μL Ab cocktail (the antibodies and dilutions are in Table 2.3) and incubated at 4°C for 30 min. To detect Treg cells, stained cells were fixed and permeabilised using the Foxp3 Fix/Perm Buffer Set (Biolegend). Anti-Foxp3 antibody was then added to the suspensions for 30 min at RT. An unstained sample, single colour-stained controls (SCC), bead compensation controls (BD CompBeads; BD Biosciences), isotype controls and fluorescence minus one (FMO) controls were included in all runs.

After staining, cells were washed once with 200 μL FACS buffer before resuspending in 100 μL of Fixation Buffer (Biolegend) for 20 min at RT in the
dark to fix the stained cells. Cells were then washed twice, resuspended in 150 μL FACS buffer and filtered through 50 μm Nitex gauze into FACS tubes.

2.2.8.3 Flow cytometric analysis

Stained cells were analysed using a FACS Canto or FACS LSR II (BD Biosciences) with FACS Diva software (BD Biosciences) and the acquired data was analysed with FlowJo (TreeStar Inc. OR, USA) software.

2.2.10 High performance liquid chromatography (HPLC)

2.2.10.1 Preparation of tissue

After harvest, the vagina and ILN were immediately placed in 200 μL complete protease inhibitor cocktail and stored at -80°C until use. A dounce homogeniser (Glas-Col, IN, USA) with a Teflon probe and a glass tube was used for this process were used to homogenise tissues. They were thawed on ice and homogenised 3 times for 30 s with 30 s intervals. The sides of the glass tube were washed with 100 μL protease inhibitor. The tissue homogenate was collected and centrifuged at 1500 g for 15 min at 4°C. The supernatants were mixed with 20% (w/v) ice-cold trichloroacetic acid (Sigma-Aldrich) in a 3:1 ratio and centrifuged again at 16000 g for 15 min at 4°C. The clarified supernatant was stored at -80°C until analysis.
A 50 μl of the tissue lysate supernatant prior to addition of trichloroacetic acid was retained for a protein assay to normalised for protein content.

2.2.10.2 HPLC analysis

To determine the extent of conversion of L-Trp to Kyn, samples were analysed by HPLC (Agilent 1200 HPLC system; Agilent Technologies, CA, USA) with a Hypersil 3 μm ODS C18 column (Phenomenex, CA, USA) and eluted with 100 mM chloroacetic acid and 9% acetonitrile at a rate of 0.5 ml/min for L-Trp and Kyn. L-Trp was detected by UV absorbance at 280 nm, peaking at ~10 to 12 min, while Kyn eluted after ~6 to 7 min at 364 nm. The concentration of L-Trp and Kyn were calculated in Excel based on the L-Trp and Kyn standards that were included in each run.

2.2.11 Enzyme-linked immunosorbent assay (ELISA)

2.2.11.1 Preparation of vaginal tissue

Vaginae from mock and infected mice were isolated and placed in a 2 ml microcentrifuge tube containing 250 μL complete protease inhibitor (Roche). A rotor/stator homogeniser (Tissue Tearor) was used to homogenise the tissue on ice. Samples were homogenised twice for 20 s with 20 s intervals. The homogenates centrifuged at 1500 g for 10 min at 4°C. The supernatants were
frozen at -80°C until use. A protein assay was also performed to normalise the ELISA results based on total protein content.

2.2.11.2 Multiplex ELISA

Multiplexed plate ELISA was performed using the Quansys Q-Plex Mouse Cytokine Screen Infrared (Quansys Biosciences, UT, USA) to quantify various cytokines and chemokines in the mouse vagina. Briefly, samples and standards were diluted in Quansys Q-Plex buffer and 50 μL of each were plated in separate wells containing 12 spots, each coated with a capture Ab specific for one cytokine/chemokine. Plates were then incubated for 1 h on an orbital shaker at 120 r.p.m., washed 3 times and 50 μL of detection Ab was added to each well and incubated for 1 h. After washing 3 times, streptavidin-Dylight IR dye was added and incubated for 15 min. Plates were washed 6 times and the substrate mix was added. Plates were scanned on the Li-Cor Odyssey Infrared Imaging System (Li-Cor Biotechnology, NE, USA) and captured images were analysed using Quansys Q-view software (Quansys Biosciences).

2.2.12 Protein assay

Total protein concentration was assessed by a BCA protein assay (Thermo Fisher Scientific). Supernatants that were derived from tissue homogenisation were diluted in distilled water to a final volume of 25 μL in a 96-well flat-bottom plate. Negative controls (i.e.: PBS) and seven bovine serum albumin (BSA)
standards (1:5 serial dilutions from 15.625 to 1000 μg/ml) were included. The freshly made reaction mix with 20 μL CuSO₄ (Sigma-Aldrich) per ml BCA reagent was added to each sample (200 μL). Samples were then incubated for 30 min at 37°C and the absorbance at 562 nm was measured using a FLUOstar Omega Microplate Reader (BMG Labtech, Germany).

2.2.13 Genotyping

2.2.13.1 Tissue digestion

For molecular genotyping, genomic DNA was extracted from tail clips or ear punches from WT and IDO⁻/⁻ mice. A 1-2 mm tail section or ear punch tissue biopsy were placed into a microcentrifuge tube. The tissue was immersed in 100 μL of tissue digestion buffer containing 1 μL of 20 mg/ml protein kinase (New England Biolabs) and left overnight at 56°C. Fully digested tissues were centrifuged at 18000 g for 10 min at RT and the clarified supernatant was mixed with 50 μL of 5M NaCl. The solution was vortexed until it looked uniformly cloudy and then centrifuged at 18000 g for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge containing 150 μL cold isopropanol. The tube was shaken vigorously and then centrifuged at 18000 g for 10 min at 4°C to sediment the DNA. This pellet was washed twice with 70% ethanol and centrifuged at 18000 g for 3 min at 4°C. At this point, the supernatant was discarded and 100 μL ultra-pure water was added to the DNA pellet. To enhance DNA relaxation, the tube was transferred to a 56°C water bath for 30 min and the
concentration of extracted DNA was then measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

2.2.13.2 PCR, agarose gel electrophoresis and imaging

Following DNA extraction, end-point reverse transcriptase PCR was conducted to amplify the gene of interest (i.e., WT and knock out IDO gene products). Forward and reverse primer sequences were obtained from Jackson Laboratories (ME, USA) (Table 2.5). A master mix was prepared for each assay containing the following reagents to obtain a final reaction volume of 10 μL: 2 μL of 5x PCR buffer (Bioline), 1 μL forward/reverse primer, 4.9 μL of nuclease-free water, 0.1 μL of Taq DNA polymerase enzyme (Bioline) and 1 μL of extracted DNA. PCR amplification was performed in a Bio-Rad TC-100 (Bio-Rad) following the program outlined in Table 2.6. PCR products were stored at 4°C or immediately loaded into an agarose gel for further analysis.

To perform electrophoresis, a 1% agarose gel in TBE buffer was prepared and then transferred into the electrophoresis equipment (Takara, Japan). PCR products were mixed with 1 μl of 6x stain/loading DNA dye (EZ-VISION Three; Amresco) and loaded into the wells of the agarose gel. A 1 kb ladder (Promega, WI, USA) was used to determine the size of the PCR products. Samples were run for 60–75 min at 100 volts. The gel was transferred to a G:BOX CHEMI 16 Bioimaging system (SYNGENE, UK) to visualise the IDO gene bands from the WT and IDO⁻/⁻ samples. Figure 2.1 shows a gel electrophoresis picture representing WT and IDO⁻/⁻ bands. To functionally delete the IDO gene, exons 3-5, which encode the critical catalytic sites of the enzyme, has been replaced with
beta-galactosidase and neomycin resistance genes. Furthermore, a translational "stop" codon (TAG) has added into exon 2 as previously described (Mellor et al., 2003a). Thus, the designed primers for IDO<sup>−/−</sup> gene detect sequence fragments of this manipulated IDO gene in mice.

Figure 2.1. Gel electrophoresis photograph of WT and IDO<sup>−/−</sup> bands
Table 2.5. Sequences of primers for the amplification and detection of IDO gene in WT and IDO<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO gene in WT mice</td>
<td>TGG AGC TGC CCG ACG C</td>
<td>TAC CTT CCG AGC CCA GAC AC</td>
</tr>
<tr>
<td>IDO gene in IDO&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>CTT GGG TGG AGA GGC TAT TC</td>
<td>AGG TGA GAT GAC AGG AGA TC</td>
</tr>
</tbody>
</table>

Table 2.6. PCR program used for genotyping IDO<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>PCR program</th>
<th>Temperature</th>
<th>Time</th>
</tr>
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<tbody>
<tr>
<td>1 First denaturation</td>
<td>95°C</td>
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<tr>
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<td>20 s</td>
</tr>
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<td>3 Annealing</td>
<td>65.9°C</td>
<td>20 s</td>
</tr>
<tr>
<td>4 Elongation</td>
<td>72°C</td>
<td>20 s</td>
</tr>
<tr>
<td>5 Repeated cycles</td>
<td>30 cycles</td>
<td>---</td>
</tr>
<tr>
<td>6 Completion of elongation</td>
<td>72°C</td>
<td>2 min</td>
</tr>
</tbody>
</table>


2.2.14 Gating strategy

2.2.14.1 Identification of macrophages, DCs and neutrophils in the vagina

Various gating strategies were applied to analyse the leukocyte populations in the vagina. To identify single cells and leukocytes, doublets were gated out using FSC-A and FSC-H (Figure 2.2A) and then total CD45+ leukocytes from mock-infected and virus-infected vaginas were selected (Figures 2.2B and C). From these cells, Ly6G+ neutrophils were identified (Figures 2.2D and E). To exclude non-myeloid cells such as NK, NKT and T cells, the NK1.1− CD3− population was gated from the Ly6G− subset (Figures 2.2F and G). From this population, B cells were gated out using B220 and MHC-II markers and then B220− MHC-II+/− subset was selected for identification of macrophages and DCs (Figures 2.2H and I). Macrophages and inflammatory macrophages were selected using CD11b and Ly6C markers (Figures 2.2J and K). CD11b+ and CD11b− DCs were also analysed (Figures 2.2L and M). In addition, blood-derived DC (BDC: CD11chi MHC-II+) and tissue-derived DC (TDC: CD11c+ MHC-IIhi) were identified (Figures 2.2N and O). To identify pDCs, B220+ cells from Figures 2.2H and I were further analysed and CD11c+ B220+ population was selected (Figures 2.2P and Q).

2.2.14.2 Identification of lymphocytes in the vagina

A similar gating strategy was used to identify lymphocytes in the vagina. To analyse lymphocyte subsets, total CD45+ leukocytes from mock-infected and
Figure 2.2 Macrophages, DCs and neutrophils in the vagina
virus-infected vaginae were selected as described in section 2.2.14.1. Cells of the myeloid lineage were then excluded and NK and NKT cells were identified using NK1.1 and CD3 markers (Figures 2.3A and B). CD3+ NK1.1− cells were further divided into CD4+ and CD8+ T cells (Figures 2.3C and D). To investigate B cells, B220− MHC-II+/− cells were first excluded (Figures 2.3E and F) and then B cells were identified as B220+ CD11c− cells (Figures 2.3G and H).

2.2.14.3 Identification of macrophages, DCs and neutrophils in the ILN and spleen of mock-infected and virus-infected mice

The method used for the vagina was also applied in the ILN and spleen to analyse macrophages, DCs and neutrophils. Doublets, red blood cells and debris were first excluded using FSC-A/FSC-H and FSC-A/SSC-A (Figures 2.4A–C and Figures 2.6A–C). From these cells, Ly6G+ neutrophils were selected and Ly6G− leukocytes were further excluded from non-myeloid cells (Figures 2.4D–G and Figures 2.6D–G). pDCs were identified from the B220+ population as CD11c+ (Figures 2.4H–K and Figures 2.6H–K) and then macrophages and DCs were analysed in the B220− subset (Figures 2.4L–Q and Figures 2.6L–Q).

2.2.14.4 Identification of lymphocytes in the ILN and spleen of mock-infected and virus-infected mice

As described earlier, doublets, red blood cells and debris were first gated out. Myeloid cells were also excluded (Figures 2.5A and B and Figures 2.7A and B). B cells were identified as B220+ and CD11c− (Figures 2.5C and D and Figures
Figure 2.3 Lymphocytes in the vagina
2.7C and D). NK (NK1.1+ CD3−) and NKT cells (NK1.1+ CD3+) were also identified (Figures 2.5E–G and Figures 2.7E–G). CD4+ and CD8+ T cells were also identified in the CD3+ NK1.1+ population (Figures 2.5E–H and Figures 2.7E–H).

2.2.14.5 Identification of adoptively transferred CD45.1+ cells in the vagina, ILN and spleen of mock-infected and virus-infected mice

In adoptive transfer studies, flow cytometry was used to identify CD45.1+ cells from donor animals in the vagina, ILN and spleen of i.vag mock- and WNV/HSV-2-infected recipients. In the vagina, total leukocytes were defined as CD45+ cells (Figures 2.8A and B). In the ILN and spleen, red blood cells and debris were also gated out (Figures 2.8C–F). Adoptively transferred cells in the corresponding tissues were then identified using CD45 and CD45.1 markers (Figure 2.8G and L).

2.2.14.6 Expression of CCR2, F4/80 and CD8α in macrophage and DC subsets in the vagina, ILN and spleen of mock- and WNV-infected mice

Following analysis of macrophages and DCs, these subsets were further subdivided based on their expression of CD11b, Ly6C, CCR2, F4/80 and CD8α in the vagina (Figures 2.9A–D), ILN (Figures 2.9E–N) and spleen (Figures 2.9O–X) of mock- and WNV-infected mice.
Figure 2.4 Macrophages, DCs and neutrophils in the ILN
Figure 2.5 Lymphocytes in the ILN
Figure 2.6 Macrophages, DCs and neutrophils in the spleen
Figure 2.7 Lymphocytes in the spleen
Figure 2.8 Adoptively transferred cells in the vagina, ILN and spleen
Figure 2.9 Further analysis of macrophages and DCs
2.2.14.7 Accumulation of beads in the spleen of mock- and WNV-infected mice

To investigate bead+ cells in the spleen, doublets, dead cells, debris and red blood cells were excluded as previously described. Total bead+ leukocytes from mock- and WNV-infected animals were then selected and analysed for the expression of other leukocyte markers (Figures 2.10A–D).

2.2.15 Statistical analysis

Data were analysed using Microsoft Excel and GraphPad Prism 5 (GraphPad Software Inc., CA, USA). GraphPad Prism was also used to generate graphs and to determine statistical significance. To compare two groups, an unpaired, two-tailed Student’s t-test was conducted, while a one-way ANOVA with a Tukey-Kramer post-test was performed to compare three or more groups. For survival studies, a Mantel-Haenszel logrank test was used. A p value \( \leq 0.5 \) was considered significant (*), with \( p \leq 0.01 \) very significant (**) and \( p \leq 0.001 \) extremely significant (***).
Figure 2.10 Beads* subset in the spleen
Chapter 3

Host immune response to intravaginal infection with WNV and HSV-2
Chapter 3:

Host immune response to intravaginal infection with WNV and HSV-2

3.1. Introduction

Sexually transmitted infections (STI) are a major global concern. Based on World Health Organization (WHO) estimates, it is estimated that a million people become infected everyday with sexually transmitted bacterial, fungal and parasitic pathogens (WHO, 2012). Approximately 490 million curable STI, namely, those due to syphilis, gonorrhea, trichomoniasis and Chlamydia, occur each year. Furthermore, millions of viral STI also occur annually. HIV, herpes simplex viruses (HSV-1 and HSV-2), human papillomaviruses (HPV) and Hepatitis B viruses (HBV) are among the most common human infectious viral pathogens (WHO, 2012). Even though there are vaccines for HPV and HBV, both HIV and HSV viruses are, at present, incurable (Frazer et al., 2011, Makidon et al., 2008, Schiffer et al., 2012). Furthermore, HSV-2 infection is associated with significant increased risk of HIV acquisition among both men and women (Johnston et al., 2011, Freeman et al., 2006). Therefore, there is a need for greater understanding of the immune response in the female reproductive tract, as relatively little is known of the role played by effector immune cells at the site of local infection in the initiation of immune response.
Animal models recapitulate many of the features of human disease. To date, the most commonly studied animal model of STI is the murine model of HSV-2 infection. In this model, intravaginal (i.vag) inoculation results in productive replication within epithelial cells and subsequent invasion of the virus to ganglionic sensory neurons in the peripheral nervous system (PNS) followed by neurons of the CNS and death (Chentoufi and Benmohamed, 2012, Parr and Parr, 2003). Notwithstanding, HSV-2 infection in humans is associated with neurological symptoms that are generally, but not always, mild and self-limiting (Berger and Houff, 2008). In this context, murine models do not accurately mirror human HSV-2 infection because this virus is lethal in mice. In addition, HSV-2 destroys the murine vaginal epithelium; therefore, it is difficult to examine the early immune response at the local area. Recently, a novel murine vaginal model of infection using neurotropic WNV has been established and is characterised by productive, but self-limiting, infection in the vagina with a robust immune response at the local infection site and draining LN (Burke et al., 2004).

The experiments described in this chapter show that vaginal epithelial infection with either of the two viruses elicits robust primary immunity to infection. The results here also show a strong immune response, both locally and within the draining LN that is associated with increased inflammatory leukocyte and lymphocyte infiltration. More strikingly, this response represented a dramatic change in the distribution of the mucosal CD11c⁺ DCs, but not CD11b⁺ or F4/80⁺ macrophages at or near the infected epithelial sites. Furthermore, adoptive transfer studies showed that these cells originate from the bone marrow.
3.2 Results

3.2.1 Morbidity and mortality following i.vag inoculation with WNV and HSV-2

To evaluate the kinetics of i.vag WNV and HSV-2 infection in C57BL/6 mice, mice were inoculated with $3.6 \times 10^7$ plaque forming units (PFU) WNV, the highest WNV stock titre available in our lab. Furthermore, in our lab, several HSV-2 doses including $10^4$ and $10^5$ PFU have been used to delineate the disease severity and pathology scores. All mice that were infected with the higher dose ($10^5$ PFU HSV-2) succumbed to infection, while 19% survived when infected with a lower dose ($10^4$ PFU HSV-2) (Yeung, 2012). To confirm these data, groups of 10 mice (8 weeks old) were infected with either $10^4$ or $10^5$ PFU HSV-2. Mice were monitored for any symptoms of illness over a time course of 25 days. WNV infection elicited no mortality and mice did not display any overt symptoms of illness throughout the time course (Figure 3.1A). However, HSV-2 i.vag infection resulted in dramatic morbidity and mortality. Following i.vag inoculation with either $10^5$ or $10^4$ PFU of HSV-2, more than 90% of mice appeared ill and succumbed to infection starting from day 8 (D8) to D19 post-infection (p.i.).

Animals were examined for the progression of vaginal lesions and overall illness. Mice were monitored and scored daily on a five-point scale from 0 to 5 based on clinical pathological signs in the vagina (Gill et al., 2006). Infected mice first developed redness and swelling in the genital region. This area gradually became ulcerated and was followed by significant hair loss. Severe swelling and increased ulceration of the vaginal tissue was occasionally accompanied by ataxia.
and hind limb paralysis (Figure 3.1B). At this stage of illness, mice were sacrificed. In all sick mice, these symptoms were associated with a ruffled coat and body weight loss (Figure 3.1C).

### 3.2.2 Kinetics of HSV-2 i.vag infection

To understand the kinetics of HSV-2 infection, levels of vaginal infection following primary inoculation were determined using primers detecting mRNA encoding for glycoprotein B of the HSV-2 virus (Namvar et al., 2005). Viral mRNA in the vagina of both groups of mice that were infected with $10^4$ or $10^5$ PFU was detectable as early as D1 p.i. In those mice inoculated with the higher dose, D1 p.i. was the peak expression day as HSV-2 mRNA gradually declined from D3 p.i. onwards. At D7 p.i., HSV-2 mRNA expression almost returned to the baseline (Figure 3.2A). In contrast, when using the lower dose of HSV-2, mRNA expression in the vagina of mice increased by ~3-fold at D2 and peaked at ~8-fold at D3 p.i. As with the high dose, very little viral mRNA was detected at D7 or D8 p.i (Figure 3.2B).

To investigate the localisation of HSV-2 infection in the vagina, cross-sections of mock-infected and HSV-2-infected vaginae were labelled for HSV-2 Ag. Immunofluorescent (IF) labelling in the infected vagina indicated that HSV-2-infected cells were in the epithelial layer at D1 and D3 p.i. in both groups. The higher dose of HSV-2 showed more extensive viral spread at D1 p.i., whereas more infected epithelial cells were observed at D3 p.i. in those mice infected with lower dose. Epithelial infection of HSV-2 on the relevant peak expression days with high and low titre are depicted in Figure 3.3A and 3.3B. There was only a
Figure 3.1

Morbidity and mortality following i.vag inoculation with WNV and HSV-2

(A) Kaplan–Meier survival curve for the mice infected with WNV 3.6 x 10^7 PFU (green) and HSV-2 with 10^4 (blue) and 10^5 PFU (red) over 25 days. (B) Pathology of mice infected with 10^4 (blue) or 10^5 PFU (red) of HSV-2 over 25 days. ***p≤0.001 applied to compare WNV and HSV-2 survival curves. WNV i.vag infection had no overt sign of pathology and all infected mice survived after this time point. Pathology of i.vag HSV-2 infection quantified by the disease score as follows: 0, no signs of infection; 1, slight redness of genital area; 2, redness and swelling of external vagina; 3, severe swelling of the external vagina and hair loss in the surrounding area; 4, ulceration, severe swelling and redness of the vaginal tissue; and 5, continued ulceration, swelling and redness, occasionally accompanied by paralysis of the hind legs (mice were euthanised at this point). The disease score was similar between 10^4 and 10^5 PFU at all time points. Data is expressed as the mean ± SEM of 3 independent experiments of 5 to 10 mice each. (C) Relative changes in the weight of mice that infected with i.vag 10^4 (blue) and 10^5 PFU (red) of HSV-2 over a 25-day time course. Each line represents the percentage weight change of one mouse, relative to their respective weight at d0 p.i. Data is representative of 3 independent experiments, with 5 to 10 mice each.
Figure 3.2

Quantification of HSV-2 infection in the vagina

Relative HSV-2 mRNA expression in the vagina of mice infected with (A) $10^5$ or (B) $10^4$ PFU HSV-2 for 8 days. HSV-2 mRNA was expressed relative to levels apparent in the vagina at D1 p.i. Data shown is the mean ± SEM of at least 4 mice per group and comprise 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; **$p \leq 0.01$ and ***$p \leq 0.001$ compared to HSV-2 D0 p.i.
slight and discrete area of the epithelium with positive staining for HSV-2 Ag at D5 p.i. and by d7 p.i., there were no detectable infected cells in either group (data not shown).

Most strikingly, HSV-2 infection was associated with a significant cytopathic effect that was accompanied by a large-scale sloughing of the infected epithelium and subsequent basal layer destruction. Virus-positive labelling in the urethra and the surrounding area in some vaginas at D5 p.i. was also observed, suggesting that the infection, which was not limited to the vaginal epithelium, had travelled to the sensory ganglia and reproductive tract (Figure 3.3C). As expected, no HSV-2+ cells were observed in mock-infected controls, relative to isotype-stained sections (Figure 3.3D). Image analysis software was used to provide a semi-quantitative measurement of the area occupied by infected cells during the course of i.vag HSV-2 infection (D0–D7 p.i.). As a general strategy, the software was set to recognise the total area of the vaginal section, as well as the HSV-2 infection, which were defined by corresponding colours and then the area of HSV-2 infection was calculated as a proportion of the measured total area. Figure 3.3E shows the percentage of the area occupied by HSV-2 infection in high and low dose inoculation throughout the infection period. Similar to the IF observations, HSV-2 vaginal infection with 10⁵ PFU resulted in high epithelial infection at D1 p.i. with more than 3% of the total area involved in HSV-2 infection. The HSV-2+ infected area at D3 and D5 p.i. decreased to 2.5% and 0.5%, respectively. In contrast, with the lower dose of HSV-2 infection (10⁴ PFU), only 1.5% of total area was seen to be infected at D1 p.i. This proportion increased to peak at D3 p.i. with ~3% of the total area infected, followed by a sharp decline by D5 p.i., in which only 0.5% of total area showed vaginal HSV-2
infection. Vaginal infection at D7 p.i. was undetectable in both groups of mice infected with either $10^5$ or $10^4$ PFU HSV-2 (Figure 3.3E).

Vaginae from mock-infected mice exhibited a continuous and normal epithelial structure, similar to that observed in untreated mice. This indicates that the physical process of inoculation caused no damage or inflammation to the vagina (Figure 3.3D). Therefore, it was used as a mock-infected control in all experiments.

### 3.2.3 Kinetics of WNV i.vag infection

The level of epithelial infection in the vagina was investigated by real-time RT-PCR and IF following inoculation with $3.6 \times 10^7$ PFU of WNV. Figure 3.4A shows that WNV RNA expression in the vaginal tissue consistently started from D1 p.i. and increased by D3 p.i. with ~7-fold more mRNA copies relative to D1 p.i., reaching the peak of infection on D5 with ~10-fold more WNV RNA copies in the vaginal homogenate. Interestingly, WNV was cleared by D7 p.i. as there was no detectable mRNA in the vagina at this time-point.

A mouse monoclonal antibody recognising the non-structural protein 1 (NS-1) of WNV was used to investigate virus spread in the vaginal epithelium of WNV-infected mice. WNV-NS1$^+$ cells were first detected at D1 p.i., with the peak of infection occurring on d5 p.i. (Figure 3.4B), followed by a dramatic decline thereafter as no epithelial cell was WNV-NS1$^+$ at D7 p.i., corresponding closely to the kinetics of HSV-2 mRNA expression. As expected, no WNV-NS1$^+$ cells were detected in mock-infected controls, relative to isotype-stained tissue
Figure 3.3

**Kinetics of HSV-2 i.vag infection**

Immunofluorescent staining for HSV-2 antigen (FITC; green) and nuclei (DAPI; blue) in vaginal cross-sections from mice infected i.vag with $10^5$ at (A) D1, $10^4$ at (B) D3 (i.e., at peak expression for each dose) and $10^5$ PFU of HSV-2 at (C) D5 p.i. No HSV-2$^+$ cells were observed in mock-infected, or D7 p.i. mice, relative to rabbit IgG isotype-stained sections (D). (E) Percentage of HSV-2$^+$ vaginal epithelial cells in mice infected with $10^5$ (red) or $10^4$ (green) PFU of HSV-2. All images are representative of at least 4 mice per group. Bars indicate the mean ± SEM of 3 to 5 mice per group and comprise 4 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; ***$p$≤0.001 compared to (HSV-2 $10^5$ PFU) D0 p.i. ###$p$≤0.001, relative to (HSV-2 $10^4$ PFU) D0 p.i.
Nuclei HSV-2 D1 p.i. 10^5 PFU

Nuclei HSV-2 D3 p.i. 10^4 PFU

Nuclei HSV-2 D5 p.i. 10^5 PFU

Nuclei HSV-2 D0 p.i.

E

% Total area

Day post infection

Graph showing the percentage total area over days post infection, with different infection doses: 10^4 PFU HSV-2 and 10^5 PFU HSV-2.
sections (Figure 3.4C). In contrast to HSV-2, infection with WNV was confined to the epithelial layer and no positive staining was observed in any other parts of the vagina at any time-point.

3.2.4 Infiltration of effector leukocytes to the vagina after WNV infection

Infection of the vaginal epithelium elicited significant leukocyte recruitment. To identify the infiltrating immune effector cells, vaginae were excised with the urethra still attached. The urethra served as an anatomical landmark during histological examination of the vagina. The urethra was distinct from the vagina as it had a smaller lumen. We previously detected a novel population of infiltrating leukocytes termed MHCII⁺ vaginal dendritic cells (vDCs) during WNV infection. vDCs participated in initiation of the immune response against WNV by forming clusters in the subepithelial region of the vagina (Burke et al., 2004). To further elucidate the characteristics of vDCs and other immune response initiators, extensive IF staining of snap-frozen vaginal sections was undertaken.

3.2.4.1 CD11c expression in the vaginal mucosa of WNV-infected mice

It was important to determine the identity of aggregated cells below the infected area of epithelium. There is a great overlap in surface marker expression between the monocyte, macrophage and dendritic cell subsets because they
Figure 3.4

*Quantification of WNV infection of the vagina*

(A) WNV mRNA expression in the vagina following i.vag. inoculation with 3.6 x 10^7 PFU WNV at D0 – D7 p.i., as indicated. Expression is relative to levels in the vagina at D1 p.i. (B) Immunofluorescent staining for WNV-NS1 (FITC; green) and nuclei (DAPI; blue) of a vaginal cross-section at D5 p.i. No WNV-NS1⁺ cells were detected in mock-infected, D7 WNV p.i. and mouse IgG1 isotype-stained tissue sections (C). Images are representative of at least 4 mice per time point. Bars represent the mean ± SEM of 4 mice per group over 4 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05 and **p≤0.01 compared to WNV D0 p.i.
A

Relative gene expression

Day post WNV infection

B

Nuclei WNV D5 p.i. 3.6 x 10^7 PFU

200 μm

C

Nuclei WNV D0 p.i.

200 μm
originate from common myeloid progenitor cells. Despite the fact that many APCs share expression of several markers, CD11c is a well-known marker for DCs, which differentiate them from monocytes. CD11c is expressed on the surface of all conventional murine DC, Langerhans cells and plasmacytoid DC (Nakano et al., 2001). Figure 3.5A shows that a small number of CD11c+ cells were distributed in the stroma and below the epithelial basal layer in the mock-infected vagina. However, an acute inflammatory reaction was observed following i.vag WNV infection including an increase in the number of CD11c+ cells at D3 and D5 p.i., compared to mock-infected mice (Figure 3.5B–C). This response was observed in all mice inoculated with WNV. Clusters of CD11c+ DCs were typically located in the mucosa underlying regions of the epithelium that were infected. NS1 staining was representative of viral infection. There were increased numbers of CD11c+ cells in all infected vaginas compared to mock-infected controls. Indeed, the majority of CD11c+ DCs exhibited an aggregation below the infected epithelium. Due to the clustering, underlying regions were markedly thicker than that of the uninfected epithelium, peaking at D5 p.i. Figure 3.5D shows the percentage area occupied by WNV epithelial infection and CD11c+ cells throughout the infection period. Compared to D0 p.i. or mock-infected controls, there was a significant increase in CD11c+ cells as early as D1 p.i., continuing until D5 p.i. before reducing slightly at D7 p.i. Image analysis of WNV-infected epithelium showed a significant increase at D3 and D5 p.i. only.
**Figure 3.5**

**Localisation of CD11c and WNV-NS1 in the vagina following WNV infection**

Immunofluorescent staining for WNV-NS1 antigen (FITC; green), CD11c surface marker (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from mice infected i.vag. with $3.6 \times 10^7$ PFU WNV at (A) D0, (B) D3 and (C) D5 p.i. Neither WNV-NS1$^+$ nor CD11c$^+$ cells were observed in mouse IgG1 and Armenian hamster IgG isotype-stained sections (not shown). (D) Percentage of WNV-NS1$^+$ vaginal epithelial cells in mice infected with $3.6 \times 10^7$ PFU (green) and proportion of the area filled with CD11c$^+$ cells (red). All images are representative of at least 3-5 mice per time point. Bars indicate the mean ± SEM of 3-5 mice per group and comprise 4 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to (CD11c) D0 p.i. ###p≤0.001, relative to WNV D0 p.i.
To determine if aggregates in the vagina contained other APCs, vaginal sections were stained for CD11b and F4/80. These markers are expressed on the surface of resident and inflammatory DCs and macrophages (Murray and Wynn, 2011, Sajic et al., 2005, Iijima et al., 2011).

As illustrated in Figures 3.6 (A–C and E–G), respectively, CD11b\(^+\) and F4/80\(^+\) cells increased over the WNV time course peaking at D5 p.i. CD11b\(^+\) and F4/80\(^+\) cells were distributed widely throughout the vaginal tissue with a tendency to cluster around the infected epithelial area, particularly at D5 p.i. However, CD11b\(^+\) and F4/80\(^+\) cells did not form significant aggregates underneath infected areas, unlike CD11c\(^+\) cells. In contrast, few CD11b\(^+\) and F4/80\(^+\) cells were found in the vaginal epithelium and lumen, suggesting these cells were principally comprised of innate phagocyte populations, including neutrophils. Neutrophils are defined as CD11b\(^{\text{high}}\) and F4/80\(^{\text{low}}\) (Narni-Mancinelli et al., 2011); thus, it is likely that the small population in the lumen, adjacent to the infection, represent activated neutrophils.

Image analysis using images from at least three distinct spots of infection revealed similar kinetic profiles for CD11b\(^+\) and F4/80\(^+\) markers (i.e., peaking at D5 p.i.) in the vagina, with ~5% of the tissue occupied by each marker at D5 p.i. (Figures 3.6D and H).
**Figure 3.6**

Localisation of CD11b and F4/80 in the vaginal mucosa after WNV infection

Immunofluorescent staining for WNV-NS1 antigen (FITC; green), CD11b surface marker (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from mice infected i.vag. with $3.6 \times 10^7$ PFU WNV at (A) D0, (B) D3 and (C) D5 p.i., (panels on the left). (D) Percentage of WNV-NS1$^+$ vaginal epithelial cells (green) and proportion of the area filled with CD11b$^+$ cells (red). Panels on the right, represent immunofluorescent staining for WNV-NS1 antigen (FITC; green), F4/80 surface marker (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections of WNV infected vagina at (E) D0, (F) D3 and (G) D5 p.i. (H) Percentage of WNV-NS1$^+$ vaginal epithelial cells (green) and proportion of the area filled with F4/80$^+$ cells (red). Immunofluorescent staining of vaginal sections with mouse IgG1, rat IgG2a and rat IgG2b (corresponding isotypes) elicited no specific staining for WNV-NS1, CD11b and F4/80 markers (not shown). All images are representative of at least 3-5 mice per time point. Bars indicate the mean ± SEM of 3-5 mice per group and comprise 4 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ compared to (CD11c or F4/80) D0 p.i. ####$p \leq 0.001$, relative to WNV D0 p.i.
3.2.4.3 Co-localisation of CD11c/CD11b and F4/80 in the WNV-infected vaginal mucosa

To further investigate if the clusters of CD11c+ cells that aggregated in the vaginal sub-epithelium adjacent to the infection expressed other APC markers, more extensive IF double staining using CD11b, F4/80 and CD11c were carried out. As shown in Figure 3.7A–E, both CD11b+ and F4/80+ cells were present separately in the vagina. The numbers of these cells increased over the time course and stayed relatively high at D7 p.i., and CD11b+ and F4/80+ cells did form clusters as previously shown for CD11c+ DCs, although not as extensively. On the other hand, as illustrated in Figure 3.7F–J it was clear that CD11b+ cells were present in the clusters underneath the epithelium where the CD11c+ DCs were the majority. Collectively, co-staining of vaginal sections showed a large number of cells recruited to the vagina in response to viral infection. Moreover, many of the infiltrating cells expressed either CD11b+, F4/80+ or CD11c+ separately on their surface with dual labelling (CD11b+ and F4/80+ or CD11c+ and CD11b+) only evident in a small percentage of cells. Interestingly, the aggregates principally comprised of CD11c+ DCs and, to a lesser extent, CD11b+ and F4/80+ cells.

3.2.5 CD11c expression in the vaginal mucosa following HSV-2 infection

IF labeling in the vagina after WNV infection using several APC markers showed aggregation of immune cells below the epithelium. Since the clusters associated with WNV-infected epithelium were found to be mostly CD11c+ and
Co-localisation of CD11b, CD11c and F4/80 in the vaginal mucosa after WNV infection

Immunofluorescent staining for CD11b surface marker antigen (FITC; green), F4/80 (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from mice infected i.vag with $3.6 \times 10^7$ PFU WNV at (A) D0, (B) D1 (C) D3 (D) D5 and (E) D7 p.i., (upper panels). Lower panels represent immunofluorescent staining for CD11b surface marker antigen (FITC; green), CD11c (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections of WNV infected vagina at (F) D0, (G) D1, (H) D3, (I) D5, and (J) D7 p.i. Immunofluorescent staining of vaginal sections with rat IgG2a and Armenian hamster IgG (corresponding isotypes) elicited no specific staining for CD11b and CD11c markers (not shown). All images are representative of at least 3-5 mice per time point. Bars indicate the mean ± SEM of 3-5 mice per group and comprise 4 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *$p \leq 0.05$ and ***$p \leq 0.001$ compared to (CD11b) D0 p.i. # p $\leq 0.05$, ## $p \leq 0.01$ and ### $p \leq 0.001$, relative to (CD11c or F4/80) D0 p.i.
not CD11b+ or F4/80+, we elected to investigate the expression and localisation of CD11c+ as an indication of vDC in HSV-2-infected vagina. As with WNV, i.vag infection of HSV-2 (10^5 PFU) elicited infiltration of CD11c+ DCs (Figure 3.8 A–E). CD11c+ DCs were typically located below the epithelium and adjacent to the HSV-2 infection site. HSV-2 labelling showed extensive epithelial infection, peaking at D1 and 3 p.i. with ~3% of the whole area representing infection (Figure 3.8F). Epithelial infection reduced significantly by D3 p.i. with only a small number of epithelial cells still infected at this time-point. HSV-2 was completely cleared from the vagina by D7 p.i. Similar to that observed in WNV infection, vaginal inoculation with HSV-2 was coincident with a rapid and dramatic infiltration of CD11c+ DCs to the vagina. Aggregation of DCs clearly began as early as D1 p.i. with the peak of infiltration at D5 p.i., that declined substantially by D7 p.i. Image analysis showed that at D1 p.i., CD11c+ cells comprised 1.5% of the total area, increasing during infection to ~4% at the peak day and dropping to ~1% at D7 p.i. (Figure 3.8F). Interestingly, unlike WNV infection, the HSV-2 infection and infiltration of DCs peaked asynchronously, with the peak of DC clustering lagging behind the peak HSV-2 infection by approximately 2 days.

3.2.6 Dynamics of macrophages, DCs and neutrophils in the vagina following i.vag WNV and HSV-2 infection

Flow cytometry was used to further investigate the phenotype of cells that infiltrated the vagina in both models of infection. Mice were inoculated i.vag with
**Figure 3.8**

**Localisation of CD11c and HSV-2 in the vagina following HSV-2 infection**

Immunofluorescent staining for HSV-2 antigen (FITC; green), CD11c surface marker (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from mice infected i.vag with $10^5$ PFU HSV-2 at (A) D0, (B) D1 (C) D3 (D) D5 and (E) D7 p.i. Neither HSV-2$^+$ nor CD11c$^+$ cells were observed in sheep IgG and Armenian hamster IgG isotype-stained sections (F). (G) Percentage of HSV-2$^+$ vaginal epithelial cells in mice infected with $10^5$ PFU (green) and proportion of the area filled with CD11c$^+$ cells (red). All images are representative of at least 3-5 mice per time point. Bars indicate the mean ± SEM of 3-5 mice per group and comprise 4 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; ***$p \leq 0.001$ compared to (CD11c) D0 p.i. ###$p \leq 0.001$, relative to HSV-2 D0 p.i.
3.6 x 10^7 PFU of WNV or 10^5 PFU of HSV-2. WNV-infected mice were then euthanised on days 0, 3 and 5 p.i., and HSV-2-infected mice were sacrificed on days 0 and 5 p.i. Single cell suspensions were prepared from the excised vagina and were stained and analysed by flow cytometry for leukocyte subsets. The changes in the number and proportion of major lymphocyte subsets in the vagina were also examined. The percentage and numbers of leukocytes (CD45^+ cells) were expressed as a proportion of the total live vaginal cells. Leukocyte subpopulations were calculated as the proportion of live CD45^+ cells in the vagina.

Intravaginal inoculation with WNV induced a slight increase in the number of leukocytes at D3 p.i., relative to mock-infected mice, and the percentage of CD45^+ cells in the vagina almost doubled at this time-point relative to at D0 p.i. As shown in Figure 3.9A–B and Figure 3.10A–B, vaginal infection with either WNV or HSV-2 at D5 p.i. was associated with a more dramatic increase in the total number and percentage of CD45^+ cells in the vagina.

The small increase in the number and proportion of CD45^+ cells was reflected in all the leukocyte subsets in the vagina as all subsets were slightly increased at D3 p.i. after WNV infection. At D5 p.i., which was the peak of leukocyte infiltration, there was a significant increase in the number and percentage of all subsets investigated (Figures 3.9C–D).

A significant increase was observed in the percentage of total macrophages (Ly6G^- B220^- CD3^- CD11b^+ CD11c^-) and inflammatory macrophages (Ly6G^- B220^- CD3^- CD11b^+ CD11c^- Ly6C^hi), in both models of infection. Indeed, the percentage of total macrophages and inflammatory
macrophages in the WNV-infected vagina rose from 0.5% to 4% and from 0.25% to 2% of CD45+ cells, respectively (Figure 3.9D). In addition, 80–90% of both total and inflammatory macrophages were MHC II+. Cell numbers in these subsets also increased by ~20- and ~12-fold on D5 p.i., respectively, which reflects the substantial recruitment of macrophages to the vagina (Figure 3.9C). HSV-2 i.vag infection showed similar kinetics as the percentages of total and inflammatory macrophages and increased by 5- and 25-fold, respectively (Figure 3.10D). Furthermore, the number of total macrophages increased from $1 \times 10^4$ to $6 \times 10^4$ and the number of inflammatory macrophages rose from $1 \times 10^3$ to $4 \times 10^4$ (Figure 3.10C).

Because of the critical role of DCs in antigen presentation, CD45+ Ly6G− B220− CD3− leukocytes were analysed for CD11b and CD11c expression. Of note, CD11b and CD11c expression on the cell surface led to the identification of two distinct populations of CD11c+ DCs, specifically, CD11b+ and CD11b DCs.

The number and percentage of CD11b+ DCs in both the WNV- and HSV-2-infected vagina significantly increased over this time, peaking at D5 p.i. However, the number of CD11b− DCs was not statistically different in either model at this time. Interestingly, the number of CD11b− DCs in the WNV-infected vagina increased at D3 p.i. before declining to baseline at D5 p.i., in contrast, the numbers in this subset in the HSV-2-infected vagina remained unchanged at both time points (Figure 3.9C–D and 3.10C–D).

Blood-derived DCs (BDC: Ly6G− B220− CD3− CD11chi MHC-II+) and tissue-derived DCs (TDC: Ly6G− B220− CD3− CD11c+ MHC-IIhi) were also evident in the infected vagina (Dakic et al., 2004, Kissenpfennig et al., 2005,
Davison and King, 2011). BDC and TDC were only a small portion of vaginal DCs; however, a significant increase in both the numbers and percentage of cells in these subsets were observed in the WNV-infected vagina at D5 p.i. On the other hand, in the HSV-2-infected vagina, the percentage and number of TDCs was unchanged while BDCs displayed a significant increase in the numbers at D5 p.i. (Figure 3.9C–D and 3.10C–D).

Neutrophils (CD45$^+$ B220$^-$ CD3$^-$ MHC-II$^+$ CD11b$^{hi}$ Ly6G$^+$) infiltrated in large numbers and comprised a large proportion of the leukocytes in the vagina of mice infected with either virus at all time points. In the mock- and WNV/HSV-2-infected vagina, neutrophils constituted ~0.5% and ~1% of total vaginal leukocytes. There was a significant and similar increase in numbers and percentage of neutrophils in the WNV-infected vagina, peaking at D5 p.i.; however, this increase was non-significant in the HSV-2-infected vagina (Figure 3.9C–D and 3.10C–D).

### 3.2.7 Dynamics of lymphocyte subsets in the vagina following i.vag

**WNV and HSV-2 infection**

The contribution of other cellular subsets, especially lymphocytes, to the immune response against WNV and HSV-2 in the vagina was also assessed by flow cytometry. To do this, the number and proportion of B cell (CD45$^+$ Ly6G$^-$ CD3$^-$ NK1.1$^-$ MHC-II$^+$ CD11c$^-$ B220$^+$), CD8$^+$ T cell (CD45$^+$ Ly6G$^-$ B220$^-$ CD3$^+$ NK1.1$^-$ CD4$^-$ CD8$^+$), CD4$^+$ T cell (CD45$^+$ Ly6G$^-$ B220$^-$ CD3$^+$ NK1.1$^-$ CD4$^+$ CD8$^+$), NK cell (CD45$^+$ Ly6G$^-$ B220$^-$ CD3$^+$ NK1.1$^-$) and NKT cell (CD45$^+$ Ly6G$^-$
Figure 3.9

**Leukocyte subsets in the vagina following WNV i.vag infection**

Following i.vag infection, the vagina of mock- and WNV-infected mice were removed, enzymatically digested and processed for flow cytometric analysis on D0, D3 and D5 p.i. (A) number of total leukocytes (CD45⁺) in the mock- and WNV-infected vagina, (B) proportion of total leukocytes (CD45⁺) as in (A), (C) number of leukocyte subsets including total macrophages, defined as Ly6G⁻ B220⁻ CD3⁻ CD11b⁺ CD11c⁻, inflammatory macrophages (Ly6G⁻ B220⁻ CD3⁻ CD11b⁺ CD11c⁻ Ly6C<sup>hi</sup>), CD11b⁺ DCs (Ly6G⁻ B220⁻ CD3⁻ MHC-II⁺ CD11b⁺ CD11c⁺), CD11b⁻ DCs (Ly6G⁻ B220⁻ CD3⁻ MHC-II⁺ CD11b⁻ CD11c⁺), TDC (Ly6G⁻ B220⁻ CD3⁻ CD11c⁺ MHC-II<sup>hi</sup>), BDC (Ly6G⁻ B220⁻ CD3⁺ CD11c<sup>hi</sup> MHC-II⁺) and neutrophil cells (CD45⁺ B220⁻ CD3⁻ MHC-II⁺ CD11b<sup>hi</sup> Ly6G⁺), and (D) proportion of leukocyte subsets as in (B). Percentages and numbers are expressed as a proportion of the total number of live CD45⁺ cells in the vagina. Data represents the mean ± SEM of 3-5 mice per group, and comprise 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to D0 p.i. or mock-infected mice.
Figure 3.10

Leukocyte subsets in the vagina following HSV-2 i.vag infection

Following i.vag infection, the vagina of mock- and HSV-2-infected mice were removed, enzymatically digested and processed for flow cytometric analysis on D0 and D5 p.i. (A) number of total leukocytes (CD45⁺) in the HSV-2-infected vagina, (B) percentage of total leukocytes (CD45⁺) as in (A), (C) number of leukocyte subsets including total macrophages, defined as Ly6G⁻ B220⁻ CD3⁻ CD11b⁺ CD11c⁻, inflammatory macrophages (Ly6G⁻ B220⁻ CD3⁻ CD11b⁺ CD11c⁻ Ly6Cahi), CD11b⁺ DCs (Ly6G⁻ B220⁻ CD3⁻ MHC-II⁺ CD11b⁺ CD11c⁺), CD11b⁻ DCs (Ly6G⁻ B220⁻ CD3⁻ MHC-II⁺ CD11b⁻ CD11c⁺), BDC (Ly6G⁻ B220⁻ CD3⁻ CD11cahi MHC-II⁺), TDC (Ly6G⁻ B220⁻ CD3⁻ CD11cahi MHC-II⁺) and neutrophil cells (CD45⁺ B220⁻ CD3⁻ MHC-II⁺ CD11bi Ly6G⁺), and (D) proportion of leukocyte subsets as in (B). Percentages and numbers are expressed as a proportion of the total number of live CD45⁺ cells in the vagina. Data represents the mean ± SEM of 3-5 mice per group, and comprise 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to D0 p.i. or mock-infected mice.
B220^- CD3^+ NK1.1^+ subsets were investigated. As D5 p.i. was the most prominent day for effector cell infiltration to the vagina in both models, only data from D0 and D5 p.i. are displayed for clarity.

B cells indirectly play a critical role in mediating Th1 antiviral protection in response to viral challenge in the vagina. Upon activation, B cells undergo expansion and differentiate into plasma cells. Plasma cells secrete large quantities of specific Ab and indirectly mediate non-cytolytic IFN-\(\gamma\)-dependent antiviral protection, which is, in turn, regulated by helper CD4^+ T cells (Iijima et al., 2008a). Although the extent of B cell activation and differentiation was not investigated in this study, when enumerated, there was a small population of B cells present in the mock-infected vagina (Figure 3.11A and 3.11C) constituting ~5% of total leukocytes; however, this subset increased sharply to ~5 x 10^3 cells at D5 p.i. in both models of infection. However, the proportion of B cells relative to total leukocytes declined from ~5% to ~1–2%, 5 days after infection (Figure 3.11B and 3.11D).

CD4^+ T cells are critically important in immune responses against viral genital infection. CD4^+ T cells interact with DCs during antigen presentation and once activated, produce large amounts of IFN-\(\gamma\) in the vagina. The number of CD4^+ T cells substantially increased by16-fold in the WNV-infected vagina by D5 p.i. This subset was also a large proportion of leukocytes in the HSV-2-infected vagina, although quantities were slightly lower than in the WNV infection model at D5 p.i. Despite the sizeable increase in number, the proportion of CD4^+ T cells decreased during infection.

Analysis of CD8^+ T cells revealed that both the numbers and proportion of
this subset were very low in both models of infection and did not significantly change during infection (Figure 3.11A–D).

NK cells are a substantial part of the innate immune system. NK cells rapidly respond to virally infected cells in the mucosa by cytotoxic activity and IFN-γ production (Fawaz et al., 1999). During infection, there was ~20-fold increase in the number of NK cells in the WNV- and HSV-2-infected vagina in comparison to mock-infected mice. The percentage of NK cells increased to 10 and 15% of the total leukocytes in WNV- and HSV-2-infected vagina at D5 p.i., respectively (Figure 3.11A–D).

Natural killer T (NKT) cells are a subset of T cells which have characteristics of both T cells and NK cells. NKT cells produce IFN-γ as well as granzyme to lyse virally infected epithelial cells (Mattner et al., 2005). In the vagina, they are present in low numbers in mock-infected mice and increased 2-fold in the WNV/HSV-2-infected vagina. Although ~6% of leukocytes in the vagina at D0 were NKT cells, their proportion had reduced significantly to under 0.5% by D5 p.i. (Figure 3.11A–D).

3.2.8 Dynamics of macrophages, DCs and neutrophils in the ILN following i.vag WNV and HSV-2 infection

Immune responses to vaginal infection are initiated in the iliac lymph nodes (ILN) that drain the vaginal mucosa (King et al., 1998, Kwant-Mitchell et al., 2009). To further analyse the immune response of i.vag WNV and HSV-2
**Figure 3.11**

**Cellularity of lymphocytes in the vagina following i.vag WNV and HSV-2 infection**

Following i.vag infection, the vagina of mock- and WNV/HSV-2-infected mice were removed, enzymatically digested and processed for flow cytometric analysis on D0 and D5 p.i. (A) numbers and (B) proportions of B cells (CD45+ Ly6G− CD3− NK1.1− MHC-II+ CD11c− B220+), CD4+ (CD45+ Ly6G− B220− CD3+ NK1.1− CD4+CD8−) and CD8+ (CD45+ Ly6G− B220− CD3+ NK1.1− CD4− CD8+) T cells, NK (CD45+ Ly6G− B220− CD3− NK1.1+) and NKT (CD45+ Ly6G− B220− CD3+ NK1.1+) cells in the WNV-infected vagina at D0 and D5 p.i. (C) numbers and (D) proportions of lymphocyte subsets in the HSV-2- infected vagina at D0 and D5 p.i., as in (A) and (B). Percentages and numbers are expressed as a proportion of the total number of live CD45+ cells in the vagina. Data represents the mean ± SEM of 3-5 mice per group, and comprise 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to D0 p.i. or mock- infected mice.
infection, the dynamics of major leukocyte populations in the ILN were assessed.

To compare these two models of infection, the kinetics of leukocyte expansion and infiltration in the ILN at D0, 1, 3, 5 and 7 p.i. were analysed. The number of total leukocytes increased significantly at D3 p.i. by 4-fold for both viruses. In HSV-2-infected mice, the total number of cells in the ILN continued to expand to peak at ~6-fold at D5 p.i., and declined slightly at D7 p.i. However, in WNV-infected mice, total numbers remained elevated and did not change significantly between D3 and D7 p.i. (Figure 3.12A).

The number of total macrophages (Ly6G− B220− CD3− CD11b+ CD11c−) peaked at D3 p.i. for WNV by ~6-fold and increased over 100-fold for HSV-2 at D5 p.i. Inflammatory macrophages (Ly6G− B220− CD3− CD11b+ CD11c− Ly6C\text{hi}) peaked significantly at D3 and D5 p.i. for both WNV and HSV-2 models, respectively, by ~10- and 15-fold (Figure 3.12B and C). The same pattern was also observed for CD11b+ and CD11b− DCs in the ILN, as these subsets peaked at D3 p.i. for WNV (~6-fold) and D5 p.i. for HSV-2-infected mice (~6-fold) (Figure 3.12D and E).

The number of BDCs in WNV-infected mice was highest at D7 p.i. (10-fold); however, in HSV-2-infected mice, BDCs increased to peak at 10-fold by D5 p.i. (Figure 3.12F). Although the peak numbers of TDCs occurred at D3 p.i. for both models, WNV infection elicited an approximate 7-fold increase in this subset while in HSV-2 infection, TDCs only increased ~5-fold. TDC numbers declined after D3 p.i. in both models of infection (Figure 3.12G).

Neutrophils comprise a small portion of cells in the ILN. In WNV-infected
mice, neutrophils peaked at D3 p.i. by ~8-fold before returning to baseline levels at D7 p.i. In contrast, neutrophil numbers in HSV-2-infected mice increased at D5 and peaked at D7 p.i. by ~10-fold more cells compared to the mock-infected mice (Figure 3.12H).

The percentage of macrophages almost doubled as a proportion of leukocytes over the 7-day time course (~6% of total live ILN cells) in WNV-infected mice at D1 p.i. before going back to baseline levels at D5 p.i. By D7 p.i., the proportion plummeted to ~1% of the total leukocytes. Interestingly, in the HSV-2 model, the proportion of macrophages continued to expand and peaked at D7 p.i. at ~6%. The peak proportion of inflammatory macrophages occurred at D3 p.i. in the WNV model, while only a significant decrease was observed at D1 p.i. in HSV-2-infected mice (Figure 3.13A and B).

Similar to macrophages, after an initial peak at D1 p.i. (~4%), CD11b+ DCs in WNV-infected mice gradually declined over the course of infection (Figure 3.13A). Conversely, this subset in the HSV-2 infection model peaked at D7 p.i. (~2.5%) (Figure 3.13B). There was no significant change in the proportion of CD11b+ DCs in both models.

The percentage of BDC gradually declined in WNV-infected mice, but it did not change over the course of HSV-2 infection. In contrast, the percentage of TDC in WNV-infected mice showed an early peak at D1 p.i., while HSV-2-infected mice showed a significant decline in the TDC proportion over this period (Figure 3.13A and B).

Throughout the time course, the percentage of neutrophils was very low
Figure 3.12

Total cellularity of leukocyte subsets in the ILN following WNV and HSV-2 i.vag infection

Following i.vag infection, the draining LN of mock- and WNV/HSV-2-infected mice were isolated and processed for flow cytometric analysis. (A) number of total leukocytes and (B) total macrophages, (C) inflammatory macrophages, (D) CD11b+ DCs (E) CD11b- DCs, (F) BDC, (G) TDC and (H) neutrophil cells in the WNV- and HSV-2-infected mice for 1, 3, 5 and 7 days post infection. The comprehensive definition of each subset is defined in figure legends 3.9 and 3.10. Subset numbers are expressed as a proportion of the total number of live ILN cells. Data represents the mean ± SEM of 3-5 mice per group, and comprise 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to HSV-2 D0 p.i. mice; #p≤0.05, ##p≤0.01 and ###p≤0.001 relative to WNV D0 p.i. mice; ^p≤0.05, ^^p≤0.01 and ^^^p≤0.001 relative to WNV and HSV-2 at the same time point.
and mostly unchanged in both models of infection, except at D3 post-WNV infection, where a significant increase was seen. The proportion of neutrophils in the ILN of the HSV-2 infection model remained unchanged throughout the course of infection (Figure 3.13A and B).

3.2.9 B and T cell dynamics in the ILN following i.vag WNV and HSV-2 infection

B and T cell lymphocyte numbers in the ILN also increased as a result of general ILN expansion following infection.

The number of B cells in the i.vag WNV and HSV-2 infection models increased substantially throughout the time course and peaked by D5 p.i. at ~50-fold. With respect to proportions, ~60% of total live cells in the WNV and HSV-2 models were B cells, peaking at D5 and D7 p.i., respectively (Figure 3.14A and B).

While the percentage of CD4⁺ T cells declined significantly in both models (from ~22% at D0 to 10% and 12% at D7 p.i. in WNV- and HSV-2-infected mice, respectively), the number of CD4⁺ T cells went up significantly to peak at D5 p.i. Of note was the higher number of CD4⁺ T cells in the ILN of WNV-infected mice at D5 and D7 p.i. compared to ILN of HSV-2-infected mice. (Figure 3.14 C and D).

Analysis of CD8⁺ T cells revealed lower numbers of CD8⁺ versus CD4⁺ T cells at all time points during infection. The percentage of CD8⁺ T cells decreased
Figure 3.13

Proportion of leukocyte subsets in the ILN following WNV and HSV-2 i.vag infection

Following i.vag infection, the draining LN of mock- and HSV-2-infected mice were isolated and processed for flow cytometric analysis. (A) percentage of leukocyte subsets in ILN of i.vag WNV-infected mice and (B) percentage of leukocyte subsets in ILN of i.vag HSV-2-infected mice. Both graphs represent total macrophages, inflammatory macrophages, CD11b+ DCs, CD11b− DCs, BDC, TDC and neutrophils on 0, 1, 3, 5 and 7 days post infection. The comprehensive definition of each subset is defined in figure legends 3.9 and 3.10. Subset percentages are expressed as a proportion of the total number of live ILN cells. Data represents the mean ± SEM of 3-5 mice per group, and comprise 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to WNV or HSV-2 D0 p.i. mice.
in the ILN of HSV-2-infected mice during infection, while in the WNV model, CD8+ T cells percentage increased slightly to peak at D3 p.i. before declining to the baseline levels at D5 and 7 p.i. The number of CD8+ T cells increased approximately 4-fold in both models at D3 p.i. (Figure 3.14 E and F). In HSV-2-infected mice, numbers of CD8+ T cells remained elevated at D5 p.i. before declining at D7 p.i.; however, in the WNV model, a decrease in CD8+ T cells was observed after D3 p.i.

3.2.10 The origin of leukocytes infiltrating the vagina and ILN following i.vag WNV and HSV-2 infection

APCs, including macrophages and DCs, play a crucial role in the initiation and regulation of immune responses (Iijima et al., 2011). They not only activate lymphocytes, but also migrate to the draining LN where they can secrete cytokines and chemokines, which, in turn, recruit more effector cells from bone marrow and periphery to the site of infection (Banchereau and Steinman, 1998).

To test whether viral infection of the vaginal mucosa triggered the recruitment of DCs, macrophages and other immune effector cells from the bone marrow, 5 x 10^6 unsorted bone marrow mononuclear cells from mock-infected congenic CD45.1+ (B6.SJL-PtprcPep3b/BoyJ) mice were labelled with CellTracker™ Violet and were adoptively transferred intravenously (i.v.) into mock-infected, i.vag WNV- or HSV-2-infected recipients at D4 p.i. All recipient mice were then sacrificed 18 hours after transfer on D5 p.i. The number and phenotype of donor CD45.1+ cells in the vaginal mucosa, ILN and spleen of
**Figure 3.14**

*B and T cells in the ILN following i.vag WNV and HSV-2 infection*

Following i.vag infection, the draining LN of mock- and WNV/HSV-2-infected mice were isolated and processed for flow cytometric analysis. (A) number of B cells, (B) percentage of B cells as in (A), (C) CD4⁺ T cell numbers, (D) percentage of CD4⁺ T cells as in (C), (E) number of CD8⁺ T cells and (F) percentage of CD8⁺ T cells as in (E) in the WNV/HSV-infected mice on days 0, 1, 3, 5 and 7 post infection were identified. The comprehensive definition of each subset is defined in Figure 3.11. Percentages and numbers are expressed as a proportion of the total number of live ILN cells. Data represents the mean ± SEM of 3-5 mice per group, and comprise 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 compared to D0 p.i. or mock-infected mice.
recipients was determined by flow cytometry and the profile of major subsets from mock-infected and virus-infected mice was investigated. D5 p.i. was chosen for analysis based on the day at which most of the immune effector populations maximally infiltrate into vagina and ILN.

As shown in Figure 3.15A, the number of CD45.1+ cells that was obtained from vagina was small; however, it was still possible to identify macrophages, DCs and lymphocyte subsets in the vagina. The total number of CD45.1+ cells in the vagina increased ~ 4 to 5-fold in both infection models relative to mock-infected mice (Figure 3.15A). Furthermore, a significant increase in total and inflammatory macrophages, as well as CD11b+ DCs, was seen in both the WNV- and HSV-2-infected vagina relative to mock-infected mice. No difference was observed in BDCs, TDCs or CD11b- DCs. Indeed, these subsets comprised only a few cells in comparison to the other populations in the vagina (Figure 3.15B). When enumerated, B cells underwent a 3- to 4-fold expansion after vaginal viral infection; however, no CD45.1+ CD4+ or CD8+ T cells were found in the vagina. Interestingly, while neutrophils decreased 4-fold in the HSV-2-infected vagina compared to D0 p.i., they remained unchanged in WNV-infected mice (Figure 3.15C).

The ILN were also examined for the presence of CD45.1+ donor cells to determine if bone marrow-derived cells infiltrated the draining LN. In contrast to the vagina, there was a dramatic increase in the number of CD45.1+ cells observed in ILN after virus infection in both models (Figure 3.16A). Strikingly, macrophages increased ~8-fold in WNV-infected mice and ~10-fold in HSV-2-infected mice at D5 p.i. Of note, there was a significantly higher number of
Figure 3.15

Bone marrow originated leukocyte and lymphocyte subsets in the i.vag WNV and HSV-2 infected vagina

Unsorted bone marrow mononuclear cells from mock-infected congenic CD45.1\(^+\) (B6.SJL-Ptpre\(^a\)Pep3\(^b\)/BoyJ) mice were isolated and labeled with CellTracker\textsuperscript{TM} Violet and adoptively transferred to mock-, i.vag WNV- or HSV-2-infected recipients on D4 p.i. 18 hours after adoptive transfer, vaginal tissues were isolated and processed for flow cytometric analysis. (A) number of total adoptively transferred leukocytes (CD45.1\(^+\)) in the mock-, WNV- and HSV-2-infected vagina, (B) number of leukocyte subsets including total macrophages, inflammatory macrophages, CD11b\(^+\) and CD11b\(^-\) DCs, BDC and TDC, and (C) number of B cells, CD4\(^+\) and CD8\(^+\) T cells and neutrophils. The comprehensive definition of each subset is defined in Figures 3.9, 3.10 and 3.11. Numbers are expressed as a proportion of the total number of live CD45\(^+\) cells. Data represents the mean ± SEM of 3-7 mice per group, and comprise 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *\(p\leq0.05\), **\(p\leq0.01\) and ***\(p\leq0.001\) compared to D0 p.i. or mock-infected mice.
A

![Graph A]

B

![Graph B]

C

![Graph C]
macrophages in the ILN of HSV-2-infected mice, relative to WNV-infected mice. Both infection models exhibited a significant increase in the number of inflammatory macrophages as well as all other DC populations, with the exception of TDC which only increased in HSV-2-infected mice (Figure 3.16B).

Lymphocytes constituted ~80–90% of total CD45.1+ cells in the ILN. The number of B cells as well as CD4+ and CD8+ T cells significantly increased in both WNV and HSV-2 infection models, relative to mock-infected mice. Neutrophils could not be detected in the ILN, as this population is generally small in the ILN (Figure 3.16C).

Since only a small number of donor CD45.1+ cells were recovered from the vagina and ILN, the spleen was analysed for the presence of donor cells, as this organ acts as one of the main reservoirs of leukocytes in the body.

Surprisingly, most of the adoptively transferred cells were found in the spleen with no difference between infected and mock-infected mice (Figure 3.17A). Similar to the vagina and ILN, total macrophages and inflammatory macrophages showed a significant increase in the spleen of WNV-infected mice, although these populations were unchanged in HSV-2-infected mice. CD11b+ DCs increased a little only in the HSV-2 i.vag model, whereas numbers of CD11b- DCs and BDCs did not change in any of the groups (Figure 3.17 B). Data from TDCs were not shown as this population is not found in the spleen.

Furthermore, there was no difference in the migration of CD45.1+ lymphocytes or neutrophils to the spleen at D5 after WNV/HSV-2 infection (Figure 3.17 C).
Figure 3.16

Bone marrow originated leukocyte and lymphocyte subsets in the ILN of i.vag WNV and HSV-2 infected mice

Unsorted bone marrow mononuclear cells from mock-infected congenic CD45.1⁺ (B6.SJL-PtpraPep³b/BoyJ) mice were isolated and labeled with CellTracker™ Violet and adoptively transferred to mock-, i.vag WNV- or HSV-2-infected recipients on D4 p.i. 18 hours after adoptive transfer, draining LNs were isolated and processed for flow cytometric analysis. (A) number of total adoptively transferred leukocytes (CD45.1⁺) in the ILN of mock-, WNV- and HSV-2-infected mice, (B) number of leukocyte subsets including total macrophages, inflammatory macrophages, CD11b⁺ and CD11b⁻ DCs, BDC and TDC, and (C) number of B cells, CD4⁺ and CD8⁺ T cells and neutrophils. Comprehensive definition of each subset is defined in Figures 3.9, 3.10 and 3.11. Numbers are expressed as a proportion of the total number of live CD45⁺ cells. Data represents the mean ± SEM of 3-7 mice per group, and comprise 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to D0 p.i. or mock-infected mice.
Unsorted bone marrow mononuclear cells from mock-infected congenic CD45.1+ (B6.SJL-Ptpr^aPep^b/BoyJ) mice were isolated and labeled with CellTracker™ Violet and adoptively transferred to mock-, i.vag WNV- or HSV-2-infected recipients on D4 p.i. 18 hours after adoptive transfer, spleen tissues were isolated and processed for flow cytometric analysis. (A) number of total adoptively transferred leukocytes (CD45.1+) in the spleen of mock-, WNV- and HSV-2-infected mice, (B) number of leukocyte subsets including total macrophages, inflammatory macrophages, CD11b+ and CD11b– DCs, BDC and TDC, and (C) number of B cells, CD4+ and CD8+ T cells and neutrophils. The comprehensive definition of each subset is defined in Figures 3.9, 3.10 and 3.11. Numbers are expressed as a proportion of the total number of live CD45+ cells. Data represents the mean ± SEM of 3-7 mice per group, and comprise 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to D0 p.i. or mock-infected mice.
Collectively, these data demonstrate enhanced infiltration of leukocytes to the vagina and ILN compared to mock-infected mice. It also indicates that most of the recruited macrophages, DCs and lymphocytes home to spleen instead of infiltrating either the site of infection or the draining LN.

3.3 Discussion

Reproductive tract infections are important global health priorities. In Australia, the seroprevalence of HSV-2 in 2006 among adults is 12%, lower than the USA rate of 21.9% (Cunningham et al., 2006). When the rate of poorer countries or specific ethnic groups are taken into account, this percentage is much larger. Apart from HIV, which is perhaps the best known viral STI, there is a dearth of data on other sexually transmitted viruses including HSV-2. Despite the fact that viral STIs have a major negative impact on health worldwide, mechanisms involved in the initiation of the immune response in the genital tract is poorly understood. In this chapter, the immune response against two different but complementary models of vaginal viral infection, employing lethal DNA (HSV-2) and non-lethal RNA (WNV) viruses were investigated.

The contribution of MHC II+ DCs in the initiation of the virus-specific response has been previously investigated in this lab (Burke et al., 2004). In the present study, we used immunofluorescence and flow cytometry to identify the localisation and kinetics of this subset(s).

Representative of multiple repeat experiments showed that both STI models were remarkably similar in terms of the initiation of immune response by
macrophage and DC recruitment to the vagina. The kinetics of leukocyte infiltration were equivalent not only at the local infection site, but also in the draining LN, with a few remarkable differences. In addition, WNV and HSV-2 also recruited similar numbers of immune effector cells that originated from bone marrow.

The typical experimental model of viral STI is HSV-2 infection of the mouse. Intravaginal challenge of C57BL/6 mice with $10^5$ PFU of HSV-2 was accompanied by severe illness and pathology. Virus was eradicated from the epithelium in all mice by D7 p.i., even though less than 10% of mice survived infection. Although, other HSV-2 inoculation doses lower than $10^4$ PFU were not examined in this study, similar symptoms were observed when using $10^5$ PFU and a 10-fold lower viral dose ($10^4$ PFU) (Figure 3.1A–C). This dose-independent high lethality may be a consequence of the epithelial disruption and CNS attack by HSV-2 (Parr and Parr, 2003) (Figure 3.3C).

On the other hand, inoculation with WNV, which is also a neurotropic virus, did not result in any signs of disease in this mouse strain (Figure 3.1A). Indeed, intravaginal challenge of C57BL/6 mice with high titre WNV ($3.6 \times 10^7$ PFU) did not result in detectable morbidity or mortality. This titre would be lethal if inoculated by other routes, including intraperitoneal, intranasal, intravenously or intradermal routes (Burke, 2005, Burke et al., 2004). Similar to HSV-2, WNV was cleared from the vagina by D7 p.i. However, no epithelial breaches were observed in WNV infection, confirming that the epithelial layer remains intact over the infection time course (Burke et al., 2004, King et al., 2007) (Figure 3.5A–B).
Therefore, while WNV productively infected the vaginal mucosa, infection was resolved by D7 p.i., and epithelium remained intact. In addition, i.vag infection triggered the infiltration of leukocytes to the site of infection and its draining LN, peaking at D5 p.i. Thus, given the highly effective immune response in WNV infection, the antiviral mechanisms and kinetics of the immune response in the vagina was further investigated.

Intravaginal WNV infection elicited robust recruitment of CD11c+ DCs (Figure 3.5A–C), with a significant number of CD11b+ and F4/80+ macrophages infiltrating the vagina, peaking at D5 p.i. (Figure 3.6A–F). DCs and macrophages declined at D7 p.i., when WNV-NS1 protein and WNV RNA were no longer detectable in the vagina, indicating clearance of the virus (Figure 3.4A–B). In addition, dual labelling of vaginal sections with CD11b+, F4/80+ and CD11c+ showed that cell clusters underneath the epithelium consisted of mainly CD11c+ DCs. However, other DCs/macrophages that expressed CD11b+ and/or F4/80+ surface markers were also present (Zhao et al., 2003) (Figure 3.7A–J). There is evidence that shows eosinophils which express moderate levels of F4/80 on the surface are also present in the genital tract (Care et al., 2013, Ghosn et al., 2010). Eosinophils are suggested to have antiviral activities by secreting ribonucleases such as the eosinophilic cationic protein (ECP) (Shamri et al., 2012). Although the presence and quantity of eosinophils were not directly assessed here, this investigation can be also extended further to include eosinophils.

Both WNV and HSV-2 infection exhibited similar results in the vagina with regards to the recruitment of CD11c+ DCs. Both models of infection showed increasing numbers of effector DCs infiltrating the vagina to contain the virally
infected epithelium (Figure 3.5A–C and 3.8A–E).

Flow cytometry of the vagina of both models of infection showed an overall increase in the total number and proportion of leukocytes by D3 and D5 p.i., relative to the mock-infected mice. Indeed, leukocytes were 8 and 10% of the total cells in the vagina of WNV- and HSV-2-infected animals, respectively. A significant increase in the number and percentage of total and inflammatory macrophages was also evident in both models. Inflammatory macrophages, which are defined by high expression of Ly6C, have been shown to accumulate in response to viral and bacterial infection (Jakubzick et al., 2008, Lim et al., 2011, Getts et al., 2012b, Iijima et al., 2007).

Furthermore, Iijima and colleagues reported that infiltrating macrophages give rise to both CD11b+ and CD11b− DCs in the HSV-2-infected vagina (Iijima et al., 2011). These results were consistent with our finding showing that inflammatory macrophages as well as CD11b+ DCs increased following i.vag infection. In contrast, CD11b− DCs, which comprised only a small population, did not change significantly at D5 p.i. in either model (Figure 3.9C–D and 3.10C–D).

In functional studies performed by Iijima and colleagues, macrophages (CD11b+ CD11c−) and CD11b+ DC (CD11b+ CD11c+) isolated from the HSV-2-infected vagina induced minimal T cell proliferation, indicating that both subsets are monocyte-derived APCs in the vagina, as they were unable to activate naive T cells. They also suggested that macrophages infiltrate the infected vagina as emergency responders and some give rise to tissue-resident DCs after clearance of viral infection (Iijima et al., 2011).
Tissue-derived and blood-derived DC subsets are defined based on CD11c and MHC II expression (Davison and King, 2011, Dakic et al., 2004, Kissenpfennig et al., 2005). Intravaginal WNV infection was associated with an increase in both DC subsets, whereas in HSV-2 infection, the TDC population stayed at baseline levels (Figure 3.9C–D and 3.10C–D). This finding was similar to that of reports by other groups showing that the BDC population increases in response to infection while the TDC subset remains unchanged (Davison and King, 2011, Iijima et al., 2011).

Neutrophils are the most abundant granulocytes in the vaginal mucosa. They are involved in resolving primary genital HSV-2 infection but play only a limited role in preventing HSV-2 spread to the sensory ganglia (Milligan et al., 2001). Along with macrophages and DCs, neutrophils increase in number and proportion in the WNV- and HSV-2-infected vagina; however, this increase was not significant in the HSV-2 model (Figure 3.9C–D and 3.10C–D). Similar to our results, Milligan reported that neutrophils were recruited to the HSV-2-infected vagina as early as D1 p.i. and remained at the site of infection until the virus was cleared. Furthermore, depletion of neutrophils resulted in significantly higher virus titre at D3 to 7 p.i. but only slightly delayed resolution of the primary genital infection (Milligan, 1999).

Lymphocytes are also important for controlling viral infection. Innate immune cells such as NK and NKT cells are required for protection against genital HSV-2 infection. NK cells infiltrate the vagina and lyse HSV-infected cells (Koelle et al., 1998). T cells are also present at the site of viral infection. As previously reported, CD4+ T cells but not CD8+ T cells formed clusters at the site of vaginal HSV-2 infection and were required for virus clearance (Iijima et al.,
Furthermore, the Th1-related cytokine, IFN-γ, is secreted early after infection by NK cells and later by CD4+ T cells and is critical to control viral infection (Parr and Parr, 2003, Shrestha et al., 2006).

CD4+ T cells and NK cells were the largest lymphocyte subsets in the WNV- and HSV-2-infected vagina. From D0 to D5 p.i., there was a dramatic rise in the number of these populations, as well as in NKT cells. However, neither the number nor proportion of CD8+ T cells changed over this time course. CD4+ T cells comprised ~20% of the total vaginal leukocytes in infected and mock-infected mice whereas the percentage of NK cells increased in the vagina of mice infected with either WNV or HSV-2. In addition, B cells independently protect mice by producing virus-specific antibodies. However, in knockout mice lacking B cells, mice still eventually clear the virus (Dudley et al., 2000).

Interestingly, the percentages of B cells and NKT cells in the WNV- and HSV-2-infected vaginae at D5 p.i. were lower than that of mock-infected counterparts.

Taken together, while there was a similar leukocyte response to vaginal infection in both models, there were greater numbers of leukocytes in the vagina of WNV-infected mice relative to mice infected with HSV-2 (Figure 3.11A–D).

Infection of the vaginal epithelium triggers a significant immune response in the ILN, which drain the vaginal mucosa. The ILN are believed to be the site where the immune response to vaginal infection is initiated (King et al., 1998, Banchereau and Steinman, 1998). DCs have a central role in ILN expansion by initiating T lymphocyte activation and differentiation into T helper type 1 (Th1) cells, Th2 cells and cytotoxic T lymphocyte (CTL) effectors (Iwasaki and Medzhitov, 2004).
In the present study, the overall immune response in both models of vaginal infection again was similar in the draining LN. Interestingly, despite the fact that HSV-2 infection is lethal in mice, the number of total and inflammatory macrophages, CD11b⁺ and CD11b⁻ DCs, TDC and neutrophils were higher in WNV-infected mice, at least until D3 p.i. Total leukocyte numbers were identical in both models. Surprisingly, neutrophils and total macrophages were significantly higher in HSV-2-infected mice after D3 p.i. with over 4- and 20-fold more cells at D5 p.i. compared to WNV-infected mice (Figure 3.12A–H).

Altogether, percentages of leukocyte subsets in both WNV and HSV-2 infection models decreased throughout the time course, with the exception of total macrophages and CD11b⁺ DCs, which were significantly higher at D7 post-HSV-2 infection. (Figure 3.13A–B).

Other groups have shown that B cells are involved in the control of local virus replication after primary genital infection by secreting natural antibodies, although this protection failed to protect mice against re-infection (Harandi et al., 2001a, Dudley et al., 2000, Parr and Parr, 2000). Furthermore, CD8⁺ T cell-deficient mice were eventually able to protect against lethal HSV-2. In contrast, CD4⁺ T cell-deficient mice had impaired HSV-2-specific IFN-γ production and succumbed rapidly to genital HSV-2 challenge. Taken together, these results emphasise that CD4⁺ T cells are critical for immune protection against lethal genital HSV-2 re-infection (Harandi et al., 2001a, Nakanishi et al., 2009, Dobbs et al., 2005).

In this study, B and CD4⁺ T lymphocytes were the major populations in the ILN. Similar to the vagina, CD8⁺ T cells were a smaller proportion of the ILN relative to other lymphocyte subsets. Mice infected with WNV had significantly
more CD4+ T cells and less CD8+ T cells than HSV-2-infected mice at D5 p.i. On the other hand, the proportion of B cells increased slightly over a 7-day period while the percentage of T cells reduced, except in the percentage of CD8+ T cells in WNV-infected mice that was unchanged after D3 p.i. (Figure 3.14A–F).

To determine whether precursor bone marrow cells could give rise to effector cells following viral genital infection in the vagina and ILN, whole bone marrow cells from a mock-infected congenic (CD45.1+) host were injected into recipient (CD45.2+) WNV- and HSV-2-infected mice. The adoptive transfer of bone marrow cells showed that at least some of the cells that accumulated in the ILN and infected vagina were derived from the donor animal and that the bone marrow gave rise to a proportion of immigrant cells in response to viral stimulation. Consistent with this observation, vaginal epithelial DCs have been shown to be repopulated by progenitors of bone marrow origin (Iijima et al., 2007, Diao et al., 2004). Furthermore, other groups demonstrated that macrophages and DCs are generated from blood monocytes. Monocytes are a population of mononuclear leukocytes that can be divided into two main subsets: a short-lived “inflammatory subset” that is recruited to the site of infection where it initiates immune responses, and a “resident subset,” with a longer half-life that infiltrate normal tissues such as lung and spleen (Geissmann et al., 2003, Iijima et al., 2011). This may explain why few of the adoptively transferred myeloid lineage cells homed to non-inflamed tissue in the mock-infected recipients, whereas inflammatory macrophages and CD11b+ DCs were significantly higher in the infected inflamed tissues, i.e., in the ILN and vagina of infected mice (Figure 3.15A–C and 3.16A–C). Notwithstanding, most of the transferred cells migrated
to the spleen, which shows little sign of inflammatory changes at these time points.

Taken together, the experiments described in this chapter further developed previous findings demonstrating that infection of the vaginal epithelium initiates strong immunity to i.vag WNV and HSV-2 infection both at the site of infection and within the draining LN, and is characterised by recruitment of inflammatory cells, an increase in the major lymphocyte subsets, not only at the local infection site but also in the ILN. Most strikingly, this response included substantial accumulation of subepithelial CD11c+ DCs, but not macrophages, to surround virally infected epithelial cells. Presumably, DCs play an important role in the protective immune response by clustering below the epithelium, taking up and presenting viral Ag to lymphocytes to initiate a robust adaptive response against vaginal infection (Iijima et al., 2007, Iijima et al., 2008a, Iijima et al., 2011, Iwasaki, 2003, Zhao et al., 2003). The observation that adoptively transferred bone marrow cells are found in the infected vagina and ILN, suggested the immune effector cells originating from bone marrow precursors gave rise to lymphocytes, macrophages, neutrophils and DCs that can preferentially migrate to these sites of relatively short notice. The data presented in the following chapter closely examines the role that chemokines play in recruiting inflammatory macrophages and DCs to the inflamed tissues to initiate the immune response.
Chapter 4:

Induction of chemokines following WNV and HSV-2 infection and identification of infiltrating effector cells
Chapter 4:

Induction of chemokines following WNV and HSV-2 infection and identification of infiltrating effector cells

4.1 Introduction

In the previous chapter, we investigated the immune response in the vagina and draining LN after i.vag WNV and HSV-2 inoculation. Both infections elicited robust infiltration of macrophages, DCs and lymphocytes to the site of vaginal infection as well as the ILN. Of interest, clusters of CD11c+ cells (but not CD11b or F4/80) were observed underneath the infected epithelium. Furthermore, adoptive transfer experiments showed that the effector cells recruited to the vagina and ILN originated from the bone marrow. Subsequently, we searched for important factors mediating this infiltration and thus discussed in this chapter.

Chemokines are a large family of small proteins that regulate the trafficking of leukocytes toward the inflamed/infected microenvironment and organise the architecture of the primary and secondary immune response (Stein and Nombela-Arrieta, 2005, Satish et al., 2009, Shi and Pamer, 2011, Lim and Murphy, 2011, Esche et al., 2005). In general, chemokines can be distinguished based on their “inflammatory” or “homeostatic” functions (Lukacs, 2001). Inflammatory chemokines are considered to be key factors for attracting a diverse array of innate immune effector cells including macrophages, DCs, neutrophils
and NK cells. Chemokines affect target cells by binding to and activating specific G-protein coupled receptors on the extracellular surface of cells (Satish et al., 2009, Landin et al., 1996, Murdoch and Finn, 2000). CCL2, also known as MCP-1 (monocyte chemoattractant protein-1), is an inflammatory chemokine that is produced by a variety of cell types after stimulation, although monocytes and macrophages are the principal source of this chemokine (Ansari et al., 2006, Ansari et al., 2011, Tsui et al., 2007). CCL2 can also be expressed by virus-infected mucosal epithelial cells, and, therefore, can induce the migration and infiltration of monocytes, memory T lymphocytes and NK cells to the site of infection (Fernandez et al., 2007, Gill et al., 2008, Williams, 2006).

CCL20 (MIP-3α; macrophage inflammatory protein-3), on the other hand, can function as either a homeostatic or inflammatory chemokine. CCL20 is expressed in mucosal environments such as the Peyer’s Patches (PP), the respiratory tract and in vaginal epithelium under steady state conditions or following inflammation/infection (Williams, 2006, Kallal et al., 2010, Berlier et al., 2006, Cremel et al., 2005, Iwasaki and Kelsall, 2000). CCL20 is mainly expressed by epithelial cells and recruits B cells, immature DCs (i.e., DCs that are specialised in the uptake and processing of antigen) and effector/memory T cells via its binding of the CCR6 receptor (Schutyser et al., 2003). Thus, these data suggest that immature DCs as well as a diverse array of leukocytes ranging from lymphocytes to neutrophils, Tregs and even inflammatory Th17 cells are recruited to the mucosa (Comerford et al., 2010, Kleinewietfeld et al., 2005, Kochi et al., 2010, Lim et al., 2008). Thus, CCL20 is an unusual chemokine because it is not only expressed and induced at high basal levels (Iwasaki and Kelsall, 2000), mainly due to the presence of commensal microflora in the gut which provides
tolerance to such unharmed bacteria, but also able to play a significant role in organizing inflammatory response (Schutyser et al., 2003, Kwon et al., 2002). As diverse set of leukocytes share CCR6 expression on the surface, it has been suggested that other factors such as CCL20/CCR6 axis may play an important role in balancing the anti- or pro-inflammatory immune response and decide whether to enhance or inhibit the maturation and activation process (Cook et al., 2014, Esplugues et al., 2011, Yamazaki et al., 2008). Notwithstanding, while extensive research has explored the roles that chemokines play in homeostasis and inflammation, the extent of its role in the female genital mucosa remains largely unclear. The aims of the present study were to define the expression, induction and function of chemokines, specifically CCL20 and CCL2, and their importance in the recruitment of macrophages, DCs and lymphocytes to the genital mucosa and secondary lymphoid organs following i.vag infection with WNV.

4.2 Results

4.2.1 Kinetics of WNV i.vag infection in BALB/c mice

BALB/c mice have been extensively used to investigate the effect of CCL20 in several experimental models including infection, inflammation, autoimmune disease and cancer models (Schutyser et al., 2003, Ansari et al., 2011, Kallal et al., 2010, Iwasaki and Kelsall, 2000, Furumoto et al., 2004). Furthermore, BALB/c mice have been used in STI models of intravaginal infection with WNV and HSV-2 (Parr et al., 1994, McDermott et al., 1984, Burke
et al., 2004). Finally, since BALB/c mice are more susceptible to WNV than C57BL/6, it was thought that this strain would provide a) more robust measurement and b) a comparative insight into infection in this murine STI model. To understand the kinetics of infection, the level of WNV vaginal infection following inoculation with $3.6 \times 10^5$ PFU of WNV was measured using real-time RT-PCR and IF staining. This inoculation titre was adopted from a report by Burke and colleagues which demonstrated that i.vag infection of BALB/c mice with $3.6 \times 10^5$ PFU of WNV was associated with low mortality (12%) and substantially protected surviving mice from WNV rechallenge (Burke et al., 2004).

Figure 4.1A shows that WNV RNA copies in the vaginal tissue rose significantly at D3 p.i., increasing ~10-fold compared to D1 p.i., and peaking ~30-fold higher at D5 p.i. WNV infection was resolved by D7 p.i. and as for C57BL/6 mice, there was no detectable mRNA in the vagina (Figure 3.4A). However, there were more mRNA copies detected in the BALB/c mice at D3 and D5 p.i. than in the C57BL/6 model. IF staining using a mouse monoclonal antibody to label non-structural protein 1 (NS-1) of WNV was used to localise virus spread in the vaginal epithelium. Infection peaked at D5 p.i. (data not shown). Figure 4.1B demonstrates that WNV infection was localised to the epithelium.
Figure 4.1

Quantification of WNV infection of the BALB/c vagina

(A) WNV mRNA expression in the BALB/c vagina following i.vag. inoculation with 3.6 x 10^5 PFU WNV at D0 – D7 p.i., as indicated. Expression is relative to levels in the vagina at D1 p.i. (B) Immunofluorescent staining for WNV-NS1 (FITC; green) and nuclei (DAPI; blue) of a vaginal cross-section at D5 p.i. No WNV-NS1+ cells were detected in mock infected, D7 WNV p.i. and mouse IgG1 isotype-stained tissue sections (not shown). Images are representative of at least 4 mice per time point. Bars represent the mean ± SEM of 4 mice per group and comprise 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post- test; *p≤0.05 and **p≤0.01 compared to D0 p.i.
A

Relative WNV mRNA expression

Day post WNV-infection

B

Nuclei WNV 3.6x10^5 PFU D5 p.i.
4.2.2 Infiltration of effector leukocytes to the vagina after i.vag infection of BALB/c mice

4.2.2.1 Expression of CD11c in the vaginal mucosa after WNV infection

To investigate the recruitment of innate immune effector cells and localisation of WNV in the vaginal mucosa of BALB/c mice, vaginal sections from mock- and WNV-infected BALB/c mice were labelled for WNV-NS1 antigen as well as CD11c. As with C57BL/6 mice, IF staining indicated that there was significant infiltration of CD11c+ DCs to the site of epithelial WNV infection. Intravaginal WNV infection was followed by an increase in the number of CD11c+ cells underneath the epithelium starting from D1 p.i. and increasing to peak at D5 p.i. As opposed to the C57BL/6 strain where WNV inoculation resulted in mild and dispersed epithelial infection (Figure 3.5 and 3.6), vaginal WNV infection in BALB/c mice were associated with severe epithelial infection on D5 p.i. However, like the C57BL/6 mouse, although no WNV was detected on D7 p.i., the number of DCs remained relatively high at this time point. Here, again, WNV was contained in the epithelial layer and no viral infection was detected in any other parts of the vagina at any time point (Figure 4.2A–E).

Image analysis was used to measure the area occupied by CD11c+ and WNV-infected epithelial cells during the course of i.vag infection (D0-D7 p.i.) (Figure 4.2F). There was a significant increase in CD11c+ cells from D3 until D5 p.i. compared to mock-infected controls before a gradual decrease at D7 p.i. Image analysis of epithelial WNV infection showed an increase at D3 p.i., with a peak at D5 p.i., with ~1.5% of the tissue area staining positive for WNV-NS1.
**Figure 4.2**

*Localisation of CD11c and WNV-NS1 in the BALB/c vagina following WNV infection*

Immunofluorescent staining for WNV-NS1 antigen (FITC; green), CD11c surface marker (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from BALB/c mice infected i.vag. with $3.6 \times 10^5$ PFU WNV at (A) D0, (B) D1 and (C) D3 (D) D5 (E) D7 p.i. (F) Percentage of vaginal epithelial cells that were WNV-NS1$^+$ in BALB/c mice infected with $3.6 \times 10^5$ PFU (green) and proportion of the area filled with CD11c$^+$ cells (red). Neither WNV-NS1$^+$ nor CD11c$^+$ cells were observed in mouse IgG1 and Armenian hamster IgG isotype-stained sections (G). All images are representative of at least 4 mice per time point. Bars indicate the mean ± SEM of 4 mice per group and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ compared to D0 p.i. #*$p \leq 0.05$ and ###$p \leq 0.001$, relative to WNV D0 p.i.
4.2.2.2 Localisation of CD11b and F4/80 in the vaginal mucosa

To further identify cells accumulating under the infected epithelium, vaginal sections were stained for other APC surface antigens, namely, CD11b and F4/80. These markers are found on macrophages as well as resident and inflammatory DCs (Murray and Wynn, 2011, Sajic et al., 2005). As Figures 4.3 (A–E) illustrate, CD11b$^+$ and F4/80$^+$ cells increased following i.vag WNV infection, peaking at D5 p.i., and, strikingly, remained in high numbers at D7 p.i. This finding is in accordance with previous observations in C57BL/6 where CD11b$^+$ and F4/80$^+$ cells were distributed in the vaginal stroma with a tendency to accumulate near infected epithelial sites; however, more aggregations of F4/80$^+$ cells were evident compared to the C57BL/6 strain. CD11b$^+$ cells (possibly neutrophils) were also present in the vaginal lumen adjacent to the epithelium.

Image analysis using images from at least three distinct spots of infection revealed a similar kinetic profile for CD11b$^+$ and F4/80$^+$ cells in the vagina (i.e., peaking at D5 p.i.), with ~ 4 and 5% of the whole area occupied by each marker, respectively. Although there was a slight reduction in the presence of both markers, they still remained elevated at D7 p.i. relative to controls (Figure 4.3F).

4.2.2.3 Co-localisation of CD11c and CD11b in the vagina after i.vag WNV infection

Co-labelling of vaginal sections revealed typical aggregations of CD11c$^+$ cells in the sub-epithelium adjacent to the areas of infection. As Figure 4.4A–E illustrate, both CD11b$^+$ and CD11c$^+$ cells are present in the vaginal
**Figure 4.3**

*Co-localisation of CD11b and F4/80 in the BALB/c vaginal mucosa after WNV infection*

Immunofluorescent staining for CD11b surface marker antigen (FITC; green), F4/80 (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from BALB/c mice infected i.vag with $3.6 \times 10^5$ PFU WNV at (A) D0, (B) D1, (C) D3, (D) D5 and (E) D7 p.i. (F) Percentage of vaginal cells that were CD11b$^+$ (green) and proportion of the area filled with F4/80$^+$ cells (red). Immunofluorescent staining of vaginal sections with rat IgG2a and rat IgG2b (corresponding isotypes) elicited no specific staining for CD11b and F4/80 (G). All images are representative of at least 4 mice per time point. Bars indicate the mean ± SEM of 4 mice per group and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; **p ≤ 0.001 and ###p ≤ 0.001, compared to D0 p.i.
microenvironment. Similar to mock-infected C57BL/6 mice, there were small numbers of CD11c+ and CD11b+ cells in the vagina, however, a portion of CD11b+ cells were located in the lumen. As described in the Chapter 3, they were more likely to be neutrophils, since these phagocytic cells are dominant in the vaginal epithelium and the lumen (Nandi and Allison, 1993). Following infection, the number of both cell types gradually increased and subsequently peaked at D5 p.i., with CD11b+ and CD11c+ cells comprising ~3.5 and ~5% of the total area, respectively. Interestingly, CD11c+ cells increased in the vagina during the infection time course and demonstrated clusters of DCs below the epithelium. Quantification of the markers at D7 p.i., demonstrated a reduction in the number of CD11b+ and CD11c+ cells compared to at D5 p.i., although both cells were still highly expressed, occupying ~2% and ~3% of the whole area, respectively, relative to D0 p.i. (Figure 4.4F).

Together, i.vag WNV inoculation resulted in a radical redistribution of cells and an acute inflammatory response in the vagina as shown by the infiltration of a large number of APCs. More specifically, these cells included CD11b+, F4/80+ or CD11c+ cells recruited to the vagina in response to viral infection. Although a few cells dual-labelled for surface markers (CD11c and CD11b or CD11b and F4/80), most of the cells only singly expressed CD11b, F4/80 or CD11c. The dominant cell type was the CD11c+ DC followed by CD11b+ and F4/80+ macrophages/DCs, as observed in C57BL/6 mice infected i.vag with WNV/HSV-2 (Figure 4.3A–E and 4.4A–E).
4.2.3 Cellular response of the ILN following i.vag WNV infection of BALB/c mice

As discussed in Chapter 3, vaginal infection is associated with a significant increase in the number of leukocytes that originated from bone marrow. In addition, other groups have described the ILN as the primary site for the initiation of immune responses to vaginal infection (King et al., 1998, Kwant-Mitchell et al., 2009, Johnston et al., 2000). To further analyse the immune response following intravaginal WNV infection in BALB/c mice, the leukocyte populations in the ILN were assessed. This lab has previously reported that WNV vaginal infection in BALB/c mice was associated with an increase in cell numbers in the ILN with major leukocyte subsets peaking at D3 p.i., however, proportion and number of specified populations gradually declined at D5 p.i. and thereafter (Burke, 2005). This observation was also in accordance with our findings as described in the chapter 3 for WNV i.vag infection in C57BL/6 mice (Figure 3.12–14). In other words, most of major ILN subsets in C57BL/6 mice, such as total and inflammatory macrophages, CD11b+ DCs, TDCs, neutrophils and CD8+ T cells peaked at D3 p.i. or had relatively similar numbers compared to D5 p.i. Therefore, based on these data (Burke, 2005) and the results in Chapter 3, the ILNs were isolated from BALB/c mice and analysed for leukocyte subsets by flow cytometry at D0, 1 and 3 p.i. only.

No significant change was observed in total ILN numbers prior to D3 post-WNV infection. At D3 p.i., however, total cell numbers increased ~3-fold while total macrophages increased ~3-fold, inflammatory macrophages increased ~8-fold and neutrophils increased ~20-fold, compared to mock-infected controls.
**Figure 4.4**

**Co-localisation of CD11b and CD11c in the BALB/c vaginal mucosa after WNV infection**

Immunofluorescent staining for CD11b surface marker antigen (FITC; green), CD11c (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from BALB/c infected vagina with 3.6 x 10^5 PFU WNV at (A) D0, (B) D1, (C) D3, (D) D5, and (E) D7 p.i. (F) Percentage of vaginal cells that were CD11b^+ (green) and proportion of the area filled with CD11c^+ cells (red). Immunofluorescent staining of vaginal sections with rat IgG2a and Armenian hamster IgG (corresponding isotypes) elicited no specific staining for CD11b and CD11c markers (G). All images are representative of at least 4 mice per time point. Bars indicate the mean ± SEM of 4 mice per group and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to D0 p.i., and #p≤0.05 and ###p≤0.001, compared to D0 p.i.
Nuclei
CD11b
CD11c
WNV D0 p.i.

Nuclei
CD11b
CD11c
WNV D1 p.i.

Nuclei
CD11b
CD11c
WNV D3 p.i.

Nuclei
CD11b
CD11c
WNV D5 p.i.

Nuclei
CD11b
CD11c
WNV D7 p.i.

% Total area
CD11b
CD11c

Day post WNV-infection

G

Nuclei
Rat IgG2a
Hamster IgG
WNV D5 p.i.
The number of CD11b\(^+\) DC and TDC subsets showed a significant rise in numbers at D1 p.i. before decreasing on D3 p.i. CD11b\(^-\) DC and BDC populations, on the other hand, did not fluctuate during this time course (Figure 4.5A- B).

Analysis of leukocyte proportions showed that total macrophages (~5% of leukocytes), CD11b\(^+\) DC (~4%) and TDC (~3%) subsets were significantly higher at D1 p.i., although, this increase was not sustained as they returned to baseline levels by D3 p.i. Percentages of inflammatory macrophages and neutrophils peaked at D3 p.i. whereas CD11b\(^-\) DC in this model underwent a significant reduction of ~50%). As with cell numbers, i.vag WNV infection did not affect the percentage of BDC in the ILN (Figure 4.5C).

Unlike macrophages, DCs and neutrophils, all lymphocyte populations in the ILN of WNV-infected BALB/c mice increased significantly in number at D3 p.i. relative to mock-infected mice. CD4\(^+\) T cells were the major population in the ILN and doubled at this time-point. Moreover, numbers of B cells and CD8\(^+\) T cells in the ILN increased ~4–5-fold on D3 p.i. (Figure 4.5D).

CD4\(^+\) T cells comprised ~70% of total cells in the ILN (Figure 4.5E). This observation contrasts the results obtained from C57BL/6 mice which showed that B cells constituted the major lymphocyte subset (Figure 3.14B). The percentage of CD4\(^+\) T cells declined significantly at D1 p.i., but returned to normal by D3 p.i. However, B cells and CD8\(^+\) T cells had different trends and peaked at D1 and D3 p.i., respectively, after i.vag WNV infection (Figure 4.5E).

Despite the fact that not all time points were available to compare the number and proportion of major leukocyte subsets in the ILN after i.vag WNV
infection, both C57BL/6 and BALB/c mouse strains were largely comparable, with a few notable discrepancies. The main differences between BALB/c and C57BL/6 mice include the abundance of different lymphocyte subsets in the ILN following i.vag WNV infection. CD4+ T cells were the major lymphocyte subset in the ILN of BALB/c mice, whereas, in the C57BL/6 strain, B cells were the dominant cell type. In BALB/c mice, both CD11b+ DC and TDC populations peaked immediately after i.vag WNV infection at D1 p.i., then returned to the same levels as mock-infected controls on D3 p.i., while these two subsets in the ILN of C57BL/6 mice continued expanding for 5 and 3 days p.i., respectively.

4.2.4 Cytokine response of the vaginal mucosa during infection with WNV of BALB/c and C57BL/6 mice

Complex cascades of pro-inflammatory and immunoregulatory cytokines may impact host susceptibility to vaginal STI (Zara et al., 2004). Cytokine induction following viral infection is the earliest and strongest immune response in the vaginal mucosa and is dominated by the expression of pro-inflammatory cytokines (Abel et al., 2005).

Accordingly, cytokine gene expression in the vagina of BALB/c and C57BL/6 mice were assessed throughout WNV infection by RT-PCR. To do this, vaginae from mock- and WNV-infected mice (3.6 x 10^5 PFU) were isolated on D0, 1, 3, 5 and 7 p.i., where, mRNA levels of IFN-γ and TNF were analysed. Nitric oxide synthase 2 (NOS2) and indoleamine 2,3-dioxygenase (IDO) mRNA expression were also examined. NOS2 and IDO enzymes are a part of the innate
Figure 4.5

Leukocyte subsets in the ILN of BALB/c mice following WNV i.vag infection

Following i.vag infection, the vagina of mock- and WNV-infected BALB/c mice were isolated and processed for flow cytometric analysis on D0, D1 and D3 p.i. (A) number of total leukocytes (CD45+) in the WNV infected vagina, (B) number of leukocyte subsets including total macrophages defined as Ly6G− B220− CD3− CD11b+ CD11c−, inflammatory macrophages (Ly6G− B220− CD3− CD11b+ CD11c− Ly6C<sup>hi</sup>), CD11b<sup>+</sup> DCs (Ly6G− B220− CD3− MHC-II<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>+</sup>), CD11b<sup>−</sup> DCs (Ly6G− B220− CD3− MHC-II<sup>−</sup> CD11b<sup>−</sup> CD11c<sup>−</sup>), BDC (Ly6G− B220− CD3− CD11c<sup>hi</sup> MHC-II<sup>−</sup>), TDC (Ly6G− B220− CD3− CD11c<sup>−</sup> MHC-II<sup>hi</sup>) and neutrophil cells (CD45<sup>+</sup> B220− CD3− MHC-II<sup>−</sup> CD11b<sup>hi</sup> Ly6G<sup>+</sup>). (C) percentage of leukocyte subsets as in (A). (D) numbers and (E) proportion of B cells (CD45<sup>+</sup> Ly6G− CD3− NK1.1<sup>−</sup> MHC-II<sup>−</sup> B220<sup>−</sup>), CD4<sup>+</sup> (CD45<sup>+</sup> Ly6G− B220− CD3− NK1.1<sup>−</sup> CD4<sup>+</sup> CD8<sup>−</sup>) and CD8<sup>+</sup> (CD45<sup>+</sup> Ly6G− B220− CD3− NK1.1<sup>−</sup> CD4<sup>−</sup> CD8<sup>+</sup>) T cells in the ILN WNV infected mice at D0, D1 and D5 p.i. Percentages and numbers are shown as a proportion of the total number of live CD45<sup>+</sup> cells in the ILN. Data is the mean ± SEM of 4 mice per group, and comprise 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to D0 p.i. or mock-infected BALB/c mice.
immune system that are highly induced by IFN-γ and TNF in response to a variety of stimuli, including viral infection (Thomas and Stocker, 1999, Benencia et al., 2003). Furthermore, studies have shown that the enzymatic activity of IDO and NOS2 are inversely regulated; in other words, the expression of each enzyme is inhibited by the induction of the other (Alberati-Giani et al., 1997).

IFN-γ expression in the vagina of BALB/c and C57BL/6 mice did not change until D5 p.i., when it increased significantly by ~100 and ~300-fold, respectively, relative to mock-infected mice (Figure 4.6A). IFN-γ induction in C57BL/6 mice in response to infection was significantly higher than in BALB/c at this time point. Furthermore, IFN-γ expression continued to rise in BALB/c, peaking at D7 p.i., while it dropped back to baseline levels in C57BL/6 mice. On the other hand, i.vag WNV infection did not induce TNF in C57BL/6 mice, while in BALB/c mice, infection elicited a significant increase in this cytokine that peaked at D5 p.i. by ~7-fold higher in the vagina than in mock-infected mice (Figure 4.6B).

Of note, the relative expression of IDO mRNA in the vagina of both strains was similar to their relative expression pattern of IFN-γ mRNA. Therefore, in WNV-infected BALB/c mice, IDO mRNA was upregulated significantly on D7 p.i., whereas the mRNA was highly induced (~7500-fold) on D5 p.i in C57BL/6 mice relative to mock-infected controls (Figure 4.6C). NOS2 mRNA levels only increased in C57BL/6 mice at D5 p.i. where it was upregulated ~5-fold. However, NOS2 mRNA was significantly higher in the vagina of BALB/c mice compared to C57BL/6 mice, peaking at D5 p.i. (~45-fold) and it remained elevated at D7 p.i., with an ~40-fold increase compared to D0 p.i. (Figure 4.6D).
4.2.5 Chemokine and chemokine receptor expression in the vaginal mucosa following i.vag WNV infection

Chemokines play a central role in the recruitment and localisation of leukocyte subsets not only during inflammation/infection but also during steady-state. Chemokines carry out their functions by binding to specific G-protein-coupled receptors on the surface specific cell populations. This is leads to the migration of macrophages, DCs, lymphocytes and other effector immune cells to the site of inflammation/infection and also to the secondary lymphoid organs, for example, the draining LN. Therefore, the local mRNA expression of several chemokine and chemokine receptors that are thought to be involved in the infiltration and initiation of immune response in the vagina were investigated.

CCL3, also known as macrophage inflammatory protein-1α (MIP-1α), belongs to the CC chemokine family, which is involved in the recruitment and activation of polymorphonuclear leukocytes, particularly those of the myeloid lineage. Monocytes, immature DC, neutrophils, NK cells, T cells and B cells respond to CCL3 and migrate to sites of infection and inflammation (Maurer and von Stebut, 2004, Peretti et al., 2005). Significant upregulation of CCL3 was detected in the WNV-infected vagina of BALB/c and C57BL/6 mice at D5 p.i. at similar levels (~4-fold relative to mock-infected mice), followed by a decrease on D7 p.i. This reduction was more dramatic in BALB/c mice as it returned to the baseline levels (Figure 4.7A).

The chemotactic activity of CCL5 or RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) mediates the trafficking and
Figure 4.6

Relative gene expression of IFN-γ, TNF, IDO and NOS2 in the WNV infected vagina

Relative mRNA expression of (A) IFN-γ, (B) TNF, (C) IDO and (D) NOS2 in the vagina of mice infected i.vag with 3.6 × 10^5 PFU of WNV at D0, D1, D3, D5 and D7 p.i. Expression is relative to levels in the vagina at D0 p.i. Data is plotted as the mean ± SEM of 4 mice per group and is representative of 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; ***p≤0.001 compared to mock-infected C57BL/6 mice. ##p≤0.01 and ###p≤0.001 relative to mock-infected BALB/c mice. ^^^p≤0.001 relative to C57BL/6 and BALB/c strains at the same time point.
homing of T cells such as Th1 cells as well as monocytes, NK cells, DCs, basophils, eosinophils and mast cells to the site of inflammation/infection. CCL5 is produced by cells including macrophages, epithelial cells, platelets, fibroblasts and endothelial cells and exerts its effect via CCR1, CCR3, CCR4 and CCR5 receptors (Appay and Rowland-Jones, 2001, Balistreri et al., 2007, Levy, 2009). WNV infection also upregulated CCL5 expression in the vagina at D5 p.i., with similar kinetics in both strains (~50–60-fold, relative to D0 p.i.). This level increased slightly to peak at D7 p.i. (~75-fold) in BALB/c animals. In contrast, CCL5 expression in the WNV-infected C57BL/6 vagina reduced slightly at D7 p.i. (Figure 4.7B).

CCL19 and CCL21 are the only known chemokines that exhibit strong specificity for the CCR7 receptor. CCL19 and CCL21 are expressed by various subsets of immune cells. CCR7 and its ligands are involved in the homing and localisation of various B and T cell subpopulations and DCs to the LN. It has been shown that DCs mature following infectious or inflammatory stimuli, which is characterised by upregulation of CCR7. These mature DCs then traffic to the LN via the afferent lymphatics. Once in the LN and in the presence of other co-stimulatory factors, T cells undergo antigen-specific activation by establishing close physical contact with mature DCs (Zlotnik and Yoshie, 2000, Förster et al., 2008, Rot and von Andrian, 2004).

CCR7 mRNA expression levels were significantly elevated on D3 and D5 p.i. in both strains; however, BALB/c mice had more mRNA copies of CCR7 in the WNV-infected vagina after D1 p.i. compared to C57BL/6 mice (Figure 4.7C). The relative expression of mRNA encoding the CCR7 ligand, CCL19, increased
significantly on D1 p.i. in BALB/c mice ~30-fold and decreased to baseline levels thereafter (Figure 4.7D). However, in C57BL/6 mice, a small but significant increase was observed at D5 (~5-fold), compared to d0 p.i. mRNA for CCL21, another CCR7 ligand, was also induced in the vagina of WNV-infected mice; it peaked at D3 p.i. by ~2-fold in C57BL/6 mice while in BALB/c mice, CCL21 mRNA was only upregulated significantly on D5 p.i., also by ~2-fold. The CCL21 mRNA expression returned to baseline levels at D7 p.i. (Figure 4.7E).

As described earlier, the CCR6–CCL20 receptor-ligand pair is responsible for the chemoattraction of immature DC, effector/memory T cells and B cells and plays a role at mucosal surfaces under homeostatic and inflammatory conditions. Therefore, given the role of CCL20 in the recruitment of immature DCs to infection site, the expression of mRNA encoding this chemokine and its receptor was analysed.

As illustrated in Figure 4.8A–B, expression of CCR6 and CCL20 mRNA in the WNV-infected C57BL/6 mice did not change throughout the time course. Surprisingly, a significant downregulation was observed for CCR6 gene expression in the vagina of BALB/c mice at D1 p.i., although expression returned to the same level as mock-infected mice by D7 p.i. With respect to CCL20 gene expression in BALB/c mice, significant upregulation was observed at D3 and D5 p.i., upregulated ~5- and 12-fold, respectively. However, by D7 p.i., CCL20 mRNA copies decreased to the level of mock-infected control mice.

CCR2 and its ligand, CCL2, belong to the inflammatory chemokine family. Because inflammatory monocytes/macrophages, as well as T and NK cells, require CCR2 to infiltrate into the infected microenvironment, the gene
Figure 4.7

Chemokine and chemokine receptor mRNA expression in the vagina following WNV infection

Relative mRNA expression of (A) CCL3, (B) CCL5, (C) CCR7, (D) CCL19 and (E) CCL21 in the vagina of mice infected i.vag with 3.6 x 10^5 PFU of WNV at D0, D1, D3, D5 and D7 p.i. Expression is relative to levels in the vagina at D0 p.i. Data is plotted as the mean ± SEM of 4 mice per group and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 compared to mock-infected C57BL/6 mice or D0 p.i. #p ≤ 0.05, ##p ≤ 0.01 and ###p ≤ 0.001 relative to mock-infected BALB/c mice or D0 p.i. ^p ≤ 0.001, ^^p ≤ 0.001 and ^^^p ≤ 0.001 relative to C57BL/6 and BALB/c strains at the same time point.
expression of this pair were also examined in the WNV-infected vagina of C57BL/6 and BALB/c mice.

Levels of CCR2 expression in the vagina of both strains of mice prior to D5 p.i. was not significant, however, CCR2 gene expression was upregulated in C57BL/6 mice by more than 2-fold at D5 and 7 p.i. In contrast, CCR2 mRNA showed a trend toward downregulation in BALB/c animals compared to mock-infected controls after D3 p.i. (Figure 4.8C).

WNV infection elicited significant expression of CCL2 at D5 p.i. (~35-fold) in C57BL/6 mice and declined straight after (i.e., D7 p.i.). Interestingly, as opposed to CCL20 expression patterns, CCL2 expression levels did not change over the infection course in i.vag WNV infected BALB/c mice (Figure 4.8D).

4.2.6 Recruitment of CCR6+ cells to the vagina and ILN following i.vag WNV infection in BALB/c mice

From the chemokine and chemokine receptor gene expression data (Figure 4.8A–D), it is clear that the CCR6-CCL20 (receptor-ligand) pair was only one whose expression was altered in BALB/c mice and not C57BL/6 animals following vaginal WNV infection. Thus, to further understand the effect of CCL20, we sought to determine the role of this chemokine by selective antibody-mediated neutralisation. CCL20 was blocked by intravenously (i.v.) injecting anti-CCL20 antibody at 6 and 48 hours post-WNV infection. These time points represented the time before the establishment of immune response and prior to the
Figure 4.8

Relative mRNA expression of CCR6-CCL20 and CCR2-CCL2 (Chemokine-receptor pair) in the WNV infected vagina

Relative mRNA expression of (A) CCR6, (B) CCL20, (C) CCR2 and (D) CCL2 in the vagina of mice infected i.vag with 3.6 x 10^5 PFU of WNV at D0, D1, D3, D5 and D7 p.i. Expression is relative to levels in the vagina at D0 p.i. Data is plotted as the mean ± SEM of 4 mice per group and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; **p≤0.01 and ***p≤0.001 compared to mock-infected C57BL/6 mice or D0 p.i. ##p≤0.01 and ###p≤0.001 relative to mock-infected BALB/c mice or D0 p.i. ^p≤0.001, ^^p≤0.001 and ^^^p≤0.001 relative to C57BL/6 and BALB/c strains at the same time point.
first significant infiltration of immune effector cells as well as upregulation of CCL20. A group of 4 mice received the isotype control antibody (polyclonal goat IgG). On D3 p.i., all groups of BALB/c mice, including mock-, WNV-infected alone, WNV-infected/anti-CCL20 injected and WNV-infected/isotype control injected mice, were sacrificed. The vagina was excised and analysed via real-time PCR and IF. The ILN were also isolated and examined for the presence of macrophages, DCs and major lymphocyte subsets.

4.2.6.1 Cytokine and chemokine gene expression following CCL20 neutralisation

To investigate the effect of CCL20 neutralisation in the vagina, WNV RNA expression was analysed in all groups of mice. As Figure 4.9A illustrates, there was no difference in WNV expression between WNV-infected, WNV-infected/anti-CCL20 or WNV-infected/isotype control mice.

Analysis of IFN-γ expression showed that there was a reduction in IFN-γ mRNA in the anti-CCL20 and isotype control groups, compared to mice that were just infected with WNV; however, this was not statistically significant (Figure 4.9B). Moreover, no change was observed in the CCL20 gene expression between all groups investigated. Although a slight reduction in the mRNA copies of CCL19 and CCL21 was observed in the vagina of anti-CCL20 and isotype control mice, these were not also significant in comparison to the vagina of WNV-infected mice (Figure 4.9C–E). Finally, CCL2 expression was elevated in all groups (~5- to 7-fold) relative to mock-infected control mice, however, no
difference was seen in the vagina of each of the WNV-infection groups (i.e., WNV-infected, anti-CCL20 and isotype control) at D3 p.i. (Figure 4.9F).

4.2.6.2 Kinetics of i.vag WNV infection following CCL20 neutralisation

As described, to investigate the localisation of WNV infection, BALB/c mice were inoculated intravaginally with $3.6 \times 10^5$ PFU of WNV. Vaginae from all groups (mock, WNV infected, anti-CCL20 and isotype control) were harvested at D3 p.i. and examined for WNV-NS1. Semi-quantitative analysis of the area occupied by WNV-NS1 staining was carried out. Images from at least three distinct areas of vaginal tissue exhibited that no WNV-infected cells were present in mock-infected control. The extent of epithelial infection in WNV infected and isotype control group did not differ as $\sim1.5\%$ of the epithelial mucosa was WNV-NS1$^+$ (Figure 4.10E). Strikingly, the anti-CCL20 group had significantly greater epithelial infection ($\sim2.5\%$) relative to the other WNV-infected groups. Therefore, neutralisation of CCL20 suggests a detrimental effect on the mucosal immune system. Higher WNV spread in the vaginal epithelium of CCL20-neutralised mice may be interpreted as a failure to control WNV infection of these mice in the vagina (Figure 4.10A–E).

4.2.6.3 Localisation of CD11b in the vaginal mucosa following CCL20 neutralisation

The presence of CD11b$^+$ macrophages and DCs in the vaginal mucosa was shown in Chapter 3. Additionally, Iwasaki and co-workers showed that CD11b$^+$
Figure 4.9

Relative gene expression following CCL20 neutralisation in the WNV-infected vagina

Relative mRNA expression of (A) WNV, (B) IFN-γ, (C) CCL20, (D) CCL19, (E) CCL21 and (F) CCL2 in the vagina of i.vag WNV-infected BALB/c mice (with 3.6 x 10^5 PFU) following injection with anti-CCL20 antibody or polyclonal goat IgG isotype control at D0 and D3 p.i. Expression is relative to levels in the WNV-infected vagina at D3 p.i. or mock-infected controls at D0 p.i. Data is plotted as the mean ± SEM of 4 mice per group and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test.
cells in the Peyer’s patches express CCR6 and migrate toward CCL20 (Iwasaki and Kelsall, 2000). Therefore, to determine this also occurs in the vagina, vaginal sections were stained with anti-CD11b antibody. The results showed that CD11b-expressing cells increased at D3 p.i. in all WNV groups relative to mock-infected controls, as expected. However, no significant differences were observed in CD11b labelling in the vagina of any of the WNV-infected mice (Figure 4.10F–J).

4.2.6.4 CD11c expression in the vaginal mucosa following CCL20 neutralisation

Given the role of CCL20 in the recruitment of immature DCs to inflamed/infected area and the aggregation of CD11c+ DCs in the subepithelial mucosal vagina, it was of interest to investigate the presence of CD11c+ cells in the vagina of WNV-infected, anti-CCL20 and isotype control mice. As Figure 4.10K illustrates, no clusters of CD11c+ cells were evident in the mock-infected control mice. Quantification of CD11c in the vagina showed that in WNV-infected and isotype control vaginas, CD11c occupied ~4% of the total area in these sections (Figure 4.10J). The CCL20 blockade in the WNV-infected mice led to a reduction in the accumulation and cluster sizes of CD11c+ cells under the WNV-infected vaginal epithelium at D3 p.i. (Figure 4.10L–N). However, quantification of total CD11c+ cells in these sections show that there was a nonsignificant reduction in CD11c+ cells in mice treated with anti-CCL20 antibody (Figure 4.10O).
**Figure 4.10**

*Localisation of WNV, CD11b and CD11c in the infected vaginal mucosa after anti-CCL20 administration*

WNV-infected BALB/c mice were injected i.v. with anti-CCL20 antibody or polyclonal goat IgG isotype control. Vaginae from mock- and WNV-infected mice were isolated on D3 p.i. and analysed for WNV-NS1, CD11b and CD11c markers. Panels on the left represent immunofluorescent staining for WNV-NS1 antigen (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in the vaginal cross-sections from mock-infected (A), WNV-infected (B), WNV-infected + anti-CCL20 antibody (C) and WNV-infected + isotype control (D). (E) proportion of the area filled with WNV-NS1+ in the vagina. Panels located in the centre exhibit immunofluorescent staining for CD11b antigen (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in the vaginal cross-sections from mock- (F), WNV-infected (G), WNV-infected + anti-CCL20 antibody (H) and WNV-infected + isotype control (I). (J) proportion of the area filled with CD11b+ cells in the vagina. Panels on the right display immunofluorescent staining for CD11c antigen (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in the vaginal cross-sections from mock- (K), WNV-infected (L), WNV-infected + anti-CCL20 antibody (M) and WNV-infected + isotype control (N). (O) proportion of the area filled with CD11c+ cells in the vagina. Also, immunofluorescent labeling of vaginal sections with mouse IgG1, rat IgG2a and Armenian hamster IgG (corresponding isotypes) elicited no specific staining for WNV-NS1, CD11b and CD11c markers (not shown). All images are representative of at least 4 mice per group. Bars indicate the mean ± SEM of 4 mice per group and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, compared to WNV-infected mice at D3 p.i.
Nuclei WNV-NS1

Mock-infected BALB/c

WNV-infected D3 p.i.

WNV-infected + α-CCL20 Ab

WNV-infected + Isotype Ab

E

% Total area

0 1 2 3

Mock  WNV  α-CCL20  Isotype

F

G

H

I

J

% Total area

0 1 2 3 4

Mock  WNV  α-CCL20  Isotype

K

L

M

N

O

% Total area

0 1 2 3 4 5

Mock  WNV  α-CCL20  Isotype
4.2.6.5 Co-localisation of CD11c and WNV-NS1 in the vaginal mucosa following CCL20 neutralisation

Figures 4.11A–D depict dual-labeling of WNV infection in the vaginal epithelium as well as the aggregation of CD11c+ DCs in higher magnification. As shown in section 4.2.6.2, there was more WNV-NS1 staining in anti-CCL20 group relative to the WNV-infected and isotype control groups, confirming greater epithelial infection in CCL20-neutralised mice. Furthermore, area of the vagina that occupied by CD11c+ cells diminished slightly, (but not significantly) in CCL20-treated mice (Figure 4.11E). As before, neither WNV infection nor accumulation of CD11c+ DCs were observed in the mock-infected controls.

Collectively, IF staining of vaginal sections in mock-, WNV-infected, CCL20-neutralised and isotype control BALB/c mice show that CCL20 significantly contributed to the initiation of immune response against WNV as neutralisation of this chemokine was associated with a significant increase in the number of epithelial cells that are infected with WNV. Although, CD11b expression was similar in all WNV-infected groups, the CD11c+ cell clusters in the genital mucosa were smaller. Therefore, this decrease in accumulation may be indicative of a failure of CCL20-neutralised mice to control and contain the infection in the vaginal epithelium.
Figure 4.11

Localisation of CD11c and WNV-NS1 in the WNV-infected BALB/c vagina following CCL20 neutralisation

WNV-infected BALB/c mice were injected i.v. with anti-CCL20 antibody or polyclonal goat IgG isotype control. Vaginae from mock- and WNV-infected mice were isolated on D3 p.i. and stained for WNV-NS1 and CD11c markers. (A) immunofluorescent staining for WNV-NS1 antigen (FITC; green) and CD11c surface marker (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in the vaginal cross-sections from mock- (A), WNV-infected (B), WNV-infected + anti-CCL20 antibody (C) and WNV-infected + isotype control (D). (E) proportion of the area filled with WNV-NS1⁺ (green) and CD11c (red) in the vagina. Additionally, immunofluorescent labeling of vaginal sections with mouse IgG1 and Armenian hamster IgG (corresponding isotypes) elicited no specific staining for WNV-NS1 and CD11c markers (not shown). All images are representative of at least 4 mice per time point. Bars indicate the mean ± SEM of 4 mice per group and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, compared to WNV-infected mice at D3 p.i.
Nuclei CD11c D0 p.i. Mock-infected

Nuclei WNV-NS1 CD11c D3 p.i. WNV-infected

Nuclei WNV-NS1 CD11c D3 p.i. WNV-infected + anti-CCL20

Nuclei WNV-NS1 CD11c D3 p.i. WNV-infected + isotype

E

% Total area

WNV CD11c

Mock WNV α-CCL20 Isotype

*
4.2.7 Cellular response of i.vag infected mice to CCL20 in the ILN

IF images presented above suggest a role for CCL20 in recruiting CD11c+ subset to the site of WNV infection. In addition, several groups have shown that myeloid DCs, particularly a CD11b+ subset that is located in the subepithelial dome of murine PP and B cells, can migrate towards the follicle-associated epithelium in response to a CCL20 gradient under steady state or infectious conditions (Iwasaki and Kelsall, 2000, Salazar-Gonzalez et al., 2006, Kallal et al., 2010). One aim of this study was to examine the effect of CCL20 neutralisation to the major myeloid and lymphoid populations in the ILN. Thus, flow cytometry was used to test the response of these subsets to the i.vag WNV infection following systemic neutralisation of CCL20. The total number of ILN cells increased significantly 3 days after i.vag WNV infection; however, other groups that received anti-CCL20 or the isotype control had even higher numbers of ILN cells at D3 p.i., compared to the mock-infected control (Figure 4.12A). This pattern was reflected in the number of total macrophages, inflammatory macrophages, CD11b+ and CD11b− DCs, as mice from the anti-CCL20 and isotype control groups had significantly increased numbers in comparison to WNV-infected mice at D3 p.i., (Figure 4.12B). Comparing WNV-infected mice with the anti-CCL20 and isotype control groups, no differences were observed in the number of cells in other subsets including BDC, TDC, CD8+ DC, pDC and neutrophils. B and T cell numbers were also elevated as a result of general ILN expansion. Similar to what was observed in some leukocyte subsets, CD4+ and CD8+ T cells but not B cells significantly increased in number when mice were injected with anti-CCL20 or isotype control antibodies (Figure 4.12C).
Similar to the numbers, the percentage of total macrophages and inflammatory macrophages increased in all groups relative to mock-infected controls, although CD11b− DCs, BDC and TDC percentages in the ILN decreased following WNV infection. Of note, the percentages of other leukocyte subsets including CD11b+ DC, CD8+ DC, pDC and neutrophils as well as lymphocytes (B cells, CD4+ and CD8+ T cells) were unchanged between WNV-infected groups (Figure 4.12D–E).

Taken together, these data show that several subsets of leukocytes such as macrophages, CD11b+ and CD11b− DCs as well as CD4+ and CD8+ T lymphocytes increased in the ILN of WNV-infected mice that received neutralising anti-CCL20 antibody.

As suggested earlier, these observations are in agreement with the ability of CCL20 to recruit the aforementioned subsets to the site of infection. Therefore, in the absence of CCL20, effector subsets that express CCR6 were not able to migrate to the vagina and remained in the draining LN. Interestingly, the same response was observed in WNV-infected mice that were injected with the isotype control, although why this was the case is not clear which suggests more investigation to provide more insights into the characteristics of isotype control antibody and it’s effect on the immune system.
Figure 4.12

Cellularity in the ILN of i.v. WNV-infected BALB/c mice following CCL20 neutralisation

WNV-infected BALB/c mice were injected i.v. with anti-CCL20 antibody or polyclonal goat IgG isotype control. ILN from mock- and WNV-infected mice were isolated on D3 p.i. and analysed for leukocyte and lymphocyte subsets in the ILN of mock- (black), WNV-infected (red), WNV-infected + anti-CCL20 antibody (blue) and WNV-infected + isotype control (green), by flow cytometry. (A) total numbers of leukocytes in the ILN, (B) numbers of macrophages, DCs and neutrophils (C) numbers of B and T cell subsets, (D) percentages of macrophages, DCs and neutrophils and (E) percentages of B and T cells at D3 p.i. Subset numbers and proportions are calculated from the total number of live ILN cells. Data is the mean ± SEM of 4 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 compared to mock-infected controls or D0 p.i.; #p ≤ 0.05 and ##p ≤ 0.01 relative to WNV-infected mice at D3 p.i.
4.2.8 CCL2 and CCL20 localisation in the WNV-infected vagina of C57BL/6 mouse

While CCL20 expression in the vagina and ILN of i.vag WNV-infected BALB/c mice reflected its inflammatory milieu, it was of interest to investigate the presence of this chemokine in the vagina of C57BL/6 mice. Additionally, i.vag WNV infection of C57BL/6 mice was associated with the mRNA upregulation of CCL2 and its corresponding receptor, CCR2 (but not the chemokine-receptor pair, CCL20-CCR6) (Figure 4.8 A–D). Therefore, to investigate the expression of CCL2 and CCL20 at the protein level, the vagina of mock- and WNV-infected C57BL/6 mice were harvested on days 0, 1, 3, 5 and 7 p.i. and the tissue stained. As Figure 4.13 shows, CCL20 protein expression was only detected at D5 p.i., with CCL20 staining occupying less than 0.2% of the total area in the WNV-infected vagina. Remarkably, CCL20 protein was typically located in the epithelium and only epithelial cells expressed this chemokine after infection.

CCL2 was upregulated slightly at D3 p.i., peaking at D5 p.i. with an ~5-fold increase relative to D0 p.i. Even though CCL2 protein decreased at D7 p.i., ~0.35% of the tissue still stained positively for CCL2 at this time-point. Surprisingly, most of the CCL2-expressing cells were distributed in the vaginal stroma. Interestingly, CCL2 expression was higher in those areas that were close to the epithelial sites of infection. There was a little expression of CCL2 by epithelial cells as well. As expected, there was no detectable expression of CCL2 and CCL20 in mock-infected control animals (Figure 4.13A–F).
4.2.9 Cellular response to WNV infection following CCL2 neutralisation

We previously showed that CCL2 and CCR2 mRNA were upregulated only in the vagina of C57BL/6 mice (but not in BALB/ mice), peaking at D5 post-WNV infection (Figure 4.8C–D). Furthermore, IF labelling of CCL2 in the vagina demonstrated that there was a significantly higher amount of this protein after infection that also peaked at D5 p.i. Other groups have shown that CCL2 recruits Ly6C\textsuperscript{hi} monocyte/macrophages as well as CD11b\textsuperscript{−} and CD11b\textsuperscript{+} DCs to site of infection in the skin, CNS and vaginal mucosa (Davison and King, 2011, Iijima et al., 2011, Lim et al., 2011). Therefore, to further investigate the role of this chemokine in vaginal infection with $3.6 \times 10^7$ PFU of WNV, CCL2 was neutralised systemically by administering anti-CCL2 antibody daily until and including D4 p.i. The isotype control (Armenian Hamster IgG) was administered to another group. Cells in the vagina, ILN and spleen of each group that received anti-CCL2 and isotype control were isolated on D5 p.i. and compared to mock- and WNV-infected C57BL/6 mice by flow cytometry.

4.2.9.1 Cellular response in the WNV-infected vagina following CCL2 neutralisation

CCL2 neutralisation elicited a dramatic decrease (~40%) in the total number of leukocytes that infiltrated the vagina at D5 p.i. (Figure 4.14A). Moreover, the infiltration of total macrophages, which comprised the majority of
Figure 4.13

Co-localisation of CCL2 and CCL20 protein expression in the WNV-infected C57BL/6 vagina

Immunofluorescent staining for CCL20 chemokine (FITC; green), CCL2 chemokine (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from mice infected i.vag with $3.6 \times 10^7$ PFU WNV at (A) D0, (B) D3 and (C) D5. D5 with higher magnification and (D) D7 p.i. (F) proportion of the area filled with CCL20+ (green) and CCL2+ (red) cells in the vagina. Immunofluorescent labeling of vaginal sections with Armenian hamster IgG and rat IgG1 (corresponding isotypes) elicited no specific staining for WNV-NS1 and CD11c markers (not shown). All images are representative of at least 4 mice per time point. Bars indicate the mean ± SEM of 4 mice per group and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *$p \leq 0.05$ and **$p \leq 0.01$ compared to mock-infected controls or D0 p.i. for CCL2; ##$p \leq 0.01$ relative to mock-infected controls or D0 p.i. for CCL20.
Nuclei CCL2 CCL20 WNV D0 p.i.

Nuclei CCL2 CCL20 WNV D3 p.i.

Nuclei CCL2 CCL20 WNV D5 p.i.

Nuclei CCL2 CCL20 WNV D7 p.i.

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**% Total area**

Day post WNV-infection

**Significance:**

- *: p < 0.05
- **: p < 0.01
total vaginal leukocytes, and neutrophil subsets were significantly impaired in CCL2-neutralised mice; however, no significant change was observed in the number of inflammatory macrophages, CD11b⁺ or CD11b⁻ DCs, BDC or TDC populations (Figure 4.14B).

Thus, the presence of CCR2⁺ subsets, as well as CD8⁺ DC and pDC, were analysed in the vagina. As Figure 4.14C illustrates, more than 95% of macrophages expressed CCR2 (from a comparison of Figure 4.14B) and the numbers of these cells was significantly reduced when CCL2 was neutralised. Similarly, almost all of the inflammatory macrophages expressed CCR2. However, there was no difference in the number of these cells present in the WNV, anti-CCL2 and isotype control vagina. Surprisingly, a macrophage subpopulation that expressed intermediate levels of Ly6C was reduced after CCL2 neutralisation, relative to WNV infected or isotype control mice. In addition, other DC subsets including CD8⁺ DC (defined as Ly6G⁻ B220⁻ CD3⁻ NK1.1⁻ MHC-II⁺ CD11c⁺ CD8⁺) and pDC (Ly6G⁻ CD3⁻ NK1.1⁻ B220⁺ CD11c⁺) subsets were in very low numbers and thus were not detectable in this model of infection at D5 p.i. (data not shown). Further analysis of neutrophils showed that CCR2 was not expressed on their surface, suggesting that CCL2 indirectly affects the recruitment of these cells (data not shown).

Within lymphocyte subsets, CD4⁺ T cells constituted the majority of lymphocytes in the vagina. This subset underwent a major reduction in response to CCL2 neutralisation. Although B cells and NKT cells only comprised a small population in the vagina, they also significantly decreased in number in CCL2-neutralised mice. Notably, while NK cells were present in large numbers in the
vagina (the second largest lymphocyte subset), CCL2 neutralisation did not affect their numbers. CD8⁺ T cells were present in very low numbers and did not change in the vagina of all WNV-infected groups at D5 p.i. (Figure 4.14D).

Despite a significant reduction in total macrophage numbers in the CCL2-neutralised mice, the percentage of this subset in the vagina remained unchanged compared to WNV-infected animals (Figure 4.14E). Additionally, percentages of inflammatory macrophages, CD11b⁺ and CD11b⁻ DCs, BDC, TDC, CCR2⁺ macrophages, CCR2⁺ inflammatory macrophages and Ly6C⁺ CCR2⁺ macrophages were also unchanged within WNV, anti-CCL2 and isotype control mice. The only reduction in percentages observed was in the neutrophil population which underwent a ~40% reduction in CCL2-neutralised mice relative to WNV-infected or isotype control mice.

The percentage of CD4⁺ T cells in the vagina also significantly decreased after anti-CCL2 antibody administration by ~25% compared to WNV-infected mice at D5 p.i.; however, no change was seen in the percentages of other lymphocytes including B cells, CD8⁺ T cells and NKT cells in the vagina (Figure 4.14E – G).

Taken together, these observations clearly show that CCL2 directs particular immune responses in the local infected area. It was interesting to note that CCR2⁺ macrophages were inhibited from infiltrating the vagina, although Ly6C⁹ inflammatory macrophage numbers were similar in all WNV-infected mice.

These results highlight the importance CCL2 not only in the initiation of
Figure 4.14

Cellular response in the WNV-infected vaginal mucosa following CCL2 chemokine neutralisation

C57BL/6 mice infected with $3.6 \times 10^7$ PFU WNV were injected i.v. with anti-CCL2 antibody or rat IgG1 isotype control, as described in section 2.2.8. Vaginae from mock and WNV-infected mice were isolated on D5 p.i. and analysed for leukocyte subsets in the vagina of mock- (black), WNV-infected (blue), WNV-infected + anti-CCL2 antibody (red) and WNV-infected + isotype control (green), by flow cytometry.

(A) total numbers of CD45$^+$ leukocytes in the vagina, (B) numbers of macrophages (Mϕ), DCs and neutrophils (C) numbers of CCR2$^+$ macrophages, (D) numbers of lymphocyte subsets, (E) percentages of macrophages, DCs and neutrophils, (F) percentages of CCR2$^+$ macrophages and (G) percentages of lymphocytes at D5 p.i. Subset numbers and proportions are calculated the total number of CD45$^+$ live cells in the vagina. Data is the mean ± SEM of 3 – 6 mice per group, and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; $^*p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ compared to mock-infected controls or D0 p.i.; $^#p \leq 0.05$, $^{##}p \leq 0.01$ and $^{###}p \leq 0.001$ in comparing WNV-infected or isotype controls to CCL2-neutralised mice at D5 p.i.
immune response but also in priming adaptive immunity following mucosal infection.

**4.2.9.2 Cellular response in the ILN following CCL2 neutralisation in WNV-infected mice**

To further define the immune response in WNV-infected, anti-CCL2 and isotype control groups, the kinetics of leukocyte subset changes in the ILN were similarly analysed. Total cell numbers in the WNV-infected groups expanded as a general response to the vaginal infection, although a significant reduction was observed when CCL2 was neutralised following infection, relative to WNV-infected or isotype control mice (Figure 4.15A).

No changes in the total number of macrophages, inflammatory macrophages or neutrophils were observed in the ILN of all WNV-infected groups at D5 p.i. In contrast to vagina, CCL2 neutralisation affected CD11b+ and CD11b– DCs, BDC and TDC subsets, as the numbers of these cells decreased significantly in the ILN. From these observations, it was clear that in the ILN, CCL2 neutralisation affected DCs more than macrophages. Even though CCR2 was expressed on ~30 and 90% of total and inflammatory macrophages, respectively, the cell numbers did not change between any of the groups of mice that were infected with WNV. Similar to in the vagina, Ly6C+ CCR2+ macrophages decreased when CCL2 was neutralised. The numbers of CCR2+ F4/80+ macrophages (defined as Ly6G– B220– CD3– NK1.1– CD11b+ CD11c– CCR2+ F4/80+) also remained unchanged in the anti-CCL2 mice, compared to
WNV-infected or isotype control animals. Interestingly, however, numbers of F4/80⁺ DCs (Ly6G⁻ B220⁻ CD3⁻ NK1.1⁻ CD11c⁺ F4/80⁺) as well as CD8⁺ and plasmacytoid DCs were significantly reduced following CCL2 neutralisation (Figure 4.15B–C).

As Figure 4.15D shows, almost all of the lymphocyte populations examined significantly decreased in the CCL2-neutralised mice relative to the control animals. When enumerated, the greatest decrease was observed in the B cell population, which was the major lymphocyte subset in the ILN. In addition, CD4⁺ and CD8⁺ T cells as well as NK and NKT cells in CCL2-neutralised mice, underwent a significant reduction in their numbers, compared to WNV-infected or isotype control groups.

Despite the remarkable effect of CCL2 on the recruitment of several leukocyte subsets in the ILN, the percentage of these populations in the ILN did not change between the WNV-infected, anti-CCL2 and isotype control groups (Figure 4.15E–G).

Collectively, although lymphocyte subsets were similarly reduced in the ILN in CCL2-neutralised mice relative to the control groups, macrophages infiltrated the ILN in the same number in all WNV-infected groups. Strikingly, DC subsets appeared to be the most affected leukocyte subtype observed as their numbers significantly decreased following CCL2 blockade.
**4.2.9.3 Cellular response in the spleen**

Apart from the role of spleen in removing aged erythrocytes, recycling iron and supplying erythrocytes after hemorrhagic shock, another major function of this organ is to elicit and regulate immune responses against infection. The spleen is a reservoir for undifferentiated monocytes that give rise to effector APCs in infected tissue (Swirski et al., 2009, Mebius and Kraal, 2005). Moreover, different subpopulations of macrophages and DCs, which are differentiated based on CD11b, CD8α, CD4 and CD205 expression, have been shown to be present in the spleen (Vremec et al., 2000, Kamath et al., 2000). In order to evaluate if the spleen plays a role in i.vag WNV infection in the presence or absence of CCL2, the spleen of mock- and WNV-infected mice (WNV, anti-CCL2 and isotype control) were isolated and analysed for the major leukocyte populations at D5 p.i.

As described above, CCL2 neutralisation was associated with dramatic reductions in the number of cells in several leukocyte populations in the vagina and ILN (Figure 4.14A–D and 4.15A–D). However, no significant change was observed in either the total spleen cell numbers (Figure 4.16A) or the number of cells within each leukocyte subsets at D5 p.i. (Figure 4.16B–D). Furthermore, even though the number of neutrophils and B cells decreased and increased, respectively, following i.vag WNV infection, this fluctuation was similar in all the WNV, anti-CCL2 and isotype control groups (i.e., no significant effect of CCL2 neutralisation observed).

CCL2 blockade also had no effect on the subset percentages in mock- and WNV-infected groups at D5 p.i., with the exception of neutrophils and B cells (Figure 4.16E–G). Together, these observations show that CCL2 only affects
Cellularity in the ILN of i.vag WNV-infected C57BL/6 mice following CCL2 chemokine neutralisation

WNV-infected C57BL/6 mice were injected i.v. with anti-CCL2 antibody or rat IgG1 isotype control, as described in section 2.2.8. ILN from mock- and WNV-infected mice were isolated on D5 p.i. and analysed for leukocyte subsets in the ILN of mock- (black), WNV-infected (blue), WNV-infected + anti-CCL2 antibody (red) and WNV-infected + isotype control (green), by flow cytometry.

(A) total numbers of leukocytes in the ILN, (B) numbers of macrophage (Mφ), DC and neutrophil subsets, (C) numbers of CCR2+ macrophages and other DC subsets, (D) numbers of lymphocytes, (E) percentages of macrophage, DC and neutrophil subsets subsets, (F) percentages of CCR2+ macrophages and other DCs and (G) percentages of lymphocytes at D5 p.i. Subset numbers and proportions are calculated from the total number of ILN CD45+ live cells. Data is the mean ± SEM of 3 – 6 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; ***p≤0.001 compared to mock-infected controls or D0 p.i.; #p≤0.05 and ###p≤0.001 in comparing WNV-infected or isotype controls to CCL2-neutralised mice at D5 p.i.
**Figure 4.16**

*Cellularity in the spleen of i.vag WNV-infected C57BL/6 mice following CCL2 chemokine neutralisation*

WNV-infected C57BL/6 mice were injected i.v. with anti-CCL2 antibody or rat IgG1 isotype control, as described in section 2.2.8. Spleens from mock- and WNV-infected mice were isolated on D5 p.i. and analysed for leukocyte subsets in the spleen of mock- (black), WNV-infected (blue), WNV-infected + anti-CCL2 antibody (red) and WNV-infected + isotype control (green), by flow cytometry.

(A) total numbers of leukocytes in the spleen, (B) numbers of macrophage (Mϕ), DC and neutrophil subsets, (C) numbers of CCR2⁺ macrophages and other DC subsets, (D) numbers of lymphocytes, (E) percentages of macrophage, DC and neutrophil subsets, (F) percentages of CCR2⁺ macrophages and other DCs and (G) percentages of lymphocytes at D5 p.i. Subset numbers and proportions are calculated from the total number of CD45⁺ live spleen cells. Data is the mean ± SEM of 3 – 6 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; **p ≤ 0.01 and ***p ≤ 0.001 compared to mock-infected controls or D0 p.i.
neutrophils and B cells in the spleen following i.vag WNV infection.

4.2.10 Cellular response to WNV infection following polystyrene beads administration

The results described above indicate that i.vag WNV infection induces the recruitment of inflammatory macrophages, DCs and lymphocytes not only to the vaginal mucosa, but also to its draining LN. This cell trafficking was impaired when CCL2 was neutralised. In addition, several groups, including ours, have shown that inflammatory monocyte subsets, specifically Ly6C<sup>hi</sup> macrophages, egress from bone marrow and traffic to the site of viral infection in a CCR2-dependent fashion (Shi and Pamer, 2011, Getts et al., 2008, Iijima et al., 2011, Serbina and Pamer, 2006, Davison and King, 2011). Indeed, the systemic administration of fluorescent polystyrene beads (a technique employed in numerous studies) can be used to label inflammatory monocyte/macrophage populations and has provided important insights into the migratory behaviour of these cells in steady-state, inflammatory and infectious conditions (Tacke and Randolph, 2006, Ginhoux et al., 2006, Jakubzick et al., 2008, Tacke et al., 2006, Randolph et al., 1999).

Therefore, to track monocyte/macrophage migration in vivo, mock- and WNV-infected mice were injected with 5.8 x 10<sup>10</sup> fluorescent polystyrene beads in 250 µL PBS on a daily basis starting from D1 until D4 p.i. Furthermore, to further investigate the combinatory effect of beads and CCL2, a group of WNV-infected mice were injected i.v. (daily from D1 to D4 p.i.) with beads and anti-
CCL2 antibody at the same time. Then, the vagina, ILN and spleen of mock- and WNV-infected groups including WNV alone, WNV-infected animals that received beads and WNV-infected mice that were injected with both beads and CCL2 blocking antibody, were isolated on D5 p.i. and analysed by flow cytometry.

4.2.10.1 Cellular response in the WNV-infected vagina

Polystyrene beads administration was associated with a significant reduction in the total number of vaginal leukocytes at D5 p.i. Interestingly, this reduction was of a similar magnitude as seen in the vaginae of WNV-infected mice that received both beads and anti-CCL2 antibody. Mock-infected animals and mock-infected animals that received polystyrene beads did not show any difference in the kinetics of all the leukocyte subpopulations examined. Therefore, only the mock-infected control group is shown in the graphs for clarity (Figure 4.17A).

The number of total and inflammatory macrophages dropped by ~50% when mice were injected with beads or beads/anti-CCL2 relative to WNV-infected mice. When enumerated, neutrophils were also shown to decrease but to a lesser extent than macrophages. Enumeration of DCs demonstrated that there were similar numbers of CD11b+, CD11b−, BDC and TDC cells in the WNV, beads and beads/anti-CCL2 groups (Figure 4.17B).

Further analysis indicated that CCR2+ inflammatory cells were the populations that were most affected by beads administration. As mentioned
previously, almost all macrophages express CCR2 on their surface. Accordingly, numbers of CCR2\(^+\) macrophages, including Ly6C\(^{hi}\) and Ly6C\(^+\) subpopulations, decreased significantly when compared to WNV-infected mice. Again, there were similar numbers of CCR2\(^+\) macrophages in the beads and beads/anti-CCL2 groups of mice (Figure 4.17C). In addition, similar to the results for CCL2 neutralisation in the previous section, CD8\(^+\) DCs and pDCs were present in low numbers in the vagina and did not change following beads administration (data not shown).

While beads administration decreased the number of macrophages in the vagina, no statistically significant change was observed in the number of cells in various lymphocyte subsets (i.e., B cells, CD4\(^+\) T cells, CD8\(^+\) T cells, NK cells or NKT cells in the vagina at D5 p.i. (Figure 4.17D). Furthermore, in contrast to anti-CCL2 antibody alone (Figure 4.14D), the combination of beads and anti-CCL2 antibody did not result in a significant reduction of CD4\(^+\) T cells (Figure 4.17D). Despite a significant reduction in the number of CCR2\(^+\) macrophages, the relative percentage of these populations, as well as other leukocyte subsets were comparable between all groups of WNV-infected mice at D5 p.i. (Figure 4.17E–G), unlike changes in subset percentages seen in mice treated with anti-CCL2 antibody alone (Figure 4.14E–G). Strikingly, no beads\(^+\) cells were detectable above background in the vagina of any group.

Taken together, injection of polystyrene beads significantly reduced the number of macrophages, particularly CCR2\(^+\) macrophages, and to a lesser extent, neutrophil numbers. The relative percentages of these subpopulations in the vagina were similar among all groups of WNV-infected mice at D5 p.i.
Figure 4.17

Cellular response in the WNV-infected vaginal mucosa following beads administration

C57BL/6 mice infected with 3.6 x 10^7 PFU WNV were injected i.v. with beads or beads + anti-CCL2 antibody, as described in section 2.2.8. Vaginae from mock- and WNV-infected mice were isolated on D5 p.i. and analysed for leukocyte subsets in the vagina of mock- (black), WNV-infected (blue), WNV-infected + beads (red) and WNV infected + beads + anti-CCL2 antibody (green), by flow cytometry.

(A) total numbers of CD45^+ leukocytes in the vagina, (B) numbers of macrophage (Mφ), DC and neutrophil subsets, (C) numbers of CCR2^+ macrophages, (D) numbers of lymphocytes, (E) proportion of macrophage, DC and neutrophil subsets, (F) percentages of CCR2^+ macrophages and (G) percentages of lymphocytes at D5 p.i. Subset numbers and proportions are calculate from the total number of CD45^+ live cells in the vagina. Data is the mean ± SEM of 3 – 6 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; ***p≤0.001 compared to mock-infected controls or D0 p.i.; #p≤0.05, ##p≤0.01 and ###p≤0.001 relative to WNV-infected controls at D5 p.i.
4.2.10.2 Cellular response in the ILN after polystyrene beads administration

As expected, cell numbers in the ILN significantly increased following i.vag WNV infection at D5 p.i., although ILN cell numbers in mice that received beads were ~30% lower. As before, administration of polystyrene beads had no effect on mock-infected controls (Figure 4.18A).

Treatment of WNV-infected mice with beads or a combination of beads and anti-CCL2 antibody induced different kinetics with respect to the number of cells in various leukocyte subsets in the ILN, compared to the vagina. The number of CD11b+ DCs, BDC and CD8+ DCs were reduced in the beads and beads/anti-CCL2 groups of mice, while the numbers of macrophage, including CCR2+ populations, as well as the other DC subsets and neutrophils, were comparable in the ILN between each of the WNV-infected groups (Figure 4.18B–C).

Surprisingly, beads injection was also associated with significant reduction in the number of lymphocytes. B cells were the most affected subset, with a decrease of ~30%, followed by CD4+ and CD8+ T cells. Even though NK and NKT cells comprised only a small number of lymphocytes, cell numbers in these subsets still decreased as a result of beads administration. Of note, injection of beads and anti-CCL2 together in WNV-infected mice did not have any effect on the number of cells in the ILN, as similar total cell numbers were observed in this group and WNV-infected mice (Figure 4.18D).

Similar to the vagina, the relative percentages of various leukocyte subsets did not differ significantly between WNV-infected controls and WNV-infected mice that received beads or a combination of beads and CCL2 neutralising
antibody (Figure 4.18E–G).

Therefore, in contrast to the vagina, only CD11b− DC, BDC and CD8+ DC numbers reduced as a result of beads or beads/anti-CCL2 administration. In addition, a decrease in cell numbers in all lymphocyte subsets examined were observed when mice were injected with polystyrene beads. Moreover, the percentages of each leukocyte subset in the ILN remained unchanged between WNV-infected, beads-injected and beads/anti-CCL2 injected-mice at D5 p.i. As for the vagina, beads+ cells were not detectable above the background noise in the ILN.

4.2.10.3 Cellular response in the spleen

Previous results from this chapter suggested that several subsets of macrophages, DCs and lymphocytes were prevented from being recruited to the vagina and ILN in response to WNV infection following beads administration. In addition, attempts to detect bead+ cells in the vagina and ILN were unsuccessful. As described earlier, innate and adaptive immune cells also reside in the spleen, and are therefore another reservoir for such subsets (Mebius and Kraal, 2005). Furthermore, studies by Tacke and colleagues as well as studies in our laboratory (Terry, 2012) have shown that a large number of fluorescent beads injected into the circulation are deposited in the spleen (Tacke et al., 2006). Therefore, to investigate the fate of leukocytes that took up beads in the i.vag WNV infection model, we flow cytometrically analysed different splenic subsets.

Examination of splenic cells demonstrated that the changes in percentage
**Figure 4.18**

*Cellularity in the ILN of i.vag WNV-infected C57BL/6 mice following beads injection*

WNV-infected C57BL/6 mice were injected i.v. with beads and beads + anti-CCL2 antibody, as described in section 2.2.8. ILN from mock- and WNV-infected mice were isolated on D5 p.i. and analysed for leukocyte subsets in the ILN of mock- (black), WNV-infected (blue), WNV-infected + beads (red) and WNV-infected + beads + anti-CCL2 antibody (green), by flow cytometry.

(A) total numbers of leukocytes in the ILN, (B) numbers of macrophage (Mϕ), DC and neutrophil subsets, (C) numbers of CCR2\(^+\) macrophages and other DC subsets, (D) numbers of lymphocytes, (E) percentages of macrophage, DC and neutrophil subsets, (F) percentages of CCR2\(^+\) macrophages and other DCs and (G) percentages of lymphocytes at D5 p.i. Subset numbers and proportions are calculated from the total number of ILN CD45\(^+\) live cells. Data is the mean ± SEM of 3 – 6 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; ***\(p \leq 0.001\) compared to mock-infected controls or D0 p.i.; #\(p \leq 0.05\) and ###\(p \leq 0.001\) relative to WNV-infected controls at D5 p.i.
and absolute numbers of total spleen cells after infection were insignificant. Moreover, excluding a few differences between mock- and WNV-infected groups, no difference was observed in the number or proportion of leukocyte populations in the spleen of mock-infected, WNV-infected or WNV-infected mice treated with beads or beads/anti-CCL2 at D5 p.i., (Figure 4.19A–G).

Next, the distribution of the beads in the spleen was determined. Bead⁺ cells appeared to comprise ~5% of total spleen cells in mock- and WNV-infected mice at D5 p.i. As there was no significant difference in the number of total spleen cells between the mock-infected control and the mock-infected mice that received beads, only the mock-infected control group is shown in the graphs for clarity (Figure 4.19A).

Strikingly, B cells constituted ~65% of all bead⁺ cells in the spleen of both mock- and WNV-infected mice (Figure 4.20A). Some CD4⁺ T cells also took up beads, as did CD8⁺ T cells although these were a very small population. Further analysis of macrophage and DC subsets revealed that almost all of the investigated populations had phagocytosed beads (Figure 4.20B). Although there was a trend towards elevated numbers of bead⁺ subsets in the spleen of WNV-infected mice compared to mock-infected animals, the only significant difference in the infected group of mice was confined to the neutrophil population (Figure 4.20A–B).

Overall, these results demonstrate that beads in the circulation are either attached to or taken up by leukocytes, mainly B cells and to a lesser extent monocytes/macrophages and CD4⁺ T cells. Since no beads⁺ cells were found in the vagina or ILN, it seems likely that these subsets may instead accumulate in the
**Figure 4.19**

**Cellularity in the spleen of i.vag WNV-infected C57BL/6 mice following beads injection**

WNV-infected C57BL/6 mice were injected i.v. with beads and beads + anti-CCL2 antibody, as described in section 2.2.8. Spleens from mock- and WNV-infected mice were isolated on D5 p.i. and analysed for leukocyte subsets in the spleen of mock- (black), WNV-infected (blue), WNV-infected + beads (red) and WNV-infected + beads + anti-CCL2 antibody (green), by flow cytometry.

(A) total numbers of leukocytes in the spleen, (B) numbers of macrophage (Mϕ), DC and neutrophil subsets, (C) numbers of CCR2+ macrophages and other DC subsets, (D) numbers of lymphocytes, (E) percentages of macrophage, DC and neutrophil subsets, (F) percentages of CCR2+ macrophages and other DCs and (G) percentages of lymphocytes at D5 p.i. Subset numbers and proportions are calculated from the total number of live CD45+ spleen cells. Data is the mean ± SEM of 3 – 6 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to mock-infected controls or D0 p.i.
Mock- and WNV-infected C57BL/6 mice were injected i.v. with beads, as described in section 2.2.8. Spleens were then isolated on D5 p.i. and analysed for beads’ leukocyte and lymphocyte subsets in the spleen of mock- (black), WNV-infected (red) mice, by flow cytometric assay.

(A) total numbers of beads’ leukocytes, as well as B cells, CD4+ and CD8+ T cells and (B) numbers of macrophages, DCs and neutrophils in the spleen. Subset numbers and proportions are calculated from the total number of spleen CD45+ live cells. Data is the mean ± SEM of 3 – 6 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; **p≤0.01 compared to mock-infected controls or D0 p.i.
spleen. However, the mechanisms that mediate this effect are unknown. Furthermore, whether this reduction in migration to the vagina and ILN results in poorer control of virus locally is a question that remains to be investigated.

4.3 Discussion

Very little is known about immune responses to viral infections of the vagina. Despite extensive research exploiting STI viruses such as SIV (simian immunodeficiency virus), HIV and HSV, there is a relative dearth of data on what mechanisms are involved in the initiation and priming of effective immunity to the vaginal infection. As described in Chapter 3, we discussed innate and adaptive immune responses in the genital mucosa and draining LN of C57BL/6 mice using two different but complementary neurotropic viruses, WNV and HSV-2. Our results confirmed that, in C57BL/6 mice, i.vag HSV-2 infection was associated with dramatic morbidity and mortality, whereas WNV proved to be non-lethal. Despite these contrasting outcomes, i.vag WNV and HSV-2 infection in C57BL/6 mice had comparatively similar leukocyte kinetics not only at the local site of infection but also in the ILN. Moreover, our results were in accordance with previous studies that have been performed in our laboratory showing that vaginal infection of C57BL/6 mice with either virus elicited Th1-type immunity, which was characterised by the induction of IFN-γ, IL-12 and TNF (Yeung, 2012, Watanabe et al., 2004, Mosmann and Coffman, 1989). On the other hand, BALB/c mice, which are a prototypical Th2-type strain (Brenner et al., 1994, Halford et al., 2004, Waris et al., 1996), are more sensitive to i.vag WNV infection as Burke and colleagues reported that i.vag inoculation with $3.6 \times 10^5$
PFU WNV resulted in 12% mortality among BALB/c mice, whereas, all C57BL/6 animals survived even when challenged with 100-fold more virus (Burke et al., 2004). Therefore, due to the distinctive susceptibility of these two mouse strains, we aimed to investigate the observed differential immune response in C57BL/6 and BALB/c mice by examining leukocyte infiltration to the vagina and ILN as well as cytokine and chemokine expression in the local microenvironment.

Thus, comparison of cytokine and chemokine production together with leukocyte recruitment in these two different strains is of interest. Furthermore, identifying common anti-viral mechanisms in the vagina is important to understand general anti-viral defences against common sexually transmitted viruses.

The WNV infection course in BALB/c mice was similar to C57BL/6 animals; however, WNV RNA was 3-fold higher at its peak at D5 p.i. in BALB/c mice (Figure 4.1 and 3.4). Moreover, as mentioned above, Burke showed that i.vag WNV infection resulted in 12% mortality among BALB/c mice, confirming higher susceptibility of BALB/c mice to infection. Intravaginal WNV infection elicited significant infection of epithelial cells in the BALB/c mice during the time course. Virus was strictly limited to the epithelium and was substantially eradicated from the vaginal mucosa by D7 p.i. (Figure 4.2) (Burke, 2005, Burke et al., 2004, Yeung, 2012). Taken together, despite the increased susceptibility of this strain, i.vag. WNV inoculation in BALB/c mice was characterised by a mild and self-limiting disease course. Importantly, the epithelial layer remains intact, similar to C57BL/6 mice; however, infection in the BALB/c vagina is much more extensive.
DCs play a pivotal role in the initiation of immune responses against viral vaginal infections. These cells have been shown to mature upon exposure to viruses or immunostimulants such as TLR9 agonist, CpG oligodeoxynucleotides (ODN) (Zhao et al., 2003, Iijima et al., 2008b, Shen and Iwasaki, 2006). This process, which is associated with functional changes including increased expression of the co-stimulatory molecules CD80 and CD86 and of MHC-I and II molecules, changes them from stationary cells with poor T cell-stimulating ability into activated cells that migrate to the draining LN where they interact with T cells to initiate adaptive immune responses (Steinman et al., 1999, Banchereau et al., 2000, Banchereau and Steinman, 1998, Iwasaki, 2003).

Together with others (Iwasaki, 2003), we showed that CD11c+ DCs accumulated under the vaginal epithelium and formed clusters under the WNV-infected epithelial cells (Figure 4.2). Iwasaki showed the same clusters of CD11c+ cells in vaginal HSV-2 infection. Other studies have shown these cell clusters were often also MHC-II+ (Burke et al., 2004, Sajic et al., 2005).

CD11b+ and F4/80+ DCs/macrophages were present in small numbers in mock-infected mice but were recruited in large numbers to the vaginal stroma in response to viral infection. Additionally, dramatic increases in the number of CD11b+ and F4/80+ cells in the vagina were seen, peaking at D5 p.i. IF staining showed that on D7 p.i., when virus was cleared from the vagina, aggregation of these subsets below the epithelium were still evident (Figure 4.2–4.4). Several other groups have also demonstrated that the infiltrating vaginal DCs express CD11b and F4/80 (Iwasaki, 2003, Zhao et al., 2003, Sajic et al., 2005, Iijima et al., 2007). A more recent study indicated that inflammatory monocytes gave rise
to CD11b\(^+\) and CD11b\(^-\) DCs in the vaginal mucosa following HSV-2 infection (Iijima et al., 2011). We also saw both CD11b\(^+\) and F4/80\(^+\) DCs in the vagina following i.vag WNV infection; however, we saw poor co-labeling for CD11c, CD11b and F4/80 in the vagina. This could be due to inability to amplify the antibody labelling of both markers simultaneously. Overall, infiltration of CD11c\(^+\), CD11b\(^+\) and F4/80\(^+\) cells to the WNV-infected vagina in the BALB/c mice was closely comparable to that of observed in i.vag WNV- and HSV-2-infected C57BL/6 mice described in Chapter 3 of this study (Figure 3.5–3.7 and 4.2–4.4).

The ILN is considered to be the primary site for the initiation of the genital immune response (Johnston et al., 2000, King et al., 1998, Kwant-Mitchell et al., 2009). The ILN of WNV-infected BALB/c mice had a similar immune response to the ILN of C57BL/6 animals, confirming that viral vaginal infection leads to general expansion in the ILN, Therefore, the number of cells in several leukocytes subsets increased by D1 and D3 p.i. compared to mock-infected BALB/c control mice (Figure 3.12–3.14 and 4.5) (Burke, 2005, Johnston et al., 2000, King et al., 1998).

However, there were notable distinctions between BALB/c and C57BL/6 mice. Most importantly, CD4\(^+\) T cells constituted the majority of the lymphocytes in BALB/c mice whereas B cells were the biggest population in the ILN of C57BL/6 animals, perhaps reflecting their differential genetic background which favour Th1- or Th2-type immune responses and differences in cytokine/chemokine secretion (Watanabe et al., 2004, López et al., 2001).

Therefore, these observed differences and similarities prompted us to
examine cytokine and chemokine expression following i.vag WNV infection in these two genetically distinct mouse strains.

IFN-γ is one of the main pro-inflammatory cytokines that is mainly secreted by NK cells and CD4+ and CD8+ T cells. It is involved in the activation of macrophages, promotion of cell-mediated responses and control of microbial and viral infections (Schroder et al., 2004, Abbas and Lichtman, 2004). As expected, mRNA expression of IFN-γ, which traditionally considered to be a Th1 cytokine, increased significantly in C57BL/6 mice but not in BALB/c animals (Watanabe et al., 2004).

TNF is a pro-inflammatory cytokine that is expressed predominately by macrophages, T cells, NK cells and granulocytes, although virtually all kinds of cells can do so (Ellerin et al., 2003, Taylor, 2001, Keystone and Ware, 2010, Herbein and O'Brien, 2000). It has been shown that the expression of TNF following viral infection is associated with enhanced chemokine production by lung mucosal epithelial cells (Veckman et al., 2006). Furthermore, TNF can inhibit IFN-γ-priming of macrophage IL-12 production which enhances Th1-type immune responses (Hodge-Dufour et al., 1998) Thus, in Th1 responses, TNF may act as an additional macrophage-activating factor, although, in mixed Th1 + Th2 or Th0 responses, it may cause tissue damage and limit the extent and duration of the inflammatory response by blocking or down-regulating Th1 cytokine production (Hernandez-Pando and Rook, 1994).

Significant expression of TNF in the vagina of BALB/c mice was observed. Considering the impaired production of IFN-γ in these mice following WNV infection, this may reflect the Th1 inhibitory effects of TNF in this
scenario.

In addition, both mouse strains upregulated inflammatory enzymes such as IDO and NOS2. As described in Chapter 1, IDO is a cytosolic haem enzyme that catalyses the conversion of the least abundant essential amino acid L-Trp to N-formylkynurenine. Once induced at the site of infection, IDO depletes L-Trp from the microenvironment. Thus, it has a role in inhibiting viral growth by limiting the availability of this amino acid (King and Thomas, 2007, Yeung et al., 2012). NOS2, on the other hand, produces large amounts of nitric oxide (NO) by catabolising L-arginine. NO is a gaseous free radical that mediates vital physiological functions, including host defence.

Both IFN-γ and TNF have the ability to induce IDO and NOS2 expression. Moreover, it has been shown that upregulation of one enzyme results in the downregulation of the other (Thomas and Stocker, 1999, Benencia et al., 2003, Alberati-Giani et al., 1997, King and Thomas, 2007, Yeung et al., 2012, Karupiah et al., 1993, Lane et al., 1997). IDO expression was induced only in C57BL/6 mice, whereas NOS2 was upregulated in BALB/c animals, demonstrating that WNV infection elicits differential inflammatory responses. These results also support the fact that IDO and NOS2 can inhibit the expression of one another (Figure 4.6).

As described in section 4.2.5, there is immense overlap in the chemokine-mediated infiltration of effector cells in response to CCL3 and CCL5. For instance, each of these chemokines are involved in the recruitment of NK cells, T cells, B cells and myeloid cells. Thus, even though the role of these chemokines in i.vag infection was not widely investigated in this chapter, it can be assumed that
these chemokines responsible to at least some degree for infiltration of granulocytes, macrophages, DCs, B cells, T cells and NK cells to the site of infection (Appay and Rowland-Jones, 2001, Levy, 2009, Maurer and von Stebut, 2004, Peretti et al., 2005).

CCL3 and CCL5 significantly increased with very similar kinetics in both strains over the 7-day WNV time course, although CCL5 expression in BALB/c mice remained elevated at D7 p.i. Considering that CCL5 chemokines are involved in the recruitment of T cells to the site of infection, it is not clear why more extensive vaginal infection occurs in the BALB/c strain rather than the C57BL/6 animals (Figure 4.7).

T cell activation by DCs is critical for the initiation of adaptive immune responses and protection against pathogens. It has been suggested that the CCR6-CCL20 and CCR7-CCL19 and -CCL21, receptor-ligand pairs have the ability to differentially regulate the migration of immature and mature DCs, respectively (Comerford et al., 2010, Rescigno, 2006, Förster et al., 2008). The local expression of CCL20 correlates with the infiltration of DCs in the intestine and PP (Anjuere et al., 1999, Nishi et al., 2003, Iwasaki and Kelsall, 2000, Rescigno, 2006, Salazar-Gonzalez et al., 2006). On the other hand, the upregulation of CCR7 by maturing DCs is essential for them to migrate away from the site of mucosal inflammation/infection and towards CCL19 and CCL21 that are expressed in the draining LN (Sozzani et al., 1998, Saeki et al., 1999).

The differential mRNA expression of CCR7 and its ligands, CCL19 and CCL21, may suggest that each mouse strain uses different pathways to recruit and activate immature DCs in the vagina following WNV infection. Expression of
CCR7 mRNA was over 2-fold higher in BALB/c mice at D5 and D7 p.i. than in C57BL/6 mice. Also, CCL19 and CCL21 were significantly increased in BALB/c mice at D1 and D5 p.i., respectively, while, C57BL/6 mice had higher upregulation (over 2-fold) only in CCL21 at D3 p.i. (Figure 4.7).

The expression of CCR6 and CCL20 mRNA did not change during the time course in C57BL/6. Surprisingly, relative expression of CCR6 within the BALB/c vaginal mucosa decreased significantly at D1 p.i. and gradually went back to the levels in mock-infected control mice by D7 p.i. This may indicate the downregulation and/or emigration of CCR6+ immature DC subsequent to local virus infection. In contrast, CCL20, increased significantly and peaked ~10-fold higher than in mock-infected mice at D5 p.i. and in itself may be indicative of the increased local infection in the BALB/c mouse (Figure 4.8).

Although the expression of the chemokine-receptor pairs were not extensively examined in the ILN, the expression profile of these in the vagina suggests that immature DCs becoming less responsive to CCL20 upon maturation due to the downregulation of CCR6 could be directed towards the ILN following CCR7 upregulation. It is important to consider, however, the local aggregation of lymphatic tissue in the vagina may also subserve this purpose (Gillgrass et al., 2005b).

Several studies have found that in CCR7-deficient mice, DCs have impaired capacities to migrate to the draining LN following mucosal lung or intestine Ag stimulation/infection (Song et al., 2009, Hintzen et al., 2006, Jang et al., 2006, Salazar-Gonzalez et al., 2006).
Collectively, this evidence support the idea that differential expression of inflammatory cytokines and chemokines results in distinct innate and adaptive immune responses that are elicited at the site of infection and the draining LN.

As described earlier, inflammatory monocytes, which are identified by the expression of CD11b, Ly6C and high levels of CCR2, are rapidly recruited to sites of infection in response to CCL2 chemotactic gradients (Geissmann et al., 2003, Iijima et al., 2011). Furthermore, several reports have demonstrated the importance of this chemokine-receptor pair in host defence, as mice lacking CCR2 have enhanced susceptibility to many pathogens (Sato et al., 2000, Iijima et al., 2011, Lim et al., 2011, Tsou et al., 2007). Interestingly, Sato and co-workers also provided evidence showing that CCR2-deficient mice have impaired DC subsets that would normally stimulate Th1 responses, leading to a striking Th2-biased non-healing phenotype in response to *Leishmania Major* (Sato et al., 2000).

Remarkably, neither CCR2 nor CCL2 expression changed during i.vag WNV infection in BALB/c mice, while, C57BL/6 mice had significant increases in the levels of CCR2 (~2-fold) and CCL2 (~35-fold) mRNA expression on D5 p.i. (Figure 4.8).

Moreover, CCL2 and CCL20 protein expression in the mock- and WNV-infected C57BL/6 vaginal sections was also investigated. Mock-infected control mice did not express either CCL2 or CCL20 protein, however, there was a significant increase in the vaginal areas that stained positively for CCL2 on D5 p.i. (~0.6%), and to a much lesser extent, CCL20 was upregulated at this time point (~0.2%). This observation confirmed the differential expression of these
chemokines at mRNA and protein level in the WNV-infected vagina (Figure 4.8 and 4.13).

Differential expression of CCL2 and CCL20 in C57BL/6 and BALB/c mice prompted us to study the effect of these chemokines in the vaginal mucosa following WNV infection. Firstly, CCL20 neutralising Ab was used to block the recruitment of CCR6+ DCs in BALB/c mice.

Relative expression of WNV, IFN-γ, CCL20, CCL19, CCL21 or CCL2 mRNA did not change significantly following CCL20 neutralisation, although a decreasing trend was seen for IFN-γ, CCL20, CCL19 and CCL21 (Figure 4.9). Unlike mRNA expression, IF staining for WNV-NS1 showed a higher epithelial infection in CCL20-neutralised mice compared to WNV-infected or isotype control mice. CD11c+ and CD11b+ cells in each WNV-infected group (WNV alone, WNV + anti-CCL20 and WNV + isotype) occupied ~4 and ~3% of total tissue area, respectively. It has been reported that CCL20 expression by epithelial cells recruit CCR6+ Langerhans cells and DCs via IL-1β and NF-κB signalling pathway in a vaginal epithelium model (Berlier et al., 2006, Cremel et al., 2005). Moreover, another study showed that Gr1+ monocytes (DC precursor) and CD11c+ MHCII+ DCs that recruited into the buccal mucosa or skin by adjuvant stimulation, are responsible for CD8+ T cell crosspriming. This infiltration of DCs was CCL20-CCR6 dependent because; either CCL20 neutralisation or monocyte depletion impaired the CD8+ T cell priming (Le Borgne et al., 2006). In our study, CD11b+ cells had similar distribution in all WNV-infected groups of mice, however, clusters of CD11c+ cells in the genital mucosa were smaller in size in CCL20-neutralised mice. Therefore, this decrease in the accumulation of CD11c+
cells and concomitant increase in the WNV viral load in the BALB/c vaginas might be the evidence that CCL20 neutralisation leads to an impaired control of viral mucosal infection and may suggest a higher susceptibility of anti-CCL20 treated mice to i.vag WNV infection (Figure 4-10–4-11).

Changes in leukocyte numbers in the ILN were also investigated. No reduction in the cell number and proportion of different subsets, including macrophages, DCs, B and T cells were observed in the anti-CCL20-treated mice. Interestingly, cell numbers in the major leukocyte subsets in the ILN significantly increased in the WNV-infected isotype control mice compared to the WNV-infected group and this increase also occurred in the anti-CCL20 group. Therefore, the cell numbers from the ILN of mice treated with anti-CCL20 similar to the isotype control mice (Figure 4.12).

Taken together, CCL20 neutralisation clearly demonstrated that other factors must be involved in the massive recruitment and accumulation of macrophages, DCs and lymphocytes to the site of infection in the vaginal mucosa to establish the immune response in the ILN. Therefore, future studies should compare leukocyte infiltration to the vagina and ILN of WNV infected mice in different time points (e. g., D5 p.i.). It also would be beneficial to take advantage of using CCL20 deficient (CCL20<sup>−/−</sup>) mice in this regard.

As C57BL/6 but not BALB/c mice significantly expressed CCR2 and CCL2, the role of CCL2 in C57BL/6 mice was also investigated using CCL2-neutralising Ab injected i.v. into C57BL/6 mice following i.vag WNV infection (Figure 4.8).
CCL2 blocking impairs the recruitment and homing a variety of monocytes to the site of WNV infection and its draining LN in a WNV model of ear infection (Davison and King, 2011). Moreover, in CCL2-neutralised mice, Ly6C^hi CCR2^+ monocytes are inhibited from infiltrating the WNV-infected brain (Getts et al., 2008). In addition, CCR2 deficient mice (CCR2^−/−) fail to accumulate in the HSV-2-infected vagina due to the lack of the recruitment of the inflammatory monocytes (Iijima et al., 2011). As with the other groups, in our study, CCL2 neutralisation affected the vagina and ILN of i.vag WNV-infected mice differently. Total leukocyte numbers decreased when CCL2 was neutralised in the vagina and ILN. Of note, there was a dramatic reduction (over than 50%) in the cell numbers of several macrophage subsets and neutrophils but not CCR2^+ inflammatory macrophages or DC populations in the vagina. On the other hand, the ILN exhibited a significant reduction in CCR2^+ Ly6C^hi macrophages as well as in cell numbers of several DC subsets.

CD8^+ T cells only comprised a small population in the vagina and did not change following CCL2 administration. However, CD4^+ T cells, B cells and NKT cells all decreased following anti-CCL2 antibody administration. On the other hand, in the ILN, there was a reduction in cell number in all of the lymphocyte subsets examined. The percentages of each leukocyte subset in the vagina and ILN did not change upon CCL2 blockade, with the exception of vaginal neutrophils and CD4^+ T cells. Moreover, analysis of subsets in the spleen showed that there were no changes in the numbers or proportions of various leukocyte populations (Figure 4.14–4.16).

Collectively, these experiments showed that CCL2 only affects leukocyte
recruitment to the vagina and its draining LN but not to the spleen. Although several studies have shown that CCL2 is responsible for the recruitment of CCR2+ inflammatory macrophages (Geissmann et al., 2003, Getts et al., 2008, Iijima et al., 2011, Lim et al., 2011, Narni-Mancinelli et al., 2011, Serbina and Pamer, 2006), in our model of infection, this effect was only observed in other CCR2+ macrophages but not inflammatory macrophages. Moreover, CCL2 blockade was associated with drastic reductions in several other leukocyte subsets, indicating that this chemokine is involved in the recruitment, either directly or indirectly of a diverse array of macrophages and DCs as well as B and T cell expansion in the vagina and ILN. There is an uncertainty in the viral load of WNV in the vagina of CCL2-neutralised animals compared to the wild type counterparts and thus it should be included in the future studies. It is important to further investigate whether CCL-2 blockade and impairment of leukocyte infiltration impacts virus resolution in the WNV-infected vagina. In addition, using dual chemokine neutralisation (CCL2-CCL20) would be of interest to give more insight into role of complex network of early antiviral immune response in the genital mucosa.

In our laboratory, there has been significant focus on the impact of i.v. injection of fluorescent polystyrene beads in WNV encephalitis. These beads have been used as markers for migrating myeloid lineage cells, however, administration of polystyrene beads also reduces inflammatory (Ly6Chi) monocyte infiltration into the infected brain and results in long-term survival (Terry, 2012).

Therefore, the use of beads administration to investigate the migration of cells in the i.vag model of WNV infection was further examined. In addition, to investigate the combinatory effect of beads and CCL2, a group of WNV-infected
mice were injected i.v. with beads and anti-CCL2 antibody at the same time.

Strikingly, similar to CCL2 neutralisation, beads injection alone affected the migration of immune cells to the vagina and ILN. Thus, there was a reduction in the number of neutrophils and CCR2⁺ macrophages, including inflammatory macrophages, in the infected vagina at D5 p.i. However, BDC, CD11b⁻ and CD8⁺ DCs decreased in numbers in the ILN after beads administration. Numbers of B and T cells in the vagina were not statistically significant in all WNV-infected groups, although these subsets in the ILN decreased in response to beads administration. Similar to the effect of CCL2 neutralisation in the spleen of WNV-infected mice, injection of beads also had no effect on the cell subsets examined in the spleen (Figure 4.17–4.19).

In the vagina, WNV-infected mice that received beads and anti-CCL2 antibody at the same time had similar results to mice that received beads only. In the ILN group, receiving beads and anti-CCL2 antibody, however, there was no change in the total number of cells or B and T cells compared to WNV-infected mice while beads alone resulted in reduction in B and T cell numbers. These results raise the question of whether the biochemical and charge characteristics of the beads interact with anti-CCL2 antibody. We are currently investigating beads-protein interactions at the molecular level to find out why mixing beads and CCL2 antibody diminishes their immunomodulatory effects.

Notwithstanding, further investigation of bead⁺ cells in the spleen revealed that the number of bead⁺ cells in the spleen correlated with the quantity of reduced cell numbers of different leukocyte and lymphocyte subsets in the ILN and vagina (Figure 4.20). Besides the primary function of B-lymphocytes in humoral
immunity, they are also involved in antigen presentation (Chen and Jensen, 2008, Kurt-Jones et al., 1988, Morris et al., 1994, Rivera et al., 2001). Therefore, B cells are able to internalise foreign Ags to process and provide appropriate signal transduction via BCR (Dal Porto et al., 2004, Geisberger et al., 2006, Zhao et al., 2003, Zhu et al., 2008). The observation in this study that 65% of all beads+ cells in the spleen are comprised of B cells may reflect this point that beads have been taken up mostly by B cells. However, whether this internalisation of beads results in a proper immune response remains unclear and necessitates further investigation. The above-mentioned finding of bead+ subsets in the spleen suggests that cells take up beads in the circulation and are subsequently sequestered in the spleen and thus do not infiltrate into the sites of infection/inflammation and the draining LN.

This chapter describes in detail the comparative dynamics of WNV infection and immune response in BALB/c and C57BL/6 mice. These data also show the differential Th1- and Th2-type cytokine and chemokine responses in each strain following i.vag WNV infection and confirm that the administration of immune-modulating agents during infection dramatically reduce innate and adaptive immune responses in the vagina and ILN but do not apparently affect the spleen.
Chapter 5

Role of IDO in intravaginal
HSV-2 infection
Chapter 5:

Role of IDO in intravaginal HSV-2 infection

5.1 Introduction

In Chapter 3, we showed that viral infection was associated with significant upregulation of the pro-inflammatory cytokine, IFN-γ, as well as the chemokines CCL2, CCL3 and CCL5, in the vaginal mucosa. These cytokines and chemokines have a pivotal role in the recruitment of innate and adaptive immune cells, particularly, NK and CD4+ T cells, respectively (Maurer and von Stebut, 2004, Appay and Rowland-Jones, 2001, Maghazachi et al., 1994, Wong and Fish, 1998, Levy, 2009). NK cells participate in innate immune responses by secreting IFN-γ, which can inhibit intracellular pathogens and promote the differentiation of activated CD4+ T cells into Th1 helper cells (Robertson, 2002, Orange et al., 1995). Upon activation, CD4+ T cells can also produce large amounts of IFN-γ in the HSV-2-infected genital mucosa (Harandi et al., 2001a, Chan et al., 2011, Svensson et al., 2012, Abbas et al., 1996).

One outcome of IFN-γ production in the microenvironment is the expression of IDO. The IDO gene has IFN-γ-activated sites (GAS) and interferon-stimulated response elements (ISREs) which are stimulated by IFN-γ (Murakami et al., 2013). In Chapter 4, we showed that IDO mRNA is expressed in the vagina following WNV infection with kinetics similar to the pattern of IFN-γ gene expression. As described in Chapter 1, IDO is one of the initial enzymes in the kynurenine pathway of L-tryptophan (L-Trp) catabolism. Upon production of active enzyme, IDO catalyzes the oxidative conversion of L-Trp, the least-
abundant, essential amino acid, into N-formylkynurenine, which is further metabolised into stable-end product, kynurenine (Kyn) (Thomas and Stocker, 1999, King and Thomas, 2007). This process depletes L-Trp from the local microenvironment and is associated with the accumulation of biologically active Kyn metabolites (Yeung et al., 2012). Several studies have shown that IDO is antiviral against cytomegalovirus, vaccinia, dengue, measles and hepatitis B viruses in vitro (Becerra et al., 2009, Bodaghi et al., 1999, Mao et al., 2011, Terajima and Leporati, 2005, Obojes et al., 2005). More specifically, Adams and colleagues, reported that IFN-γ-driven IDO activity was responsible for the inhibition of HSV-1, and HSV-2 in human cell lines cultures (Adams et al., 2004a, Adams et al., 2004b, Yeung et al., 2012) Even though IDO is upregulated in human patients and experimental animal models of viral infection (Becerra et al., 2009, Larrea et al., 2007, Heyes et al., 1992), its antiviral function in vivo has yet to be confirmed since there are other reports indicating that depletion of L-Trp and concomitant increased levels of Kyn and other downstream metabolites may dampen virus-specific T cell responses by inducing regulatory T cells (Tregs) and inhibiting effector Th1 cells (Boasso et al., 2007, Li et al., 2008, Manches et al., 2008, van der Sluijs et al., 2006, Hryniewicz et al., 2006, Mellor and Munn, 2004, Huang et al., 2013).

There has been significant focus on the role of IDO in i.vag WNV and HSV-2 infections in our laboratory. However, previous attempts to block IDO using 1-methyl tryptophan (1-MT), the most commonly used IDO inhibitor, found that 1-MT had minimal or no effect on the inhibition of IDO activity in the i.vag infection models (Yeung, 2012). Therefore, given that the role of IDO in the context of STI infections has not been fully investigated in vivo, this chapter seeks
to explore the antiviral and immunosuppressive effects following the induction of IDO by i.vag HSV-2 infection in IDO-gene knockout mice.

5.2 Results

5.2.1 Kinetics of IFN-γ and IDO mRNA expression following HSV-2 infection in the vaginal mucosa

In the previous chapter, we showed that IFN-γ and IDO mRNA were highly upregulated in the vagina of i.vag WNV-infected C57BL/6 mice, peaking at D5 p.i. (Figure 4.6). To investigate if IFN-γ and IDO mRNA were expressed in the HSV-2 infected vaginal mucosa, C57BL/6 mice were infected i.vag. with $10^5$ PFU of HSV-2. Vaginae were isolated and the cDNA examined for IFN-γ and IDO mRNA expression at D0, 1, 2, 3, 5, 6, 7 and 8 p.i. Expression of IFN-γ and IDO mRNA had similar kinetics of expression following vaginal infection. Strikingly, the mRNA of both genes were markedly upregulated at D2 p.i. before decreasing on D3 p.i. but they then rapidly increased to peak ~300- and ~17000-fold higher at D5 p.i., respectively. The expression of both genes gradually declined thereafter. No IFN-γ and IDO mRNA were detected in mock-infected mice at any time-point (Figure 5.1A–B).
Figure 5.1

Relative gene expression of IFN-γ and IDO in the HSV-2 infected vagina

Relative mRNA expression of (A) IFN-γ and (B) IDO in the vagina of C57BL/6 mice infected i.vag with 10^5 PFU of HSV-2 at D0, D1, D2, D3, D5, D6, D7 and D8 p.i. Expression is relative to levels in the vagina at D0 p.i. Data is plotted as the mean ± SEM of 3-5 mice per group and represents 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05, **p≤0.01, ***p≤0.001, compared to mock-infected mice.
5.2.1 Localisation of IDO protein in the HSV-2-infected vagina

It was clear that HSV-2 infection was associated with a dramatic increase in IDO gene expression. To determine if IDO protein was present in the infected genital mucosa, vaginal sections from HSV-2-infected mice were labelled for IDO and HSV-2 protein. Here, labelling for HSV-2 showed extensive epithelial infection that peaked at D1 p.i. with ~4% of the total tissue area staining positive for HSV-2. Staining was localised to the epithelial cells (Figure 5.2B). HSV-2 infection gradually decreased during the infection time course, as no epithelial infection was observed in the vaginal mucosa at D7 p.i. (Figure 5.2B–F). On the other hand, IDO protein was first detected at, and peaked on, D2 p.i., where ~2% of the tissue stained positive for IDO (Figure 5.2C). It was notable that IDO was mainly expressed by infected epithelial cells, as shown by the co-localisation of the red and green signals, and to a much lesser extent, in the stroma (Figure 5.2C–F). HSV-2 infection decreased in the vaginal mucosa from D2 p.i. onwards, however, the IDO protein expression first declined at D3 p.i. before increasing again at D5 p.i. Of interest, IDO expression was observed in cells in the stroma at this time, although infected and uninfected epithelial cells also expressed IDO (Figure 5.2C–F). Even though virus infection was resolved by D7 p.i., IDO expression was still evident at this time-point (< 1% IDO⁺) (Figure 5.2F–G).

Collectively, IF staining for IDO the vagina showed the same pattern as IDO mRNA induction, i.e., there were two waves of IDO expression in the vagina that peaked at D2 and D5 p.i.
**Figure 5.2**

**Localisation of IDO protein and HSV-2 in the vagina following HSV-2 infection**

Immunofluorescent staining for HSV-2 antigen (FITC; green), IDO protein (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in vaginal cross-sections from mice infected i.vag with $10^5$ PFU HSV-2 at (A) D0, (B) D1, (C) D2, (D) D3, (E) D5 and (F) D7 p.i. Neither HSV-2$^+$ nor IDO$^+$ cells were observed in sheep IgG and normal rabbit IgG isotype-stained sections (not shown). (G) Percentage of vaginal epithelial cells that were HSV-2$^+$ in mice infected with $10^5$ PFU (green) and proportion of the area filled with IDO$^+$ cells (red). All images are representative of at least 3-5 mice per time point. Data indicate the mean ± SEM of 3-5 mice per group and represent 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; **$p$$\leq$0.01 and ***$p$$\leq$0.001 compared to D0 p.i., and #$p$$\leq$0.05 and ###$p$$\leq$0.001, compared to D0 p.i.
A nuclei IDO D0 p.i.

B nuclei HSV-2 IDO D1 p.i.

C nuclei HSV-2 IDO D2 p.i.

D nuclei HSV-2 IDO D3 p.i.

E nuclei HSV-2 IDO D5 p.i.

F nuclei HSV-2 IDO D7 p.i.

G

% Total area

0 1 2 3 4 5

0 1 2 3 4 5 6 7

Day post HSV-2 infection

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- *** p < 0.001
- ** p < 0.01
- * p < 0.05
- # p < 0.1
- #* p < 0.01
- ** p < 0.001
5.2.2 Localisation of IDO protein in the ILN of i.vag HSV-2-infected mice

As IDO was present in the infected vagina, it was also of interest to see whether IDO protein was expressed in the ILN. No IDO protein expression was observed in the lymphoid cells within the ILN; however, IDO staining was evident in vessel-shaped segments in mock- and HSV-2-infected mice. Several studies have shown that endothelial cells, which line the interior surface of blood and lymphatic vessels, can express IDO as a result of infection and inflammation (Beutelspacher et al., 2006, Wang et al., 2010). Thus, these results suggest that endothelial cells express IDO protein in the ILN.

IDO expression in the ILN was higher on D2 and D5 p.i. Despite the fluctuation in the IDO expression during infection, there was no significant change in the percentage of the IDO+ areas in the ILN (Figure 5.3A – G).

Overall, IDO did not increase significantly in the ILN following i.vag HSV-2 infection, suggesting that IDO activity was only critical at the primary site of vaginal infection. Notwithstanding, at D2 and D5 p.i. when IDO mRNA and protein dramatically increased in the vagina, there was a slight corresponding trend in the ILN.
Figure 5.3

Localisation of IDO protein and HSV-2 in the draining ILN following i.vag HSV-2 infection

Immunofluorescent staining for HSV-2 antigen (FITC; green), IDO protein (Alexa-Fluor 594; red) and nuclei (DAPI; blue) in the draining ILN cross-sections from mice infected i.vag with $10^5$ PFU HSV-2 at (A) D0, (B) D1, (C) D2, (D) D3, (E) D5 and (F) D7 p.i. No HSV-2 staining was observed in ILN sections at any time point. Neither HSV-2$^+$ nor IDO$^+$ cells were observed in sheep IgG and normal rabbit IgG isotype-stained sections (not shown). (G) Proportion of the area filled with IDO$^+$ cells (red). All images are representative of at least 3-5 mice per time point. Data indicate the mean ± SEM of 3-5 mice per group and represent 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test.
A![Image](93x603 to 291x752)

B![Image](302x603 to 499x752)

C![Image](93x437 to 291x586)

D![Image](302x437 to 499x586)

E![Image](93x270 to 291x419)

F![Image](302x270 to 499x419)

G![Image](197x115 to 395x254)

Nuclei

HSV-2

IDO

D0 p.i.

Nuclei

HSV-2

IDO

D1 p.i.

Nuclei

HSV-2

IDO

D2 p.i.

Nuclei

HSV-2

IDO

D3 p.i.

Nuclei

HSV-2

IDO

D5 p.i.

Nuclei

HSV-2

IDO

D7 p.i.

G

% Total area

Day post HSV-2 infection

IDO
5.2.3 IDO protein activity in the vagina and ILN following i.vag HSV-2 infection

Taking into consideration that IDO is an enzyme that catalyses the catabolism of L-Trp into several metabolites, including Kyn, and since it’s activity is tightly regulated post-translationally, it was necessary to examine if the induced IDO protein was enzymatically active within the primary and secondary sites of the immune response. Thus, L-Trp and Kyn levels in vaginal and ILN homogenates of HSV-2-infected mice were investigated by HPLC. Analysis of vaginal homogenates revealed that L-Trp was present in vagina of mock-infected mice at basic levels (~5 μM). However, this concentration gradually rose throughout the infection course. Importantly, the concentration of Kyn also increased during HSV-2 infection in the vagina. Low quantities of Kyn were detected from D2 to D5 p.i., before rapidly increasing to a peak at ~5 μM at D6 p.i. Interestingly, at this time point, L-Trp concentration also increased significantly to peak at ~30 μM. Furthermore, even though L-Trp and Kyn levels in the vagina decreased after D6 p.i., Kyn was still significantly elevated above baseline (Figure 5.4A). Overall, the Kyn/L-Trp ratios increased over this time to a peak at D8 p.i., significantly, differed from the baseline ratios (Figure 5.4B).

As expected, the concentration of L-Trp remained unchanged and Kyn was not detected in the vagina of mock-infected mice. This corresponded with the lack of IDO mRNA and protein in these control mice.

Minimal IDO enzymatic activity was observed in the ILN homogenates before D6 p.i. At D6 p.i., Kyn increased significantly to 0.5 μM and continued to increase to peak at D8 p.i. No significant change in L-Trp was observed during
the time course, except at D7 p.i. when the L-Trp concentration was ~2-fold higher compared to mock-infected controls (Figure 5.4C). The ratios of Kyn/L-Trp peaked at D2 p.i. and again at D5 and D6 p.i., corresponding to mRNA peaks of IDO and IFN-γ in the vagina (Figure 5.4D).

Taken together, these results confirmed the enzymatic activity of the IDO protein in the vagina and ILN of HSV-2-infected mice. It was also interesting to observe that L-Trp levels increased at D6 and D7 p.i. in the vagina and ILN, respectively. These elevated L-Trp levels were consistently associated with rapid increases in the concentration of Kyn within these tissues.

5.2.4 Kinetics of HSV-2 mRNA expression in wild type and IDO gene knockout mice

Studies thus far have demonstrated that IDO mRNA and protein are highly upregulated in the HSV-2-infected vagina and that this protein is active, as Kyn and more importantly Kyn/L-Trp ratio increased following infection. Therefore, it was of interest to evaluate the role of IDO in the mucosal immune response following i.vag inoculation with HSV-2 by using IDO gene knock out (IDO−/−) mice and their respective C57BL/6 wild type (WT) counterparts. To verify that IDO−/− mice were genuinely IDO-deficient, tail or ear clips were taken of both strains of mice and genotyped. Genotyping studies confirmed that all IDO−/− mice contained a specific band (on the gel electrophoresis image) at the 280 bp (base pair) region and lacked the WT IDO band, which was evident in the WT controls.
Figure 5.4

**IDO activity in the vagina and ILN of i.vag HSV-2-infected mice**

(A) L-Trp (black circles) and Kyn levels (red squares) in the vagina, (B) ratios of Kyn/L-Trp in the vagina, (C) L-Trp (black circles) and Kyn levels in the draining ILN and (D) the respective ratios of Kyn/L-Trp in the ILN of mice infected with $10^5$ PFU of HSV-2 for the time points indicated. L-Trp and Kyn levels are normalized by total tissue protein concentrations and expressed as μM of total protein after homogenisation of the vagina and ILN per time point. Data indicate the mean ± SEM of 3-5 mice per group and represent 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ compared to D0 p.i.
in the 430 bp region (Figure 2.1). This confirmed the IDO gene defect in the IDO−/− mice.

Age-matched WT and IDO−/− mice were infected with 10⁴ or 10⁵ PFU HSV-2 i.vag. Then, mRNA encoding for glycoprotein B of HSV-2 (Atanasiu et al., 2013) was detected using real-time RT-PCR. Unfortunately, all time points could not be analysed due to the limited availability of IDO−/− mice.

Viral mRNA in the vagina of WT and IDO−/− mice was detectable as early as D1 p.i. In mice inoculated with the higher dose, D1 p.i. was the peak expression day as HSV-2 mRNA gradually declined from d3 p.i. onwards. Interestingly however, no difference was observed in the kinetics of infection between the WT and IDO−/− mice (Figure 5.5A). When infected with the lower HSV-2 dose, mRNA expression in the vagina of WT mice peaked at ~8-fold at D3 p.i., compared to at D1 p.i. Although levels of HSV-2 message in the vagina of IDO−/− mice tended to be higher at D2 and D3 p.i., there was no significant difference between WT and IDO−/− mice at any point in the time course (Figure 5.5B).

5.2.5 Morbidity and mortality following i.vag inoculation with HSV-2

Preliminary results from above studies illustrated no differential change in the viral mRNA copies in the vagina of WT and IDO−/− mice. To further investigate the kinetics of i.vag HSV-2 infection, groups of 10 age-matched WT and IDO−/− mice were inoculated with either 10⁴ or 10⁵ PFU HSV-2 and monitored
Figure 5.5

Quantification of HSV-2 infection in the vagina of WT and IDO−/− mice

Relative HSV-2 mRNA expression in the vagina of WT (black circles) and IDO−/− (red squares) mice infected with (A) 10^5 or (B) 10^4 PFU HSV-2 for indicated time points. HSV-2 mRNA expression is relative to levels apparent in the vagina at D1 p.i. Data shown is the mean ± SEM of at least 3-5 mice per group and represents 3 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test.
for any sign of illness over a time course of 25 days. Intravaginal infection resulted in significant morbidity and mortality in both WT and IDO<sup>−/−</sup> mice. Following i.vag infection with 10<sup>5</sup> and 10<sup>4</sup> PFU of HSV-2, more than 90% of mice appeared ill and succumbed to infection from D8 p.i. (Figure 5.6A–B). Animals were also examined for the progression of vaginal lesions and overall illness as well as weight loss. Mice were monitored daily and scored on a five-point scale from 0 to 5 based on the several signs of pathology (Gill et al., 2006). Interestingly, i.vag HSV-2 infection progressed similarly in both WT and IDO<sup>−/−</sup> mice and no difference in clinical illness was observed even when the lower dose was used (Figure 5.6C–F).

Taken together, analysis of several parameters including mortality rates, weight loss and pathology scores show that there were no differences between infected WT and IDO<sup>−/−</sup> mice. Moreover, the progression of disease in both strains of mice was identical when two different doses of HSV-2 were used. Therefore, IDO does not have any discernable effect on HSV-2 mRNA levels in the vagina or on disease development.

5.2.8 Gene expression of immunoregulatory factors

The results above indicate that IDO gene deficiency has no effects on the viral mRNA expression in the vagina, disease pathology or survival rate following HSV-2 infection. Since IDO did not appear to be antiviral here, we investigated immunoregulatory role in the infection, as recently, there has been increasing evidence that IDO-related factors can play a pivotal role in orchestrating
Figure 5.6

**Morbidity and mortality following i.vag HSV-2 infection**

Kaplan–Meier survival curve for the WT (black squares) and IDO<sup>−/−</sup> (red triangles) mice infected with (A) $10^5$ and (B) $10^4$ PFU of HSV-2 over 25 days. (C) Relative changes in the weight of WT (black squares) and IDO<sup>−/−</sup> (red triangles) mice that infected with i.vag $10^5$ and (D) $10^4$ PFU of HSV-2 over a 25-day time course. (E) Pathology scores of WT (black squares) and IDO<sup>−/−</sup> (red triangles) mice infected with $10^5$ and (F) $10^4$ PFU of HSV-2 over 25 days. Pathology of i.vag HSV-2 infection quantified by the disease score as follows: 0, no signs of infection; 1, slight redness of genital area; 2, redness and swelling of external vagina; 3, severe swelling of the external vagina and hair loss in the surrounding area; 4, ulceration, severe swelling and redness of the vaginal tissue; and 5, continued ulceration, swelling and redness, occasionally accompanied by paralysis of the hind legs (mice were euthanised at this point). All data are expressed as the mean ± SEM of 3 independent experiments of 5 to 10 mice for each group. Each line represents the percentage weight change of one mouse, relative to their respective weight at d0 p.i. Data represents 3 independent experiments, with 5 to 10 mice each.
immunoregulatory responses against viral infections (Veiga-Parga et al., 2011). The aryl hydrocarbon receptor (AhR) has emerged over the last decade as a critical regulator of the immune system (Pot, 2012). AhR, a cytosolic transcription factor, is expressed in cells involved in both innate and adaptive immune responses (Platzer et al., 2009, Kiss et al., 2011). AhR modulates regulatory responses by participating in the generation of FoxP3+ Treg cells. Treg cells are a subpopulation of CD4+ T cells that constitutively express CD25 as well as the transcription factor FoxP3 (Forkhead box P3), which is currently considered as the most accurate marker for Tregs (Sakaguchi, 2005, Fontenot et al., 2003). Moreover, Kyn, the first L-Trp degradation metabolite, can specifically bind to and activate AhR (Mezrich et al., 2010, Nguyen et al., 2013, Rutz and Ouyang, 2011, Veiga-Parga et al., 2011). Activated AhR, then, promotes the development a subset of CD4+ T cells that produce the anti-inflammatory cytokine, IL-10 (Apetoh et al., 2010, Rutz and Ouyang, 2011).

Therefore, in an effort to delineate the role of immunoregulatory factors downstream of IDO, AhR and IL-10 mRNA expression in the HSV-2-infected vagina (10^5 PFU) of both WT and IDO−/− mice were analysed.

There was no significant difference in AhR mRNA expression in the vagina of WT mice throughout infection. Interestingly, however, there was a significant upregulation in the AhR message at D1 p.i. in the vagina of IDO−/− mice as 2-fold more mRNA copies were present compared to mock-infected controls. Despite the significant upregulation in the HSV-2-infected IDO−/− vagina compared to WT mice at D1 p.i., AhR expression in IDO−/− counterparts downregulated after this time-point to regain the same levels as seen at D0 p.i. (Figure 5.7A).
Unlike the AhR gene expression, HSV-2 infection elicited a different response from IL-10 mRNA. In the absence of IDO (i.e., IDO⁻/⁻ mice), IL-10 mRNA gradually increased at D2 p.i., and decreased the day after. However, IL-10 mRNA rose again to 40-fold compared to mock-infected controls, peaking at D5 p.i. IL-10 expression had decreased sharply at D7 p.i., although it was still elevated relative to control mice. IL-10 mRNA expression in WT was different to IDO⁻/⁻ mice, particularly at D2, D5 and D7 p.i. when IL-10 mRNA was lower in WT mice than in KO animals. More specifically, IL-10 was only upregulated in WT mice by 10-fold at D5 p.i. relative to D0 p.i. Thus, at this time-point, IL-10 mRNA was 4-fold lower in WT mice than in their IDO⁻/⁻ counterparts (Figure 5.7B).

Collectively, AhR mRNA expression was similar in both WT and IDO⁻/⁻ mice, except on D1 p.i., in which message in the vagina of IDO⁻/⁻ was significantly higher than in WT mice. Furthermore, IL-10 expression in the vagina of WT mice was only upregulated at D5 p.i. However, in IDO⁻/⁻ mice, IL-10 mRNA had similar kinetics to IFN-γ and IDO mRNA expression, which were seen in HSV-2 infected vagina of WT mice (i.e., upregulation on D2 followed by downregulation on D3 p.i. before reaching to its peak levels at D5 p.i., Figure 5.1). These results suggest that IL-10 may compensate for the loss of IDO expression to modulate immunoregulatory responses in the vagina.
**Figure 5.7**

*Relative gene expression of AhR and IL-10 in the HSV-2-infected vagina*

Relative mRNA expression of (A) AhR and (B) IL-10 in the vagina of mice infected i.vag with $10^5$ PFU of HSV-2 at D0, D1, D2, D3, D5 and D7 p.i. Expression is relative to levels in the vagina at D0 p.i. Data is plotted as the mean ± SEM of 3-5 mice per group and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; ***$p \leq 0.001$, compared to mock-infected WT mice. #$p \leq 0.05$ and #$$p \leq 0.001$ relative to mock infected IDO$^-$ mice. ^$p \leq 0.05$ and ^$$p \leq 0.001$ relative to WT and IDO$^-$ mice at the same time-point.
5.2.6 Cytokine and chemokine response in the WT and IDO$^{-}$vaginal mucosa during HSV-2 infection

While there was no difference in i.vag HSV-2 infection of WT and IDO$^{-}$ mice with respect to viral infection, survival rate, pathology and AhR (a receptor for Kyn) gene expression, a lack of IDO did affect IL-10 gene expression and this prompted us to look at other chemokines/cytokines. Accordingly, multiplex ELISA was used to assess protein expression of several cytokines and chemokines in the vagina throughout infection. To do this, vaginas from WT and IDO$^{-}$ mice were isolated and homogenised and the concentration of 12 pro- and anti-inflammatory cytokines and chemokines on D0, 1, 2, 3, 5 and 7 p.i. were analysed.

5.2.6.1 Cytokines

IFN-$\gamma$ is the main inducer of IDO (Alberati-Giani et al., 1997, Ansari et al., 2006, Becerra et al., 2009, Bodaghi et al., 1999, Mao et al., 2011, Obojes et al., 2005, Terajima and Leporati, 2005, Yeung et al., 2012). As previously shown in this chapter, i.vag HSV-2 infection generated two waves of IFN-$\gamma$ mRNA expression at D2 and D5 p.i. in C57BL/6 mice (Figure 5.1A). Expression of this cytokine in the vaginal homogenates of WT and IDO$^{-}$ mice was also rapidly upregulated on D1 p.i. to peak at $\sim$40 and $\sim$30 pg/mg of total protein, respectively. A second wave of IFN-$\gamma$ protein expression was observed at D5 p.i., followed by a decline to the baseline levels at D7 p.i. (Figure 5.8A).
The pro-inflammatory activities of TNF, which can be expressed by almost all cells, are well established (Ellerin et al., 2003, Taylor, 2001, Keystone and Ware, 2010, Herbein and O’Brien, 2000, Tabb et al., 2013). However, a number of reports have indicated a critical role for this cytokine in regulating and limiting the extent and duration of the inflammatory response in vivo (Hodge-Dufour et al., 1998, Masli and Turpie, 2009, Tabb et al., 2013). TNF protein was only expressed at very low quantities in both WT and IDO−/− mice following (10⁵ PFU) HSV-2 infection and its concentration remained below 2 pg/mg of total protein throughout the infection (Figure 5.8B).

Granulocyte macrophage colony-stimulating factor (GM-CSF), which is expressed by T cells, macrophages, endothelial cells, as well as stromal cells in the bone marrow, is a cytokine and hematopoietic growth factor that promotes myeloid cell development and maturation as well as DC differentiation (Abbas and Lichtman, 2004, Hercus et al., 2009, Laia and Martin, 2010). There is also growing evidence supporting its role in some inflammatory reactions and in the host response to mucosal infections in the pulmonary, GI and genital tracts (Fernandez et al., 2007, Laia and Martin, 2010, Paine et al., 2000). However, the concentration of GM-CSF remained unchanged at ~2.5 pg/mg at all time points in the vagina of HSV-2-infected WT and IDO−/− mice (Figure 5.8C).

IL-12 is mostly produced by activated APCs including monocytes, macrophages and DCs both in infected tissues and in secondary lymphoid organs (Romagnani, 2000, Komastu et al., 1998, Trinchieri et al., 2003). This pro-inflammatory cytokine is a potent inducer of IFN-γ production by the activation and differentiation of Th1 cells, and is thus critically involved in the cellular response and clearance of the intracellular pathogens such as HSV-2 (Malmgaard
IL-12 was present in mock-infected control mice at ~15 pg/mg protein. After i.vag HSV-2 infection, enhanced production of IL-12 was seen in WT mice at D2 p.i., where there was 2-fold more IL-12 in WT mice than IDO−/−. No significant difference was observed at other time-points (Figure 5.8D).

IL-17 is a prominent pro-inflammatory cytokine, produced mainly by T cells, and boosts the antiviral immune response following viral infections (Ge and You, 2008, Pappu et al., 2010, Ryzhakov et al., 2011). In the presence of IL-17, other cells such as epithelial, stromal and endothelial cells can increase the production of pro-inflammatory cytokines and chemokines, leading to the recruitment of effector immune cells to the site of infection (Gaffen, 2008, Pappu et al., 2012, Reynolds et al., 2010, Ryzhakov et al., 2011). IL-17 was expressed in both WT and IDO−/− mice at ~5 pg/mg in mock-infected mice and this concentration remained constant during infection in both strains of mice (Figure 5.8E).

Anti-inflammatory cytokines act in concert with specific cytokine inhibitors and receptors to regulate the immune response to strike a balance between inflammation/pathogenesis and infection control. It is also notable that anti-inflammatory cytokines have at least some pro-inflammatory properties, which is dependent on the timing of cytokine release, local milieu, the presence of competing elements, cytokine receptor density and local responsiveness to cytokines (Opal and DePalo, 2000, Wilke et al., 2011). Among others, IL-4, IL-6 and IL-10 are key anti-inflammatory cytokines and were thus investigated in this study.
IL-4 is primarily expressed by Th2 CD4⁺ T cells and leads to the suppression of Th1 cells by inhibiting the differentiation of Th0 cells to Th1 effector cells. IL-4 secretion, along with IL-6 and IL-10, induces strong antibody responses, particularly IgE responses, but inhibits several functions of phagocytic cells (monocytes, macrophages, dendritic cells and neutrophils) (Romagnani, 2000, Opal and DePalo, 2000, Abbas and Lichtman, 2004). IL-4 was present at very low concentrations in the vagina of mock-infected control mice. Although infection did not change the expression pattern of this cytokine, IL-4 did tend to increase in WT mice starting from D3 p.i. On the other hand, IDO⁻⁺ mice downregulated IL-4 production as there was ~3-fold less IL-4 protein in the vagina at D7 p.i. relative to mock-infected animals (Figure 5.8F).

IL-6 has both pro- and anti-inflammatory effects. It is produced by macrophages, T cells, endothelial cells and fibroblasts after inflammatory/infectious stimulation. When secreted, IL-6 inhibits the synthesis of the pro-inflammatory cytokines while having little effect on the production of anti-inflammatory cytokines such as IL-10 (Opal and DePalo, 2000, Scheller et al., 2011). HSV-2 infection was associated with significant IL-6 upregulation in both WT and IDO⁻⁺ mice at D1 p.i. IL-6 secretion in WT mice decreased after D1 p.i. and returned to the baseline levels by D3 p.i. Although a similar decrease was also observed in IDO⁻⁺ mice, IL-6 was still significantly higher in these mice at D5 p.i. at 15 pg/mg, ~7-fold higher than in WT mice (Figure 5.8G).

IL-10, an important anti-inflammatory cytokine, is primarily produced by CD4⁺ Th2 cells, monocytes, Treg cells and B cells (Tsai et al., 2013, Wilson and Brooks, 2011). It inhibits the production of Th1 cytokines and chemokines and induces the downregulation of surface markers such as MHC II, TNF receptor and
B7 accessory molecules (Opal and DePalo, 2000, Sabat, 2010, Sun et al., 2010). IL-10 was differentially produced in WT and IDO⁺/⁻ mice. Following i.vag HSV-2 infection, IL-10 was upregulated in WT mice as early as D1 p.i. and remained elevated until D3 p.i. This response in IDO⁺/⁻ mice was delayed, since IL-10 levels only increased on D5p.i. with 3-fold higher levels compared to at D0 p.i. At D5 and D7 p.i., the concentration of IL-10 was similar in both WT and IDO⁺/⁻ mice at ~3 pg/mg (Figure 5.8H).

5.2.6.2 Chemokines

The mRNA expression profile of CCL3, CCL5 and CCL2 following i.vag WNV infection was studied in Chapter 4. There is immense overlap in the infiltration of effector cells in response to CCL2, CCL3 and CCL5. As previously mentioned, each of these chemokines are involved in the recruitment of NK cells, T cells, B cells, myeloid cells as well as macrophages and DCs to the site of infection (Appay and Rowland-Jones, 2001, Levy, 2009, Maurer and von Stebut, 2004, Peretti et al., 2005, Iijima et al., 2011, Geissmann et al., 2003).

CCL2 is secreted by a variety of haematopoietic and non-haematopoietic cells, although monocytes and macrophages are the major source of CCL2 (Satish et al., 2009, Ansari et al., 2011). HSV-2 infection elicited a dramatic increase in the production of CCL2 at D1 p.i. (~900 pg/mg in WT mice and ~700 pg/mg in IDO⁺/⁻ mice). This production increased again at D3 and D5 p.i. after a sharp decline at D2 p.i. The concentration of CCL2 was significantly higher in IDO⁺/⁻ mice at D5 p.i. compared to their WT counterparts, although by D7 p.i., the concentration of CCL2 returned to baseline levels (Figure 5.9A).
Pro- and anti-inflammatory cytokine concentrations in the HSV-2-infected vagina of WT and IDO−/− mice

Protein concentrations of pro-inflammatory (A) IFN-γ, (B) TNF, (C) GM-CSF, (D) IL-12 and (E) IL-17 and anti-inflammatory (F) IL-4, (G) IL-6 and (H) IL-10 cytokines in the vagina of WT (black circles) or IDO−/− (red squares) mice infected i.vag with 10⁵ PFU HSV-2 between 0 and 7 days, as indicated. Cytokine levels are expressed as pg per mg of the total protein after homogenisation of the vaginal tissue. Data is the mean ± SEM of 3-4 mice per group. The experiment was carried out once. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p≤0.05 and **p≤0.01, compared to mock-infected WT mice. #p≤0.05 and ##p≤0.01, relative to mock-infected IDO−/− mice.
CCL3 is produced by a variety of leukocyte subsets, particularly lymphocytes and monocyte/macrophages. CCL3 production in the infected vagina followed the same pattern in both WT and IDO⁻/⁻ mice. High concentrations of CCL3 were observed at D1 and D5 p.i. with ~125 pg/mg and 50 pg/mg, respectively. In both WT and IDO⁻/⁻ mice, CCL3 secretion dropped to the levels similar to mock-infected control mice at D7 p.i. (Figure 5.9B).

CCL5, which is generated predominantly by CD8⁺ T cells, epithelial cells, fibroblasts and platelets, was in significantly higher concentrations in WT mice at D1 p.i. (~500 pg/mg) than in IDO⁻/⁻ mice (~200 pg/mg) after HSV-2 infection. Similar to CCL2 and CCL3, CCL5 protein expression underwent a steep decline straight after its peak expression at D2 p.i. and was not significantly different over the rest of the time course (Figure 5.9C).

Keratinocyte-derived chemokine (KC, CXCL1) is a CXC-type chemokine and is expressed mainly by macrophages, neutrophils and epithelial cells (Thapa et al., 2008). It has been shown that genital epithelial cells are capable of producing CXCL1 in culture in response to HSV-2 infection (Fernandez et al., 2007). Furthermore, CXCL1 expression is also upregulated in the vagina and CNS of i.vag HSV-2-infected mice (Thapa et al., 2008). This chemokine binds to the receptor CXCR2, promoting chemotaxis and activation of neutrophils (Schumacher et al., 1992, Chensue, 2001, Rubio and Sanz-Rodriguez, 2007). Similar to the other chemokines examined during HSV-2 infection, production of CXCL1 increased at D1 p.i., followed by a decrease at D2 p.i. CXCL1 levels in WT and IDO⁻/⁻ mice were similar at all time-points except at D5p.i. when CXCL1 was found at ~2-fold higher levels in IDO⁻/⁻ mice than in WT counterparts (Figure
Taken together, the pro- and anti-inflammatory cytokine and chemokine profiles in WT and IDO⁻/⁻ mice after i.vag HSV-2 infection were largely comparable, with a few notable discrepancies, which will be discussed later.

5.2.7 Dynamics of leukocyte subsets in the ILN following i.vag HSV-2 infection

To further define and compare the immune response in WT and IDO⁻/⁻ mice during i.vag HSV-2 infection, and to determine if a deficiency in IDO affected leukocyte populations in the ILN, WT and IDO⁻/⁻ mice were inoculated with 10⁵ PFU HSV-2 and the ILNs were isolated after 1, 3, 5 and 7 days and analysed by flow cytometry. Unfortunately, due to the limited availability of IDO⁻/⁻ mice, IDO⁻/⁻ mice were only analysed at D3 and D5 p.i. as there were high levels cellular infiltration in the ILN at these time-points (Chapter 3).

As expected, i.vag HSV-2 infection resulted in a substantial increase in the number of total and inflammatory macrophages, CD11b⁺ DC, CD11b⁻ DC, BDC, TDC, pDC and neutrophils. All subsets peaked at D5 p.i. except for the neutrophils which peaked later at D7 p.i. As illustrated in Figure 5.10, no difference was observed in the number of cells in each of the leukocyte subsets examined in the ILN of WT and IDO⁻/⁻ mice at D3 and 5 p.i. (Figure 5.10A–H).

The numbers of B cells, CD4⁺ and CD8⁺ T cells as well as NK and NKT cells in the ILN were also investigated. As shown in Figure 5.11, the numbers of
**Figure 5.9**

*Inflammatory chemokine concentrations in the HSV-2-infected vagina of WT and IDO⁺/⁻ mice*

Protein concentrations of inflammatory (A) CCL2, (B) CCL3, (C) CCL5 and (D) CXCL1 chemokines in the vagina of WT (black circles) or IDO⁺/⁻ (red squares) mice infected i.vag with 10⁵ PFU HSV-2 between 0 and 7 days, as indicated. Chemokine levels are expressed as pg per mg of the total protein after homogenisation of the vaginal tissue. Data is the mean ± SEM of 3-4 mice per group. The experiment was carried out once. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test; *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001, compared to mock-infected WT mice. #p ≤ 0.05, ##p ≤ 0.01 and ###p ≤ 0.001, relative to mock-infected IDO⁺/⁻ mice. ^p ≤ 0.05 and ^^p ≤ 0.001 relative to WT and IDO⁺/⁻ mice at the same time-point.
Figure 5.10

Cellularity of leukocyte subsets in the draining ILN of WT and IDO−/− mice following i.vag HSV-2 infection

Following i.vag infection, the draining ILN of mock- and 10⁵ PFU HSV-2-infected mice were isolated and processed for flow cytometric analysis. (A) numbers of total macrophages, (B) inflammatory macrophages, (C) CD11b⁺ DCs, (D) CD11b⁻ DCs, (E) BDC, (F) TDC, (G) PDC and (H) neutrophil cells in the HSV-2-infected mice for D0, D1, D3, D5 and D7 p.i. Subset numbers are expressed as a proportion of the total number of live ILN cells. Data is the mean ± SEM of 3-5 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test and unpaired t-test with two-tailed option; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to mock-infected WT mice; #p≤0.05, ##p≤0.01 and ###p≤0.001, relative to HSV-2-infected D3 p.i. IDO−/− mice. No difference was observed between WT and IDO−/− mice at the same time-points.
cells in these subsets were largely similar in both WT and IDO−/− mice at D3 and D5 p.i. (Figure 5.11A–E). Treg cells (defined as CD3+ CD4+ CD25+ FoxP3+) were also examined in the ILN of both types of mice. As described earlier, Treg cells are a population of CD4+ T cells that express CD25 and the transcription factor FoxP3 (Sakaguchi, 2005). Treg cells can inhibit the immune response by binding to accessory molecules (e.g., CD80 and CD86) on the surface of APCs and enhancing the production of IDO, which in turn, results in robust immunosuppressive activity (Boasso et al., 2007, Boasso et al., 2005a, Fallarino et al., 2003, Munn et al., 2004b). Analysis of Treg cells in the ILN of HSV-2-infected mice showed that the kinetics of the increase were similar in both WT and IDO−/− mice, with no difference in numbers between WT and IDO−/− mice at D3 and 5 p.i. (Figure 5.11F).

Overall, analysis of ILN cellularity after i.vag HSV-2 infection demonstrated that the leukocyte response on the ILN was very similar between WT and IDO−/− mice, although only D3 and 5 p.i. were examined.

5.3 Discussion

Although there has been a lot of research about the role of IDO in immunity, little is known about its role in in vivo viral infections, and especially in STI. Moreover, from chapter 4, there is high induction of IDO, which suggests that it may play a significant role in the immune response against viral infections in the vagina. In the present chapter, the role of IDO in the induction of inflammation or immune tolerance to viral vaginal infection was assessed and the
Figure 5.11

Cellularity of lymphocyte subsets in the draining ILN of WT and IDO<sup>−/−</sup> mice following i.vag HSV-2 infection

Following i.vag infection, the draining ILN of mock- and 10<sup>5</sup> PFU HSV-2 infected-mice were isolated and processed for flow cytometric analysis. (A) numbers of B cells, (B) CD4<sup>+</sup> T cells, (C) CD8<sup>+</sup> T cells, (D) NK cells, (E) NKT cells and (F) CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> Treg cells in the HSV-2 infected mice for D0, D1, D3, D5 and D7 p.i. Subset numbers are calculated from the total number of live ILN cells. Data is the mean ± SEM of 3-5 mice per group, and represents 2 independent experiments. Statistical analysis was conducted using one-way ANOVA with a Tukey-Kramer post-test and unpaired t-test with two-tailed option; *p≤0.05, **p≤0.01 and ***p≤0.001 compared to mock-infected WT mice; #p≤0.05, ##p≤0.01 and ###p≤0.001, relative to HSV-2-infected D3 p.i. IDO<sup>−/−</sup> mice. No difference was observed between WT and IDO<sup>−/−</sup> mice at the same time-points.
ability of mice that lack the IDO gene to mount an immune response was also examined.

IFN-γ production is a key element of defence against viral infections as IFN-γ decreases virus replication and promotes the development of various immune responses (Katze et al., 2002). IFN-γ can stimulate the upregulation of IDO which catalyses the initial reaction in the kynurenine pathway, the major route of L-Trp degradation in mammals (Becerra et al., 2009).

Several groups have shown that IDO is induced in experimental animal models and human patients during infection with influenza virus (Yoshida et al., 1979), poliovirus (Heyes et al., 1992), hepatitis C (Larrea et al., 2007), LP-BM5 murine leukemia virus (Hoshi et al., 2010), dengue virus (Becerra et al., 2009) and Epstein-Barr virus (Bellmann-Weiler et al., 2008), as well as HSV-1 (Reinhard Jr, 1998). However, the role that IDO plays in vivo is poorly defined.

Although i.vag WNV and HSV-2 infection in mice resulted in significantly different disease outcomes, IDO expression was one similarity between these two differing, but complementary, models of infection.

As with WNV (described in chapter 4), i.vag infection with HSV-2 induced robust expression of IDO mRNA in the vagina. This expression was found to be largely attributable to IFN-γ, as previous studies in our lab have shown that vaginal expression of IDO in response to HSV-2 infection was abolished in IFN-γ−/− mice (Yeung, 2012). IFN-γ mRNA had two waves of upregulation at D2 and D5 p.i. before declining rapidly on D7 p.i. This pattern was similar for IDO mRNA expression (Figure 5.1A–B).

Although IDO expression peaked at D5 p.i. with $1.5 \times 10^4$-fold more mRNA in the vagina compared to mock-infected control animals, IDO protein in
the infected vagina had similar levels of expression on D2 and D5 p.i., suggesting that IDO is similarly regulated at the mRNA and protein levels (Figure 5.1B and 5.2G). Immunofluorescent staining of cross sections of the vagina early after HSV-2 infection (i.e., D1, D2 and D3 p.i.) localised IDO protein mostly to the vaginal epithelium, and interestingly, in some cases, IDO expression co-localised with HSV-2-infected epithelial cells. Strikingly, IDO protein was expressed in uninfected cells in the vaginal stroma on D5 and D7 p.i. During these later time points, HSV-2 infection decreased and was resolved (Figure 5.2A–G). Interestingly, previous studies in our lab investigating the co-localisation of IDO and WNV-NS1 proteins in the WNV-infected vagina showed that WNV-infected epithelial cells did not express IDO protein, but was solely induced in uninfected vaginal epithelial cells surrounding areas of WNV infection (Yeung, 2012).

These results suggest that IDO protein expression can be induced by both innate and adaptive immunity. In the early days following infection, the presence of viral products directly within infected cells may lead to the induction of IDO. However, from D5 p.i. onwards, when IDO was localised in the vaginal stroma, other factors, including soluble or cellular signaling mediators may be required for IDO induction, as some reports indicate that cytokines or cell-to-cell contact is also required for IDO expression (Larrea et al., 2007, Mellor and Munn, 2004, Munn et al., 2004b, Murakami et al., 2013, Yeung et al., 2012).

In contrast to the vagina, IDO seems to be expressed solely by endothelial cells in the ILN of mock- and HSV-2-infected animals. We are currently undertaking IF staining to co-localise IDO protein and endothelial cells. This will further confirm that IDO is expressed by endothelial cells in the ILN. Although this expression fluctuated during infection, changes between mock controls and
infected animals were insignificant, suggesting that IDO has little or no effect on the migration or proliferation of leukocytes in the ILN (Figure 5.3A–G). Little is known about the role of IDO in the draining ILN, however, several studies have shown the regulatory effects of IDO expression by endothelial cells during systemic infection. For example, expression of IDO by pDCs correlates with immunosuppression in the tumor draining LN (Munn et al., 2004a). Moreover, L-Trp metabolism to Kyn via IDO contributes to vascular relaxation and the regulation of blood pressure in systemic inflammation and infection (Wang et al., 2010). More interestingly, a study by Rudiger and colleagues show that IDO activity in brain endothelial cells might mediate three different effects during HSV-mediated encephalitis, including the control of L-Trp influx into the brain, the regulation of T cell responses and the direct restriction of viral growth (Rüdiger et al., 2005).

HPLC analysis of the vagina and ILN demonstrated that the enzyme was capable of decreasing L-Trp levels in both tissues to generate Kyn. However, this activity was not evident until D5 p.i., corresponding to the peak of leukocyte infiltration in response to i.vag HSV-2 infection. Kyn quantities in the vagina peaked at D6 p.i., closely correlating with the virus clearance at this site. In the ILN, Kyn levels peaked at D8 p.i. (Figure 5.4A–B). These observations suggested that IDO could contribute to the antiviral mechanisms in the vagina while reducing the inflammation and controlling the immune response in the draining LN. Considering that there was no Kyn in the mock-infected control mice, this highly elevated Kyn concentration in the vagina and ILN suggested that Kyn quantity or Kyn/L-Trp ratio was more crucial factor in regulating immune
response than just reduction in the concentration of L-Trp (Lyon et al., 2011, Darcy et al., 2011).

Interestingly, even though the vagina is colonised by commensal organisms (Round et al., 2010), and microorganisms are known to induce IDO in vivo, no IDO mRNA, protein or activity was detected in the vagina of mock-infected animals. This is in contrast to other mucosal environments including GI tract, where there is IDO activity in low levels during steady state, presumably as a result of continued exposure to foreign antigens. This activity in the GI tract is enhanced following inflammation and infectious disease (Cherayil, 2009).

To further determine the role of IDO in i.vag viral infection, the kinetics of HSV-2 infection and subsequent pathology in the vagina were investigated using IDO gene knockout mice. Surprisingly, despite the fact that active IDO was expressed locally in the site of infection and in the draining LN, no change was observed in the vaginal virus titre in IDO−/− mice. Furthermore, the survival rate and disease severity in IDO−/− mice were similar that of WT mice (Figures 5.5 and 5.6). These observations contrast with other studies showing that the absence of IDO activity enhances the clearance of influenza virus-infected macrophages (Huang et al., 2013) or reduces viral replication in animal models of HIV-1 encephalitis (Potula et al., 2005) and LP-BM5 murine leukemia virus infection (Hoshi et al., 2010).

Taken together, these data show that IDO does not have a major role in inhibiting virus growth and lacking IDO does not affect the survival rate or disease pathology in the i.vag HSV-2 model of infection using 2 different viral titres. Whether this slight differential observation may exist at virus titres lower than these such as $10^3$, $10^2$ and $10^1$ PFU of HSV-2, would be useful to explore in
the future. Unfortunately, the lower titres were not tested in this study due to the lack of IDO knockout mice. Viral doses used in the previous studies showed maximum immune responses in the vagina and ILN. Therefore, to investigate the IDO activity and its effect on the vaginal immune response, it was first necessary to apply higher viral doses to match the results with other studies.

As described earlier, several groups have reported that IDO is involved in the production of Treg cells (Mezrich et al., 2010, Fallarino et al., 2006). Although the exact pathway by which IDO induces the generation of Treg cells has been debated, both L-Trp depletion and the direct effects of Kyn and other metabolites have been suggested (Frumento et al., 2002, Fallarino et al., 2006).

Furthermore, the IDO-AhR interaction was of interest in light of the observation that IDO may be upregulated by AhR (Jux et al., 2009, Vogel et al., 2008) and that Kyn and related metabolites may be AhR agonists (Denison and Nagy, 2003, DiNatale et al., 2010). In addition, AhR mediates the production of the anti-inflammatory cytokine IL-10 via a subset of CD4+ T cells (Apetoh et al., 2010, Rutz and Ouyang, 2011, Nguyen et al., 2013). Therefore, it is likely that, among other pathways, IDO is indirectly involved in IL-10 induction.

Therefore, the induction of AhR and IL-10 mRNA were analysed in the vagina. Interestingly, except D1 p.i. when AhR mRNA in IDO−/− mice were higher than in their WT counterparts, the level of AhR expression in the vagina of both strains of mice did not change throughout infection. Despite similarity in AhR levels, IL-10 was significantly higher in IDO−/− mice during i.vag HSV-2 infection, especially at D2, D5 and D7 p.i. (Figure 5.7).
It is tempting to speculate that the increase in IL-10 gene expression within the vaginal mucosa during infection is a compensatory response to IDO deficiency.

Based on the observation that IL-10 mRNA in IDO−/− mice was elevated during infection, it was hypothesised that other cytokines and chemokines within the vaginal tissue were also differentially expressed. Cytokines are involved in the differentiation of various leukocytes, for example, the differentiation of naïve CD4+ T cells into effector Th1, Th2, Th17 cells or immunosuppressive Treg cells, depending on the nature of the antigenic stimulation (e.g., intracellular/extracellular pathogen) and the predominant cytokine milieu. Differentiation into Th1 cells is associated with the production of IL-12 by macrophages and DC, which then subsequently secrete large amounts of IFN-γ, IL-2 and TNF, in addition to GM-CSF, and small levels of IL-10.

In contrast, production of IL-4 by activated T-cells after receiving signal leads to Th2 differentiation and inhibition of Th1 cells by the producing of high levels of IL-4, -5 and -13, as well as IL-6 and IL-10. Thus, the primary function of Th1 cells is the activation of macrophages as well as stimulating the production of opsonising and complement-fixing antibodies by B cells, cell cytotoxicity and the induction of cell-mediated immunity. On the other hand, Th2 cells mainly evoke robust antibody responses (especially IgE), eosinophil differentiation and activation and provide a form of phagocyte-independent inflammation (by inhibition of phagocytic cell functions) (Romagnani, 2000).

As previously mentioned, there is extensive overlap in the types of immune cells that infiltrate in response to CCL2, CCL3 and CCL5. Once expressed, these inflammatory chemokines recruit a diverse array of effector cells
including NK cells, T cells, B cells, myeloid cells as well as macrophages and DCs to the site of infection (Appay and Rowland-Jones, 2001, Levy, 2009, Maurer and von Stebut, 2004, Peretti et al., 2005, Iijima et al., 2011, Geissmann et al., 2003). In addition, the influx of neutrophils from the blood and their interaction with the vascular endothelium at the site of infection or damage is an early step of acute inflammation. CXCL1, which is proposed be a functional homologue of human IL-8, is a CXC-type chemokine and is associated with neutrophil recruitment and inflammation (Rubio and Sanz-Rodriguez, 2007, Schumacher et al., 1992).

In our studies, surprisingly, the cytokine and chemokine response over the course of infection in WT and IDO\(^{-}\) mice did not differ significantly. Analysis of pro-inflammatory cytokines, namely IFN-\(\gamma\), TNF, GM-CSF, IL-12 and IL-17 and chemokines including CCL2, CCL3, CCL5 and CXCL1 showed similar responses in both WT and IDO\(^{-}\) mice. IFN-\(\gamma\) was upregulated in two waves, on D1 and D5 p.i.; however, no difference was observed between the two strains of mice. In addition, the protein levels of other predominantly inflammatory cytokines including TNF, GM-CSF and IL-17 did not change during the infection time course and remained at the baseline levels. Although, IL-12 protein levels in WT mice were elevated on D2 p.i., it was not significantly higher than levels in IDO\(^{-}\) mice (Figure 5.8A–E).

Infection with HSV-2 resulted in significant upregulation of chemokines, peaking at D1 p.i. in both WT and IDO\(^{-}\) mice. However, CCL2 and CXCL1 were higher in KO mice than WT mice at D5 p.i. On the other hand, CCL5 in WT mice was significantly higher than IDO\(^{-}\) mice on D1 p.i. Interestingly, no difference was observed in CCL3 in both types of mice at all time points (Figure 5.9).
Taken together, while the expression of pro-inflammatory cytokines and chemokines had some differential expression at particular time-points, the overall pattern of their expression was largely comparable in both strains.

Corresponding to the similar pro-inflammatory cytokine and chemokine concentrations in the vagina between the two mouse strains, the cellular response in the ILN did not differ between WT and IDO−/− mice. In other words, the numbers of cells of the main IFN-γ producers including NK cells, CD4+, CD8+ T cells and NKT cells, were similar in the ILN of both types of mice (Figure 5.11B–E). Numbers of macrophages and DCs as well as neutrophils were also similar in both mouse strains, at least at D3 and D5 p.i. (Figure 5.10A–H).

Analysis of the levels of anti-inflammatory cytokines (IL-4, IL-6 and IL-10) in the vagina of HSV-2-infected mice showed that the results are in accordance with the findings of other groups, emphasizing the upregulation of inflammatory cytokines/chemokines inhibits the differentiation of immune effector cells to produce anti-inflammatory cytokines. Accordingly, as IFN-γ and IL-4 are mutually inhibitory (Abbas and Lichtman, 2004, Mosmann and Coffman, 1989), the relatively low levels of IL-4 throughout infection are likely the result of high IFN-γ production in the vagina. Although IDO−/− mice had more IL-4 protein in the vagina, it declined gradually by D7 p.i., while WT mice had the opposite dynamic (Figure 5.8A and 5.8F). Moreover, IL-6 can suppress TNF expression (Scheller et al., 2011), possibly contributing to the low concentrations of this cytokine as well. Thus, in the absence of high TNF expression, IL-6 protein peaked at D1 p.i. before a steep drop to the baseline levels that was maintained for the rest of the time course. However, IDO−/− mice significantly upregulated IL-6 concentration at D5 p.i. compared to WT mice (Figure 5.8B and 5.8G). IL-10 is
known to be broadly immunosuppressive (Sabat, 2010). This cytokine is produced by CD4+ Th2 cells, monocytes, Treg cells and B cells (Tsai et al., 2013, Wilson and Brooks, 2011). IL-10 was differentially expressed in WT and IDO−/− mice. Surprisingly, IL-10 mRNA copies were significantly higher in the IDO−/− vagina over the HSV-2 infection time course. In contrast, IL-10 protein was significantly upregulated in WT mice and remained elevated until D3 p.i., before reducing gradually while this response in IDO−/− mice was delayed until D5 p.i., where, the protein increased 3-fold relative to D0 p.i. At D5 p.i. and after, the concentration of IL-10 was similar in both strains of mice (Figure 5.8H). The reason for the difference between the levels of IL-10 mRNA and protein expression is not clear.

IL-10 mRNA is induced by a wide range of cell types, however, the amount and level of the translated IL-10 protein remains undetectable for many of them (Powell et al., 2000, Couper et al., 2008). It is widely known that IL-10 is subject to the strict post-transcriptional modification (Stoecklin et al., 2008, Sharma et al., 2009, Le et al., 1997). For instance, there are several AU rich motifs in the 3′-untranslated region of IL-10 mRNA that are the targets for microRNAs (Schulte et al., 2011) or destabilising elements such as tristetraprolin (TTP, RNA binding protein that rapidly degrades mRNA containing AU-rich elements) (Stoecklin et al., 2008, Teixeira-Coelho et al., 2014). Moreover, IL-10 mRNA stability can be regulated by signals through transcription factors including Sp1 (specificity protein 1), Egr1 (Early growth response protein 1) (Sharma et al., 2009) and STAT-3 (Gaba et al., 2012) and also by TLR2 and TLR-4 that are expressed on activated macrophages (Teixeira-Coelho et al., 2014).

Even though protease inhibitor was used to protect the total protein concentration in the vagina, experimental procedures such as mechanical
homogenisation of the vaginal tissue may have a detrimental effect on the stability of the vaginal proteins and alter or degrade the protein concentration in the vagina at least to some degree. Moreover, this issue might arise from the poor sensitivity of methodological assessments, as mouse IL-10 protein quantification techniques are only able to detect changes in the IL-10 protein expression within the target tissue over the level that is expected.

Although the effect of Th2 cytokine secretion on the activation and differentiation of B cells and Treg cells was not investigated in the vagina in this series of experiments, the absolute numbers of these subsets were enumerated in the ILN. However, no difference in these subsets was observed between WT and IDO<sup>−/−</sup> mice, suggesting a similar immune response to i.vag infection in knockout and WT mice (Figure 5.11A and F). It should be noted that there might be differences at the other time-points that we were unable to investigate. Unfortunately, limited availability of IDO<sup>−/−</sup> mice prevented us from analysing all the time-points in this study.

Indeed, many reports suggest that IDO affects the function and activity of immune cells. For instance, IDO alters NK, NKT and T cell polarisation and enhances the production of Treg cells (Baban et al., 2009, Chen et al., 2008, Reeves et al., 2011, Fallarino et al., 2006). In addition, IDO can interfere with the DC maturation process (Xu et al., 2008). Importantly, IDO expression changes stimulatory DC into immunosuppressive DC (Terness et al., 2006, Mellor and Munn, 2004). While this was not directly examined in this study, based on the observation that cellularity in the ILN of both mouse strains were similar, it suggests that IDO deficiency does not affect the recruitment or expansion of
inflammatory cells. However, we did not investigate the effect of IDO on the function of these cells.

Collectively, the work presented in the current chapter provides a detailed analysis of IDO induction and activity following HSV-2 infection in the vagina. We showed that IDO mRNA and protein is expressed in the vagina in response to HSV-2 infection. Importantly, in the vagina, IDO activity had no major effect on viral replication during the early stages and had little effect on the survival of mice and the migration of leukocytes to the site of infection. IDO activity in the ILN was also found to have no effect on the proliferative T cell responses. Therefore, further investigation of the effect of L-Trp consumption and the generation of Kyn and other downstream metabolites on leukocyte function is needed. In addition, whether IDO is an in vivo antiviral molecule or whether it stimulates the immunoregulatory pathway is also of interest. This will allow us to determine how to best exploit the induction of IDO for the resolution of infection while controlling the inflammatory responses in the site of infection.
Chapter 6

General discussion
Chapter 6:

General discussion

Viral sexually transmitted infections have a major negative impact on sexual and reproductive health worldwide. Despite extensive research into the virology, prevalence and pathogenesis of STI, the mechanisms involved in the initiation and priming of immune responses in the female genital tract to viruses such as HPV, HIV and HSV, remain poorly understood. It has been confirmed that HSV-2 infection is linked with HIV acquisition (Freeman et al., 2006, Johnston et al., 2011). The seroprevalence of HSV-2 in developed and developing countries are still rising, particularly among young adults (Cunningham et al., 2006). Moreover, recent efforts to develop an effective vaccine against HSV-2 have failed (Cohen, 2010). Thus, this necessitates a better understanding of the mucosal immune response and the host-virus interaction in the vagina. This may inform antiviral approaches to ameliorate or abrogate infection.

The most commonly studied murine STI model is of HSV-2 infection. This has been extensively used in numerous studies to delineate different aspects of immune response initiation and the factors mediating the host defence against HSV-2 vaginal infection. Given i.vag HSV-2 infection in mice is not exactly analogous to the infection in humans, we employed a murine model of STI using the flavivirus, WNV, which is known to be resolved in the vaginal mucosa by D7 p.i. (Burke et al., 2004), without spread to the CNS and mortality in the C57BL/6
model, in contrast to the BALB/c mouse in which some mortality occurs.

We investigated various elements of the innate and adaptive immune responses using WNV and HSV-2 models of vaginal infection focusing particularly on the factors that mediated the initiation of immune response. Moreover, we analysed the differential induction of chemokines and cytokines in two different mouse strains, i.e., BALB/c and C57BL/6 mice. In addition, the potential role of the tryptophan-degrading enzyme, IDO, in response to vaginal HSV-2 infection was also investigated.

6.1 Comparison of the HSV-2 and WNV models of vaginal infection

HSV-2 infection in humans begins with productive vaginal epithelial infection, which rapidly spreads to the peripheral ganglia and establishes life-long latency with recurrent shedding. However, i.vag challenge of mice with HSV-2 results in severe illness and a high morbidity rate, as reported previously (Parr et al., 1994) and confirmed in Chapter 3. In our study, HSV-2 mRNA copy numbers peaked at D1 and D3 p.i, with the high ($10^5$ PFU) or low ($10^4$ PFU) viral inoculation dose, respectively. With both doses, HSV-2 infection resolved in the vagina by D7 p.i. and were comparable in terms of morbidity and mortality, as 10% of mice survived primary challenge, denoting further uncontrolled spread of HSV-2 beyond the epithelium. It is possible that the dose-independent high lethality is perhaps a result of a breach in the epithelial layer and CNS attack by HSV-2 (Parr and Parr, 2003). As with HSV-2 infection, WNV productively infected the epithelial layer and was eradicated from the vaginal mucosa by D7
Infection with HSV-2 in most individuals is associated with no or very mild symptoms. Similar to human HSV-2 infection, murine vaginal WNV infection does not cause detectable morbidity or mortality in C57BL/6 mice, even with high titre inoculation of WNV (3.6 x 10⁷ PFU). In this model, WNV infection peaked 2 days later than in HSV-2 infection at D5 p.i. and was essentially confined to the epithelial layer. WNV is a neurotropic virus and likely gains access to the CNS by retrograde axonal transport (King et al., 2007). WNV infection caused neurological disease and is highly lethal in mice if inoculated by other routes, including intraperitoneal, intranasal, intravenous or intradermal routes (King et al., 2007). Also, a previous study in this laboratory using BALB/c mice showed that i.vag WNV infection is associated with neurological involvement, resulting in 12% mortality (Burke et al., 2004), indicating the greater susceptibility of this strain to CNS infection by WNV via this route of inoculation than C57BL/6 mice. Therefore, this present study demonstrates that C57BL/6 mice are a better strain in which to investigate the early anti-viral immune response in the vaginal mucosa, as C57BL/6 mice only have a mild and self-limiting vaginal infection is cleared by D7 p.i. without epithelial barrier breakdown and with intact early defence mechanisms during infection.

Previous studies investigating the initiation of the immune response in the vagina showed that both i.vag HSV-2 and WNV infection elicited redistribution of MHC II⁺ cells underneath the epithelium at the infection sites (Burke et al., 2004, Parr et al., 1994, Parr and Parr, 1998). Moreover, Zhao and colleagues reported that CD11b⁺/CD11c⁺ sub-mucosal DCs contribute to the subepithelial
clusters under areas of HSV-2 infection (Zhao et al., 2003). Similar to these observations, we showed in this study that a large numbers of F4/80⁺, CD11b⁺ and CD11c⁺ macrophages and DCs were rapidly recruited to the site of WNV and HSV-2 infection in the vagina of both C57BL/6 and BALB/c mice and formed clusters beneath foci of infection, presumably helping to contain infection at these sites. Even though these macrophages and DCs were abundant in the sub-mucosa, the clusters of cells underneath the epithelium were mainly comprised of CD11c⁺ and not F4/80⁺, CD11b⁺ macrophages/DCs.

In order to investigate the similarities and differences in the immune response following WNV and HSV-2 infection, the kinetics of leukocyte infiltration into the vagina and its draining LN were analysed. Again, both WNV and HSV-2 infection recruited similar numbers of immune effector cells to the vagina. Despite the more inflammatory nature of HSV-2 infection, causing desquamation of the epithelial layer and severe pathology, the total macrophage numbers as well as the other leukocyte subsets in the vagina were mostly comparable in both models of infection. Other similarities in both of these models of infection were the abundance of Th1-type cells and IFN-γ-producing cells in the vagina, including CD4⁺ T cells, NK and NKT cells.

However, a few notable discrepancies were observed in this study. In the vagina of WNV-infected mice, numbers of CD11b⁺ DCs and B cells were significantly higher. Even though CD11b⁺ DCs and CD11c⁻ macrophages are considered to be monocyte-derived APCs, Iijima and co-workers demonstrated that these subsets are not required to elicit robust T cell responses in HSV-2-infected mice, as isolated CD11b⁺ DCs and CD11c⁻ macrophages from the
infected vagina were unable to activate naïve T cells (Iijima et al., 2011). Furthermore, despite the contribution of B cells to the anti-viral response by producing virus-specific antibodies, these cells are also dispensable for clearance of HSV-2 virus from the vagina (Dudley et al., 2000, Parr and Parr, 2000).

Thus, given the fact that C57BL/6 mice are more resistant to WNV infection than HSV-2, the greater numbers of B cells in C57BL/6 mice may have a more critical and effective role in the protection of mice against WNV. This is certainly true in WNV encephalitis (Diamond et al., 2003). Iijima and colleagues showed that B cells, together with DCs, might constitute “memory APCs” and help to maximise Th1-type memory responses through BCR-specific uptake of antigen (Iijima et al., 2008a). We also observed a significant increase in the numbers and percentages of neutrophils in the WNV-infected vagina but not in the HSV-2 model. This is in contrast with other reports that showed a high recruitment of neutrophils to the site of HSV-2 infection and suggested a critical involvement of neutrophils in HSV-2 clearance and inhibition of virus spread to the sensory ganglia (Milligan, 1999, Milligan et al., 2001). Although the infiltration of neutrophils was not investigated thoroughly at all time points in this study, it is hypothesised that these cells could have a more effective role in protecting the epithelial layer and clearing virus-infected cells from the WNV-infected vagina.

Both models also elicited similar levels of cellular expansion in the ILN. Previous studies have demonstrated that the immune response in the vagina is initiated in the ILN (Banchereau and Steinman, 1998, King et al., 1998). It has been shown that CD11b− CD11c+ DCs are recruited close to the vicinity of the
infected epithelium. Here, they may acquire viral antigens and on subsequent migration to the ILN they could stimulate a Th1-type immune response against HSV-2 (Zhao et al., 2003). Although monocyte-derived CD11b⁺ CD11c⁺ DCs and CD11b⁺ CD11c⁻ macrophages are dispensable for T cell responses against primary infection, these cells are required for re-stimulating Th1 CD4⁺ T cells to secrete IFN-γ (Iijima et al., 2011, Iwasaki, 2003). Although the extent of T cell stimulation and IFN-γ production were not investigated in this study, both WNV and HSV-2 models of infection elicited similar levels of infiltration of CD11b⁻ and CD11b⁺ DCs, as well as CD4⁺ T cells subsets in the ILN. Moreover, the observation that the overall initiation, peak and resolution of effector leukocytes in the vaginal mucosa was coincident with the changes in the ILN in both models of infection, suggests that, in both models, there were likely to be similar levels of viral antigen uptake in the vagina and T cell stimulation in the ILN.

Several groups have shown that APCs migrating into a foci of infection, particularly macrophages and DCs, originate in the bone marrow (Diao et al., 2004, Iijima et al., 2007, Iijima et al., 2011, Iijima et al., 2008b, Iwasaki, 2007). In line with these reports, we also showed that effector immune cells in the vagina and ILN of WNV- and HSV-2-infected mice originated, at least in part, from bone marrow precursors. Both models of infection similarly elicited the predominant involvement of APCs, including total and inflammatory macrophages, CD11b⁺ DCs and B cells in the vagina and ILN.

Taken together, even though WNV and HSV-2 are strikingly different (small ssRNA vs. complex dsDNA, respectively), a comparison between the two infection models discussed here reveal that vaginal epithelial infection in both
models initiates a robust immune response that is remarkably similar locally in the vagina and also in the associated lymphoid tissue, with few differences. Given the advantages of using the non-lethal WNV model of infection to investigate mucosal immunity, this may allow us and other groups to elucidate the immune mechanisms involved in the early protection of the genital tract to prevent STI, as well as to inform the potential development of safe and effective vaccine candidates.

6.2 Comparison of BALB/c and C57BL/6 mice during WNV vaginal infection

The murine model of HSV-2 vaginal infection was first developed in BALB/c mice using lethal WT lethal HSV-2 and a non-lethal thymidine kinase-deficient (TK–) HSV-2 strain (McDermott et al., 1984, Parr et al., 1994). Unlike C57BL/6 mice, which elicit robust Th1-type immune responses, BALB/c mice are a prototypical Th2-type strain. Previous studies in this laboratory have shown that these mice are more susceptible to WNV infection, even when infected with a 100-fold lower viral titre (Burke, 2005). Although, there is 12% mortality in BALB/c mice after intravaginal WNV infection, no evidence of recurrent WNV infection or viral shedding in the vagina is observed. More over, all surviving BALB/c mice show complete immunity against WNV rechallenge even with other routes of infection such as intradermal or intraperitoneal inoculation, which is in contrast with the HSV-2 model of infection (Burke et al., 2004). In investigation of the similarities and differences of the immune response in the vagina and ILN of WNV-infected BALB/c and C57BL/6 mice revealed that both
strains were fairly similar with respect to the initiation of the immune responses in the vagina and ILN. Thus, similar to what was observed in C57BL/6 mice, clusters of CD11c⁺ DCs, but not CD11b⁺ and F4/80⁺ macrophages/DCs, are evident immediately underneath the WNV-infected epithelium in the vagina of BALB/c mice. Moreover, in BALB/c mice, there is a similar response in the recruitment of leukocyte subsets to the ILN, including inflammatory macrophages, CD11b⁺ DCs and CD8⁺ T cells as shown in Chapter 3 for i.vag WNV-infected C57BL/6 and BALB/c mice (Burke et al., 2004). Despite the similarities however, more epithelial cells in the vagina of BALB/c mice are infected with WNV, confirming that BALB/c mice are more susceptible to vaginal WNV infection.

CD4⁺ T cells comprise the predominant lymphocyte subset in the ILN of BALB/c mice, while B cells are the major population in the ILN of WNV-infected C57BL/6 mice. These discrepancies presumably reflect the genetically distinct background of the two strains (López et al., 2001, Watanabe et al., 2004), but could influence early responses and ultimate outcomes in each strain. Furthermore, chemokine and cytokine responses reveal that these two strains respond differently to i.vag WNV infection. Typical Th1-type responses are characterised by the high production of IFN-γ (Abbas and Lichtman, 2004, Schroder et al., 2004, Mosmann and Coffman, 1989). Therefore, after i.vag WNV infection, C57BL/6 mice, which elicit the Th1-type responses, produce more IFN-γ, while BALB/c mice express more TNF, likely downregulating IFN-γ-priming and Th1 responses in this strain (Hernandez-Pando and Rook, 1994, Hodge-Dufour et al., 1998).
The profiles of the chemokine responses in both strains are similar for CCL3 and CCL5. As previously described, these chemokines are involved in the recruitment of NK cells, T cells, B cells and myeloid cells to the site of infection (Appay and Rowland-Jones, 2001, Levy, 2009, Maurer and von Stebut, 2004, Peretti et al., 2005). Therefore, it is not unexpected that there were similar numbers of cells in these subsets in the vagina and ILN of both strains.

In contrast, striking differences were observed between BALB/c and C57BL/6 with respect to the chemokines principally responsible for macrophage infiltration, DC maturation and migration to the secondary lymphoid tissue. Differential expression of CCR7-CCL19/21, CCR6-CCL20 and CCL2-CCR2 receptor-ligand pairs suggest that each mouse strain uses different pathways to recruit monocyte-derived macrophages and DCs to the site of infection, stimulate their maturation, and to elicit the migration of these cells from the site of infection towards the draining LN to prime T cells in response to i.vag WNV infection.

In this study, we observed higher expression of CCL20 in WNV-infected BALB/c mice. CCL20 is involved in the recruitment of immature DCs to the mucosal sites in both steady-state and during inflammation or infection (Williams, 2006, Kallal et al., 2010, Berlier et al., 2006, Cremel et al., 2005, Iwasaki and Kelsall, 2000). On the other hand, CCL2, which belongs to the family of inflammatory chemokines (Ansari et al., 2006, Ansari et al., 2011, Tsui et al., 2007), is highly expressed in C57BL/6 mice, indicative of the more inflammatory response in this strain following WNV infection.

Due to the differential expression of these chemokines, we conducted neutralisation studies to delineate the effect of these chemokines locally in the site
of WNV infection and at the draining LN. Therefore, the increased epithelial WNV infection in the vagina of BALB/c mice, together with a trend towards a reduction of sub-mucosal CD11c+ cells, suggest a local role for CCL20 in the vagina, although no statistically significant differences were seen between treated and mock-treated groups. On the other hand, CCL2 neutralisation in C57BL/6 mice dramatically impaired the recruitment of leukocytes not only to the vagina but also to the ILN. Interestingly, the spleen was not evidently involved in this inflammatory reaction, indicating that CCL2 likely functions locally in the infection site and associated lymphoid tissues. This may be a result of CCL2 expression driven by local infection, as occurs in the brain and skin (Davison and King, 2011, Getts et al., 2008).

The cytokine expression profiles and the kinetics of leukocyte infiltration in response to WNV infection demonstrated that C57BL/6 and BALB/c mice respond differently by the same virus to vaginal infection. This differential response must presumably arise from the distinct genetic background that triggers Th1- or Th2-type immune responses in these strains. Thus, investigating these differences in both models may be useful to delineate different aspects of immune response in the mucosal sites to understand the full spectrum of immune mechanisms available to combat STI in humans, comprising widely disparate genetic populations.
6.3 The effect of polystyrene bead administration in i.vag WNV infection

As described in Chapter 4, administration of polystyrene beads have been used to track migrating myeloid lineage cells in the circulation and in inflamed and infected tissue (Getts et al., 2008, Iijima et al., 2011, Randolph et al., 1999, Tacke and Randolph, 2006). However, in our laboratory, this technique has also been used as a therapeutic tool (Terry, 2012). It has been shown that inflammatory macrophages (Ly6C\textsuperscript{hi}) emigrate in large numbers from the bone marrow and rapidly infiltrate the infected tissue in a CCR2-dependent manner (Getts et al., 2008, Iijima et al., 2011). In an i.nas model of WNV encephalitis, i.v. injection of beads reduces the number of Ly6C\textsuperscript{hi} macrophages infiltrating into the brain, resulting in decreased brain pathology and extended survival of WNV-infected mice (Terry, 2012). It has also been reported that beads with different sizes in diameter have distinct imprints and differentially modulate immune responses. Alveolar and non-alveolar macrophages, B cells, and CD11b\textsuperscript{+} and CD103\textsuperscript{+} DC in the lung preferentially internalise 50nm nanoparticles but not 500nm microparticles and consequently inhibit adaptive allergen-specific immunity and decrease the quantity of CD11b\textsuperscript{+} MHCII\textsuperscript{hi} DCs in the draining LN (Hardy et al., 2013) On the other hand, another study showed that 500nm microparticles bearing encephalitogenic myelin epitopes prevented a mouse model of multiple sclerosis (EAE) from clinical autoimmune symptoms and induced long-term T cell tolerance (Getts et al., 2012a).

In this study, injection of 50nm polystyrene beads in i.vag WNV-infected mice reduces the recruitment of CCR2\textsuperscript{+} macrophages and neutrophils to the
However, in the ILN of infected mice, we observed decreased infiltration of a diverse array of myeloid and lymphoid lineage cells. Interestingly, 65% of all bead⁺ cells in the spleen are comprised of B cells, likely due to their ability to internalise foreign Ag and APC activity (Chen and Jensen, 2008, Kurt-Jones et al., 1988, Morris et al., 1994, Rivera et al., 2001, Getts et al., 2012a). However, changes in phenotype and activity of B-lymphocytes following beads internalisation remains to be further investigated. Therefore, bead⁺ cells become sequestered in the spleen despite the lack of cellular change occurring there during this infection. This observation suggests that cells take up beads in the circulation, become trapped in the spleen and are consequently prevented from infiltrating the infected tissue.

6.4 The role of IDO

Production of IFN-γ is the hallmark of Th1-type immune defence against viral infections (Katze et al., 2002). IFN-γ is highly expressed in the WNV-infected vagina of C57BL/6 but not BALB/c mice. As in i.vag WNV infection, one the most prominent downstream effects of IFN-γ production is the upregulation of enzymatically active IDO enzyme in the HSV-2-infected vagina. This expression is not limited to the epithelial layer; thus, some cells in the stroma also express active IDO following HSV-2 infection. Although IDO is expressed in the draining LN of the HSV-2-infected vagina, this is solely confined to endothelial cells and IDO levels in the ILN did not change during the infection time course. Even though the role of IDO-expressing endothelial cells in this model of infection is unclear, other groups have proposed both
immunosuppressive (Wang et al., 2010) or anti-viral (Rüdiger et al., 2005) effects following systemic infection or HSV-induced encephalitis, respectively. Moreover, the concentration of Kyn and the Kyn/L-Trp ratio in the vagina and ILN increased after i.vag HSV-2 infection, confirming that IDO was functional at these sites. Unexpectedly, the concentration of L-Trp also increased at some time points, correlating with the resolution of viral infection from the vagina. Previously, Wang et. al., showed that L-Trp is involved in arterial relaxation via an IDO- and endothelium-dependent mechanism following infection-induced increase in blood pressure and inflammation (Wang et al., 2010). Thus, the increased blood pressure after infection and the requirement of the L-Trp in the endothelium for Kyn production by IDO and subsequent arterial dilation could be taken to suggest that the observed increase in the levels of L-Trp in the vagina and ILN of HSV-2-infected animals may help alleviate systemic inflammation caused by HSV-2 infection.

In this study, we attempted to characterise the immunosuppressive and possible anti-viral function of IDO by using IDO gene knockout mice. However, the absence of IDO activity had minimal or no effect on the vaginal virus titre, survival rate or disease pathology. This observation is in contrast with others showing at least some degree of anti-viral activity in the IDO⁻/⁻ mice (Huang et al., 2013, Potula et al., 2005, Hoshi et al., 2010). It is possible that HSV-2 doses lower than were used in this study may exert a differential effect. Although this was not tested in this project, it would be beneficial to examine this possibility in the future.

There was extensive overlap in the cytokine and chemokine expression
profile in the vagina of HSV-2-infected WT and IDO⁺ mice. Only the IL-10, an immunoregulatory cytokine, was differentially expressed in WT and IDO⁺ animals. While IL-10 mRNA is significantly higher in IDO⁺ mice, however, expression of this cytokine at the protein level was actually higher in WT mice. Although the reason for this is unclear, we are currently investigating whether IL-10 mRNA is subject to the post-transcriptional modification (Le et al., 1997, Sharma et al., 2009, Stoecklin et al., 2008, Teixeira-Coelho et al., 2014). In addition, using other experimental procedures to measure IL-10 protein such as blotting techniques could give an insight into this paradox.

The leukocyte dynamics in the ILN in response to i.vag HSV-2 infection were also largely similar between WT and IDO⁺ mice. This similarity extended not only to the inflammatory subsets or lymphocytes but also to the number of Treg cells. However, similarity of leukocyte numbers in the ILN does not prove that these cells are also functionally similar. Even though we did not investigate the function of infiltrating cells in the ILN of infected WT and IDO⁺ mice, there are some reports indicating that IDO expression can alter the polarisation of NK, NKT and T cells (Baban et al., 2009, Chen et al., 2008, Reeves et al., 2011, Fallarino et al., 2006). Moreover, IDO interferes with the DC maturation process and changes stimulatory DC into immunosuppressive DC (Xu et al., 2008, Mellor and Munn, 2004, Terness et al., 2006). The study that showed IDO is induced in murine DCs after the ligation of B7 Ag to CTLA-4 Ig (expressed on Th-2 type CD4⁺ cells), which supports the immunoregulatory role of IDO in tolerogenic DCs. Whereas, binding of B7 antigen to CD28 (expressed on Th-1 type CD4⁺ cells), leads to the maturation of stimulatory DCs (Munn et al., 2004b, Grohmann et al., 2002).
Collectively, given that IDO is highly induced by one of the most important and effective anti-viral cytokines, IFN-\(\gamma\), it is surprising that we found minimal or no IDO-mediated anti-viral or immunosuppressive responses. Although we showed that IFN-\(\gamma\)-induced (Yeung, 2012), enzymatically active IDO protein in the vagina and ILN of i.vag HSV-2-infected animals, we were unable to determine its predominant role or its contribution to immune response against HSV-2 in this STI model of infection.

6.5 Conclusion

The observations made in this study have enabled us to propose a model describing how the immune system responds to viral infection in the vaginal mucosa (Figure 6.1). In particular, data from this study further the previous findings and show similar innate and adaptive immune responses against two genetically distinct mouse models of STI infections and by two quite different viruses. More specifically, bone marrow-derived leukocytes infiltrated the site of infection and the ILN in much the same way and the redistribution and aggregation of vaginal sub-mucosal DCs in response to a mild and self-limiting WNV infection were comparable to the highly lethal HSV-2 model. The kinetics of other leukocyte subset recruitment to the vagina and ILN of infected mice were similar in these models of infection with relatively few discrepancies. Similarly, vaginal WNV infection in Th1 and Th2 prototype mouse strains revealed that the aggregation of vaginal sub-mucosal DCs and the dynamics of leukocyte infiltration to the vagina and ILN were largely comparable. However, the cytokine and chemokine responses to i.vag WNV infection were remarkably different.
Infection of vaginal epithelial cells stimulates the expression and secretion of a number of pro-inflammatory cytokines and chemokines, including CCL2, CCL3, CCL5, TNF and IFN-\(\gamma\) in the vagina. These inflammatory factors can then recruit effector immune cells from the local microenvironment via chemotactic gradients to initiate the immune response. Moreover, inflammation can be sensed from remote sites such as the bone marrow since pro-inflammatory elements also enter the circulation. Specifically, large numbers of macrophage/DC precursors egress from the bone marrow in response to CCL2 secretion and infiltrate the vaginal mucosa and its draining LN. Although adoptive transfer experiments show that the majority of the transferred bone marrow cells accumulate in the spleen, this tissue does not seem to be involved in the inflammatory responses as there are no differences in the number of leukocytes in the spleen during vaginal infection. In the vagina, the macrophages and DCs form clusters under the epithelium at infected sites. These clusters mainly comprise CD11c\(^+\) DCs, although CD11b\(^+\) and F4/80\(^+\) macrophages are also evident. NK cells are also recruited to the site of viral infection and secrete IFN-\(\gamma\) that, in turn, helps to recruit more inflammatory cells and activate effector CD4\(^+\) T cells. CD4\(^+\) T cells themselves become the main source of IFN-\(\gamma\) production around D5 p.i. IFN-\(\gamma\), as well as the activity of other effector cells such as neutrophils and CD8\(^+\) T cells, help resolve epithelial viral infection by D7 p.i. On the other hand, IFN-\(\gamma\) production massively enhances the expression of the tryptophan-catabolising enzyme, IDO, in the vagina. Despite this significant expression, there is no difference in viral infection between WT and IDO\(^-\) mice. Thus, whether the activity of this enzyme assists the immune response in coping with this viral challenge or whether the enzyme has immunosuppressive actions in the vagina is still unclear.
HSV-2/WNV-infected vaginal epithelial cell

Bone Marrow

Blood Stream

Iliac Lymph Node

Pro-inflammatory cytokines/chemokines e.g., CCL2, CCL3, CCL5, …
Homeostatic chemokines e.g., CCL20

IFN-γ → IDO

IL-10 ?

DC
MΦ
NK
CD8^+
CD4^+

Spleen

?
C57BL/6 mice expressed high amounts of IFN-γ and IDO following vaginal infection and used CCL2 as the principal chemokine to attract myeloid and other cells to the site of infection and the ILN, while the response of BALB/c mice was characterised by the high expression of TNF and NOS2 and the use of CCL20 to recruit DCs. Despite the high induction of enzymatically active IDO in the infected vagina and ILN, the functional presence of this enzyme was associated with minimal or no effect on virus replication or leukocyte numbers in IDO gene knockout mice compared to WT mice. Nevertheless, this study provides some insight into the complex network of innate and adaptive mucosal immunity and may yet inform the eventual development of effective prophylactic and therapeutic vaccines against sexually transmitted viral infections.
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