The Immunobiology of Japanese Encephalitis Virus

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THE JOHN CURTIN
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Statement of Declaration

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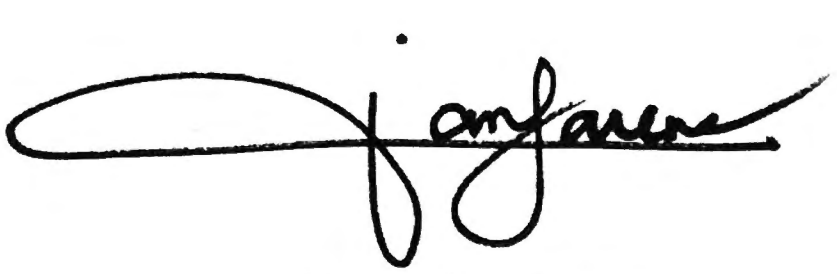

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Abstract

Japanese encephalitis virus is the leading cause of viral encephalitis in Asia. In spite of this, the immunological correlates for recovery from primary Japanese encephalitis virus (JEV) infection remain poorly defined. In this work, I have first established a mouse model for Japanese encephalitis in which a low-dose virus inoculum was administered subcutaneously into adult C57BL/6 mice. In this model, ~60% of the mice developed fatal encephalitis, concomitant with virus burden in the central nervous system (CNS). JEV infection induced a peak natural killer (NK) cell response on day 4 post-infection (pi), a peak $CD8^+$ T cell response on day 7 pi, a peak anti-JEV IgM response on day 8 pi, and an increasing anti-JEV IgG responses from day 8 pi onwards.

Second, to investigate the relative importance of immune effector cell populations, splenocyte transfer experiments, and JEV-challenge of mice with acquired or induced cellular deficiency were performed. Using mice lacking B cells ($\mu MT^{-/-}$ mice) and immune B cell transfer to wild-type mice, I find a critically important role for humoral immunity in preventing virus spread to the CNS. T cell help played an essential part in the maintenance of an effective antibody response necessary to combat the infection, since mice lacking major histocompatibility complex class II showed truncated IgM and blunted IgG responses, and the infection was uniformly lethal. On the other hand, NK cells were dispensable for protection against lethal Japanese encephalitis, while $CD8^+$ T cells, by itself, have no significant effect on the survival of JEV-infected mice.

Third, cytolytic effector pathways and interferon gamma (IFN-γ) help protect against lethal JE. Granule exocytosis and Fas-Fas ligand pathways of cytotoxicity act within the CNS to reduce disease severity, and show a redundancy in control of JEV infection. Only mice defective in both cell contact-dependent cytotoxic effector mechanisms display increased susceptibility to subcutaneous challenge with a low dose of JEV. Also, I demonstrate that IFN-γ is critical in recovery from primary infection with JEV by a mechanism involving suppression of virus growth in the CNS, but not in extraneural tissues, and that T cells are the main source of the cytokine that promotes viral clearance from the brain.

Fourth, the chemokine receptor, CCR5, serves as a host antiviral factor important against lethal JE. CCR5 deficiency resulted in increased mortality, and viral burden in the CNS. Absence of CCR5 did not only result in impaired trafficking of leucocytes into the brain, but also resulted in a multifaceted deficiency of cellular immune responses characterized by reduced NK cell activity, reduced $CD8^+$ T cell activity, and low splenic cellularity. This result is consistent with a mechanism by which CCR5 expression enhances lymphocyte activation and thereby promotes host survival after JEV infection.

Fifth, a novel immunodeficient mouse strain, *Tuara*, was established. *Tuara* mice displayed enhanced susceptibility to flavivirus infections, due to a loss-of-function mutation that was originally derived from an inbred knockout mouse strain, MHCII-$A\alpha^{-/-}$. The gene of interest was segregated from the MHCII defect, and after customized sequence capture and re-sequencing, was localized within ~50 Mb region of Chromosome 1. Among the candidate list, a G>T transversion on mouse genome reference position 52,179,547 bp of STAT1 gene was identified as the most likely causal mutation.

Finally, towards application of the insights gained on the basic immunobiology of JE, vaccination experiments using 'new generation' JE vaccines were performed. They revealed a dominant role of humoral immunity in vaccine-mediated protection of mice from lethal challenge with JEV, and in cross-protection against related viruses belonging to the JE serocomplex. $CD8^+$ T cells were not required for protection, while $CD4^+$ T cells provided substantial helper function for the protective humoral immune responses induced by vaccination. These immune correlates for homologous and heterologous protection against JE serocomplex viruses were observed in vaccination with either a live attenuated vaccine (ChimeriVax-JE), or a candidate inactivated Vero cell culture-derived JE vaccine (ccJE) formulated with Advax™, a novel immunologically active polysaccharide adjuvant.

List of manuscripts accepted or for submission as journal publication, either with principal or supplementary contribution:

Lobigs, M., **Larena, M.**, Alsharifi, M., Lee, E., & Pavy, M. (2009). Live chimeric and inactivated Japanese encephalitis virus vaccines differ in their cross-protective value against Murray Valley encephalitis virus. J. Virol. 83(6), 2436-2445.

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6th Annual Scientific Meeting Australasian Virology Society, Kingscliff, New South Wales, Australia[b]

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1st International Conference on Translational Medicine. John Curtin School of Medical Research, The Australian National University. Canberra. Australia[b]

29th Annual Meeting of American Society for Virology, Montana State University, Montana, Wyoming, USA[a]

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3rd Annual Biosecurity Symposium: Global Health Security. The National Center for Biosecurity. Canberra, Australia.

5th Scientific Meeting Australasian Virology Group, Lorne, Victoria, Australia

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List of Abbreviations

%	Percent
°C	Degrees Celsius
Ab	Antibody
ADAR	dsRNA-specific adenosine-deaminase
ADEM	Acute disseminated encephalomyelitis
AGRF	Australian Genomics Resource Facility
APC	Antigen-presenting cell
APF	Australian Phenomics Facility
Arbovirus	Arthropod-borne virus
BDV	Borna disease virus
BHK	Baby hamster kidney
bp	Base pair
BSA	Bovine serum albumin
C	Capsid protein
C6/36	*Aedes albopictus* salivary gland cells
cDNA	Complementary DNA
CLEC5A	C-type lectin domain family 5
CLR	C-type lectin receptor
CMV	Cytomegalovirus
CNS	Central Nervous System
CO_2	Carbon dioxide
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocytes
Cyp20a	Cytochrome P450, family 20, subfamily a, polypeptide 1
DDT	Dichlorodiphenyletrichloroethane
DENV	Dengue virus
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA

DTT	Dithiothreitol
E	Envelope
ECTV	Ectromelia virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENU	N-ethyl-N-nitrosourea
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorter
FasL	Fas ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
fp	Footpad
g	Gram
g	Centrifugal force
GBP	Guanylate-binding proteins
GMT	Geometric mean titer
Gzm	Granzymes
h	Hour
HBSS	Hanks' balanced salt solution
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	Heat shock protein
ip	Intraperitoneal
ic	Intracranial
ICOS	Inducible T cell co-stimulator
IFN	Interferon
IFN-α-R	Interferon-alpha receptor
Ig	Immunoglobulin
IRF	IFN regulatory factor

ISG	IFN-stimulated gene
iv	Intravenous
Jak-Stat	Janus kinase - signal transducer and activation of transcription
JEV	Japanese encephalitis virus
Kb	Kilobase
kDa	Kilodalton
KUN	Kunjin
L	Liter
LCMV	Lymphocytic Choriomenengitis virus
LGP2	Laboratory of genetics and physiology 2
LRR	Leucine-rich repeats
M	Membrane protein
M	Molar
Mb	Megabase
mAb	Monoclonal Antibody
MCMV	Mouse cytomegalovirus
MDA-5	Melanoma differentiation-associated antigen 5
MEM	Minimal essential medium
Met	Methionine
μg	Microgram
Mg	Milligram
MHC	Major histocompatibility complex
min	Minute
Ml	Millilitre
mm	Millimetre
mM	Millimolar
MOI	Multiplicity of infection
MST	Mean survival time
MVEV	Murray Valley encephalitis virus
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate

NaOH	Sodium Hydroxide
ng	Nanogram
NK	Natural killer
NO	Nitric oxide
NOD	Nucleotide binding oligomerization domain
NOS-1	Nitric oxide synthase type 1
NS	Non-structural protein
nt	Nucleotide
OAS	Oligoadenylate synthetases
OD	Optical density
ORF	Open Reading Frame
pi	Post-infection
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1
PEG	Polyethylene glycol
PFU	Plaque forming unit
PKR	Protein kinase
pmol	Picomole
prM	pre-Membrane protein
PRNT	Plaque reduction neutralization test
PRR	Pathogen recognition receptor
Prss40	Protease, Serine, 40
PSN	Penicillin-streptomycin-neomycin
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RER	Rough endoplasmic reticulum
RIG	Retinoic acid-inducible gene
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute

RT-PCR	Reverse transcriptase polymerase chain reaction
SAP	SLAM-associated protein
sc	Subcutaneous
SD	Standard deviation
SEM	Standard error of mean
SFV	Semliki Forest virus
SLEV	St. Louis encephalitis virus
SNP	Single-nucleotide polymorphisms
SNV	Single-nucleotide variation
ssRNA	single-stranded RNA
STAT	Signal transducers and activators of transcription protein
TBEV	Tick-borne encephalitis virus
TFH	Follicular helper T
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TRAIL	TNF-related apoptosis-inducing ligand
tRNA	transfer ribonucleic acid
Tyk2	Tyrosine kinase 2
U	Unit
UTR	Untranslated region
UV	Ultraviolet
VACV	Vaccinia virus
WNV	West Nile virus
µl	microliter

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Chapter 1

Introduction

Japanese encephalitis (JE) is an acute central nervous system inflammatory disease caused by an infection with Japanese encephalitis virus (JEV), a small, enveloped, plus-strand RNA virus belonging to the family *Flaviviridae*. It is the leading cause of viral encephalitis in South-East Asia, India and China, where 3 billion people are at risk of contracting the disease (Erlanger *et al*., 2009). Annually, about 35,000 – 50,000 cases of JE are reported, resulting in about 10,000 deaths and a high incidence of neuropsychiatric deficits among survivors (Solomon, 2004; Tsai, 2000). Treatment of JE patients is supportive and in the absence of availability of antiviral compounds the mainstay of protection against JE is vaccination (Halstead & Thomas, 2011). In the past decades there has been an expansion of the geographic distribution of the virus in Asia and the Asia-Pacific region (van den Hurk *et al*., 2009) and there is an urgent requirement for improved human and veterinary JE vaccines. An understanding of the immunological responses that lead to recovery from infection with JEV and account for vaccine-mediated protection is important in the design of rational approaches to new treatments and vaccines against the disease, and will be the focus of this introduction.

1.1 Classification, Epidemiology, and Biology of JEV

1.1.1 Taxonomy of flaviviruses

The *Flaviviridae* are a large family of positive-sense single-stranded RNA viruses. The name is derived from the Latin word "flavus" (meaning yellow), named after the first described human virus, the jaundice-causing Yellow Fever virus (YFV) discovered by Walter Reed in 1901. The family *Flaviviridae* is classified into 3 genera: *Flavivirus, Hepacivirus, and Pestivirus* (Heinz *et al*., 2000; Lindenbach *et al*., 2007). The *Flavivirus* genus comprise of at least 70 viruses, and is further subdivided, based on

molecular phylogenetics, into clusters, clades, and species, or based on serological criteria, into antigenic complexes or subcomplexes (Billoir *et al*, 1997; Kuno *et al*, 1998; Kuno *et al*., 2001; Lindenbach *et al*., 2007; Westaway and Blok, 1997; Zanotto *et al*., 1996). The genus mostly consists of arthropod-borne viruses causing major human diseases, including flu-like illness, hepatitis, hemorrhagic fevers, and encephalitis. The principal vectors of one group are *Ixodes* ticks, transmitting tick-borne viruses such as Powassan virus, TBEV-European subtype, and TBEV-Far Eastern subtype. Another group are transmitted by mosquitoes, which include *Aedes-borne* flaviviruses such as YFV and dengue viruses (DENV), and *Culex*-borne encephalitic flaviviruses such as JEV, West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), and St Louis encephalitis virus (SLEV) (Fig 1.1).

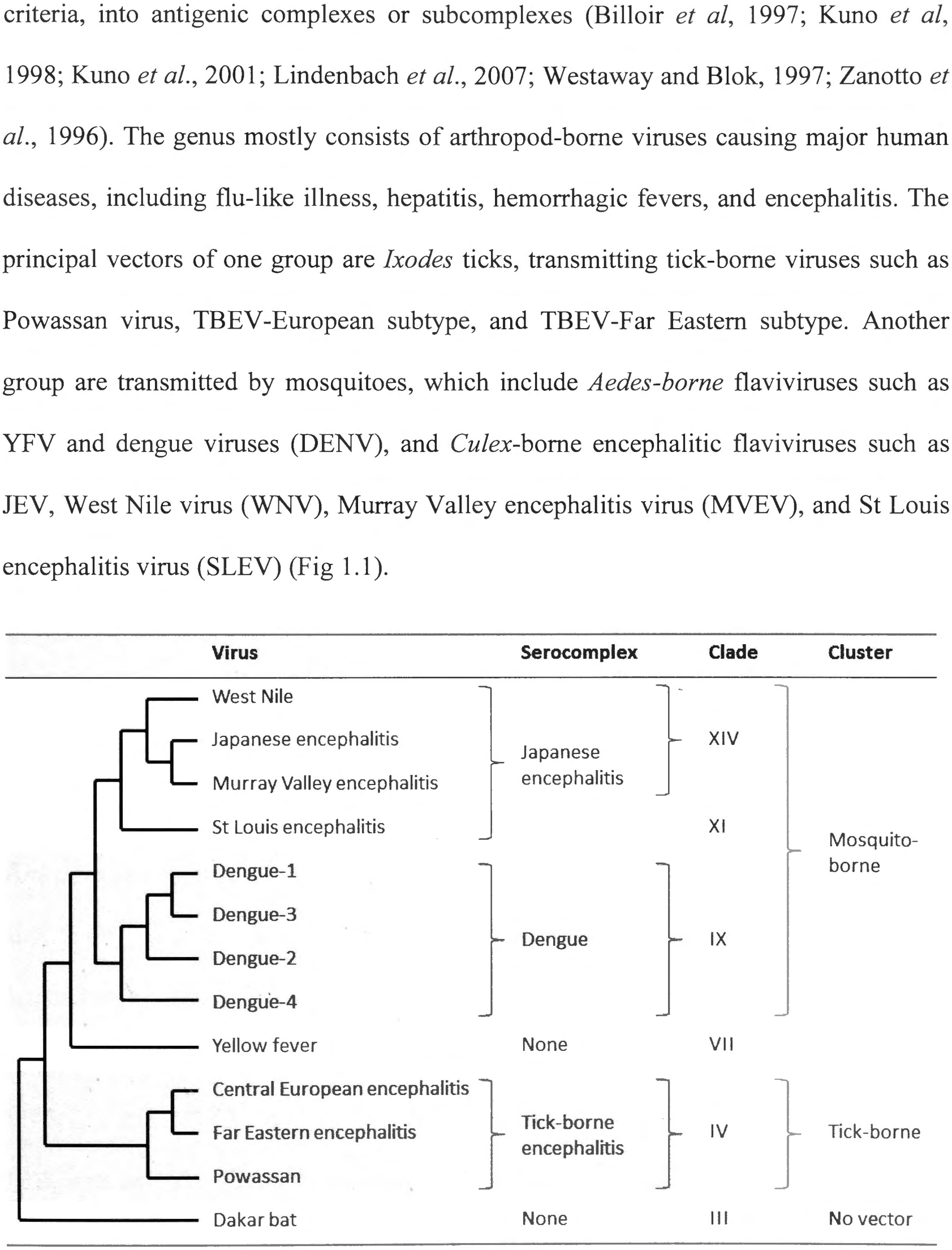


Figure 1.1 Classification of *Flavivirus* genus. Flaviviruses are classified, based on serologic criteria, into serocomplexes, or based on phylogenetic studies, into clusters, clades, or species. Figure was adapted, with some modifications, from Mukhopadhyay *et al.* (2005).

1.1.2 Geographical distribution of Flaviviruses

Encephalitic flaviviruses of the JE serocomplex have a widespread global distribution (Mackenzie *et al.*, 2004) (Fig. 1.2). JEV is largely endemic in the Asia-Pacific region encompassing Japan, extreme south-eastern part of Russia, Korea, Taiwan, and China in the northeast, to Nepal, Bhutan, Bangladesh, India, Sri Lanka, and southern Pakistan in the west, and to Southeast Asia, Papua New Guinea, and Torres Strait Islands in the southeast (van de Hurk *et al.*, 2009). In 1998, expansion of JEV towards the Australian mainland was documented with a single human case and seroconverted sentinel pigs on Cape York Peninsula (Hanna *et al*, 1999). MVEV, on the other hand, is locally endemic in Australia, Papua New Guinea, and eastern Indonesia (Knox *et al*, 2012; Marshall, 1988). WNV has a widespread distribution throughout Africa, south and central Asia, the Middle East and southern Europe (Kramer *et al.*, 2008). WNV strain Kunjin (WNV_{KUN}), is endemic in the Australasian region, while another WNV strain started to appear in New York City on 1999 (WNV_{NY99}), and rapidly spread throughout the Americas in a span of ~8 years (Kramer *et al.*, 2008). Lastly, SLEV is endemic in North and South America (Day, 2001). Regions were two or more viruses of the JE serocomplex overlap in geographic distribution include India and the Indochinese peninsula for WNV and JEV, North America for WNV and SLEV, Australia for WNV_{KUN} and MVEV, and eastern Indonesia and Papua New Guinea for WNV_{KUN}, JEV, and MVEV. Flaviviruses belonging to the JE serocomplex that are of minor medical importance and of limited geographical distribution, include Calcipacore virus in South America, MVEV subtype Alfuy in Australia, and Usutu, Koutango, and Yaounde viruses in Africa (Mackenzie *et al.*, 2004).

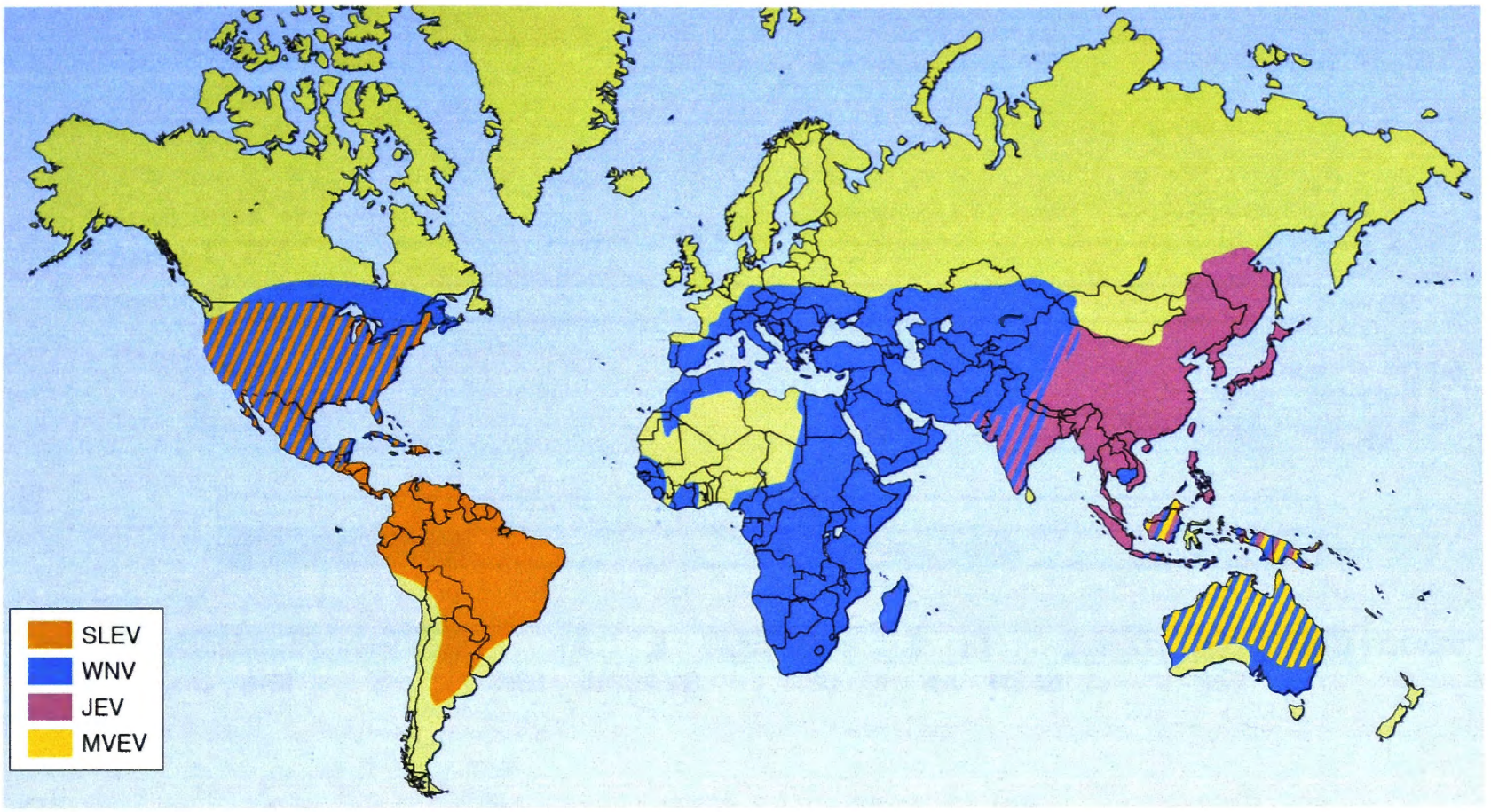


Figure 1.2 Geographic distribution of encephalitic flaviviruses of major medical importance. World map indicating the distribution of the most important encephalitic flaviviruses belonging to the JE serocomplex: SLEV, WNV, JEV, and MVEV. Figure was adapted from Mackenzie *et al.* (2004).

1.1.3 Basic structure and genome organization of flaviviruses

Flaviviruses are ~50 nm small enveloped viruses, with an electron dense core of ~30 nm (Murphy, 1980; Kuhn *et al.*, 2002; Mukhopadhyay *et al.*, 2003). The viral particles have an outer surface made up of two viral proteins, the envelope (E) and membrane (M) protein, while the core is an assembly of viral capsid (C) proteins and a positive-sense, single-stranded RNA genome of ~11,000 nucleotides in length (Sumiyoshi *et al.*, 1987). The 5' terminus carries a type 1 cap structure (m7GpppAmpN2), while the 3' terminus is non-polyadenylated (Lindenbach., 2007). The genome serves as the messenger RNA (mRNA) that codes for a single polyprotein composed of (from the amino-terminus) 3 structural proteins: C, E, and premembrane (prM), and 7 non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 1.3).

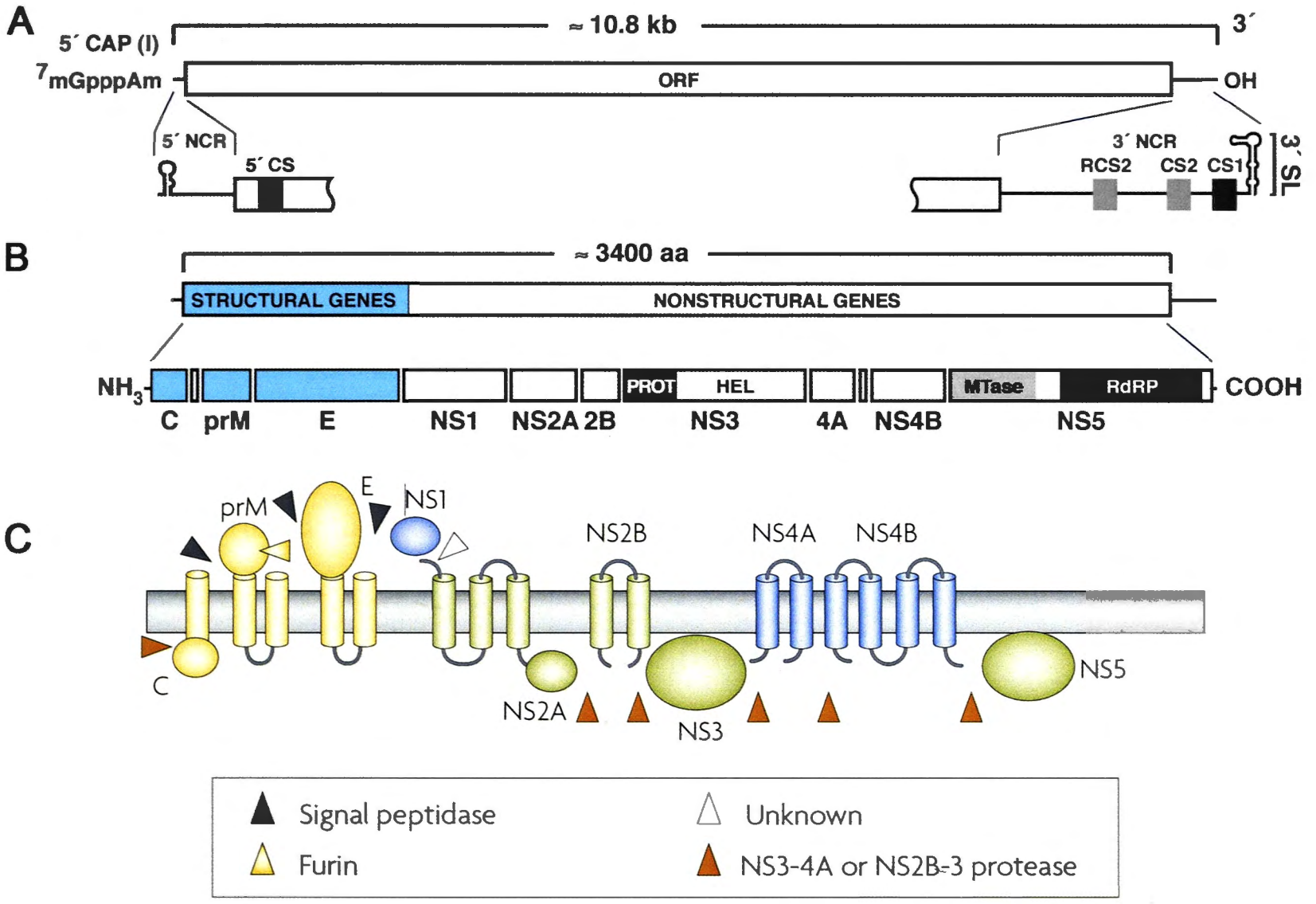


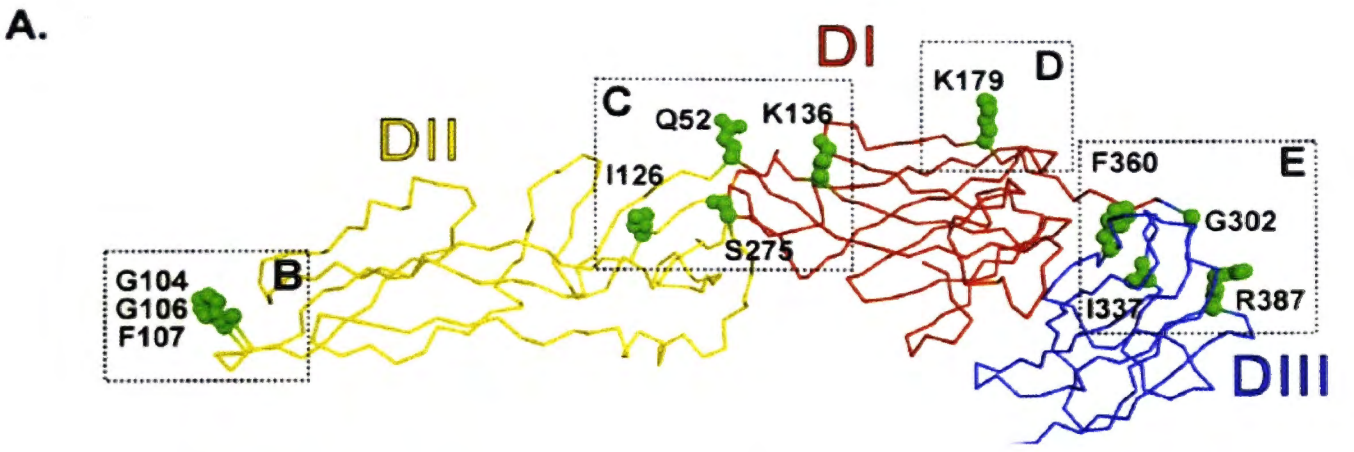
Figure 1.3 Flavivirus genome organization and protein expression. (A) Flavivirus approximate genome size of 10.8 KB, with open reading frame flanked by non-coding regions of capped 5' and non-polyadenylated 3' ends. (B) Chronological list of expressed structural and nonstructural genes, with proportional representation of individual proteins based on amino acid length. (C) Putative topology of flavivirus protein cleavage products in relation to the endoplasmic reticulum membrane, with labelled enzymes responsible for various specific cleavages. Figures adapted, with some modifications from Lindenbach *et al.* (2007) and Murray *et al.* (2008).

The NS proteins are thought to play a role in the morphogenesis of flaviviruses (Murray *et al.*, 2008) (summarized in Table 1.1). Capsid protein subunits form the nucleocapsid containing the viral genomic RNA, while the prM protein serves as the precursor of the mature M protein (Dokland *et al.*, 2004; Jones *et al.* 2003; Konishi and Mason, 1993). The latter forms a dimmer with E protein and prevents the premature fusion of the E protein during passage of immature virions through the secretory pathway (Elshuber *et al.*, 2003; Wengler and Wengler, 1989). The cleavage of prM by the cellular enzyme furin into pr and M coincides with the formation of E protein homodimers, a process important for virus maturation (Stadler *et al.*, 1997; Yu *et al.*, 2008).

Table 1.1 Functional description of flaviviral proteins.

Viral protein	Functional description
E	Receptor binding and membrane fusion Main target of neutralizing antibodies
prM	Precursor of M protein Prevents premature fusion of E protein during virus maturation
C	Structural component of the nucleocapsid, C-terminal hydrophobic anchor of nascent C protein is a signal peptide for ER translocation of prM
NS1	RNA replication Interferon inhibition
NS2A	RNA replication Genome encapsidation
NS2B	RNA replication Capsid Cleavage Cofactor for the NS2B-NS3 serine protease that cleaves at the C/prM, NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4B/NS5 junctions
NS3	RNA replication Capsid Cleavage Genome encapsidation Forms part of NS2B-NS3 serine protease that cleaves at the C/prM, NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4B/NS5 junctions Helicase and RNA triphosphatase activity
NS4A	RNA replication Interferon inhibition
NS4B	RNA replication
NS5	RNA replication RNA-dependent RNA polymerase and Methytransferase activity Interferon inhibition

The E protein plays an essential role in the early entry steps of the viral replication cycle through its receptor-binding and fusion functions (Allison *et al.*, 2001; Chu and Ng., 2004; Davis *et al.*, 2006*b*; Huang *et al.*, 2010; Lee E. *et al.*, 2006; Navarro-Sanchez *et al.*, 2003). It has three ectodomains, domain 1 (DI), DII, and DIII (Luca *et al.*, 2012). A single N-linked glycosylation site is located at amino acid position 154 in JEV, and is considered important for flavivirus particle release and infectivity, virulence, and stimulation of innate immune responses (Beasley *et al.*, 2005; Davis *et al.*, 2006*a*; Hanna *et al.*, 2005, Lee and Lobigs, 2010). Given its surface-exposed position on the virion particle, the E glycoprotein is the principal immunologic protein of JEV and related flaviviruses (Lobigs and Diamond, 2012). Mapping of the neutralizing epitopes on the E glycoprotein revealed 4 important sites: DI lateral ridge, fusion loop, DIII lateral ridge, and DI-DII hinge (Luca *et al.*, 2012) (Fig. 1.4).



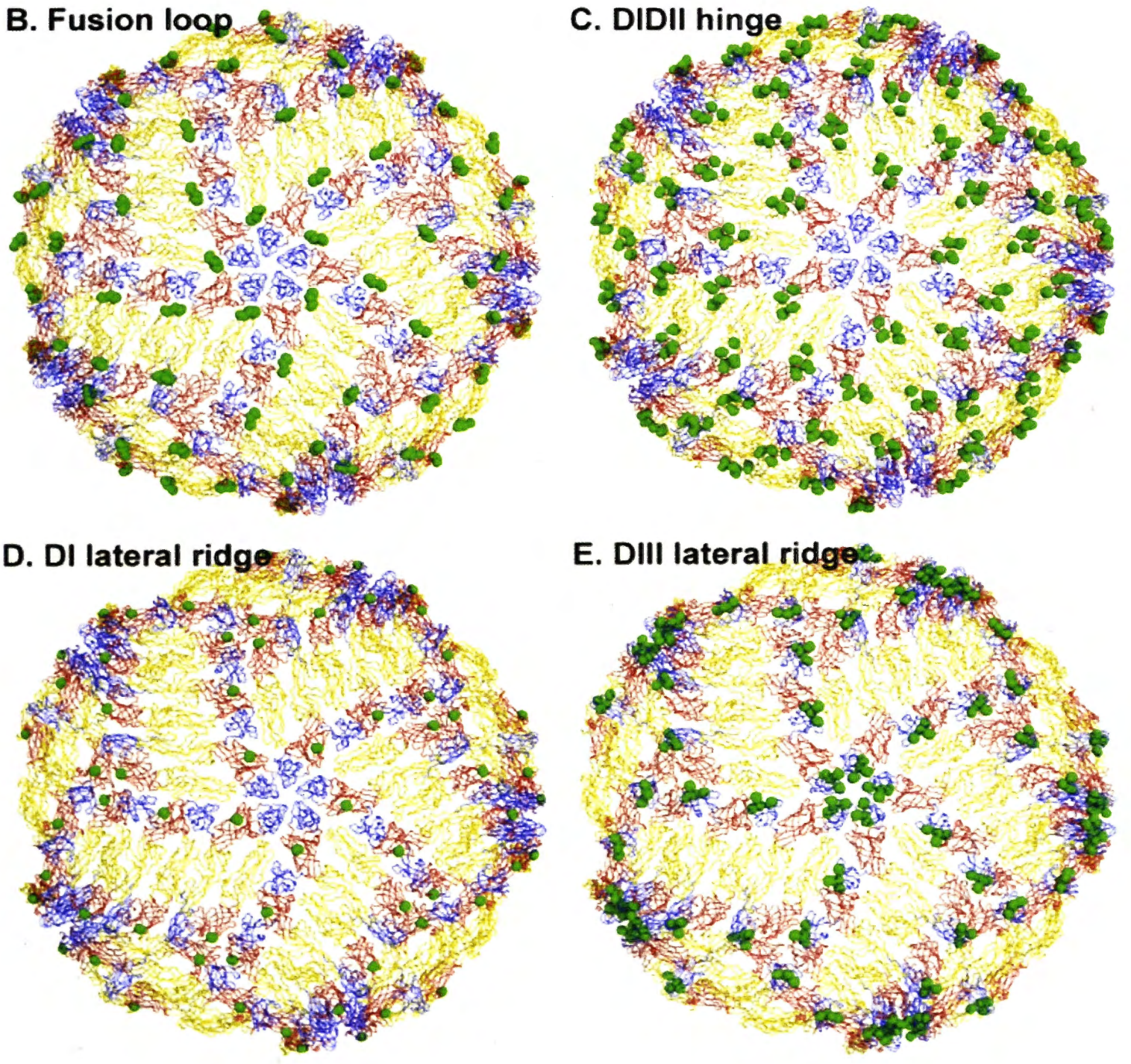


Figure 1.4 Neutralizing epitopes on JEV E glycoprotein domains. Reconstructed virion indicating neutralizing epitopes mapped on fusion loop, DI-DII hinge, DI lateral ridge, and DIII lateral ridge of JEV glycoprotein. (A) Colored-green spheres represent side chains of residues for binding sites of previously established JEV neutralizing antibodies. JEV E-specific neutralizing antibodies (B) 4G2 (G104, G106, and L107) was mapped to the fusion loop; (C) B2 (I126), NARMA3 (Q52), and 503 (Q52, I126, K136, and S275) to the DI-DII hinge; (D) A3 (K179) to the DI lateral ridge; (E) E3 (G302) and E3.3 (I337, F360, and R387) to the DIII lateral ridge (colored-green spheres as side chains of residues for binding site). Figure was adapted from Luca *et al.* (2012).

1.1.4 Replication cycle of Flaviviruses

The replication cycle commences with binding and uptake of the virion through receptor-mediated endocytosis (Chambers *et al.*, 1990; Lindenbach *et al.*, 2007) (Fig. 1.5). Candidate cellular receptors for E glycoprotein interaction include DC-SIGN and DC-SIGNR (Davis *et al.*, 2006; Mukhopadhyay *et al.*, 2005). Fusion of the viral envelope with the endosomal membrane is induced by low pH within the endosomal compartment (Allison *et al.*, 2001; Bressanelli *et al.*, 2004; Fritz *et al.*, 2008; Huang *et al.*, 2010). Following uncoating of the nucleocaspid, the viral genome is released into the cytoplasm. The RNA genome serves as the template for RNA replication, messenger RNA for viral protein translation, and genetic material for the formation of new viruses (Chambers *et al.*, 1990; Chu and Westaway, 1985; Chu and Westaway, 1992). The genome encodes for a single open reading frame of ~10,300 nucleotides, that is translated into an ~3,400 amino acid long polypeptide, with structural proteins (C, E, and prM) encoded in the amino-terminal and the non-structural protein encoded in the carboxy-terminal portion (Lindenbach *et al.*, 2007). Host and viral proteases subsequently cleave the polyprotein co- and post-translationally into 10 protein subunits. A negative sense RNA genome serves as an intermediate for RNA replication in the cytoplasm, with non-structural proteins involved in the replication complex. The virus assembles by budding through the membranes of endoplasmic reticulum, and progeny virions transit via the Golgi complex during virus egress from the infected cell (Mackenzie and Westaway, 2001).

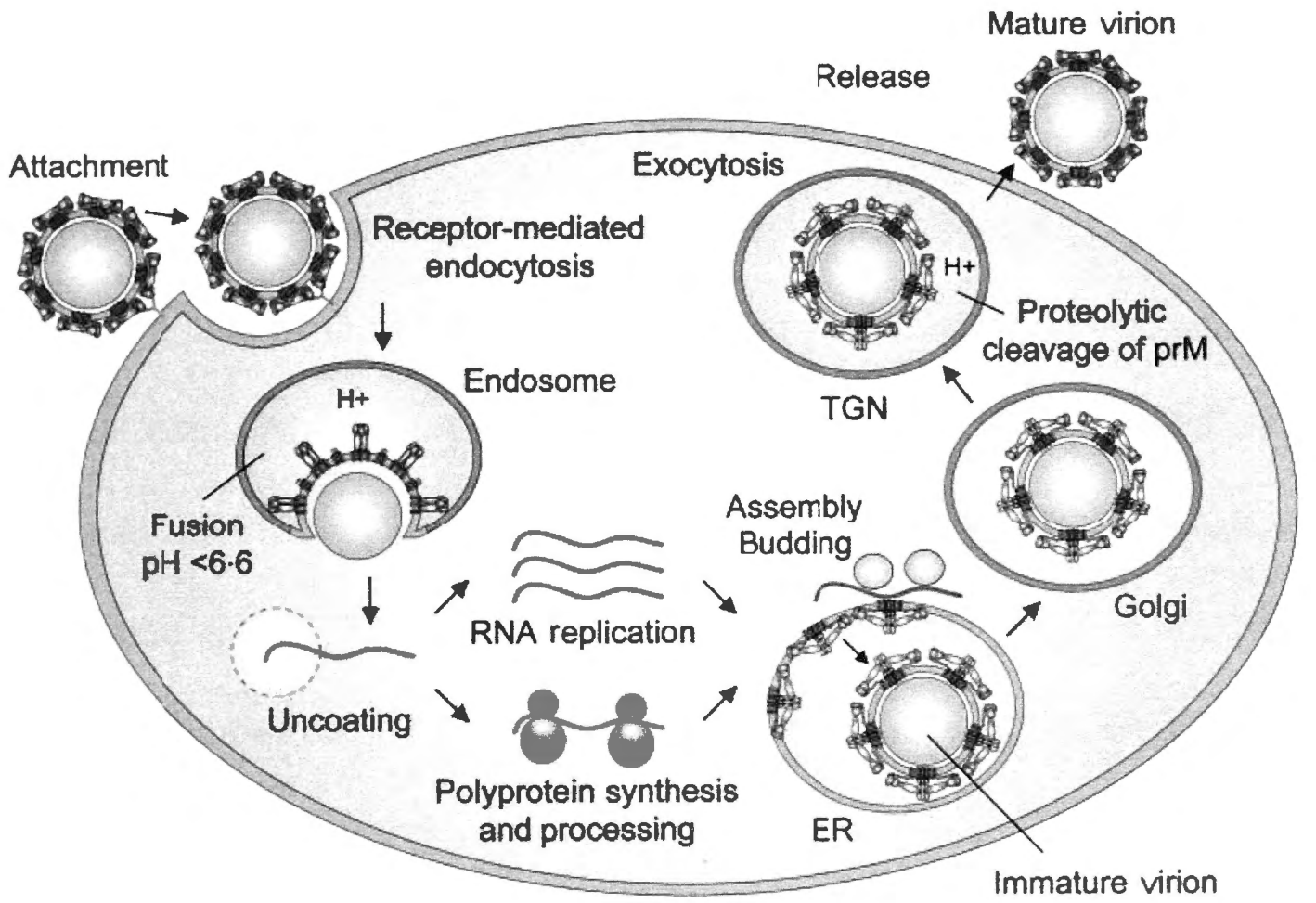


Figure 1.5 Flavivirus replication cycle. Sequential steps in flavivirus replication: attachment, fusion, uncoating, RNA replication and polyprotein synthesis, assembly, budding, and exocytosis. Figure adapted from Stiasny and Heinz (2006).

1.1.5 History of JEV discovery and spread

Description of encephalitis in humans was initially reported in Japan as early as 1871 (van den Hurk *et al.*, 2009) (Historical highlights summarized in Table 1.2). Minor epidemics occurring during summer have been reported regularly in Japan from 1873 to 1968, with major epidemics documented in 1924, 1935, and 1948 (Shiraki, 1970; Vaughn and Hoke, 1992). In 1935, JEV was first isolated from the brain of an

individual who succumbed to encephalitis (Mitamura *et al.*, 1936). It was originally designated as "type B encephalitis" to distinguish it from "type A encephalitis" (von Economo encephalitis or encephalitis lethargica). With regular "summer epidemics" every 2-3 years in Japan, Korea, and Taiwan until the middle of 1960's, historical records show its gradual spread as major epidemics in a southeast to southwest direction; commencing with mainland China in 1940, Philippines in 1950's, Vietnam in 1965, Chiang Mai Valley of Thailand in 1969, West Bengal in 1973, Nepal in 1978, Pakistan in 1983, Sri Lanka in 1985 (Chakravarty *et al.*, 1975; Grossman *et al.*, 1973*a*; Grossman *et al.*, 1973*b*; Grossman *et al.*, 1973*c*; Mackenzie *et al.*, 2007, Peiris *et al.*, 1992; Solomon, 2003; Umenai *et al.*, 1985; Vitarana *et al.*, 1988; Yamada *et al.*, 1971)

Phylogenetic analysis revealed that JEV isolates are classified into 5 genotypes (I-V) (Solomon *et al.*, 2003*b*; Mohammed *et al.*, 2011). In spite of the first case description in Japan, and the epidemiologic history of apparent southward spread, genetic studies have revealed that, on the contrary, the virus must have originated from the Malay archipelago, with subsequent outward spread all throughout Asia. This is supported by the discovery that all 5 genotypes (including the oldest genotype) were only found in a confined geographical area of Malaysia and Indonesia (Mohammed *et al.*, 2011). It is estimated that the current JEV genotype strains evolved from an ancestral strain in the Malaysia-Indonesia region in the mid 1500's, with a mean evolutionary rate of 4.35×10^{-4} (range: 3.5 to 5.3×10^{-4}) nucleotides substitution/site /year.

Table 1.2 Historical highlights of JE

Time	Event
1871	First reported cases of JE in Japan
1924	First reported major epidemic in Japan
1935	Isolation of the virus from the brain of an infected individual
1954	Licensure of mouse brain-derived inactivated JE vaccine in Japan
1965	First reported major epidemic in Vietnam
1969	First reported major epidemic in Thailand
1973	First reported major epidemic in India
1968	Development of primary hamster kidney-cell grown inactivated JEV vaccine in China
1983	Documented westward expansion of JEV into Pakistan
1987	First complete nucleotide sequence of JEV RNA genome
1988	Licensure of live-attenuated vaccine SA-14-14-2 in China
1995	First 2 cases of JE in Torres Strait Islands, Australia
1998	First report of JEV infection on the Cape York Peninsula of mainland Australia
2003	Phylogenetic analysis classified JEV into 5 genotypes
2005	Cessation of production of mouse brain-derived inactivated JE vaccine
2009	Licensure of Vero cell-grown formalin-inactivated JE vaccine in US, Europe, Australia
2010	Licensure of live-attenuated vaccine ChimeriVax-JE in Australia
2012	Crystal structure of JEV E glycoprotein determined

1.1.6 Transmission cycle

Mosquitoes of the rice paddy-breeding *Culex vishnui* subgroup are the established vectors for JEV, with *Culex tritaeniorhynchus* as the principal vector, and *Culex gelidus*, *Culex fuscocephala* and *Culex annulirostris* as secondary vectors in limited geographical areas (Buescher *et al.*, 1959*c*; Gresser *et al.*, 1958*b*; Keiser *et al.*, 2005; Mackenzie *et al.*, 2002; Peiris *et al.*, 1993; Reisen, 1981; Ritchie *et al.*, 1997; Vythilingam *et al.*, 1997). Infected mosquitoes transmit the virus to pigs and ardeid

birds, which serve as reservoir and amplifying hosts, and to humans and horses, which serve as accidental or dead end hosts (Buescher and Scherer, 1959; Scherer and Buescher, 1959; Scherer *et al.*, 1959a) (Figure 1.6). Vertical transmission of JEV in mosquitoes has also been demonstrated; however, its role in viral persistence in the field remains unknown (Rosen *et al.*, 1989; Rosen *et al.*, 1978; Takashima and Rosen, 1989). Seroconversion is observed in cattle, dogs, monkeys, and sheep but is unlikely to play a role in the transmission cycle of JEV, given its inability to exhibit detectable viremia (Vaughn and Hoke, 1992; van den Hurk *et al.*, 2009).

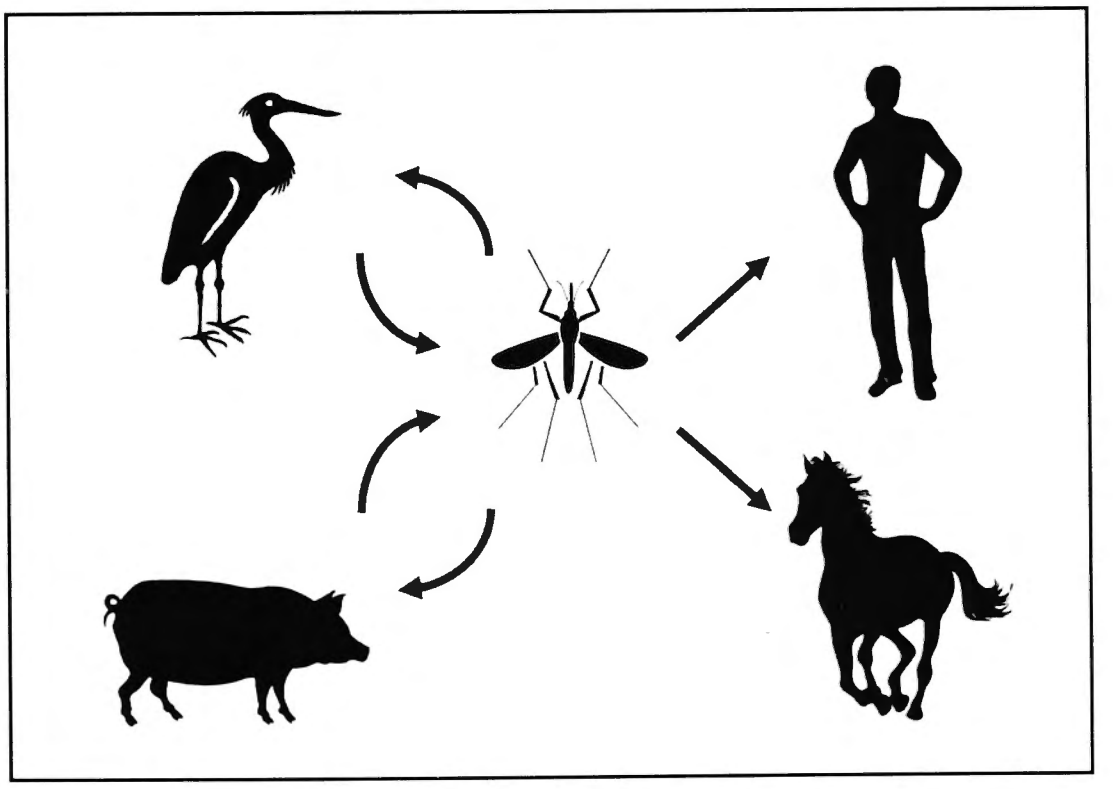


Figure 1.6 Natural transmission cycle of JEV. JEV is maintained in the environment through an enzoonotic cycle with pigs and ardeid birds. *Culex* mosquitoes serve as the vector. Infected *Culex* mosquitoes transmit JEV to humans and horses, which are dead-end or accidental hosts.

Due to the proximate location of human dwellings and pig-rearing facilities, pigs are the principal reservoir host of JEV causing human infections (Burke and Leake, 1988; Buescher *et al.*, 1959*a*; Konno *et al.*, 1966; Scherer *et al.*, 1959*d*; Scherer *et al.*, 1959*e*). Characteristics that make pigs an important amplifying host include i.) high natural infection rates, ii.) high viremia that can last up to 4 days, iii.) high porcine feeding rates of infected vectors, iv.) high birth rate, and v.) high turnover population needed for commercial use (Gresser *et al.*, 1958*a*; Scherer *et al.*, 1959f; Scherer *et al.*, 1959g). Ardeid wading birds, such herons and egrets, on the other hand, are vital for the maintenance of JEV in the environment (Buescher *et al.*, 1959*b*; Buescher *et al.*, 1959*d*; Buescher *et al.*, 1959*e*; Scherer *et al.*, 1959*c*; Rodrigues *et al.*, 1981). The propensity of ardeid birds to migrate great distances makes them a candidate reservoir host important in establishing JEV in new geographic locations (Nga *et al.*, 2004; Solomon, 2004). Other postulated mechanisms by which JEV is established in new areas include wind-blown infected mosquitoes and travel or migration of infected individuals (Ming *et al.*, 1993; Ritchie and Rochester, 2001; Sellers, 1980; Vaughn and Hoke, 1992).

Two typical patterns of JEV transmission are described. In tropical areas, such as Cambodia, Indonesia, Thailand and Vietnam, an endemic pattern with year-round transmission is observed, with occasional outbreaks during the rainy season or as a consequence of increased irrigation in rice paddy areas (Burke *et al.*, 1985*b*). In temperate areas, such as Japan, China and South Korea, an epidemic pattern is observed, with increased incidence during summer seasons. The latter was presented in a study by Konno *et al.* (1966), which earlier depicted a cyclical transmission pattern for JEV among humans, mosquitoes, and pigs (Fig. 1.7).

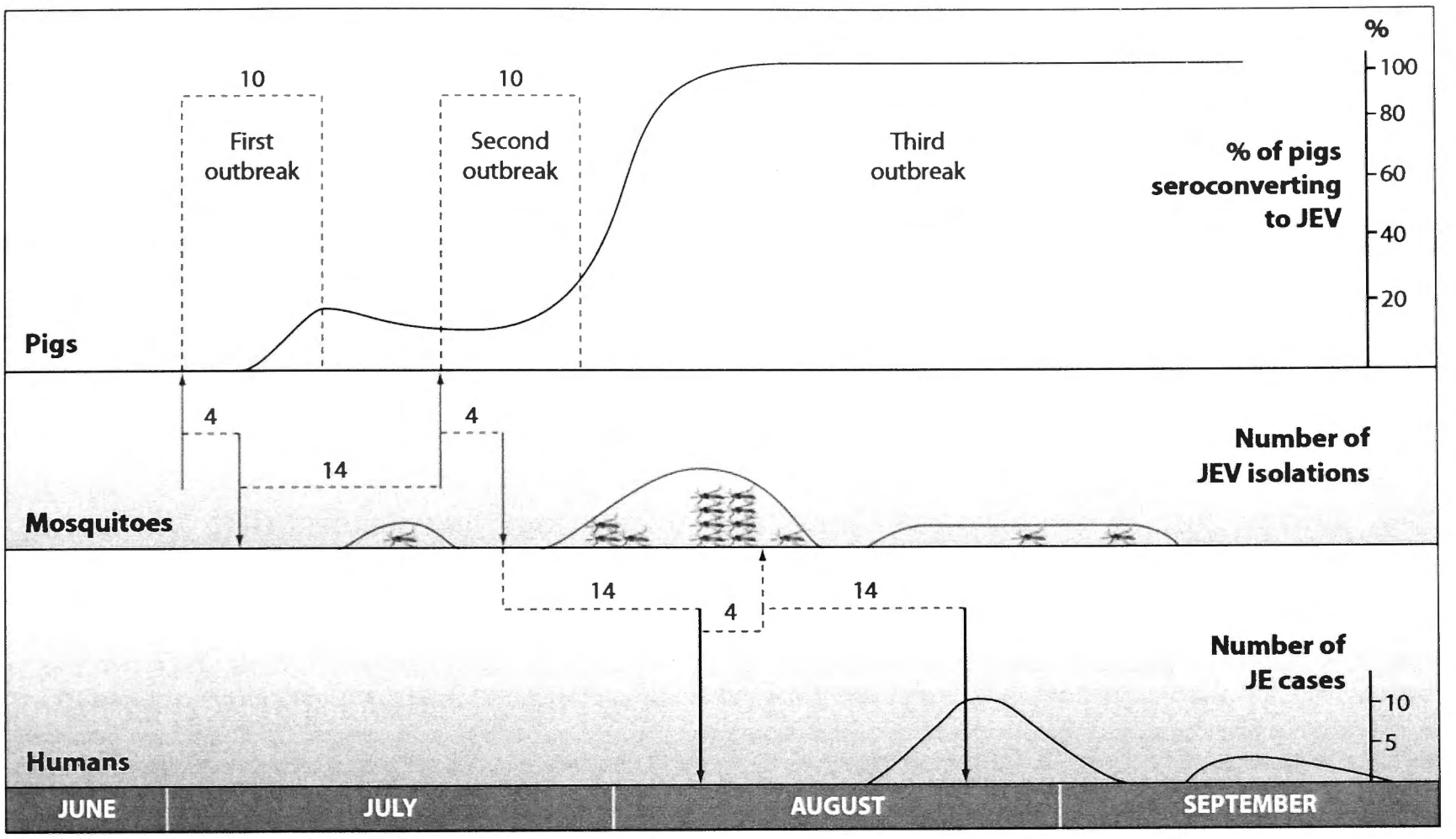


Figure 1.7 Cyclical epidemic pattern of JE. Following JEV inoculation, pigs demonstrate viremia after 4 days, and detectable antibodies after 10 days. This initial outbreak occurs in early July, with 20% of pigs becoming infected. Mosquitoes acquire JEV from viremic pigs, become infectious after 14 days external incubation period (EIP), and transmit JEV into more pigs. This second outbreak results in 100% seroconversion in pigs. A larger pool of viremic pigs results in larger population of infected mosquitoes. Fourteen days after EIP in a new batch of infected mosquitoes, the outbreak of JE in humans starts to appear. Figure adapted from Konno *et al.* (1966).

1.2 Clinical spectrum of Japanese encephalitis

1.2.1 Clinical manifestations

Infection with JEV starts with a bite of an infected *Culex* mosquito, although the possibility of transplacental transmission has been demonstrated in mice, swine and humans (Chaturvedi *et al.*, 1980; Mathur *et al.*, 1981; Morimoto *et al.*, 1972). The infection is largely subclinical with only 1:50 to 1:10,000 human infections resulting in symptomatic disease (Tsai, 2000). The clinical features of infection range from a non-specific febrile illness, aseptic meningitis and poliomyelitis-like syndrome to a severe meningoencephalomyelitis (Solomon, 2003; Solomon *et al.*, 2000; Solomon *et al.*, 1998). The incubation period is from 5 to 15 days before onset of prodromal symptoms, which include fever, generalized weakness, coryza, diarrhoea, and rigors. Afterwards, patients experience headache, vomiting, decreased sensorium and convulsion. Then a classic presentation ensues, including dull, flat, mask-like facies with wide unblinking eyes, tremor, generalized hypertonia and cogwheel rigidity. Other signs and symptoms found in a subset of patients include generalized tonic-clonic seizures, focal seizures, upper motor neuron facial nerve palsy, extrapyramidal manifestations, asymmetric paralysis and mental illness. Occasional extrapyramidal symptoms include non-intention tremors, cogwheel rigidity, head nodding and pill rolling movements, opsoclonus, myoclonus, choreoathetosis, and bizarre facial grimacing and lip smacking (Solomon *et al.*, 2000). The case fatality ratio can be as high as 50-60 % (Tsai, 2000), and one half of the survivors have long-term neurologic or psychiatric sequelae (Solomon, 2003).

1.2.2 Animal models

JE is also a veterinary disease with occasional fatal outcome in horses, and abortions and abnormal births in pigs (Halstead & Jacobson, 2003). While pigs can act as amplifier host in the transmission cycle of the virus, JEV infection of horses, like that of humans, does not generate sufficient viremia for virus transmission. The clinical course of JE in horses resembles that found in humans (Gould *et al.*, 1964; Lam *et al.*, 2005; Miyake, 1964; Yamanaka *et al.*, 2006). Bats have been shown to transmit JEV transplacentally, and to sustain low-level viremia in the lab via simulated hibernation, both of which are suggested to be a mechanism for overwintering of JEV (Sulkin *et al.*, 1964; Sulkin *et al.*, 1966). Also, simulated hibernation experiments have shown detectable viremia in JEV-infected lizards, snakes, and frogs (Doi *et al.*, 1983; Oya *et al.*, 1983).

Mice have been most extensively used as a model for studies on the pathogenesis of JEV (Kimura *et al.*, 2010), and show significant similarity to natural human infection. Age, genetic background and route of inoculation are risk factors for severe encephalitis (Grossberg & Scherer, 1966). The pathologic changes seen in mouse brain infected with JEV are similar to those observed in humans, with perivascular cuffs, cellular infiltrates, and mild vascular damage (Hase *et al.*, 1990; German *et al.*, 2006). Immunohistochemical staining reveals JEV infected neurons in multiple loci, predominantly localized in the following areas: cortex, thalamus, and hippocampus (Wang and Deubel, 2011). The initial local site of JEV replication following footpad infection probably involves dendritic cells, given the evidence that they support JEV replication (Aleyas *et al.*, 2009; Cao *et al.*, 2011; Li *et al.*, 2010). Local spread then ensues with viremia and splenic viral load, detectable only by real time RT-PCR (Wang

and Deubel, 2011). Subsequently, virus enters the brain. Putative mechanisms for virus invasion into the CNS include i) hematogenous spread, ii) entry through olfactory neurons, iii) retrograde axonal transport through peripheral nerves, iv) a "Trojan horse" mechanism through infected monocytes and v) transcytosis through the endothelial cells of the blood-brain-barrier. JEV infection of neurons is accompanied by a local inflammatory reaction. Virus clearance from the CNS is complicated by the irreplaceable nature of neurons, and the fact that neuronal damage can be caused directly by virus infection or by infiltrating leukocytes in response to the infection (Griffin, 2011).

1.3 Innate Immune response to JEV

1.3.1 Sensing of the pathogen

Host cells detect distinct conserved molecular signatures (pathogen associated molecular patterns: PAMPs) of invading viruses through germ-line encoded transmembrane or cytosolic pathogen recognition receptors (PRRs) (Bowie & Unterholzner, 2008) (Table 1.3). This initial sensing and recognition is of paramount importance in viral immunobiology, where activating intracellular signalling cascades ultimately lead to the induction of antiviral, inflammatory and adaptive immune responses. Transmembrane PRRs include C-type lectin receptors (CLRs) and the widely studied toll-like receptors (TLRs), both of which are upregulated after JEV infection (Gupta & Rao, 2011). CLRs contain carbohydrate recognition domains interacting with mannose, fucose, and glucan carbohydrate structures of pathogens (Geijtenbeek & Gringhuis, 2009). A particular CLR, C-type lectin domain family 5 (CLEC5A), is highly expressed after JEV infection and is associated with a proinflammatory profile (Gupta *et al.*, 2010). Direct interaction of JEV with CLEC5A, resulting in macrophage and microglial cell activation, has recently been reported (Chen *et al.*, 2012). CLEC5A has been implicated in the immunopathology of dengue hemorrhagic fever (Chen et al., 2008). Similarly, CLEC5A plays a role in JEV-induced inflammation and lethality (Chen *et al.*, 2012). Treatment of JEV-infected mice with monoclonal antibodies against CLEC5A resulted in reduced blood-brain-barrier damage, reduced microglial activation, reduced bystander neuronal damage, and reduced cellular infiltration into the CNS (Chen *et al.*, 2012).

Table 1.3 List of pathogen recognition receptors

Membrane-bound receptors

Toll-like receptors (TLRs)

Structure: Composed of N-terminal leucine-rich repeats (LRR), transmembrane region, and cytoplasmic Toll/Interleukin-1 receptor (TIR) domain
Expression: predominantly in macrophages, dendritic cells, and other leucocytes; all other cell types
Functions: recognition of all microbes, inflammation, Type-1 interferon induction, antimicrobial signalling
Examples: TLRs 1-13

C-type Lectin receptors (CLR's)

Group 1 CLR's
Structure: Mannose receptors with Carbohydrate binding protein, recognize high mannose structures
Expression: macrophages and dendritic cells
Functions: inflammation, activate a pathway of the complement system, mediate endocytosis of pathogens, cell-cell recognition
Examples: Mannose receptor (CD206), DEC205

Group 2 CLR's
Structure Asialoglycoprotein receptors with Carbohydrate binding protein, recognize mannose, fucose, or glucan carbohydrate structures
Expression: mainly macrophages and dendritic cells also on B cells and neutrophils
Functions: inflammation, antigen presentation, mediate endocytosis of pathogens, cell-cell recognition
Examples: DC-SIGN, CLEC2, CLEC4A, CLEC4C, CLEC4E, CLEC4K, CLEC5A, CLEC6A, CLEC7A, CLEC9A, CLEC10A, CLEC12A, CLEC12B

Cytosolic receptors

NOD-like receptors

Structure: Composed of LRRs domain; a nucleotide binding oligomerization domain (NOD); and a domain for the initiation of signaling, such as caspase recruitment domains (CARDs), pyrin, or baculovirus inhibitor of apoptosis repeat (BIR) domains
Expression: mainly leucocytes, all other cell types
Functions: recognition of bacteria and viruses, regulation of inflammation and apoptosis
Examples: NOD1, NOD2

RIG-1-like receptors

Structure: RNA helicases; composed of two N-terminal CARDs, a central DEAD box helicase/ATPase domain, and C-terminal regulatory domain
Expression: mainly leucocytes, all other cell types
Functions: intracellular recognition of viruses
Examples: Retinoic acid-inducible gene I (RIG-1), Melanoma differentiation associated-protein 5 (MDA5), LGP2

TLRs are composed of a leucine-rich repeat-containing ectodomain, a transmembrane domain and an intracellular Toll–interleukin 1 receptor (TIR) domain (Kawai & Akira, 2010) (Table 1.4). The ectodomain mediates recognition of PAMPs, while the intracellular TIR domain mediates downstream signal transduction. TLR signaling, except that via TLR3, requires the TIR-domain adaptor molecule, MyD88, and

therefore can be prevented by MyD88 knockout (Kawai & Akira, 2010). Aside from MyD88, TLR4 can activate an alternative signalling pathway, which is through adaptor proteins TIR-domain-containing adaptor protein inducing interferon-β and TRIF-related adaptor molecule (Lu *et al.*, 2008). In the absence of MyD88, bone marrow-derived macrophages and dendritic cells infected with JEV have reduced production of inflammatory cytokines interleukin (IL)-6, IL-10, IL-12, and tumour necrosis factor (TNF)-α (Aleyas *et al.*, 2009). This supports a role of TLR signalling through MyD88 in shaping the immune responses to JEV. However, this is not reflected in a markedly altered disease outcome, given that $MyD88^{-/-}$ mice only show a partial impairment in interferon (IFN)-α production and similar susceptibility to JEV in comparison to wild-type mice (Kato *et al.*, 2006), suggesting a redundancy in pathways for recognition of JEV infection.

Table 1.4 List of toll-like receptors (TLRs)

TLR	Ligand	Expression location	Adapter Protein
TLR1	Multiple triacyl lipopeptides	Cell surface	MyD88/MAL
TLR2	Lipoteichoic acid, zymosan HSP70, Lipoarabinomannan	Cell surface	MyD88/MAL
TLR3	Double-stranded RNA	Cell compartment	TRIF
TLR4	Lipopolysaccharide Lipoteichoic acid Viral glycoproteins	Cell surface	MyD88/MAL TRIF/TRAM
TLR5	Flagellin	Cell surface	MyD88
TLR6	Multiple diacyl lipopeptides	Cell surface	MyD88/MAL
TLR7	Single-stranded RNA	Cell compartment	MyD88
TLR8	Single-stranded RNA	Cell compartment	MyD88
TLR9	Unmetheylated CpG DNA	Cell compartment	MyD88
TLR10	Unknown	Cell compartment	My88
TLR11	Pofilin	Cell surface	MyD88
TLR12	Unknown	Unknown	Myd88
TLR13	Unknown	Unknown	MyD88, TAK-1

Functional TLR 10 is not found in mice. Functional TLR11, 12, and 13 are not found in humans.

Cytosolic PRRs are essential for detecting pathogens invading the cytosol. They are classified into nucleotide binding oligomerization domain (NOD)-like and retinoic acid-inducible gene (RIG)-1-like receptors (Wilkins and Gale, 2010) (summarized in Table 1.3). NOD-like receptors, NOD2 and NLRP3, recognize ssRNA and dsRNA, respectively, and have significant antiviral activity through IFN signalling. Both proteins are expected to recognize flaviviral genomic RNA, although their role in JEV infection remains to be investigated. RIG-1-like receptors, also known as RNA helicases, have a conserved DExD/H box helicase domain and a C-terminal regulatory domain among the three recently identified members, RIG-1, melanoma differentiation-associated antigen 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2) (Wilkins and Gale, 2010). The C-terminal regulatory domain serves as the recognition site for sensing ssRNA and dsRNA. Kato et al (2006) have shown that RIG-1 receptor signaling, but not that via MDA-5, is critical for the antiviral response against JEV: RIG-$1^{-/-}$, but not MDA-$5^{-/-}$ mice, display impaired type 1 IFN production and increased susceptibility to JEV infection. LGP2 was initially reported as a dominant negative regulator of RIG-1 and MDA-5 signalling (Komuro & Horvath, 2006; Murali *et al.*, 2008); however, Satoh et al (2010) have demonstrated that bone marrow-derived dendritic cells from $LGP2^{-/-}$ mice infected with JEV have an impaired production of IFN-β, indicating that LGP2 functions upstream of RIG-1 and MDA-5 to potentiate viral RNA-induced signalling as a positive regulator.

1.3.2 Type 1 interferon induction and signalling

Interferons (IFNs) are a group of cytokines first discovered based on their antiviral activity against influenza (Borden *et al.*, 2007). Three families of IFNs, type I, type II and the recently identified type III, can be distinguished. Type I IFNs include multiple

IFN-α subsets, a single IFN-β, IFN-ω, and the recently discovered IFN-ε (Hardy *et al.*, 2004). All members bind to the same cell surface receptor, and are located in a single gene cluster both in humans and in mice. In addition to their antiviral activity, type I IFNs are key to efficient establishment of the adaptive immune responses (Borden *et al.*, 2007). Type III IFNs (IFN-λ1, -λ2 and -λ3) are new members of the IFN superfamily first discovered in 2003 and shown to be related to type I IFN (Ank *et al.*, 2006). However, they differ by signalling through a receptor complex that is different from that used by type I IFNs. Numerous RNA and DNA viruses induce and are sensitive to IFN-λs, although it remains unclear if type III IFNs are important in the host response against JEV infection. Type II IFN consists of a single cytokine, IFN-γ, and its function in JEV infection is described in Section 1.3.3.

IFN production after JEV infection was initially documented in mice (Rokutanda, 1969) and later in humans (Burke and Morill, 1987). Early *in vitro* and *in vivo* animal studies depicting its significance as an antiviral compound against JEV employed the use of IFN inducers (Ghosh *et al.*, 1990; Taylor *et al.*, 1980) and recombinant IFN-α (Crance et al., 2003). Furthermore, mice deficient in IFN-α receptor infected with JEV show sustained high viremia and fulminant disease (Lee *et al.*, 2004; Lee & Lobigs, 2002; Lobigs *et al.*, 2009), demonstrating that type I IFN is a key tropism determinant of JEV.

IFN gene expression is induced by the binding of PRR-activated transcription factors to their promoters (Borden *et al.*, 2007). They include IFN regulatory factor (IRF) proteins and NF-κB (Honda *et al.*, 2006; Tenoever *et al.*, 2007). In the case of JEV infection, RIG-1-dependent IRF-3 and phosphatidylinositol-3 kinase-dependent NF-κB activation is essential for IFN production (Chang *et al.*, 2006). NF-κB-dependent and NF-κB-independent mechanisms of IFN induction after JEV infection have been suggested by

Abraham et al (2010). Binding of IFN to its cognate receptor at the cell surface triggers a signalling cascade, the Janus kinase - signal transducer and activation of transcription (JAK-STAT) pathway, ultimately triggering IFN-stimulated response element and expression of IFN-stimulated genes (ISGs). ISGs serve as mediators of IFN action directed towards initiation of antiviral and immunoregulatory functions (Borden *et al.*, 2007). Antiviral proteins associated with flaviviral infections include double-stranded RNA-activated protein kinase (PKR), the 2',5'-oligoadenylate synthetases (2'-5'-OAS), ISG15, ISG20, viperin and IFN-induced transmembrane proteins (Brass *et al.*, 2009; Hsiao *et al.*, ; Jiang *et al.*, 2010; Kajaste-Rudnitski *et al.*, 2006; Samuel *et al.*, 2006). Of these, 2'-5'-OAS proteins are the most widely studied and acts through activation of RNAse L, a potent endoribonuclease that cleaves viral RNA (Silverman, 2007). The critical role of 2'-5'-OAS in the control of West Nile virus (WNV) infection was first reported in mice (Mashimo *et al.*, 2002; Perelygin *et al.*, 2002) and recently in horses and humans, where distinct OAS1a gene polymorphisms were identified as a risk factor (Lim *et al.*, 2009; Rios *et al.*, 2010). Given the association of OAS with the flavivirus-resistance phenomenon in mice (Brinton & Perelygin, 2003), this ISG most likely also plays an important role in recovery from JEV infection. ISG-15 is an additional ISG recently documented to be involved in the control of JEV infection (Hsiao *et al.*, 2010).

Considering its antiviral action, the therapeutic potential of recombinant IFN in human cases of JE has been investigated. While an initial study suggested a benefit (Harinasuta *et al.*, 1985), a subsequent randomised double-blind placebo-controlled clinical trial did not (Solomon *et al.*, 2003*b*). The failure to observe a benefit in the larger scale study posed the question of clinical relevance of IFN treatment. It remains to be seen, whether the outcome might differ if higher doses were given, given earlier in the course of infection, or given in combination with other drugs. It is likely that the failure of IFN

therapy after an established JEV infection can be attributed to the IFN-antagonistic mechanisms of the virus itself. JEV counteracts the effect of IFN by blocking tyrosine kinase 2 (Tyk2) and Stat activation (Lin *et al.*, 2004). This is mediated by the viral NS5 protein through the activation of protein tyrosine phosphatases (Lin *et al.*, 2006). Additionally, JEV NS4A protein is reported to block IFN action through inhibiting phosphorylation of Stat 1 and Stat 2 (Lin *et al.*, 2008*a*). Moreover, aside from IFN antagonism at the level of Jak-Stat signalling, JEV is also able to inhibit a downstream antiviral molecule, viperin, by promoting its degradation via a proteasome-dependent mechanism (Chan *et al.*, 2008).

1.3.3 Cellular factors, chemokines and cytokines

Neutrophil leucocytosis is a unique feature in human cases of JE (Chaturvedi *et al.*, 1979; Singh *et al.*, 2000*a*). A neutrophil chemotactic factor derived from JEV-stimulated macrophages has been reported to induce neutrophilia (Khanna *et al.*, 1991). Additionally, an increased level of the neutrophil chemoattractant, IL-8, found in CSF and serum of JEV infected individuals is significantly associated with neutrophilia and an elevated level of IL-8 in CSF and plasma is linked with adverse clinical outcome (Singh *et al.*, 2000*b*; Winter *et al.*, 2004). This contrasts with a potentially beneficial role of neutrophils in the control of JEV infection by a mechanism involving the degradation of virus via triggering a respiratory burst and the generation of toxic radicals (Srivastava *et al.*, 1999).

Cells of the monocytic lineage and the release of soluble factors thereof have been implicated in JEV pathogenesis. Macrophages predominate the inflammatory cells infiltrating the brain parenchyma of individuals with Japanese encephalitis (Johnson *et*

al., 1985). They are permissive for JEV replication, and provide a putative mechanism for JEV entry into the CNS (Aleyas *et al.*, 2009; Hasegawa *et al.*, 1990; Mathur *et al.*, 1988; Yang *et al.*, 2004). Cathepsin L-mediated processing of the capsid protein appears to play a role in JEV replication in macrophages, since mutant virus resistant to cleavage by the protease has impaired growth in macrophage but not fibroblast or mosquito cell lines (Mori *et al.*, 2007). Microglia are a brain-resident macrophage cell population, which can be infected with JEV for prolonged periods without morphological alteration, suggesting that microglia might serve as a reservoir for viral persistence in the CNS (Thongtan *et al.*, 2010). Local immune responses initiated by microglial cells may provide protection against JEV infection of the CNS. However, microglial activation resulting in elevated levels of proinflammatory cytokines (IL-6, TNF-α, IL-1β) and chemokines (IL-8, RANTES, MCP1) in the CSF and plasma may give rise to irreversible neuronal damage and correlates with an increased mortality rate (Chen *et al.*, 2004; Chen *et al.*, 2010*b*; Ghoshal *et al.*, 2007; Ravi *et al.*, 1997; Saxena *et al.*, 2008; Winter *et al.*, 2004). Astroyctes were originally classified as a subclass of glial cells with pleiotropic functions for maintenance of CNS homeostasis, and only recently were they shown to be immunocompetent cells (Dong & Benveniste, 2001). JEV infected astrocytes are an important source of chemokines (CCL5 and CXCL10) for migration of leukocytes into the CNS (Bhowmick *et al.*, 2007; Chen *et al.*, 2010*a*).

Lastly, natural killer (NK) and γ/δ T cells form the cytotoxic arm of the innate immune pathways. Both exhibit cellular cytotoxicity by causing apoptotic lysis of virally infected cells, either through a direct cell-cell contact mechanism or, indirectly, by release of soluble cytokines, IFN-γ and TNF-α. An *in vitro* study has demonstrated the antiviral activity of IFN-γ against JEV (Hasegawa *et al.*, 1990). IFN-γ mediates its antiviral effect, at least in part, through induction of nitric oxide (NO) synthase

(Karupiah *et al.*, 1993) and an inhibitory effect of NO on JEV growth has been documented (Lin Y.L. *et al.*, 1997; Saxena *et al.*, 2000). IFN-γ, derived from γ/δ T cells is necessary for the early control of dissemination of WNV, which is closely related to JEV (Wang *et al.*, 2003a). γ/δ T cells may play a protective role at the interface of innate and adaptive immunity, since TCRδ$^{-/-}$ mice display higher susceptibility after secondary challenge with WNV compared to wild-type mice (Wang T. *et al.*, 2006). It will be interesting to uncover whether γ/δ T cells are also important in experimental models of JEV.

Table 1.5 Innate immune factors affecting outcome of JEV infection

Factor	Outcome after JEV infection
Pathogen Recognition	
Myd88	absence = ↓ production of inflammatory cytokines,
RIG-1	absence = ↓ production of type 1 IFN; ↑ disease severity
MDA-5	absence = no effect on production of type 1 IFN; no effect on disease severity
LGP2	absence = ↑ production of type 1 IFN
Interferon Induction and Signalling	
NF-κB	↑ PI3K dependent production of type 1 IFN
IRF-3	↑ RIG-1 dependent production of type 1 IFN
IFN-α	inhibits virus, no effect with treatment of human cases
IFN-α receptor	absence = ↑ disease severity
Jak-stat	inhibited by viral NS4A and NS5 proteins
ISG15	inhibits virus
Viperin	inhibited via proteosome-dependent mechanism
Cellular Factors	
Neutrophils	neutrophilia, intracellular degradation of JEV ↑ release of inflammatory cytokines; ↑ pathology
Macrophage	monocytosis; ↑ migration of monocytes to CNS; ↑ inflammatory cytokines
Microglial Cells	microgliosis; ↑ release of inflammatory cytokines; ↑ pathology
Astrocytes	↑ release of inflammatory cytokines

↑ = increase, ↓ = decrease

1.4 Adaptive Immunity

Adaptive immunity represents the second wave of immune responses and is characterized by specificity, high potency, and development of memory. For it to become activated, it requires signals from antigen presenting cells of the innate immune system. This can be directly through cell-to-cell communication or, indirectly, by recognition of soluble cytokines. Adaptive immunity is composed of the humoral and cell-mediated immune responses mediated by B and T lymphocytes, respectively. The essential contribution of the adaptive immune responses in recovery from viral infections has been evident from empirical observations in people with defective B cell or T cell development (Fulginiti *et al.*, 1968; Wilfert *et al.*, 1977; Wyatt, 1973).

1.4.1 B cells

Humoral immunity has paramount protective function in primary JEV infection. The importance of a vigorous, virus-specific, humoral immune response in ameliorating and preventing illness has been documented in human cases of JE (Burke *et al.*, 1987; Libraty *et al.*, 2002; McCallum, 1991) and in animal models by administration of antibody prior or subsequent to infection with JEV (Goncalvez *et al.*, 2008; Gupta *et al.*, 2003; Kimura-Kuroda & Yasui, 1988; Zhang *et al.*, 1989).

Mechanistically, antibodies elicited against flaviviruses exhibit their action directly by neutralization of infectivity, or indirectly by antibody-dependent cell-mediated cytotoxicity, Fc-γ-receptor-mediated clearance, or complement-mediated cytotoxicity (Pierson *et al.*, 2008). Neutralizing antibodies predominantly target the E protein of the virion, although protective antibodies against prM and NS1 proteins have also been

documented (Dewasthaly *et al.*, 2001; Kolaskar & Kulkarni-Kale, 1999; Konishi *et al.*, 1991; Konishi *et al.*, 1992*a*; Konishi *et al.*, 1992*b*; Lin *et al.*, 2008*b*; Lin *et al.*, 1998; Nam *et al.*, 1999; Seif *et al.*, 1995; Wu et al., *2003*; Wu & Lin, *2001*; Xu et al., *2004*). The latter can control JEV infection by their complement-mediated cytolytic potential (Krishna *et al.*, 2009; Lin *et al.*, 2008*b*; Lin *et al.*, 1998). Antibody neutralizes flavivirus infectivity with high efficiency mainly by interfering with early steps of the viral entry pathway, including attachment, internalisation, and fusion (Butrapet *et al.*, 1998; Crill & Roehrig, 2001; Goncalvez *et al.*, 2008; Nybakken *et al.*, 2005).

1.4.2 T cells

T cells can be classified phentoypically, on the basis of their antigen receptor usage (α/β vs γ/δ) and their co-receptor expression (CD4 vs. CD8), or functionally (cytotoxic vs helper). Generally, cytotoxic T lymphocytes (CTLs) are predominantly of $CD8^+$ and, at times, helper T (Th) cells of $CD4^+$ phenotype. T cells of the γ/δ phenotype recognize non-classical major histocompatibility complex (MHC) antigens and form part of the innate immune response as described earlier. On the other hand, $CD8^+$ and $CD4^+$ α/β T cells recognize MHC-I and MHC-II plus peptide antigen, respectively, and serve as mediators of adaptive immune responses.

1.4.2.1 $CD4^+$ T cell immune response

Multifaceted $CD4^+$ T cells contribute to controlling infection by various mechanisms, including antiviral cytokine production, activation of antigen-presenting cells, antibody class switching, direct cytotoxicity, and maintenance of $CD8^+$ T cell activity (Zhu *et al.*, 2010). Follicular helper T cells (T_{FH}) are a subset of $CD4^+$ T cells that enter B cells

follicles to provide help in germinal center formation. T_{FH} require expression of inducible T cell co-stimulator (ICOS) and SLAM-associated protein (SAP) for optimal T_{FH}-B cell interaction important in generation of long-lived plasma cells and memory B cells (Bertram *et al.*, 2002; Crotty *et al.*, 2003; Kamperschroer *et al.*, 2006) (Fig. 1.8A). Direct costimulatory interactions, mainly through CD40L-CD40, between $CD4^+$ T cells and B cells are also necessary for the optimal generation of humoral immunity (Borrow *et al.*, 1996; Sangster *et al.*, 2003). $CD4^+$ T cell-mediated help for generation of effector $CD8^+$ T cells is via various mechanisms, including paracrine release of interleukin-2 by $CD4^+$ T cells, $CD4^+$ T cell direct CD40L-CD40 interaction with antigen-presenting cell that enhances priming of $CD8^+$ T cells, and upregulation of CD25 on $CD8^+$ T cells (Swain *et al.*, 2012; Obar *et al.*, 2010; Williams *et al.*, 2006) (Fig 1.8B). On the other hand, $CD4^+$ T cell-mediated help for generation of memory $CD8^+$ T cells is via downregulation of apoptotic genes in $CD8^+$ T cells, such as TNF-related apoptosis-inducing ligand (TRAIL) and programmed cell death protein 1 (PD1) (Fuse *et al.*, 2009; Janssen *et al.*, 2003; Janssen *et al.*, 2005; Oh *et al.*, 2009). Also, CD4+ T cell direct CD40L-CD40 interaction with $CD8^+$ T cells has been shown to enhance generation of memory $CD8^+$ T cells (Mueller *et al.*, 2006).

The protective value of JEV-immune $CD4^+$ T cells has been explored in adoptive transfer experiments in mice (Biswas *et al.*, 2009). Exposure to JEV induces effective $CD4^+$ T cells immunity, characterised by T cell proliferation, production of Th1 and Th2 cytokines, and immunoglobulin class switching (Konishi *et al.*, 1995; Ramakrishna *et al.*, 2003). Putative Th epitopes that elicit virus-specific and flavivirus cross-reactive proliferative responses in immune splenocytes have been mapped in E protein (Kutubuddin *et al.*, 1991). In humans, exposure to live JEV infection or vaccination similarly induces JEV-specific and flavivirus cross-reactive $CD4^+$ T cell responses

(Aihara *et al.*, 1998; Konishi *et al.*, 1995). A region of NS3 protein (residues 193 – 324) has been identified as the dominant source of peptide determinants for $CD4^+$ T cells in a healthy JEV-endemic cohort (Kumar *et al.*, 2004*a*; Kumar *et al.*, 2004*b*). Patients with severe encephalitis had impaired NS3-specific $CD4^+$ T cell responses, indicating a critical protective role of these immune cells in the pathogenesis of JE (Kumar *et al.*, 2004*a*).

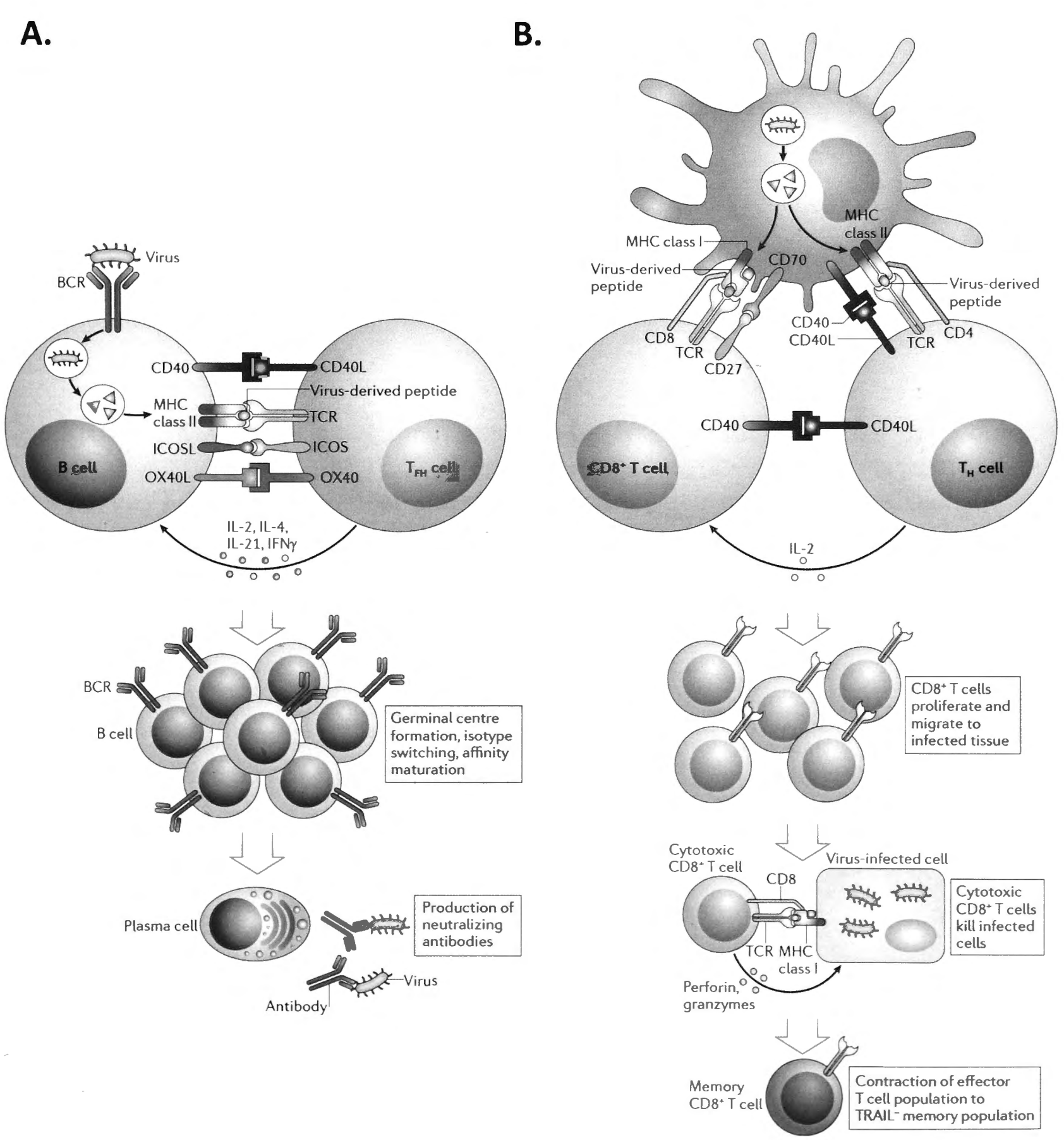


Figure 1.8 $CD4^+$ T cells helper functions. $CD4^+$ T cell-mediated help of B cells (A) is mediated through a subset of $CD4^+$ T cells called Follicular helper T (TFH) cells. In addition to release of soluble cytokines, direct interaction of TFH with B cells,

most importantly CD40L-CD40 interaction, induces germinal center formation, isotype switching and affinity maturation of antibody responses. All of which are important in the production of efficient neutralizing antibodies targeted towards eliminating viral pathogens. $CD4^+$ T cell-mediated help of $CD8^+$ T cells (B) via release of interleukin-2 (IL-2), direct CD40L–CD40 interaction, and indirectly via activation of antigen-presenting cells (APCs). Activation of APCs by $CD4^+$ T cells, via direct CD40L-CD40 interaction and cognate peptide-MHCII-II interaction, leads to enhanced priming of $CD8^+$ T cells by activated APCs. $CD4^+$ T cell-mediated help in generation of memory $CD8^+$ T cells involves downregulation of pro-apoptotic genes in $CD8^+$ T cells, such as TNF-related apoptosis-inducing ligand (TRAIL). BCR: B cell receptor, TCR: T cell receptor, TH: T helper, ICOS: inducible T cell co-stimulator, ICOSL: ICOS ligand, MHC: major histocompatibility complex. Figure was adapted from Swain *et al.* (2012).

1.4.2.2 $CD8^+$ T cell immune response

Cytotoxic $CD8^+$ T cells exert their function by lysing virally infected cells directly through Fas-FasL interaction or the perforin-granzymes exocytosis mechanism, and indirectly by release of soluble cytokines, IFN-γ and TNF-α (Russel and Ley, 2002; Mullbacher *et al.*, 2004) (Fig. 1.9). The binding of FasL, expressed on $CD8^+$ T cells, to Fas receptor on infected target cell, initiates apoptosis via the activation of a caspase cascade (Rouvier *et al.*, 1993; Nagata, 1997). FasL-Fas interaction induces the association of Fas-Associated protein with Death Domain, caspase-8, caspase-10, and c-FLIP, which altogether form the death-inducing signaling complex (DISC) (Peter *et al.*, 2007). The formation of DISC transduces a downstream signalling pathway, leading to target cell apoptosis.

Polarized release of pore-forming perforin and proteolytic granzymes by effector $CD8^+$ T cells to infected target cells initiate apoptosis via caspase-dependent and independent

mechanisms (Henkart, 1994; Heusel *et al.*, 1994; Simon and Kramer, 1994; Trapani and Smyth, 2002). It was originally thought that entry of granzymes into the target cell is mediated through the plasma membrane pores created by perforin, however recent evidence demonstrate that it is not the case (Chowdhury and Lieberman, 2008; Pipkin and Lieberman, 2007). The more accepted alternative mechanism of granzyme entry is still perforin-dependent. It speculates that the microscopic holes created by perforin induces a calcium influx, which subsequently activates a cellular membrane response leading to dynamin-dependent endocytosis of all substances, including perforin and granzymes, located at the narrow interphase between the effector and target cell (Keefe *et al.*, 2005; Pipkin and Lieberman, 2007).

To date, there are 5 identified human granzymes (Gzm) (A, B, H, K, M) and 11 mouse granzymes (A, B, C, D, E, F, G, K, L, M, N) (Granville, 2010). Among which, GzmA and GzmB are the most extensively studied. GzmA and GzmB induce apoptosis in target cells using different mechanisms, which is largely dependent on the cleavage specificities of the particular protease (Andrade, 2010). GzmB cleaves after aspartate residues (Asp-ase), making it able to activate pro-apoptotic caspases 3, 7, and 8, and activate effector proteins important in caspase-independent apoptotic pathways, such as BH3 interacting domain death agonist and inhibitor of caspase-activated DNase (Casciola-Rosen *et al.*, 2007; Cullen *et al.*, 2007). Endpoint of cell-death induced by GzmB is characterized by oligonucleosomal DNA fragmentation (Chowdhury and Lieberman, 2008). GzmA, on the other hand, cleaves target substrates containing consensus sequences with arginine or lysine at the P1 position, for instance Histone-A1 and lamins (Andrade, 2010; Lieberman and Fan, 2003; Zhu *et al.*, 2006; Zhu *et al.*, 2009). GzmA–induced death is mechanistically independent to that of GzmB, and results in single-stranded DNA breaks in target cells (Andrade, 2010).

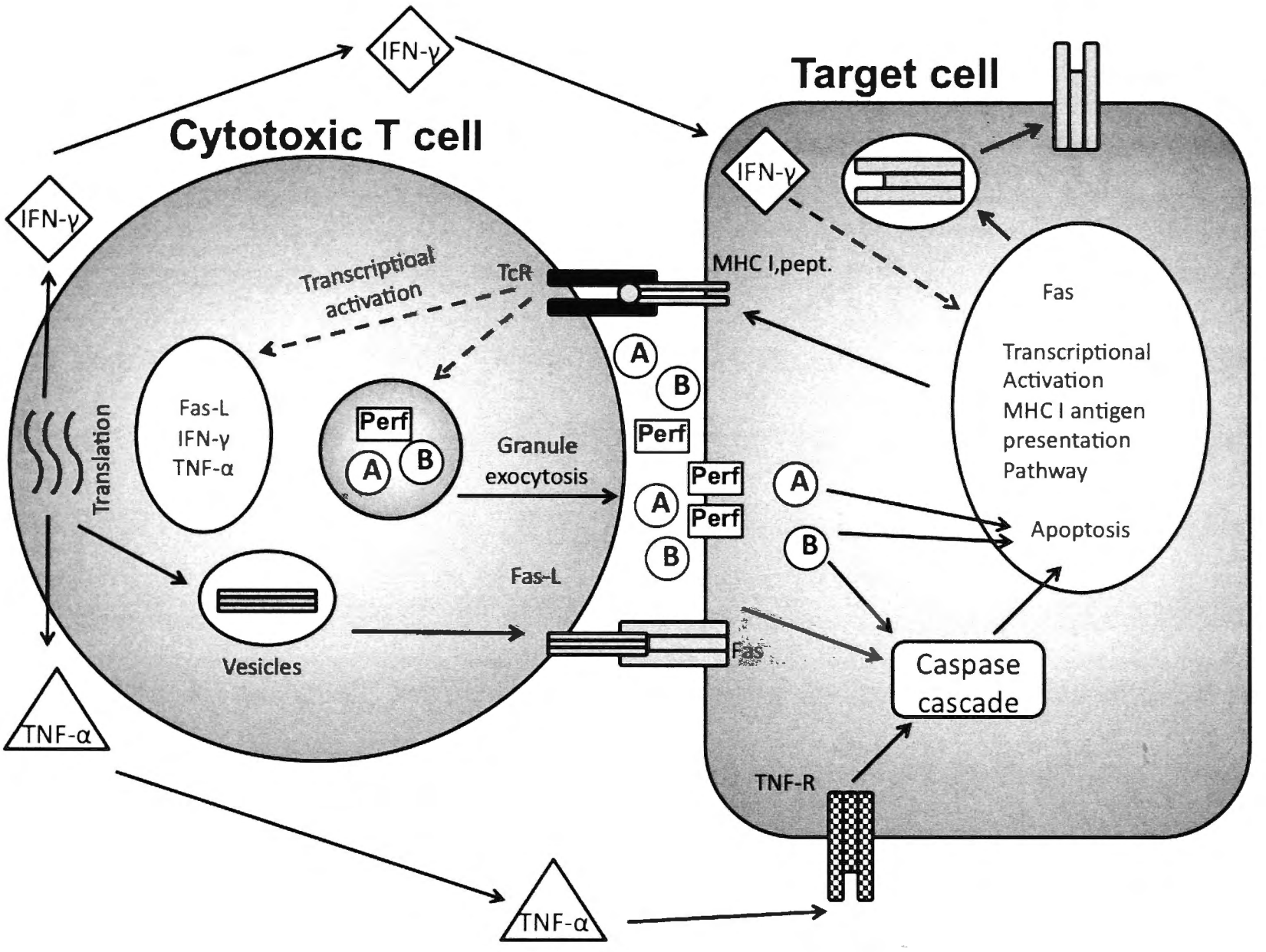


Figure 1.9 Effector pathways of cytotoxicity. T cell receptors (TCR) on the surface of $CD8^+$ T cells recognize cognate peptide-MHC-I complex presented by an infected target cell. The specific recognition activates an intracellular signal that leads to unidirectional secretion of lytic granules, containing perforin (perf) and granzymes (A or B in circles) into the synaptic cleft. Perforin is required for the uptake of granzymes by the target cell. Granzymes induce target-cell apoptosis by caspase-dependent and -independent mechanisms. Effector-target cell FasL-Fas interaction can trigger apoptosis mediated through activation of a caspase cascade. Simultaneously, TCR stimulation induces target cell production of soluble antiviral cytokines, tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ). Recognition of TNF-α by the TNF-α receptor expressed by target cells triggers apoptosis via the caspase cascade. IFN-γ, on the other hand, enhances target cell transcriptional activation of Fas and molecules of the MHC-I antigen presentation pathway, which creates a positive feedback loop of enhancing target cell apoptosis. The figure was adapted, with some modifications, from Mullbacher *et al*. (2004).

Early reports demonstrated JEV-specific $CD8^+$ T cell proliferative responses and cytolytic activity in humans and mice after vaccination or exposure to live JEV infection (Konishi *et al.*, 1995; Konishi *et al.*, 1997; Konishi *et al.*, 1998*a*; Murali-Krishna *et al.*, 1995*a*; Murali-Krishna *et al.*, 1994; Murali-Krishna *et al.*, 1995*b*). Peptide determinants recognized by JEV-immune $CD8^+$ cells are starting to be identified: they include a H-$2K^d$-restricted E protein-derived peptide (CYHASVTDI) (Takada *et al.*, 2000) and a H-$2D^b$-restricted NS4B protein-derived peptide (SAVWNSTTA) (Trobaugh *et al.*, 2010). In humans, the $CD8^+$ T cell response against JEV appears to be biased to peptide determinants derived from the NS3 protein (Kumar *et al.*, 2004*b*; Kumar *et al.*, 2003), as was first reported for the CTL response against the closely related Murray Valley encephalitis virus (MVEV) in mice (Lobigs *et al.*, 1994). This response against determinants in NS3 protein is broadly flavivirus cross-reactive and paradoxically recognises disparate epitopes from JEV and distantly related flaviviruses, but ignores more similar peptides from "self" and other virus families (Regner *et al.*, 2001), suggesting that primary sequence homology is not always the crucial factor in peptide recognition in the cross-reactive cellular immune responses against flaviviruses.

A dominant protective role of $CD8^+$ T cells in JEV infection was initially reported by Murali-Krishna et al (1996); however, this involved co-injection of a large number of splenocytes with virus into the brain and required co-transfer of $CD4^+$ T cells. A series of detailed studies have uncovered a conflicting role of $CD8^+$ T cells in recovery from infection with encephalitic flaviviruses (Table 1.6). While essential for virus elimination from the CNS and survival in mouse models of West Nile encephalitis (Shrestha and Diamond, 2004; Shrestha *et al.*, 2006*a*; Wang *et al.*, 2003b; 2004), a disease-potentiating effect of CTLs was documented in mice infected with MVEV (Licon Luna

et al., 2002). Notably, mice genetically deficient in the Fas- or perforin-dependent pathways of cytotoxicity show greatly increased susceptibility to virulent lineage I WNV infection (Shrestha and Diamond, 2007; Shrestha *et al.*, 2006*a*), but do not differ from wild-type mice in susceptibility to infection with lineage II WNV strain, Sarafend (Wang *et al.*, 2004), and are more resistant to infection with MVEV (Licon Luna *et al.*, 2002). These findings highlight a difference in pathogenesis between even closely related flaviviruses belonging to the JEV serocomplex (Mullbacher *et al.*, 2004) that most likely involves a difference in the capacity of the cellular immune response to resolve the virus infections in the CNS.

Table 1.6 Impact of cell-mediated immunity on pathogenesis

Knock-out mice or Treatment	WNV lineage 1	WNV lineage 2	MVEV
$CD8^+$ T cell-deficient or depleted	↑	↑	nt
β2m-deficient	↑	↑	↓
Perforin deficient	↑	no effect	no effect
Granzyme A & B deficient	nt	↑	no effect
Perforin and Granzyme A & B deficient	nt	↑	no effect
Fas ligand deficient	↑	no effect	↓
Perforin x Fas Ligand deficient	nt	↑	↓
IFN-γ-deficient	↑	no effect	↑

↑ = increase, ↓ = decrease, nt = not tested. Figure adapted, with some modifications, from Lobigs et al. (2009)

1.4.2.3 Modulation of MHC-I

MHC-I is expressed on virtually all mammalian cells and the cell surface expression of this class of restriction elements for CTLs is up-regulated as a consequence of infection with JEV and other flaviviruses in a diverse range of cell types from different species (King and Kesson, 1988; Kesson *et al.*, 2002; Lobigs *et al.*, 2003). Flavivirus-induced up-regulation of MHC-I cell surface expression is, at least in part, IFN-independent (Abraham *et al.*, 2010; Kesson & King, 2001; Mullbacher & Lobigs, 1995), and also includes that of non-classical MHC-I (Abraham *et al.*, 2008). Although the physiological relevance of this phenomenon in virus transmission remains unclear, it has been proposed that the process may contribute to reduced NK cell activity, which is inhibited by engagement with MHC-I by NK cell inhibitory receptors (Hershkovitz *et al.*, 2008; Momburg *et al.*, 2001). It has also been hypothesised that flavivirus-induced up-regulation of MHC-I leads to transient T cell autoimmunity (given the increase in "self" antigen presentation), followed by subsequent suppression of "self"-reactive T cell activity, and that flavivirus infection or live vaccination of humans in the tropics could contribute to the observed lower incidence of overt autoimmunity in the tropics than in temperate climates, where flaviviruses are not endemic (Lobigs *et al.*, 1996).

1.5 Control and Prevention of JE

No approved therapeutic agent is available for JE. Treatment is mainly supportive, for instance, using mannitol to decrease increased intracranial pressure (Solomon *et al.*, 2002). Clinical trials evaluating the efficacy of corticosteroids and IFN-α on improving the outcome of patients with JE have shown discouraging results (Hoke *et al.*, 2002; Solomon *et al.*, 2003*a*). The only means to combat JE, therefore, is through effective means of control and prevention.

Given that the life cycle of arboviruses alternates in stages between mosquito vectors and animal or human hosts, vector control and mass vaccination are two suitable approaches available for control and prevention. Chemical insecticides used in vector control were noted for their early successes (Walker and Lynch, 2007; World Health Organization, 2006). For instance, dichlorodiphenyletrichloroethane (DDT) is regarded as the instrumental insecticide that paved the way for the elimination of arthropod-borne diseases, such as yellow fever and malaria, in North America (Gubler, 2004; Karunamoorthi, 2011). However, issues pertaining to its toxicity in humans and adverse environmental impact, such as bioaccumulation, brought forward its ban from use (Eskenazi *et al.*, 2009; van den Berg, 2009). Due to safety issues and environmental concerns of chemical insecticides, recent alternatives were developed, including Release of Insects containing a Dominant Lethal (RIDL)-based or Wolbachia-based strategies. Both strategies are designed to shorten the lifespan of mosquitoes, thereby preventing the development of vector-capable adult mosquitoes. The RIDL strategy employed by Oxitec (United Kingdom) requires mass release of genetically modified mosquitoes that carry a dominant lethal gene (Alphey *et al.*, 2002; Thomas *et al.*, 2000). The Wolbachia-based strategy involves mass release of mosquitoes infected with natural

strains of *Wolbachia*, an easily transmissible bacterium that causes life shortening in mosquitoes (Fu *et al.*, 2010; Moreira *et al.*, 2009; McMeniman *et al.*, 2009; Xi *et al.*, 2005).

Experience with yellow fever vaccination revealed that eradication of arboviruses is not likely possible, for two main reasons: constant presence of reservoir or maintenance hosts in the environment, and constant presence of primary and alternative vectors maintaining the virus in both urban and rural cycles (Barrett and Higgs, 2007). It is then thought that the most cost-effective way of controlling spread of arboviral diseases is through mass vaccination.

1.5.1 JE Vaccines

Vaccination is the most cost-effective measure in controlling the spread of JE (Jelinek, 2008). In countries were routine immunization against JE were implemented, the dramatic drop in annual incidence of JE was observed (Erlanger *et al.*, 2009). For the past 50 years, the formalin-inactivated mouse brain-derived JEV was the only licensed vaccine for international use. However, due to the perceived issues of safety, the use of the vaccine was suspended, and its production was ceased since 2005 (Appaiahgari *et al.*, 2010). It is though that the recommendation was done in the anticipation of the arrival of new generation JE vaccines. For a new JE vaccine to get through the regulatory process, it has to display non-inferiority to formalin-inactivated mouse brain-derived JE vaccine. This is based on an established surrogate marker for JE vaccine efficacy, which is $PRNT_{50}$ titre (50% plaque reduction and neutralization test) of at least 1:10 (Hombach *et al.*, 2005, Markoff, 2000).

1.5.1.1 Inactivated mouse brain-derived JE vaccine

In 1954, a formalin-inactivated mouse brain-derived JE vaccine (JE- VAX®) was licensed in Japan (Fischer *et al.*, 2010; Scholer *et al.*, 2009). Its formulation was subsequently modified through the decades, with the vaccine containing inactivated JEV strain Beijing-1 and/or Nakayama. The internationally licensed vaccine is an inactivated JEV Nakayama-NIH strain produced in The Research Foundation for Microbial Diseases of Osaka University (Biken, Osaka, Japan), and distributed worldwide in the recent past by Sanofi Pasteur as JE-VAX. Each one ml dose formulation contains 500 μg of gelatin, <100 μg of formaldehyde, <0.0007% v/v Polysorbate 80, <50 ng of mouse serum protein, and 0.007% thimerosal preservative. Aside from effective use in military personnel and tourists from JE-nonendemic to JE-endemic countries, JE- VAX® vaccine was instrumental in the dramatic decline of JE incidence in JE-endemic countries, such as in Japan, South Korea, Taiwan, and Thailand (Fischer *et al.*, 2010).

Two large clinical trials have demonstrated the efficacy of JE-VAX® vaccine. The first study demonstrated a vaccine efficacy rate of 80% after one year of follow-up in children receiving a two-dose regimen of the vaccine, while a single dose vaccine was not efficacious (Hsu *et al.*, 1971). The second study demonstrated a combined vaccine efficacy rate of 91% after two years of follow-up in children receiving monovalent vaccines (containing either Nakayama or Beijing-1 strain), while no difference was noted between monovalent vs. bivalent vaccines (Hoke *et al.*, 1988).

Twenty percent of JE- VAX® vaccine recipients were associated with local adverse events, such as localized erythema, tenderness, and swelling at the injection site, while

10% of vaccine recipients experienced mild systemic effects, such as fever, chills, headache, myalgia, rash, and gastrointestinal symptoms (Defraites *et al.*, 1999; Poland *et al.*, 1990). The frequency of severe hypersensitivity reaction is estimated to range from 10 to 260 cases per 100,000 vaccine recipients (Fischer *et al.*, 2010). These serious, but rare, systemic allergic reactions include generalized urticaria, angioedema of the extremities, face, and oropharynx, with or without accompanying bronchospasm, respiratory distress, and hypotension. History of anaphylaxis, urticaria, or other allergies provides a 2 to 11 times likelihood of developing a reaction after JE- VAX® vaccination (Berg *et al.*, 1997; Plesner *et al.*, 2000; Sakaguchi *et al.*, 2001*b*). A study in Japan has demonstrated gelatin, a vaccine stabilizer, as the likely compound that induces IgE-mediated immediate hypersensitivity reaction in children (Sakaguchi *et al.*, 1997; Sakaguchi *et al.*, 1998; Sakaguchi *et al.*, 2001*a*). Approximately 0.1-2 cases per 100,000 JE-MB recipients demonstrated moderate to severe neurologic symptoms, including encephalitis, gait disturbances, Parkinsonism, and seizures (Fischer *et al*, 2010). Cases of fatal acute disseminated encephalomyelitis (ADEM) were reported in Korea and Japan, and in 2005, this was one of the cited reasons that prompted Japanese officials to suspend the used of JE-MB vaccine (Ohtaki *et al.*, 1995; Matsui *et al.*, 2002; Takahashi *et al.*, 2000). However, the WHO Global Advisory Committee on Vaccine Safety found no evidence of increased risk for ADEM after JE-MB vaccination (Fischer *et al.*, 2010).

1.5.1.2 Inactivated Beijing-3 strain JE vaccine

In 1968, a primary hamster kidney cell culture-derived JEV Beijing-3 strain (P-3) was developed in China (Scholer *et al.*, 2009). It was widely used in China until it was gradually replaced by a live, attenuated (SA14-14-4) vaccine. No large-scale

randomized control trials were done to fully evaluate the safety and immunogenicity of the P-3 vaccine. A single case-control study revealed a limited protection of 78% after 2-dose primary vaccination plus annual boosters (Luo *et al.*, 1994). The limited efficacy and requirement of multiple doses was seen as the basis for the Chinese authorities to shift to using the SA14-14-2 vaccine (Scholer *et al.*, 2009).

1.5.1.3 SA14-14-4 JE vaccine

In 1988, Chinese authorities licensed SA14-14-2, a live attenuated JE vaccine produced at the Chengdu Institute of Biological Products (CDIBP), China. SA14-14-2 was generated by serial passages of SA14 strain in primary hamster kidney cell culture, and in live mice and hamsters, with subsequent plaque purifications in primary chick embryo cell culture (Appaiahgari *et al.*, 2010; Halstead and Thomas, 2011). Q-One Biotech (UK) has determined the SA14-14-2 to be free of adventitious viruses after quality assurance check of the master seed vaccine and vaccine lots. It has been administered to more than 200 million children in China, and has recently been licensed in India, Sri Lanka, Nepal, and South Korea (Appaiahgari *et al.*, 2010).

Three small case-controlled studies (Hennessy *et al.*, 1996; Bista *et al.*, 2001; Ohrr *et al.*, 2005) demonstrated the efficacy rate of SA14-14-4 at > 95%. No large randomized trial was done to assess vaccine effectiveness; however, epidemiologic observational studies have revealed significant disease reduction in areas where the vaccine was used (Zhou *et al.*, 1999, Zhou *et al.*, 2001). A cluster-randomized control trial by Liu *et al.* (1997) has shown that SA14-14-4 vaccine, relative to unvaccinated controls, was not associated with increased adverse events. In addition, the WHO's Global Advisory Committee on Vaccine Safety has acknowledged the excellent safety and efficacy profile of the SA14-14-4 vaccine. In over a million children followed up in safety

studies, approximately 5-10% present with transient fever, 1-3% present with local reactions, rash or irritability, and no reported cases of acute encephalitis or anaphylactic reactions (Halstead *et al.*, 2004; Liu *et al.*, 1997). The excellent safety profile was also supported by a post-marketing surveillance study of SA14-14-2 vaccine use in South Korea (World Health Organization, 2005).

1.5.1.4 ChimeriVax-JE vaccine

ChimeriVax-JE is a recent live attenuated recombinant JE vaccine produced by replacing the E and prM genes of YF-17D with JEV vaccine strain SA14-14-4 (Chambers *et al.*, 1999; Monath *et al.*, 2012). The construct was created at the St Louis University Health Sciences Center and Acambis Inc., and was recently licensed for production by Sanofi Pasteur. ChimeriVax-JE is highly stable, and its infection and replication is restricted to mosquito vectors (Monath *et al.*, 1999; Bhatt *et al.*, 2000). Its safety, immunogenicity, and protective capacity have been demonstrated in preclinical studies (Monath *et al.*, 1999).

Two randomised control phase 3 clinical trials revealed noninferiority of a single dose of ChimeriVax-JE relative to 3 dose regimen of inactivated mouse brain-derived JE vaccine (Torresi *et al.*, 2010). Long-term immunogenicity after single dose ChimeriVax-JE immunization was demonstrated in a study done by Nasveld et al. (2010), which showed seroprotection in 99% and 97% of participants at 1 and 6 months post-immunization, respectively. Furthermore, 87% of the participants, who were seroprotected at month 6, were still be protected at month 60, while 96% of the participants, who received a booster dose at month 6, were still be protected at month 60.

A series of clinical studies did not show serious or life-threathening adverse events that were related to ChimeriVax-JE immunization (Halstead and Thomas, 2011). The rate of mild adverse events in participants receiving Chimerivax-JE was lower relative to control YF-17D immunization (Monath *et al.*, 2002; Monath *et al.*, 2003), or similar compared to hepatitis A vaccination (Chokephaibulkit *et al.*, 2010). A large post-marketing surveillance study will be needed to fully assess the safety of ChimeriVax-JE vaccine.

1.5.1.5 Alum-adjuvanted Vero cell culture-derived JE vaccine

An inactivated Vero cell culture-derived JE vaccine (ccJE) is manufactured by Intercell Biomedical (Livingston, United Kingdom) and is distributed by Novartis Vaccines (Fischer *et al.*, 2010; Schioler *et al.*, 2007). The inactivated vaccine is adjuvanted with aluminium hydroxide (Alum), and is derived from the attenuated SA14-14-2 JEV strain. Each 0.5-mL dose contains 6 μg of purified, inactivated JEV proteins, 0.1% aluminum hydroxide, and does not contain gelatin stabilizers, antibiotics, or preservative thimerosal. Given that a correlate for protection was established for JE vaccines, no efficacy study was needed for licensure of Alum-adjuvanted ccJE vaccine. Instead, a noninferiority to JE-MB vaccine study was done, which revealed comparable immunogenicity (Fischer *et al.*, 2010). Durability of protective neutralizing antibodies after a 2-dose regimen of Alum-adjuvanted ccJE vaccine was 95% at 6 months and 83% at 12 months (Schuller *et al.*, 2008). In contrast, another study performed in a different vaccinee population, revealed a less durable protective neutralizing antibody response of 83% after 6 months, 58% after 1 year, and 48% after 2 years (Dubischar-Kastner *et al.*, 2009).

A pooled analysis of seven studies on Alum-adjuvanted ccJE vaccine revealed a frequency of 3% of local adverse events, which was comparable to controls and better than for the JE- VAX® vaccine (14%) (Dubischar-Kastner *et al.*, 2009). Mild systemic symptoms were comparable in Alum-adjuvanted ccJE, JE- VAX®, and placebo control groups (36% vs 40%, respectively). Only 1% of subjects receiving Alum-adjuvanted ccJE vaccine presented with serious advents, while no serious anaphylactic reactions or neurologic adverse events were recorded (Dubischar-Kastner *et al.*, 2009). However, given that only a small population has received the vaccine, a post-marketing surveillance study is underway to fully evaluate vaccine safety.

1.5.2 Vaccine Adjuvants

An adjuvant is a substance added to a vaccine formulation intended to enhance the quality of the immune response to the antigen. It is derived from the Latin word "adjuvare," meaning "to help". In the 1920's, the word adjuvant was first used by Gaston Ramon of the Institut Pasteur, upon observing that the immune response in horses given diphtheria toxin was enhanced when local inflammation is present at the inoculation site (Lombard and Moulin, 2007). An adjuvant, when added to a vaccine formulation, offers the following benefits: i.) increase potency of weak immunogen, ii.) improve immunogenicity of vaccines when given to aged or immunocompromised individuals, iii.) reduce dose of antigen used in vaccine formulation (antigen-sparing capacity), thereby lowering costs of vaccines, and iv.) enable various modes of vaccine delivery (Coffman *et al.*, 2010). The latter is exemplified by mucosal vaccination using pertussis or cholera toxin adjuvants, which potentiates Th2 responses, and thereby increases neutralizing IgA and IgE antibody responses at mucosal sites.

For the past ~70 years, Alum has been the only adjuvant licensed for international use (Fig. 1.10). Multiple attempts were done to introduce novel adjuvants through the years, and yet failed due issues of safety and reactogenecity. However, this changed in recent years when empirical studies were replaced with in-depth investigations uncovering the molecular mechanisms of adjuvant mode of action, resulting in series of new generation adjuvants entering or about to enter the market (Coffman *et al.*, 2010). A web-based vaccine adjuvant database has summarized a total of 104 adjuvants, both licensed and candidates included (Sayers *et al.*, 2012). Adjuvants can be classified, based on their mode of action, into: i.) antigen delivery systems, ii.) immune potentiators, and iii.) combination systems (Table 1.7).

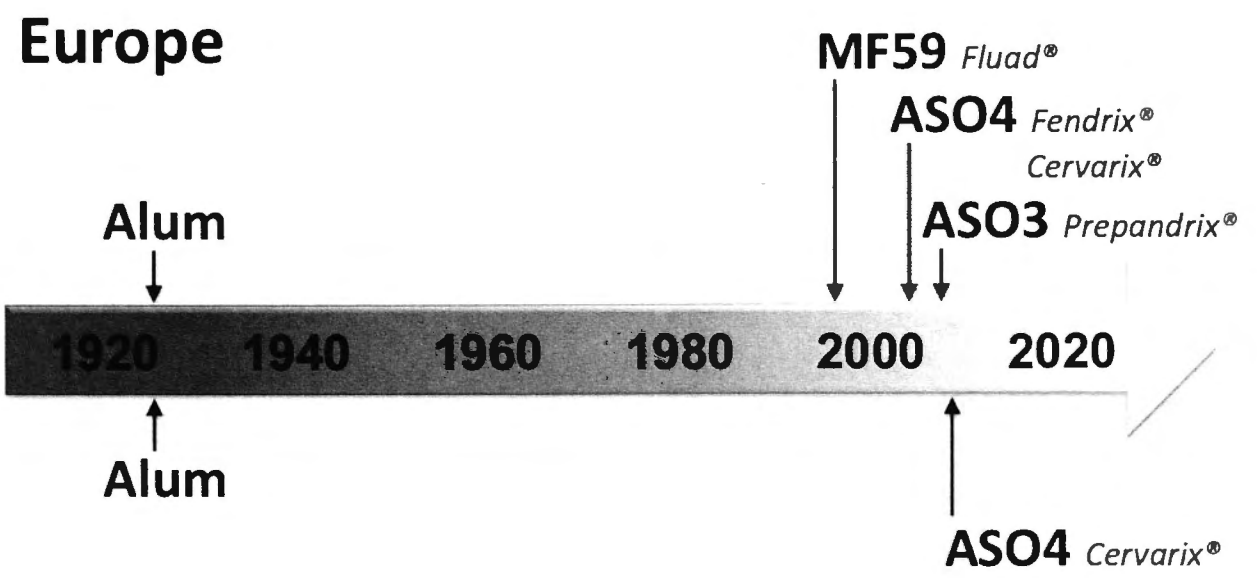


Figure 1.10 List of licensed adjuvants in Europe and USA. Alum is the only licensed adjuvant from 1926 until 1997. Since then, a series of adjuvants have been approved for use in Europe: MF59 in 1997 for seasonal influenza vaccine (*Fluad*), ASO4 in 2005 for hepatitis B vaccine (*Fendrix*) and human papilloma virus vaccine (*Cervarix*), and ASO3 in 2008 for pandemic flu vaccine (*Prepandrix*). In the United States, ASO4 was licensed in 2009 for *Cervarix*. MF59 (Novartis) and ASO3 (GlaxoSmithKline): oil-in-water emulsion based on squalene, ASO4 (GlaxoSmithKline): Monophosphoryl lipid A + Alum.

Table 1.7 Classification of Adjuvants

Type	Mode of action	Examples
1. Antigen delivery systems	a.) Forming depots	Water-in-oil emulsion
	b.) Antigen delivery to antigen-presenting cell	Liposomes ISCOMS
2. Immune potentiator	Act directly to activate immune system	TLR agonists MPL (TLR4) Flagellin (TLR5) Imiquinod (TLR7 and/or TLR8) CpG (TLR9)
3. Combination systems	Combination of antigen delivery system and immune potentiator	Aluminum salts ASO2 ASO4

MPL: Monphosphoryl lipid A, ASO2: oil-in-water emulsion containing MPL and QS-21, ASO4: Alum and MPL, ISCOMS: immune stimulating complexes

1.5.1.1 Alum adjuvant

Alum (Aluminum salts) is the most commonly used vaccine adjuvant with a proven safety record (Exley *et al.*, 2010; Marrack *et al.*, 2009). It has been extensively used in humans, and is included in vaccine formulations targeted against diphtheria, tetanus, pertussis, hepatitis A and hepatitis B. The mechanism of action of Alum was originally believed mainly through depot formation at the injection site, that facilitates efficient antigen uptake and presentation. However, recent studies have documented alternative mechanisms, mainly through enhanced immune stimulation, and involving i.) NRLP3 inflammasome, ii.) lipid sorting, and iii.) extracellular signal-regulated kinase (ERK).

An *in vitro* study has demonstrated the ability of Alum to stimulate caspase-1 and produce the inflammatory cytokines, IL-1β and IL-18. The activation of caspase-1 by alum is mediated through the NRLP3 inflammasome (Eisenbarth *et al.*, 2008; Hornung

et al., 2008; Li *et al.*, 2007). The role of NRLP3 inflammasome was confirmed with the use of NALP3$^{-/-}$ or caspase-1$^{-/-}$ mice, which were unable to mount a substantial humoral immune response after alum-adjuvanted immunization. Alum is also able to bind to sphingomyelin and cholesterol on dendritic cell surface membranes. The binding results in lipid sorting, such as clustering of immunorecpetor tyrosine-based activation motif-containing receptors. Lipid sorting subsequently activates intracellular transduction pathways, mediated through Syk and phosphoinositide 3-kinase (Flach *et al.*, 2011). Lastly, Alum is identified to interact with another kinase, ERK, which stimulates abortive phagocytosis in dendritic cells, and thereby facilitating the uptake of a specific antigen (Flach *et al.*, 2011)

Two main disadvantages have been associated with Alum. One is the recorded frequency of adverse side effects including injection site pain and inflammation, lymphadenopathy, and eosinophilia (Goto *et al.*, 1993; Goto *et al.*, 1997), and, in some cases, its association with a chronic inflammatory disease known as macrophagic myofascitis (Gherardi *et al.*, 2001; Gherardi and Authier, 2003). Another limitation is the limited efficacy of Alum to enhance immune responses to a specified antigen (Doherty *et al.*, 2006), for instance, its failure to augment cell-mediated immune responses and failure to provide durable memory B cell responses (Kool *et al.*, 2011, Exley *et al.*, 2010; Marrack *et al.*, 2009).

1.5.1.2 Advax adjuvant

Advax is a promising adjuvant that has been shown to enhance the protective value of vaccines targeted against influenza virus, human immunodeficiency virus, African Horse Sickness virus, and *Burkholderia mallei* (Cristillo *et al.*, 2011; Cooper and

Petrovsky, 2011; Eckersley *et al.*, 2011; Layton *et al.*, 2011; Lobigs *et al.*, 2010). The delta inulin polysaccharide particle Advax is a strong candidate as an ideal adjuvant: it is safe, non-reactogenic, tolerable, immunogenic when combined with an antigen, and significantly antigen-sparing (Cooper and Petrovsky, 2011; Honda-Okubo *et al.*, 2012). Recently, it has been shown that co-administration of Advax with inactivated ccJE dramatically improved the immunogenicity of the vaccine (Lobigs *et al.*, 2010). This was reflected by solid neutralizing titres, which were known to be protective against JEV infection or heterologous challenges with MVEV or WNV.

The simple inert polysaccharide composition of Advax adjuvant also imparts it an advantage as a safe formulation (Cooper and Petrovsky, 2011). Preclinical studies have revealed that, based on footpad swelling test, local injection site reactogenicity was very minimal when using Advax adjuvant relative to recently licensed adjuvants in the market (Honda-Okubo *et al.*, 2012). The mechanism of action of Advax adjuvant is not yet completely understood. It is known to stimulate a balanced Th1 and Th2 responses, and is associated with robust and durable antibody and T cell responses (Honda-Okubo *et al.*, 2012). It is thought that Advax adjuvant does not work through enhanced activation of innate immune inflammation observed in Toll-like receptor agonists, nor does it have direct action on T cell activation. Advax adjuvant is known to bind to undefined receptors in mononuclear cells, with a consequent upregulation of co-stimulatory molecules (CD11c, CD83, CD86, MHC-II) required for efficient antigen presentation (Cooper and Petrovsky, 2006; Honda-Okubo *et al.*, 2012). It is then currently hypothesized that Advax adjuvant works through enhancing early antigen presentation, and thereby boosting adaptive immune responses.

Aims of the study:

The immunological correlates of recovery from JEV infection remains poorly defined. It is the aim of this study to establish the role of adaptive immunity in host recovery in mouse models of primary JEV infection. Furthermore, it is aimed to use these insights to establish the immunological mechanism of protection of candidate JE vaccines.

Objectives:

1. To establish a pathogenesis model of JEV infection that mimics natural infection route and dose in humans.
2. To determine the role of humoral immunity in the pathogenesis of Japanese encephalitis.
3. To determine the role of $CD4^+$ and $CD8^+$ T cell populations in the pathogenesis of Japanese encephalitis.
4. To determine the role of effector mechanisms of cytotoxicity in the pathogenesis of Japanese encephalitis.
5. To highlight the commonalities with and differences from WNV or MVEV in the role of adaptive immune pathways in recovery from JEV infection.
6. To determine the role of the chemokine, CCR5, in the pathogenesis of Japanese encephalitis.
7. To determine the impact of adaptive immune pathways in vaccine-mediated protection, and establish the mechanism of protection of a new generation JE vaccine.

Hypotheses:

In murine models of Japanese encephalitis, effectors of adaptive immunity play an important role in recovery from primary infection. Likewise, all components of the adaptive immune pathways are crucial for protection provided by JE vaccines.

Chapter 2

Materials and Methods

2.1 Viruses and cells

Cell lines were originally obtained from the American Type Culture Collection (ATCC). Vero cells (African green monkey kidney) and YAC-1 cells (Moloney leukemia virus-induced T cell lymphoma) were grown at 37°C with 5% CO_2 atmosphere in Eagle's minimal essential medium plus nonessential amino acids (MEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS). Working stocks of JEV (strain Nakayama) were infected Vero cell culture supernatants ($2x10^8$ PFU/ml) stored in single-use aliquots at -70°C. Other previously prepared viruses (Table 2.1) stored in single-use aliquots at -70°C included MVEV (strain MVE-1-51), WNV strain Kunjin (WNV_{KUN}), WNV strain New York 1999 (WNV_{NY99}), ChimeriVax-JE, yellow fever virus (YFV), dengue virus type 2 strain PUO (DENV-2), vaccinia virus Western Reserve strain ($VACV_{WR}$).

Table 2.1 List of viruses used in the study

Virus	Stock titre[a]	Preparation
JEV	$2x10^8$ PFU/ml	Vero cell culture supernatant
MVEV	$2x10^9$ PFU/ml	10% suckling mouse brain homogenate[b]
WNV_{KUN}	$1x10^9$ PFU/ml	10% suckling mouse brain homogenate[b]
WNV_{NY99}	$1x10^8$ PFU/ml	C6/36 cell culture supernatant
ChimeriVax-JE	$1x10^7$ PFU/ml	Vero cell culture supernatant
DENV-2	$5x10^6$ PFU/ml	C6/36 cell culture supernatant
YFV	$1x10^7$ PFU/ml	C6/36 cell culture supernatant
$VACV_{WR}$	$1x10^7$ PFU/ml	Infected CV1 cell lysate

[a] Stocks stored in single use aliquots at -70 °C.

[b] Homogenates in Hanks' balanced salt solution (HBSS) containing 20 mM HEPES buffer (pH 8.0) and 0.2% bovine serum albumin (HBSS-BSA)

2.2 Plaque Assay

Titration of JEV was by plaque formation on Vero cells monolayers used at 80% confluence in six-well tissue culture plates (Nunc). Samples from infected cell culture supernatants, serum, or tissue homogenates were serially diluted in ten-fold dilution steps in HBSS-BSA on ice, and 100 µl of diluted samples was inoculated on Vero cell monolayers. For virus adsorption, plates were incubated for 1 h at 37°C in an atmosphere of 5% CO2, with gentle shaking every 15 minutes. Four ml of agar overlay medium containing 1% M199 medium and Bacto-agar (Difco, Detroit, Mich.), supplemented with 10 mM L-glutamine, 2% FCS, and 100U of antibiotics [penicillin (30 µg/ml; Sigma, St. Louis, Mo.), streptomycin sulfate (50 µg/ml; Sigma), and neomycin sulfate (50 µg/ml; Sigma)] was added to each well. Following incubation for 72 to 96 h at 37°C, 1 ml of 0.03% neutral red (BDH Chemicals, Poole, England) in HBSS were added to stain the monolayers. After 16 hours incubation at 37°C, the stain and agar overlay were removed, and plaques were counted to quantify viral titer expressed in PFU (plaque forming unit).

2.3 Mice

C57Bl/6 (B/6), C57Bl/10 (B/10), CBA, Balb/c, 129x1/SvJAPB (129), B6.129P2/J ($CCR5^{+/+}$) and various knockout mice strains (summarized in Table 2.1) were bred under specific pathogen-free conditions, and supplied by the Animal Breeding Facility at the John Curtin School of Medical Research, The Australian National University (ANU), Canberra. Female mice were used in all experiments. All animal experiments were conducted with approval from ANU Animal Ethics Committee.

Table 2.2 List of knockout mice strains

Mice strain	Brief description	Reference
$\mu MT^{-/-}$	B cell-deficient mice designed via targeted disruption of a membrane exon of the immunoglobulin μ chain gene.	Kitamura *et al.*, 1991
$\beta 2m^{-/-}$	MHC-I-deficient or $CD8^+$ T cell-deficient mice designed via targeted disruption of the second exon of the β2m gene.	Koller *et al.*, 1990
MHCII-$\alpha\beta^{-/-}$	MHC-II-deficient or $CD4^+$ T cell-deficient mice designed via targeted 80 kb deletion of the entire MHC-II region.	Madsen *et al.*, 1999
MHCII-$\alpha^{-/-}$	MHC-II-deficient or $CD4^+$ T cell-deficient mice designed via targeted disruption of the H2-Aα gene.	Cosgrove *et al.*, 1991
MHCII-$\beta^{-/-}$	MHC-II-deficient or $CD4^+$ T cell-deficient mice designed via targeted disruption of the H2-Aβ gene.	Grusby *et al.*, 1991
RAG-$1^{-/-}$	Combined B and T cell-deficient mice designed via targeted disruption of the recombination activating gene-1 (RAG-1).	Mombaerts *et al.*, 1992
$Fas^{-/-}$	Fas-deficient mice designed via targeted disruption of the Fas gene.	Adachi *et al.*, 1995
$Perf^{-/-}$	Perforin (Perf)-deficient mice designed via targeted disruption of perforin gene.	Kagi *et al.*, 1994
GzmA/$B^{-/-}$	Granzymes (Gzm) A and B-deficient mice produced by crossing $GzmA^{-/-}$ and $GzmB^{-/-}$ mice.	Simon *et al.*, 1997 Ebnet *et al.*, 1995 Heusel *et al.*, 1994
$Perf^{-/-}$xGzmA/$B^{-/-}$	Perforin (Perf), GzmA, and GzmB-deficient mice produced by crossing $Perf^{-/-}$ and GzmA/$B^{-/-}$ mice.	Mullbacher *et al.*, 1999
$Fas^{-/-}$xGzmA/$B^{-/-}$	Fas, GzmA, and GzmB-deficient mice produced by crossing $Fas^{-/-}$ and GzmA/$B^{-/-}$ mice.	Rode *et al.*, 2004
IFN-$\gamma^{-/-}$	Interferon gamma (IFN-γ)-deficient mice designed via targeted disruption of the IFN-γ gene.	Dalton *et al.*, 1993
B6.129P2-$CCR5^{-/-}$	Chemokine CCR5-deficient mice designed via targeted disruption of the CCR5 gene	Kuziel *et al.*, 2003
IFN-α-$R^{-/-}$	Interferon alpha receptor (IFN-α-R)-deficient mice designed via targeted disruption of the IFN-α-R gene.	Muller *et al.*, 1994
B6.$MyD88^{-/-}$	Myeloid differentiation primary response gene 88 (MyD88)-deficient mice designed via targeted disruption of MyD88 gene	Adachi *et al.*, 1998
Balb/c.$MyD88^{-/-}$	MyD88-deficient mice on Balb/c background produced by backcrossing $MyD88^{-/-}$ mice 10 times on Balb/c wt mice.	Sutherland *et al.*,2011
TNF-α-$R^{-/-}$	Tumor necrosis factor alpha receptor (TNF-α-R)-deficient mice designed via targeted disruption of TNF-α-R gene.	Korner *et al.*, 1993

All mice are in B6 background except otherwise indicated.

2.4 Mouse inoculation and tissue collection.

For subcutaneous (sc) and intracranial (ic) inoculation, mice were anesthetized with 50 µl of a 10% ketamine–xylazole solution in phosphate-buffered saline (PBS) by intramuscular injection. Mice were infected sc via the footpad or ic with a single injection of a defined dose of JEV in 20 µl HBSS-BSA. Mouse challenge experiments using intravenous (iv) inoculation were done via the lateral tail vein with a single injection of a defined dose of JEV in 100 µl HBSS-BSA. Mice were monitored twice daily. Daily changes in 5 parameters including hair coat, posture, breathing pattern, activity, and movement were recorded (scored) as normal (0), mild (1), moderate (2), or severe (3), and severely moribund mice (clinical score > 7) and/or mice with hindlimb paralysis were euthanized by rapid cervical dislocation. For tissue processing, mice were euthanized at the time points indicated and a sterile midline vertical thoracoabdominal incision was made to expose the internal organs. After cardiac puncture for blood collection, animals were perfused with 10 ml sterile PBS. The brain and spinal cord were excised intact and collected for virus titration. For determination of virus titers, sample tissues were snap frozen on dry ice. One-half of the brain samples were placed in cell culture medium and homogenized for lymphocyte isolation.

2.5 Real-time RT-PCR.

For determination of viral burden in mouse serum and spleen samples, total RNA in 50 μl splenic homogenates (10% [wt/vol]) and 50 μl serum was extracted using Trizol (Invitrogen) as described previously (Lee *et al.*, 2004), and virion RNA content, expressed in genome equivalents, was determined by quantitative reverse transcription (RT)-PCR. For a genome copy standard, JEV RNA extracted from a Vero cell-grown virus stock and quantitated by spectrophotometry was used. RT was performed at 43°C for 90 min in a 10 μl mixture containing 2 μl sample RNA, Expand reverse transcriptase (Roche), RNase inhibitor (Invitrogen), 10 mM deoxynucleoside triphosphate, 10 pmol downstream primer (5'-TTGACCGTTGTTACTGCAAGGC-3'), 10 mM dithiothreitol (DTT), and the manufacturer's recommended buffer condition. Real-time PCR was performed using IQSybr qPCR mixture (Bio-Rad) and 0.2 nM downstream and upstream primers (5'-GCTGGATTCAACGAAAGCCACA-3') under cycling conditions of 95°C for 3 min for 1 cycle and 95°C for 30 sec, 63°C for 30 sec and 72°C for 60 sec for 40 cycles. Each sample was tested in duplicate, and genome copy numbers were determined by extrapolation from a standard curve generated within each experiment. The detection limit of the assay was $4x10^3$ RNA copies/ml.

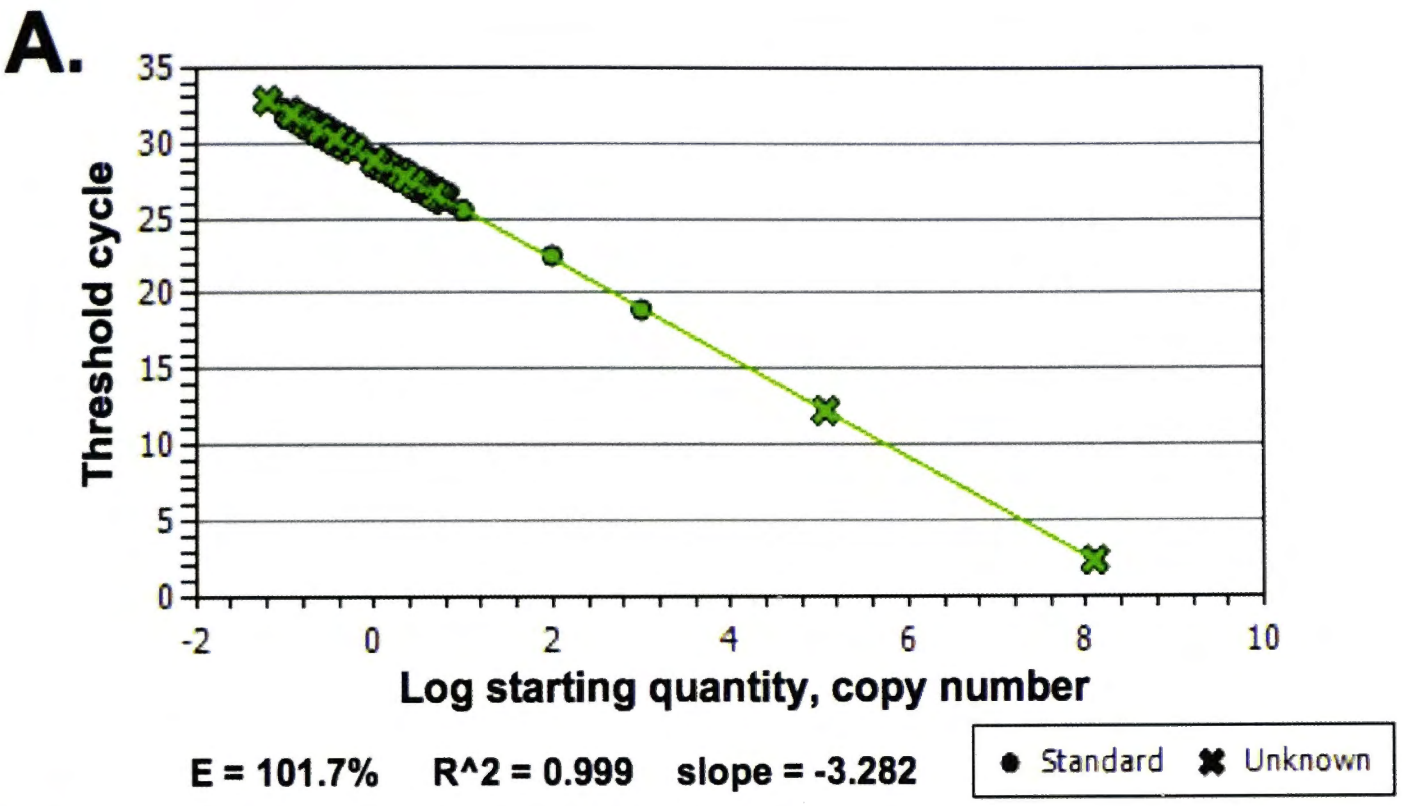


Figure 2.1 Real time RT-PCR for quantification of Japanese encephalitis viral RNA. Representative real time RT-PCR (A) standard curve and (B) plotted melt curve analysis performed for quantification of viral RNA in serum and tissues.

2.6 Histology and immunohistochemistry

After dissection, brain tissue samples were placed in 10% neutral-buffered formalin fixative and stored at room temperature. For examination of basic cell morphology and neuronal myelin integrity, 6 μm sagittal brain sections were stained with hematoxylin & eosin and luxol fast blue, respectively. Immunohistochemistry was performed with an avidin-biotin-complex technique. Triplicate sagittal sections of brain samples were incubated overnight with anti-NS1 monoclonal antibody, 4G4 (Clark *et al.*, 2007), at 4°C to stain JEV antigen. After 10 min incubation at room temperature with biotinylated rabbit anti-rat Ig (DAKO Corporation) as secondary antibody, specimens were incubated for 10 min at room-temperature with streptavidin-HRP complex (DAKO Corporation). Subsequently, a mixture of 3,3'-diaminobenzidine tetrahydrochloride-chromogen and substrate buffer (DAKO Corporation) was applied for 5 min at room temperature. Slides were examined under bright-field microscopy and brown staining of cells was indicative of positive immune reactivity.

2.7 Lymphocyte isolation from the brain

Homogenized brain samples were digested with 2 mg collagenase type I (Gibco-Life Technologies) in 5 ml MEM plus 5% FCS for 45 min at 37°C with intermittent shaking, then centrifuged at 400 *g* for 10 min. Pellets were resuspended in 2 ml 90% percoll in MEM plus 5% FCS and overlaid gently with 60%, 40% and 10% percoll (GE Healthcare) in MEM plus 5% FCS. The gradients were centrifuged at 800 *g* for 30 min at 25°C and lymphocytes collected from the 40 – 60% interface were subsequently analysed by flow-cytometry.

2.8 Cell surface and intracellular cytokine staining.

The surface marker staining employed in this thesis utilized the following reagents (all from Becton Dickinson except otherwise indicated): allophycocyanin (APC)-conjugated anti-CD8 antibody; phycoerythrin (PE)-conjugated anti-CD3, anti-CD25, anti-CD69, anti-NK1.1, anti-F4/80 (Caltag) and anti-CCR5 antibodies; fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-NK1.1, anti-F4/80 (Serotec) and anti-CD19 antibodies; and peridinin-chlorophyll-protein complex (PerCP)-conjugated anti-CD45 and anti-CD3 antibody (BioLegend). 10^5 events were acquired for each sample on a four-color FACSort flow-cytometer. Results were analyzed using Cell Quest Pro Software. For intracellular cytokine staining, $1x10^6$ splenocytes were suspended in 100 µl MEM with 5% FBS and stimulated for 16 h with 10^{-4} M H-2D^b-binding 9-mer JEV NS4B protein-derived peptide, SAVWNSTTA, in the presence of 1 µl/ml brefeldin A (eBioscience). An ectromelia virus (ECTV) H-2K^b-restricted peptide, TSYKFESV (Tscharke *et al.*, 2005), was used at 10^{-4} M as a negative-control peptide. For stimulation of *ex vivo* splenocytes with JEV, splenocytes were infected at a multiplicity of infection (MOI) of 1for 1 h at 37°C and washed twice with MEM containing 5% FBS before incubation for 16 h in MEM plus 5% FBS in the presence of brefeldin A. Enumeration of activated NK cells by intracellular staining for IFN-γ expression was performed directly on *ex vivo* splenocytes. Cells were surface stained with anti-CD8–APC or anti-NK1.1-PE antibodies before paraformaldehyde fixation and permeabilization with saponin (Biosource) according to the supplier's instruction. Cells were then stained with anti-IFN-γ–FITC (BioLegend) and/or anti-tumor necrosis factor alpha (TNF-α)–PE (Invitrogen), and washed twice with fluorescence-activated cell sorter (FACS) washing buffer (2% FBS in PBS) before assessment by FACS analysis.

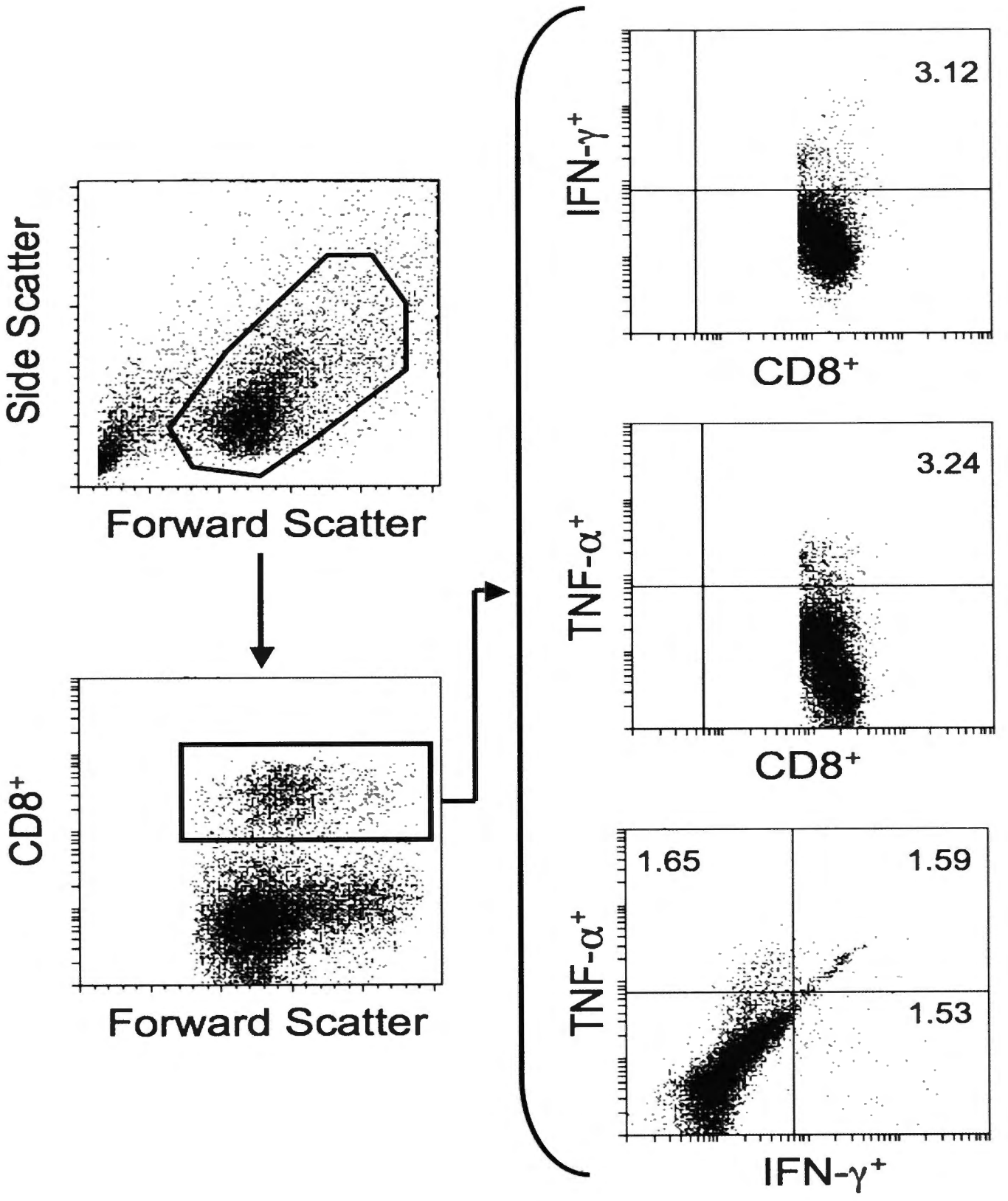


Figure 2.4 Flow cytometry gating strategy. Representative dot plot for FACS analysis of intracellular cytokine staining of IFN-γ and/or TNF-α in $CD8^+$ T cells. Lymphocytes were isolated based on forward scatter and side scatter. Subsequently, $CD8^+$ T cells were gated for analyzing intracellular expression of IFN-γ, TNF-α, or both IFN-γ and TNF-α.

2.9 *In vivo* depletion of $CD8^+$ T cells

$CD8^+$ T cells in B/6 and 129 mice were depleted by ip injection with 0.5 mg of a rat anti-mouse CD8a monoclonal antibody (2.43, IgG2b, BioXcell). Control mice were injected with 0.5 mg of an isotype control antibody (MPC-11, IgG2b, BioXcell). The antibodies were diluted in 0.5 ml PBS and injected at 3, 2 and 1 days before and at 7 days after virus infection. The efficiency of depletion was >98%, based on FACS analysis of lymphocytes in blood on day 7 pi.

2.10 *In vivo* depletion of $NK1.1^+$ cells

$NK1.1^+$ cells in B/6 mice were depleted by ip injection with 100 μg of a rat anti-mouse $NK1.1^+$ monoclonal antibody (PK136, IgG2a, BioXcell). The antibodies were diluted in 0.5 ml PBS and injected one day before, on the day, one day after virus infection, and every 4 days thereafter. Control mice were injected with corresponding volume of PBS. The efficiency of depletion was >98%, based on FACS analysis of lymphocytes in blood on days 2 and days 7 pi.

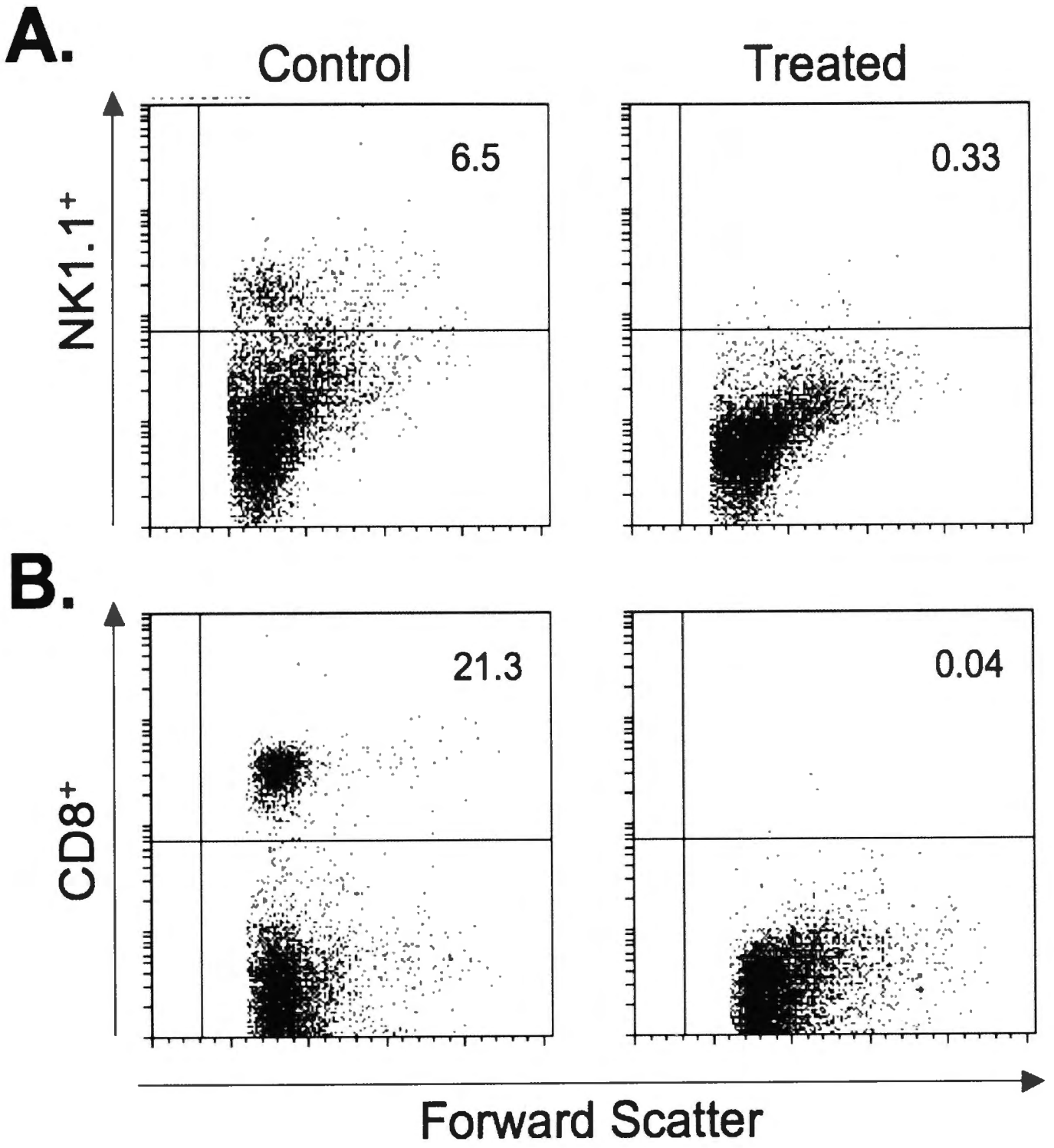


Figure 2.4 ***In vivo*** **depletion of cell popultions.** Example dot plots representing *in vivo* depletion of (A) $NK1.1^+$ and (B) $CD8^+$ T cells by ip injection of anti mouse NK1.1+ (PK136) and anti-mouse CD8a (2.43) monoclonal antibody, respectively. Control dot plots represent untreated mouse.

2.11 T cell enrichment by nylon wool separation and complement-mediated lysis.

For T-cell enrichment, $1.2x10^8$ splenocytes suspended in HBSS plus 10% FCS were loaded onto 10-ml nylon wool columns and incubated for 45 min at 37°C. Effluent from the columns was collected, and cells were pelleted by centrifugation at 400 x *g* for 5 min. This procedure resulted in a population of 90% T cells with 5% B cell contamination. To further improve the purity of T cells eluted from nylon wool columns, the T-cell-enriched splenocyte population was incubated with anti-CD45R/B220 supernatant (RA2-3A1) in minimal essential medium plus 5% FCS for 30 min at 4°C, followed by incubation with rabbit serum complement (Cedarlane Laboratories Ltd.) for 30 min at 37°C. Cells were washed twice with PBS before transfer into recipient mice. This procedure gave rise to a population of 95% T cells with 1% B-cell contamination.

2.12 Complement-mediated lysis of $CD4^+$ and/or $CD8^+$ T cells

For $CD4^+$ and $CD8^+$ T cell depletion or for further enrichment of either $CD4^+$ or $CD8^+$ T cells, total splenocytes or B cell-depleted splenocytes were incubated with a 1:3 dilution of either anti-CD4 (RL172) or anti-CD8 (31M) supernatant in MEM plus 5% FCS for 30 min at 4°C, followed by incubation with rabbit serum complement (Cedarlane Laboratories) for 30 min at 37°C. Cells were washed twice with PBS before transfer into recipient mice. The efficiency of depletion of $CD4^+$ or $CD8^+$ cells was >95% as assessed by FACS.

2.13 Positive selection of $CD19^+$ B cells by magnetic bead separation

Single-cell splenocyte suspensions were prepared by pressing the spleen tissue gently against a fine metal mesh tissue sieve. Erythrocyte lysis was by suspension of the splenocyte pellet in 4.5 ml distilled water followed immediately by the addition of 0.5 ml of 10x PBS. Lysed cells were discarded after centrifugation at 400 x *g* for 5 min. Cells were resuspended in 900 µl of buffer (PBS containing 0.5% BSA and 2mM EDTA) per 10^8 cells. Then 100 µl of $CD19^+$ microbeads were added per 10^8 total cells. Following incubation for 15 minutes at 4°C, samples were washed with 1 ml of buffer per 10^7 cells, subjected to centrifugation at 400 x *g* for 5 minutes, and resuspended in 500 µl buffer per 10^8 cells for magnetic separation. LD columns were placed in the MACS separator, and rinsed with 2 ml of buffer. The cell suspension was then loaded into the columns. Effluent from the columns, containing B-cell depleted cells, was collected, and cells were pelleted by centrifugation at 400 x *g* for 5 min. Efficiency of B cell depletion was >99% as assessed by FACS analysis

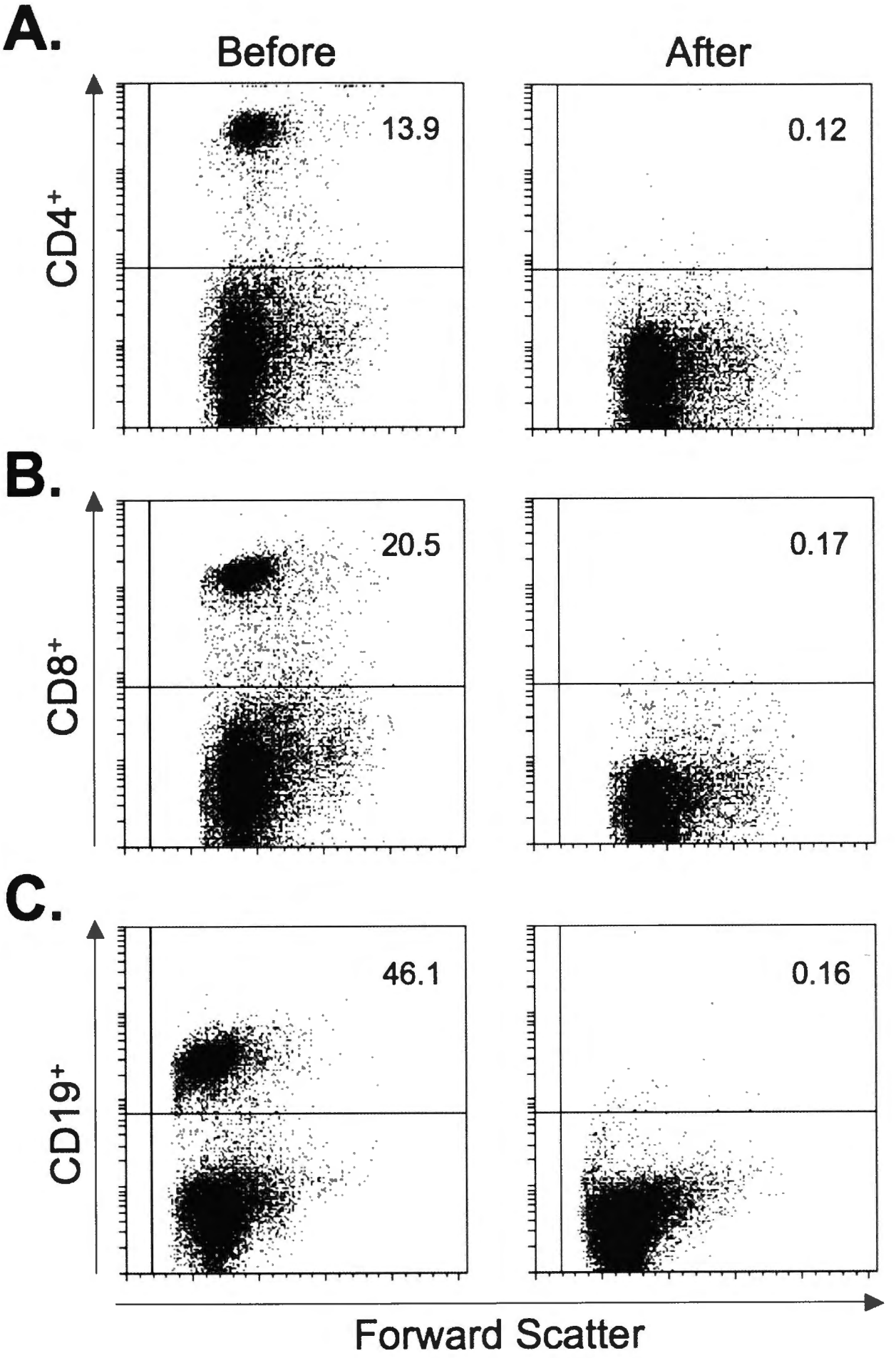


Figure 2.4 *In vitro* depletion of cell popultions. Example dot plots representing *in vitro* depletion of (A) CD4+ T and (B) CD8+ T cells by complement mediated lysis, and (C) B cells by magnetic beads separation.

2.14 Transfer experiments

Eight-week-old B/6 mice were infected with $1x10^3$ PFU of JEV, iv, and were sacrificed a week later for aseptic removal of spleens. Single-cell splenocyte suspensions were prepared by pressing the spleen tissue gently through a fine metal mesh tissue sieve. Erythrocyte lysis was by suspension of the splenocyte pellet in 4.5 ml distilled water followed immediately by the addition of 0.5 ml of 10x PBS. Lysed cells were discarded after centrifugation at 400 *g* for 5 min. Cells were purified by antibody complement depletion and/or magnetic beads separation as described earlier. Splenocytes were resuspended in 100 µl PBS and injected through the lateral tail vein of 8-week-old B/6 recipient mice. Recipient mice were challenged a day later with $1x10^3$ PFU JEV via footpad injection.

2.15 Serological tests

For titration of JEV-specific antibody isotypes in mouse serum, ELISAs were performed with HRP-conjugated rabbit anti-mouse Ig and the peroxidase substrate, 2,29-azino-di(3-ethyl-benzthiasoline sulfonate). The JEV Nakayama strain was used for ELISA antigen production as described (Colombage *et al.*, 1998). For determination of ELISA end-point titres, absorbance cut-off values were established as the mean absorbance of eight negative-control wells containing sera of naive mice plus 3 SD. Absorbance values of test sera were considered positive if they were equal to or greater than the absorbance cut-off and end-point titres calculated as the reciprocal of the last dilution giving a positive absorbance value.

2.16 Neutralization assay

Neutralization titers, measured in a 50% plaque reduction neutralisation test, were determined as previously described (Lobigs *et al.,* 2003*c*). Naive and JE-immune serum samples underwent complement-inactivation by heating at 56 °C for 30 minutes. Then, serum samples were serially diluted twofold with HBSS-BSA in 96-well trays, and 110 µl of JEV at ~200 PFU was added. Plates were incubated at 37 °C for 1 h. Duplicate aliquots at 100 µl were assayed for infective virus by plaque formation on Vero cell monolayers in six-well plates as earlier described. JEV incubated with naive serum from the same mouse strain served as negative controls, and approximately yielded 50–100 PFU per well. PRNT50 titres were calculated as the reciprocal of serum dilutions that resulted in a ≥50 % plaque number reduction.

2.17 NK cell cytotoxic assay

Mice were infected with 10^3 PFU of JEV, iv, and sacrificed at days 4 and 7 post-infection (pi). Spleens were collected and tested for NK cell cytotoxicity in a standard ^{51}Cr-release assay (Momburg *et al.*, 2001), using uninfected YAC-1 cells as targets. Splenocytes from uninfected mice were used as controls. P values were calculated from a four-point logarithmic regression curve, interpolated at an effector-to-target (e/t) cell ratio of 30.

2.18 Preparation of L-929 conditioned medium

A murine fibrosarcoma cell line, L-929, was used as a source of macrophage-colony stimulating factor (M-CSF) (Austin *et al.*, 1971; Stanley, 1985). L-929 cells derived from ATCC (ATCC no. CCL-1) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. After cells were grown to confluence, L-929 conditioned medium was harvested, and subjected to centrifugation at 400 *x g* to remove cellular debris. Supernatants were then collected and stored in 10 ml aliquots at –70°C.

2.19 Preparation of bone marrow-derived macrophages

B/6 or knock-out mice were euthanized by cervical dislocation. Aseptically, skin in the lower extremities was removed, and muscles dissected to exposed the femur, tibia, and fibula. Bones were excised through a cut in the proximal hip and distal ankle joints, and placed in dish containing complete Mixed Lymphocyte Culture media (MLC media supplemented with 1% L-glutamine, 1% NEAA, 1% pyruvate, 50 μM 2-ME, 100 units PSN, and 10% FBS). Both ends of individual bones were severed to expose the cell-filled marrow cavity. Using a 10-ml syringe, the marrow cavity was flushed with complete MLC media to expel cells. Collected cells were centrifuged for 5 minutes at 400 *x g*, and resuspended in a complete MLC supplemented with 10% L-929 supernatant. Bone marrow-derived cells were cultured at $1x10^6$ cells/well in 6-well plates, and incubated at 37°C in 5% CO_2 atmosphere. Culture media were replaced with fresh complete MLC + 10% L-929 supernatant every 48 hours. Differentiation into mature macrophages was observed approximately on day 7 after start of culture.

2.20 JEV growth inhibition by IFN-α treatment

Bone marrow-derived macrophages in 6-well plates were prepared as earlier described, and incubated with various concentrations of IFN-α (PBL Interferon Source) (250, 100, 10, 1, and 0.1 Units) for 16 hours. Following washing with complete MLC media plus 5% FBS, cells were incubated with 100 µl of JEV at MOI of 1, for 1 hr at 37°C, with intermittent gentle shaking. Cells were washed twice, and cultured with complete MLC media plus 10% L-929 supernatant for 48 hrs. Culture supernatant (100 µl) were collected at 12, 16, 24, and 48 hrs pi, and inhibition of virus growth by IFN-α was determined by plaque titration on Vero cell monolayers.

2.21 Interferon assay

Determination of IFN-α levels in infected serum or tissue culture supernatants were performed as instructed in the *VeriKine*^{TM} mouse IFN-α ELISA kit. First, an IFN-α standard stock (10,000 pg/ml) was serially diluted in twofold dilution steps (concentrations from 400 pg/ml to 12.4 mg/ml). Samples (100 µl) and IFN-α standards were added into pre-coated microplate strips. Following addition of 50 µl of diluted antibody solution to each well, plates containing the strips were covered with plastic sealers, and incubated for 1 hr at 25°C with shaking at 450 rpm, and for 20 hrs at 4°C without shaking. Plate content was discarded, and plates were washed 4 times with diluted wash buffer solution. Following addition of 100 µl of diluted HRP solution to each well, plates were covered with plastic sealers and incubated for 2 hrs at 25°C with shaking at 450 rpm. Plate content was discarded, and plates were washed 4 times with diluted wash buffer solution. Substrate (100 µl) was then added to wells, and plates incubated unsealed for 15 minutes at 25°C in the dark and without shaking. Finally, 100 µl of stop solution were added into the wells, and within 5 minutes absorbance at 450 nm was determined spectrophotometrically.

2.22 Allelic discrimination by Amplifluor SNPs genotyping

The genotyping team of the Australian Phenomics Facility (APF), headed by Dr Belinda Whittle, performed the allelic discrimination by Amplifuor SNPs genotyping. An amplification cocktail was prepared, with each 10 µl reaction volume containing 3.15 µl distilled H_20, 0.5 µl 20x amplifluor SNP FAM primer (Millipore), 0.5 µl 20x amplifluor SNP JOE primer (Millipore), 2 µl 20x SNP specific primer mix, 1 µl 10x reaction mix S plus, 0.8 µl dNTPs (2.5 mM each), 0.05 µl Platinum hotstart Taq Polymerase (Invitrogen), and 2 µl DNA samples stored in Tris-Buffered H_20. DNA from B/6, CBA and F1 mice and distilled H_20 were used as controls. Sample amplification cocktails (8 µl) were placed in 96-well plates. Plates were covered with optically clear strips, and run under cycling conditions of 95◦C for 4 min for 1 cycle, 95◦C for 10 sec, 60◦C for 20 sec, 72◦C for 40 sec for 34 cycles, and 72◦C for 3 min for 1 cycle. Plates were read in a fluorescent plate reader using the following: excitation filter 490/10 nm and emission filter 520/10 (gain 2200), and excitation filter 530/10 and emission filter 560/10 (gain 2400). Data was analysed using Fluostar optima software.

2.23 Nimblegen capture and re-sequencing

Customized sequence capture by hybridization (Roche NimbleGen 2.1M capture array) was performed by the next generation sequencing team from the Australian Genomics Research Facility (AGRF, Melbourne), headed by Matthew Tinning. Ezra Lyon, and her team from Roche NimbleGen, designed the sequence capture probe set, and provided all the array reagents to AGRF. Re-sequencing of the captured samples was done using Illumina HiSeq sequencing system at AGRF. The bioinformatics team of AGRF performed the bioinformatic analysis. Alignment was performed with Illumina CASAVA v1.8.2 and aligner module ELAND v2 (Efficient Large-Scale Alignment of Nucleotide Databases). Illumina Exome script was applied to the primary alignment folder to generate CASAVA build folder and Exome coverage statistics. Functional annotation of genetic variants including single nucleotide variations, insertions, and deletions was done using the ANNOVAR software. A second bioinformatics analysis, using an in-house software, was done by the bioinformatics team of APF.

2.24 MHCII-Aα genotyping

The genotyping team of APF, headed by Dr Belinda Whittle, performed the MHCII-Aα genotyping by PCR. Briefly, samples were prepared, with each 10 µl reaction volume contains 2.6 µl distilled H_20, 0.4 µl of 10 µM MCHII-Aα wt reverse primer (5'-GGTGTGAGCACGTACCATTG-3'), 0.2 µl of 10 µM MCHII-Aα common forward primer (5'-TGGAGACATTGGCCAGTACA-3'), 0.8 µl of 10 µM neomycin common primer (5'-GGATGATCTGGACGAAGAGC-3'), 5 µl 2x Mix (Qiagen Multiplex PCR kit), and 1 µl DNA sample. DNA samples from B/6 wt, F1, and MCHII-$A\alpha^{-/-}$ mice were used as controls. Samples were rin under cycling conditions 95◦C for 4 min for 1 cycle, 95°C for 15 sec, 60°C for 20 sec, 72°C for 30 sec for 35 cycles, and 72°C for 2 min for 1 cycle. Following amplification, samples were added with 30 µl of loading buffer, were loaded into 1.5 % agarose gel stained with gel red. The gel was electrophoresed for 10 minutes at 350 volts.

2.25 ChimeriVax-JE immunization

For challenge experiments, groups of 8-week-old B/6 or knockout mice were immunized sc with one dose of $1x10^5$ PFU of ChimeriVax-JE. A month after immunization, mice were inoculated, via the fp, with $1x10^3$ PFU of JEV. Mortality was recorded after a 28-day-observation period. For splenocyte transfer experiment, 6-week-old donor IFN-α-$R^{-/-}$ mice were immunized sc with $1x10^5$ PFU ChimeriVax-JE, and were sacrificed a month later for aseptic removal of spleens and blood extraction. Serum samples were isolated after centrifugation for 10 min at 10,000 rpm, heat inactivated for 30 min at 56°C, and stored at -70°C. Single-cell splenocyte suspensions were prepared, and T cells were enriched by nylon wool separation and complement-mediated lysis of B cells as earlier described. Groups of ten-week-old IFN-α-$R^{-/-}$ mice groups received either total splenocytes, T cells, immune serum, or immune serum plus T cells, and were challenged a day after splenocyte transfer with $1x10^2$ PFU MVEV via fp injection. Mortality was recorded in recipient mice after a 28-day-observation period

2.26 Inactivated Vero-cell culture derived-JE immunization

Jespect (inactivated JEV SA14-14-2 strain) was obtained from CSL Ltd, Australia, and was diluted in PBS admixed with 0.1% aluminum hydroxide. A formalin-inactivated Vero-cell culture-derived JE (Nakayama strain) vaccine was obtained from Kitasato Institute, Japan (Torinawa and Komiya, 2008). A low or high dose ccJE vaccine (0.5 μg and 0.05 μg, respectively) was diluted in PBS alone or admixed with 1 μg of Advax. B/6 wt or knockout mice were immunized sc with various vaccine formulations, at two weeks apart, and challenged, one month after complete of the vaccination schedule, via the fp with $1x10^3$ PFU of JEV or sc with $1x10^3$ WNV_{NY99}. Mortality was recorded after a 28-day-observation period. For splenocyte transfer experiments, B/6 or $\beta 2m^{-/-}$ donor mice were immunized twice with Advax-adjuvanted ccJE (0.5 μg), given 2 weeks apart. Splenocytes were isolated from donor mice one month, three months, or 6 months after completion of vaccine regimen. B cells were purified by complement-mediated lysis of $CD4^+$ and $CD8^+$ T cells, while $CD4^+$ T cells were purified via magnetic separation of B cells plus complement-mediated lysis of $CD8^+$ T cells. Recipients mice were challenge with a defined dose of JEV, WNV_{NY99} or MVEV, one day after splenocyte transfer.

2.27 Statistical analyses

Mortality data were plotted into Kaplan-Meier curves and assessed by the log-rank test for significance. The Mann-Whitney test was applied to assess differences in data gathered between two experimental groups. A P value of ≤ 0.05 was considered as significant.

Chapter 3

Pivotal role of antibody and subsidiary contribution of $CD8^+$ T cell to recovery from infection in a murine model of Japanese encephalitis

The findings in this Chapter has been published:

Larena, M., Regner, M., Lee, E., & Lobigs, M. (2011). Pivotal role of antibody and subsidiary contribution of CD8+ T cells to recovery from infection in a murine model of Japanese encephalitis. J. Virol 85(11):5446-55.

3.1 Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus belonging to the JEV serocomplex, which also includes the closely related Murray Valley encephalitis (MVEV) and West Nile viruses (WNV). In terms of incidence and severity of disease in humans, JEV is the most important member of this serocomplex. It is the leading cause of viral encephalitis in Asia, accounting for 35,000-50,000 cases per year and an estimated 10,000 deaths, with long-term neurologic sequelae in about one-half of the survivors (Solomon, 2004). In the past decades, there has been an expansion of the geographic distribution of the virus in Asia and emergence of virus transmission and human cases of encephalitis in Pakistan, the eastern Indonesian archipelago, New Guinea and northern Australia (reviewed in (Mackenzie *et al.*, 2004)). Vaccination is the main measure for protection against Japanese encephalitis (reviewed in reference (Beasley *et al.*, 2008)), but due to expense and logistics is not available to a large population in Asia that should be immunised.

The majority of human infections with JEV are subclinical, with the ratio of apparent to inapparent infections estimated to range from 1:25 to 1:1000 (Solomon, 2004). Host factors, rather than variation in viral virulence, are thought to dominantly determine outcome of infection in terms of disease severity (reviewed in reference (Halstead and Jacobson, 2003)). Of these factors, an understanding of the immunological responses that lead to recovery from JEV infection is important in the design of rational approaches to new treatments and vaccines.

However, insight into the immunological correlates of recovery from JEV infection is incomplete (reviewed in reference (Mullbacher *et al.*, 2003)). Among the innate

immune pathways, an essential role of type I interferons (IFN) in recovery is illustrated by the uncontrolled growth of the virus in mice lacking a functional IFN-a receptor (Lee and Lobigs, 2002). Similarly, the importance of a vigorous humoral immune response in ameliorating or preventing illness has been documented in human cases of Japanese encephalitis (Burke *et al.*, 1985*a*; McCallum *et al.*, 1991; Libraty *et al.*, 2002) and in animal models by administration of antibody prior or subsequent to infection with JEV (Goncalvez *et al.*, 2008; Gupta *et al.*, 2003; Kimura-Kuroda *et al.*, 1988; Zhang *et al.*, 1989).

In contrast, the relative contribution of the cellular immune responses to recovery from JEV infection remains unclear. A limited number of studies in mice suggest, for instance, a protective value of JEV-immune $CD4^+$ T cells by a mechanism involving enhanced antibody production (Biswas *et al.*, 2009) and a possible role of $CD8^+$ T cells in virus clearance (Murali-Krishna *et al.*, 1996), although the latter study involved co-injection of a large number of splenocytes with virus into the brain and required co-transfer of $CD4^+$ T cells. Thus, insight into the immunobiology of JEV is lagging in comparison to the significantly more detailed understanding of the role of innate and adaptive immune responses in recovery from infection with the related WNV, predominantly derived from studies on virulent lineage I North-American isolates in mice deficient in defined immune effector functions (reviewed in reference (Klein and Diamond, 2008)).

This raises the question of generality of immunological correlates identified for WNV as determinants of disease outcome for JEV and other viruses of medical importance belonging to the JEV serocomplex (Mullbacher *et al.*, 2004). For instance, the contribution of $CD8^+$ T cells to recovery from flaviviral infection is variable and can

range from protective to immunopathological outcomes (Licon Luna *et al.*, 2002; Wang *et al.*, 2003; Shrestha and Diamond, 2004). To begin to answer this question, I have established a pathogenesis model in adult C57Bl/6 (B/6) mice for Japanese encephalitis involving conditions that mimic natural infection route and dose, and report both commonality and differences with WNV in the role of adaptive immune pathways in recovery from JEV infection.

3.2 Results

3.2.1 Mouse model of Japanese encephalitis by subcutaneous infection of adult B/6 mice with a low-dose virus inoculum.

To establish a mouse model for Japanese encephalitis which resembles the natural infection route and virus dose and which allows investigation of the immunological correlates for recovery from infection, the pathogenesis of the prototype (Nakayama) strain in adult (8-week-old) B/6 mice was first characterised. After a low-dose sc challenge with 10^3 PFU of JEV, most mice presented with clinical signs of infection starting at day 10 pi, which included progressive generalized paresis, piloerection and rigidity. Severe neurological impairment demonstrated by ataxia, postural imbalance and generalized tonic-clonic seizures were evident later in the course of infection, invariably leading to fatality within 24 - 36 hrs after disease onset. A small number of mice presented with unilateral hind limb paralysis without central manifestations. A dose-insensitive survival curve was observed similar to previous findings with WNV and MVEV (Wang *et al.*, 2003; Diamond *et al.*, 2003*a*; Licon Luna *et al.*, 2002) thus, over a dose range of 10 - 10^5 PFU the mortality rate was comparable (60 - 67%; Table 1). The mean survival time (MST) of animals that succumbed to infection was 12 – 13 days, except for the 10^5 PFU dose group, where a dual pattern of early (8 days pi) and late (11 days pi) mortality was observed, overall resulting in a shorter MST. Rapid and uniform mortality was seen when mice were infected with 10^7 PFU (MST = 6.0 days). All surviving mice infected by the sc route, even those infected with 10 PFU, seroconverted and showed high JEV-specific antibody ELISA endpoint titers (≥3.5 log).

When mice were infected via the ic route, the mortality rate and average time to death were dose dependent (Table 1). Virus doses of 10 and 100 PFU gave a 100% mortality

rate (MST = 8.0 and 6.4 days, respectively), while 1 PFU gave an 80% mortality rate (MST = 10.3 days) and 0.1 PFU did not result in disease. These data suggest that, in contrast to peripheral infections, the quantity of virus directly entering the brain serves as an accurate predictor of disease outcome. Consistent with the high lethality of JEV infection of the central nervous system (CNS) (a 50% lethal dose of ≤1 PFU given ic), partial seroconversion was found in the 0.1-PFU dose group.

Viral burdens in serum, spleen, spinal cord, and brain samples were determined in order to elucidate the kinetics of growth of JEV in adult B/6 mice (Fig. 1). Virus was undetectable by plaque assay in both serum and spleen samples across all time points. A more sensitive assay utilizing real-time RT-PCR detected low levels of JEV RNA in serum and spleen samples (Fig. 2). Peak viremia occurred on day 2 pi with a geometric mean titer (GMT) of 9×10^4 RNA copies/ml of serum, whereas splenic viral burdens peaked at day 4 p.i. with a GMT of 3 × 105 RNA copies/g of tissue. Virus invasion of the brain was first apparent on day 6 in only 3 out of 9 mice (Fig. 1). This increased to 55% of the brain samples showing virus infection at day 8 pi (GMT = 1×10^4 PFU) and 60% of the virus-positive brains at day 10 pi (GMT = 4×10^4 PFU). Viral dissemination to the spinal cord occurred later in the course of infection and was first detected on day 8 pi in 33% of the samples (GMT = 1×10^3 PFU), increasing to 40% of the samples collected on day 10 p.i. (GMT = 2×10^3 PFU). Accordingly, viral entry into the CNS commenced in the brain, from where the virus spread centripetally to the spinal cord.

Sections of brain were examined for histopathological changes at day 10 pi with JEV. Hallmarks of acute viral encephalitis, including microglial nodules surrounding degenerating neurons (Fig. 3A) and perivascular leukocytic infiltration (Fig. 3B) appeared widespread in focal regions of the brain. Meningeal inflammation

characterized by mononuclear infiltration into the leptomeninges was also seen (Fig. 3C). There was no evidence of demyelination as indicated by intact myelinated axonal networks after luxol fast blue staining (Fig. 3D). Immunohistochemical staining utilizing an anti-flavivirus NS1 antibody was employed to identify specific sites of neuronal lesions within the brain parenchyma. JEV infected neurons were found in multiple loci, predominantly localized in the following areas: cerebral cortex, hippocampus, thalamus, brainstem and cerebellum (Fig. 3E). The pathological lesions observed served as a clinical correlate of disease.

Table 3.1 Effect of JEV dose on mortality and survival time of B/6 mice

Challenge route and dose[a]	Mortality (No. of deaths/total)	MST (days) ± SD	% Seroconversion of surviving mice[b]
Subcutaneous			
10 PFU	63% (5/8)	11.8 ± 1.1	100
10^2 PFU	67% (10/15)	12.8 ± 2.5	100
10^3 PFU	63% (12/19)	13.3 ± 1.7	100
10^5 PFU	60% (3/5)	9.7 ± 1.5	100
10^7 PFU	100% (5/5)	6.0 ± 0.7	
Intracranial			
0.1 PFU	0% (0/5)	-	60
1 PFU	80% (4/5)	10.3 ± 1.0	100
10 PFU	100% (5/5)	8.0 ± 1.4	
10^2 PFU	100% (5/5)	6.4 ± 0.5	

[a] Eight-week-old B/6 mice were infected with various doses of JEV via the sc or ic routes; virus dose in 20 µl HBSS/BSA. Surviving mice were monitored for 28 days.

[b] Antibody against JEV in serum of surviving mice was measured at 28 days pi by ELISA.

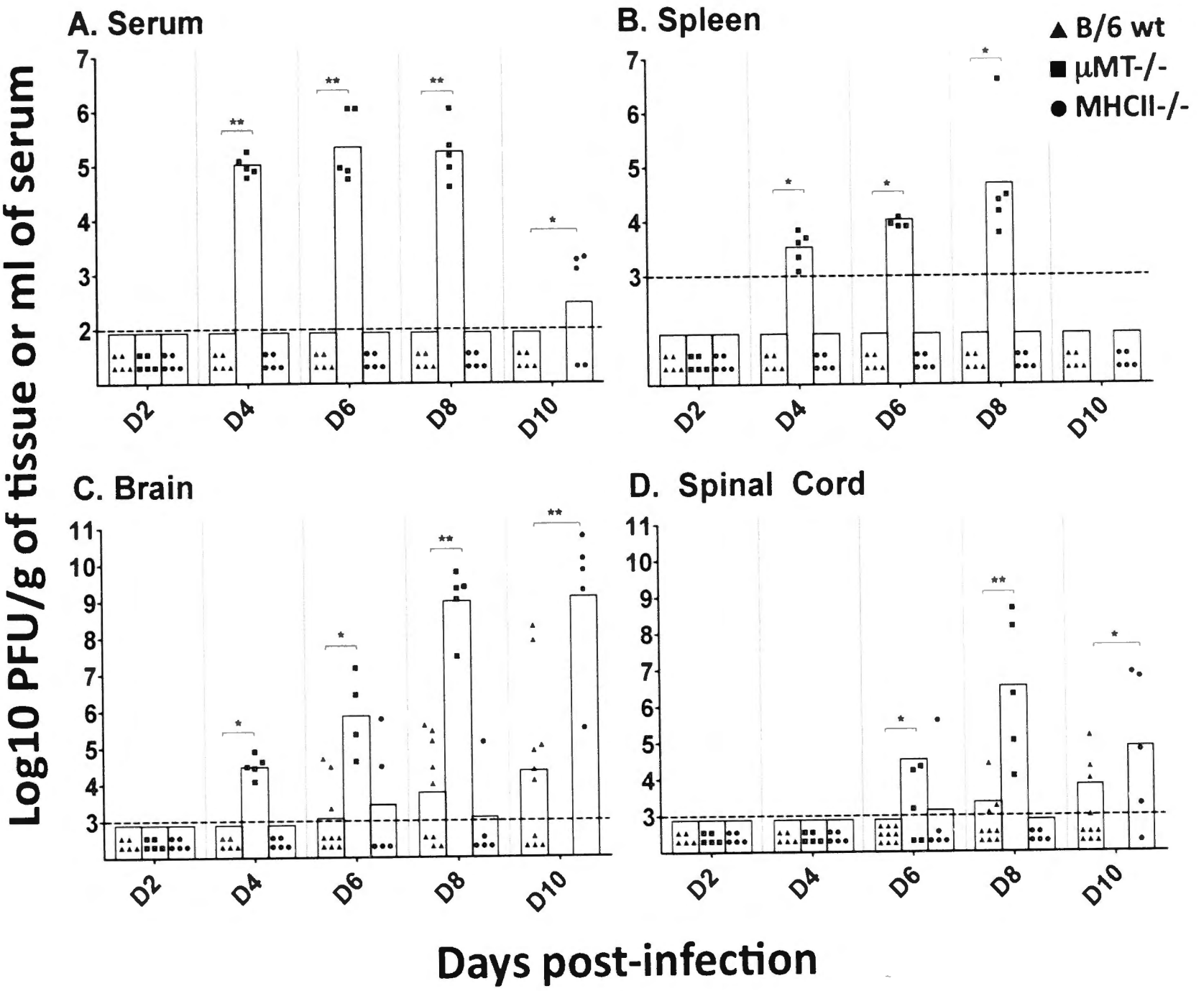


Figure 3.1 JEV burden in serum and tissues. JEV burden in serum (A), spleen (B), brain (C) and spinal cord (D) of B/6 wt, $\mu MT^{-/-}$ and MHCII-$\alpha\beta^{-/-}$ mice after infection with 10^3 PFU of JEV, s.c. At the indicated time points, animals were sacrificed and virus content in serum and tissues measured by plaque titration. Each symbol represents an individual mouse and geometric mean titers (GMT) are indicated by horizontal bars. The lower limit of virus detection is indicated by the horizontal dotted line.

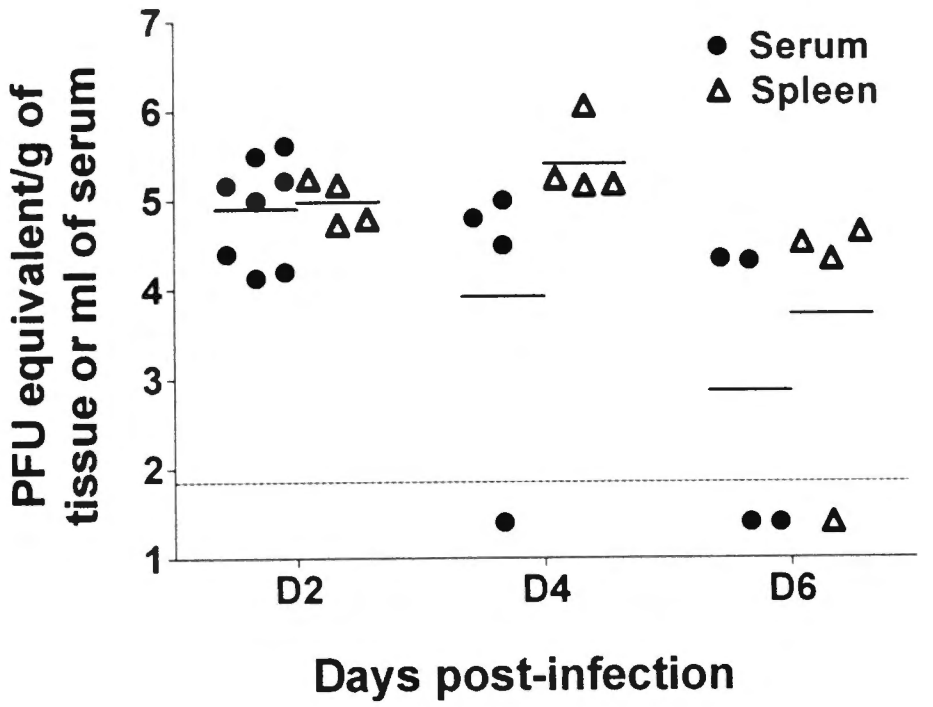


Figure 3.2 JEV RNA in serum and spleen. JEV RNA detected in serum and spleen samples of B/6 WT mice after sc infection with 10^3 PFU of JEV are shown. At the indicated time points, animals were sacrificed and the viral RNA contents of serum and spleen samples were measured by real-time RT-PCR. Each symbol represents an individual mouse, and GMTs are indicated by horizontal bars.

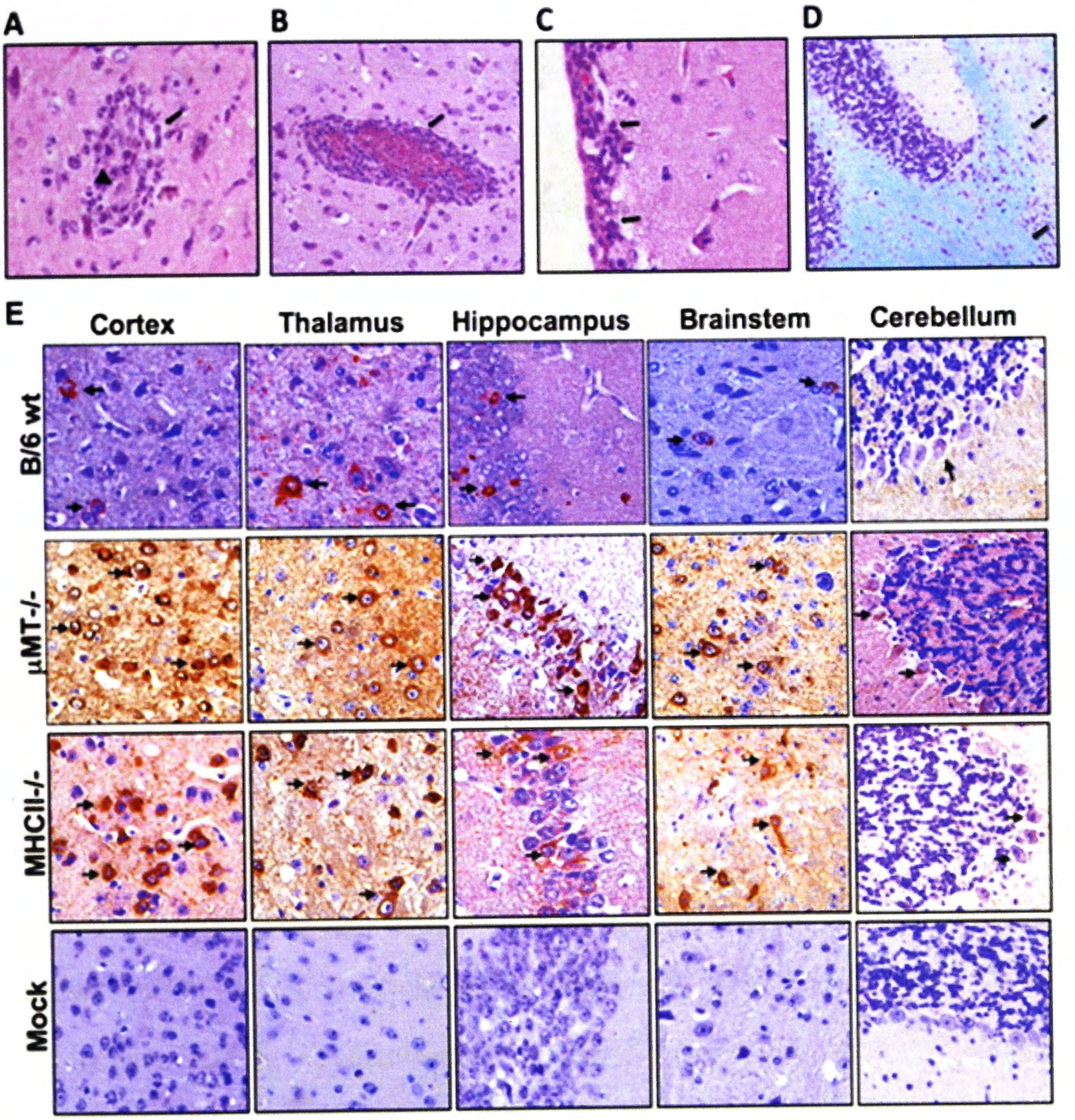


Figure 3.3 Histology and immunohistochemistry. Brain sections of B/6 wt mice at 10 days pi with 10^3 PFU of JEV, s.c. Hematoxylin and eosin stain showing (A) microglial nodules (arrow) with neuronophagia (arrow head), (B) perivascular mononuclear infiltration (arrow) and (C) leukocytic infiltration of the meninges (arrows). (D) Intact myelinated axonal projections (arrows) of cerebellum after luxol fast blue staining. (E) Detection of JEV antigen by immunohistochemistry using anti-NS1 protein. Infected cells or injured neurons (arrows) in representative sections of cerebral cortex, brainstem, thalamus, hippocampus, and cerebellum.

3.2.2 Induction of humoral and cell-mediated immunity after JEV infection in B/6 mice. JEV-specific IgM appeared at day 4 pi and peaked at day 8 pi with a mean titer of 1:450 (Fig. 4A). Both anti-JEV IgG1 and IgG2b appeared later in the course of infection and were first detectable at 8 day pi, attaining mean titers of 1:1400 and 1:275, respectively, at day 10 pi (Fig. 4B). Qualitative measurement of the antibody response revealed neutralizing activity as early as day 4 pi ($PRNT_{50}$ titer = 20 in 2 of 4 mice), concomitant to the onset of detectable anti-JEV IgM antibody, and reached a mean $PRNT_{50}$ titer of 330 (range: 80 - 640) at day 10 pi (Fig. 4C).

B/6 mice produced a robust $CD8^+$ T cell response following infection with 10^3 PFU JEV, i.v. The infection induced the characteristic up-regulation of expression of the early activation marker, CD69, which peaked on day 1 pi and that of the late activation marker, CD25, which peaked on day 4 pi (Fig. 5A). Among the $CD8^+$ T lymphocytes from JEV primed mice, ~5% were stimulated to produce IFN-γ with JEV NS4B peptide, SAVWNSTTA, also described by others to be recognized by H-$2D^b$-restricted JEV-immune $CD8^+$ T cells (Trobaugh *et al.*, 2010). Two other putative D^b-binding JEV peptides (on the basis of a predictive algorithm for MHC class I binding peptides using the SYFPEITHI database; (Rammensee *et al.*, 1999) corresponding to amino acids TAWRNRELL in the E protein failed to stimulate IFN-γ production, as did a D^b-restricted ectomelia virus (ECTV)-specific negative control peptide (Fig. 5B). NS4B peptide stimulation of JEV-immune *ex vivo* splenocytes resulted in greater IFN-γ expression than stimulation with live JEV, suggesting poor infection of splenocytes and suboptimal antigen presentation with the latter approach.

The JEV-immune $CD8^+$ T cell response in the spleen, defined here by the total number of NS4B peptide-reactive $CD8^+$ lymphocytes, first appeared at day 4 pi, reached a peak

of ~10^6 cells at day 7 pi, but markedly declined thereafter (Fig. 5C). The majority of these splenocytes were polyfunctional and expressed both IFN-γ and TNF-α followed *ex vivo* stimulation with NS4B peptide, with peak numbers of 7 x 10^5 cells at day 7 pi. $CD8^+$ T cell recruitment into the site of infection was measured by isolation of lymphocytes from JEV infected brains (Fig. 5D). $CD8^+$ T cell infiltration was already apparent at day 4 pi, showing a 2-fold increase (1.2 x 10^4 cells) relative to baseline. On day 7 pi, corresponding to the peak of the primary JEV-specific $CD8^+$ T cell response in spleen, $CD8^+$ T cell numbers in JEV infected brains reached a plateau, which was maintained until day 10 pi (>100-fold increase relative to baseline; $P = 0.002$). *Ex vivo* $CD8^+$ T cells isolated from brain at day 7 pi secreted IFN-γ both in the presence and absence of stimulation with NS4B peptide (Fig. 5E). This is in contrast to splenocytes harvested at day 7 pi, which required peptide stimulation for IFN-γ production. Considering the presence of virus in the brain at this time point, the difference may reflect antigen presentation via JEV-infected mononuclear cells present in brain-derived cell preparations, resulting in $CD8^+$ T cell activation and cytokine secretion in the absence of *ex vivo* peptide stimulation. Alternatively, brain-derived lymphocytes may show a greater background level of IFN-γ secretion than those isolated from the spleen.

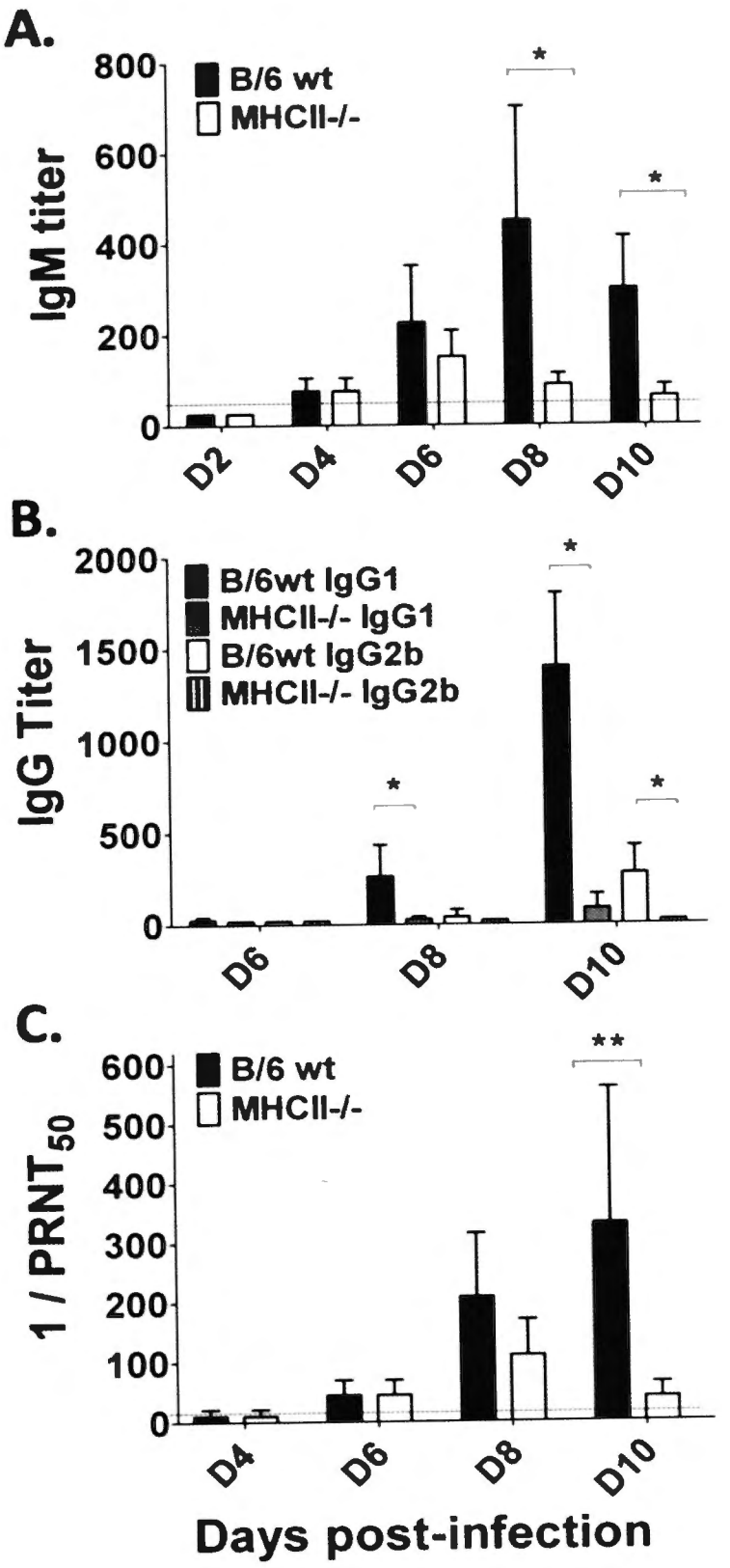


Figure 3.4 Antibody responses in B/6 wt and MHCII-$\alpha\beta^{-/-}$ mice. Eight-week-old B/6 wt and MHCII-$\alpha\beta^{-/-}$ mice were infected with10^3 PFU of JEV, s.c., and sera collected at the indicated time points. Anti-JEV IgM (A) and IgG (B) isotype antibody titers were determined by ELISA. Data presented are reciprocal mean endpoint titers representative of 4 mice per group with SD indicated by error bars. (C) Neutralizing antibody titers determined by plaque reduction neutralization assay. Data presented are mean $PRNT_{50}$ titers representative of 4 - 7 mice per group and error bars indicate the SD. Asterisks denote significance of difference between samples from wt relative to MHCII-$\alpha\beta^{-/-}$ mice (* = P <0.05; ** = P <0.01).

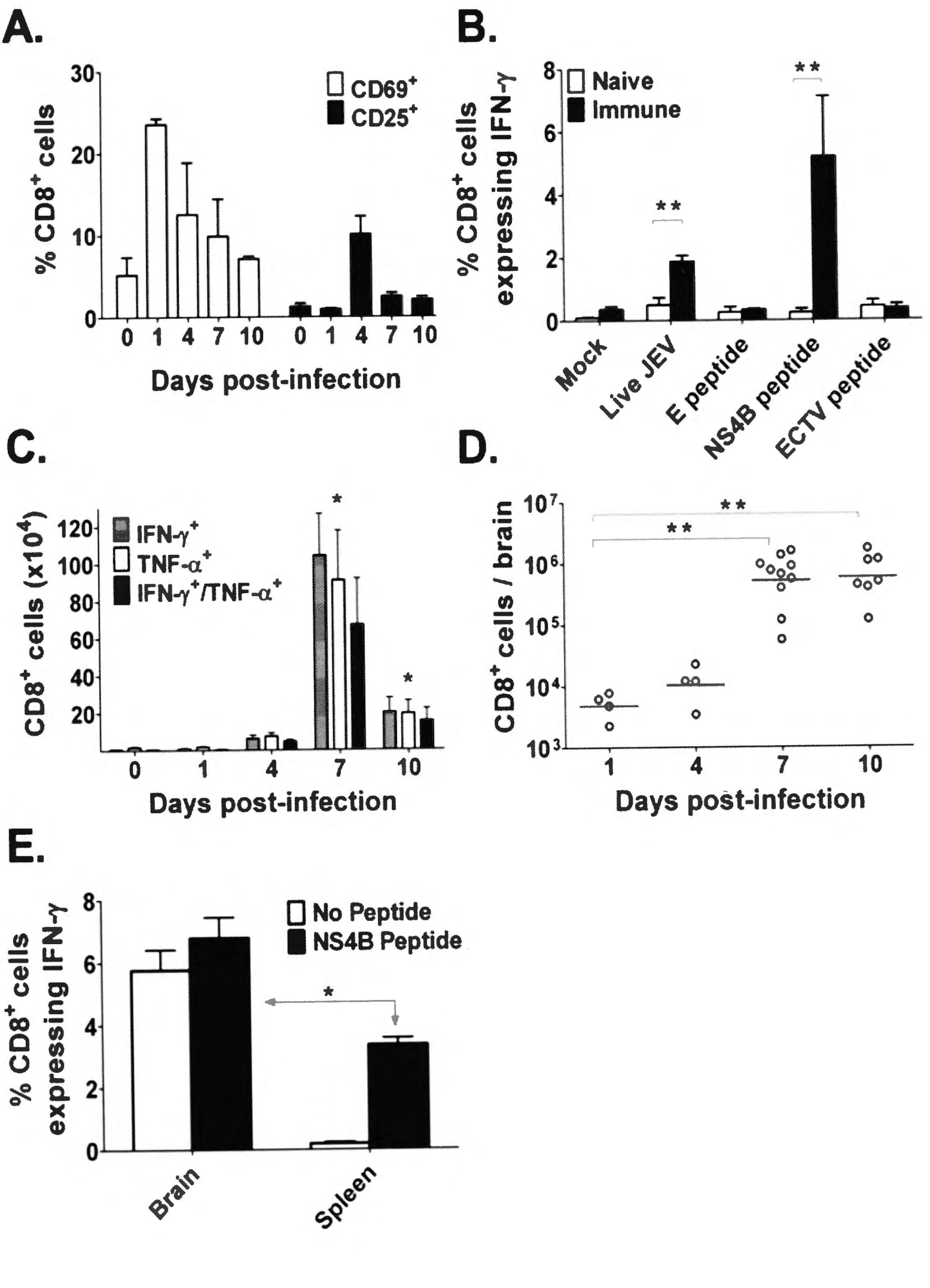


Figure 3.5 CD8$^+$ T cell response in B/6 wt mice infected with JEV. (A) Eight-week-old B/6 mice were infected with 10^3 PFU of JEV, iv, or left uninfected. Spleens (n = 3/group) were collected at the indicated time points and splenocytes were stained for CD8 and lymphocyte activation markers, CD69 or CD25. Data presented are the percentage of CD8$^+$ cells expressing the early activation marker, CD69, or the late activation marker, CD25. Error bars denote the SD and data are representative of 2

independent experiments. (B) Splenocytes harvested at 7 days pi, as above, were stimulated *ex vivo* with live JEV, D^b-restricted JEV NS4B protein-, E protein- or NS1 protein-derived peptides, a D^b-restricted ectomelia virus (ECTV) negative control peptide, or mock treated and IFN-g production in $CD8^+$ T cells measured by flow-cytometry. Means for 3 samples ± the SD are presented and data are representative of 2 independent experiments. (C) Kinetics of JEV-immune $CD8^+$ T cell activation measured after ex-vivo stimulation with D^b-restricted JEV NS4B peptide. Data show the number of $CD8^+$ T cells/spleen that express IFN-γ, TNF-α, or both cytokines following stimulation. Means for 3 samples ± the SD are presented and data are representative of 2 independent experiments. (D) Kinetics of infiltration of $CD8^+$ cells into the brain of 8-week-old B/6 wt mice infected with 10^3 PFU of JEV, sc. Lymphocytes were isolated from the brain of infected mice as described in Materials and Methods and stained for CD8 expression. Each symbol represents an individual mouse and horizontal lines indicate mean values for each time point. (E) JEV-immune $CD8^+$ T cells in spleen and brain of B/6 wt mice infected with 10^3 PFU of JEV, s.c., at 7 days pi were measured by IFN-g expression in $CD8^+$ cells following *ex vivo* stimulation in the presence or absence of JEV NS4B peptide. Means for 6 - 8 samples ± the SD are presented. Asterisks denotes significance (* = $P < 0.05$; ** = $P < 0.01$).

3.2.3 Antibody is essential for recovery from JEV infection. To assess the role of antibody in JEV pathogenesis, mortality and virus growth following infection with 10^3 PFU of JEV, sc, were assessed in $\mu MT^{-/-}$ mice, which are deficient in B cells and antibody production. The challenge was uniformly lethal and mortality occurred significantly earlier than was found in wt mice (MST = 11.1 and 12.1 days pi, respectively; $P = 0.001$; Fig. 3.6). In contrast to wt mice, JEV infection produced high viremia in all $\mu MT^{-/-}$ mice on day 4 pi (GMT = 1.5×10^5 PFU/ml) and the viremia persisted at that level until the animals succumbed to infection (Fig. 3.1). Virus was also detected in spleen of infected $\mu MT^{-/-}$ mice at 4 days pi and at later time points (Fig. 3.1). However, the viral load in spleen was mostly less than that in serum, making it unclear whether virus grew efficiently in that organ. For the same reason it is unclear, whether the viral titers detected in brains of all $\mu MT^{-/-}$ mice on day 4 pi were due to virus content in residual blood, although animals were perfused prior to organ collection. Viral load in brain increased 10- and 10,000-fold at 6 and 8 days pi, respectively, and the titers in $\mu MT^{-/-}$ mice exceeded those in wt mice by ~3 log and ~5 log on days 6 and 8 pi (Fig. 3.1). Viral dissemination into the spinal cord of $\mu MT^{-/-}$ mice occurred earlier and was more widespread than in wt mice: virus was first detected on day 6 pi in 60% of mice (GMT = 3×10^4 PFU) and was present in spinal cords of all animals on day 8 pi with JEV titers exceeding those in wt mice by 4 log. Taken together, absence of antibody in $\mu MT^{-/-}$ mice leads to uncontrolled viremia, early neuroinvasion and widespread viral dissemination in the CNS, and a uniformly lethal outcome of infection with a shorter MST than in wt mice.

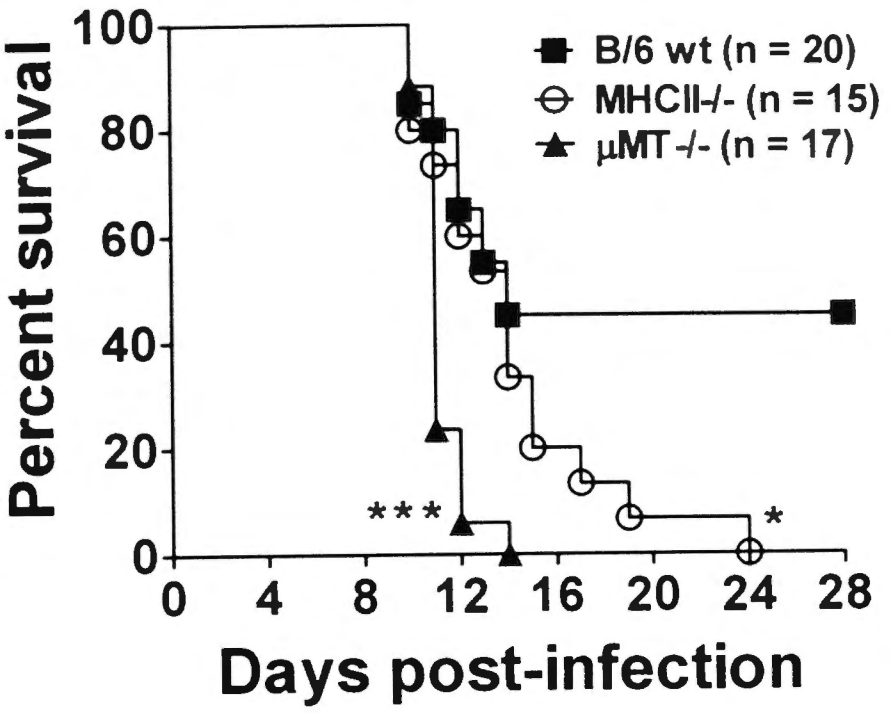


Figure 3.6 Susceptibility of B/6 wt, $\mu MT^{-/-}$ and MHCII-$\alpha\beta^{-/-}$ mice to infection with JEV. Groups of 12-week-old mice were infected sc with 10^3 PFU of JEV. Morbidity and mortality were recorded daily and surviving mice were monitored for 28 days. Data was constructed from two independent experiments. Significance of difference in mortality between wt and knock-out mice was determined by using the log-rank test: * $P = 0.028$; *** $P = 0.0002$.

3.2.4 CD4$^+$ T cell are instrumental for virus clearance but not for early virus-neutralizing antibodies. MHCII-αβ$^{-/-}$ mice are deficient in CD4$^+$ T cells which, in turn, are important in priming and memory formation of antiviral B and CD8$^+$ T cell responses and the production of inflammatory cytokines (Zhu *et al.*, 2010). All MHCII-αβ$^{-/-}$ mice succumbed to JEV infection, in contrast to a mortality rate of 55% observed for a group of age-matched wt mice (P = 0.028; Fig. 3.6). Interestingly, the MST of infected MHCII-αβ$^{-/-}$ mice suggested a dual course of disease progression, where the MST of the majority of mice was similar to that of wt controls (~12.5 days pi), while 21% of MHCII-αβ$^{-/-}$ mice displayed a later average time to death (~20 days pi). Similar to wt mice, virus was not detectable in serum and spleen during the first 8 days of infection and virus was found in brain in some mice at 6 and 8 days pi (Fig. 3.1). However, at 10 days pi, 3 out of 5 MHCII-αβ$^{-/-}$ mice developed a moderate viremia and all animals showed virus in the brain with the GMT exceeding that in wt controls by 4 log. Virus dissemination into the spinal cord was also more prominent in MHCII-αβ$^{-/-}$ than wt mice at 10 days pi (Fig. 3.1).

The lack of CD4$^+$ T cells did not prevent induction of a JEV-specific IgM response in infected MHCII-αβ$^{-/-}$ mice, but markedly truncated this response (Fig. 4A). While peaking at day 8 pi in wt mice, JEV-specific IgM declined after day 6 pi in MHC-II-αβ$^{-/-}$ mice and was significantly lower on days 8 and 10 pi than in wt (P = 0.02). Immunoglobulin class-switching to IgG was significantly blunted in MHC-II-αβ$^{-/-}$ mice with anti-JEV IgG1 titers in wt mice exceeding those in the CD4$^+$ T cell-deficient mice by ~10- and 20-fold on days 8 and 10 pi, respectively (Fig. 4B). IgG2b isotype antibodies against JEV first appeared at 10 days pi in wt mice but remained undetectable in MHCII-αβ$^{-/-}$ mice (Fig. 4B). Importantly, the neutralising activity of the humoral immune response against JEV first detectable in both mouse strains at day 6 pi

only increased marginally on day 8 pi in infected MHCII-$\alpha\beta^{-/-}$ mice and subsequently declined, while in wt mice neutralising antibody levels continued to increase. As a consequence, the $PRNT_{50}$ titer in JEV infected wt mice (mean = 330, range 80 – 640) significantly exceeded that in MHCII-$\alpha\beta^{-/-}$ mice (mean = 40, range 20 - 80) at day 10 pi ($P = 0.007$; Fig. 4C). Accordingly, the deficient humoral immune response after day 6 pi in MHCII-$\alpha\beta^{-/-}$ mice most likely is the dominant factor contributing to the late viremia and high mortality in the JEV infected host lacking $CD4^+$ T cell function.

3.2.5 Marginal contribution of $CD8^+$ T cells to the control of JEV infection.

To establish the role of $CD8^+$ T cells in recovery from primary JEV infection, B/6 mice were depleted *in vivo* of $CD8^+$ T cells and challenged with 10^3 PFU JEV, sc (Table 3.2). Only a marginal, but statistically insignificant, increase in susceptibility to JEV of mice depleted of $CD8^+$ T cells was seen compared to a group of B/6 mice treated with an isotype control antibody (84% and 67% mortality, $P = 0.07$). Similarly, the time to death was slightly earlier in $CD8^+$ T cell-depleted mice than in the control group ($P = 0.12$; Table 2). $CD8^+$ T cell depletion did not allow the generation of detectable viremia during the course of JEV infection (data not shown). However, $CD8^+$ T cells contributed to a significant reduction in virus growth in the brain and spinal cord (Fig. 3.7). Thus, depletion of $CD8^+$ T cells resulted in a ~100-fold increase in virus titers in both tissues on day 10 pi, relative to controls, accompanied by a greater number of spinal cord samples showing the presence of virus in treated relative to mock-treated animals. Thus, although $CD8^+$ T cells did not provide a significant advantage in terms of survival following JEV infection, they demonstrated a beneficial role in controlling virus growth in the CNS, with the proviso that the latter may occur at the cost of increased immunopathology.

The already high susceptibility of B/6 mice to JEV infection may have concealed a survival advantage mediated by $CD8^+$ T cells in the depletion experiment. Therefore, the contribution of $CD8^+$ T cells to recovery from JEV infection was tested in the 129 mouse strain, which shows a low mortality rate (~10%) following infection with 10^3 PFU of JEV, sc. The depletion of $CD8^+$ T cells had no impact on the survival rate of JEV infected 129 mice (Table 3.2).

Table 3.2 Effect of $CD8^+$ T cell depletion on susceptibility to JEV infection in B/6 and 129 mice

Mouse strain and treatment [a]	Mortality (No. of deaths/total) [b]	MST (days) ± SD [c]
C57Bl/6		
Isotype Control	67% (14/21)	13.0 ± 1.7
$CD8^+$ T cell-depleted	84% (16/19; $P = 0.07$)	12.1 ± 2.6 ($P = 0.12$)
129x1/SvJAPB		
Isotype Control	11% (1/9)	14
CD8+ T cell depleted	0% (0/11; $P = 0.26$)	-

[a] Eight-week-old mice were treated i.p. with 0.5 mg of $CD8^+$ T cell-depleting or isotype control antibody in 500 μl PBS on days -1, 0, +1 and +7 of sc challenge with 10^3 PFU of JEV. Surviving mice were monitored for 28 days.

[b] Survival data were analysed for significance between the treated and corresponding control groups by the log-rank test. Data were constructed from two independent experiments.

[c] Differences in mean survival times between treated and control groups were analysed for significance by the Mann-Whitney test.

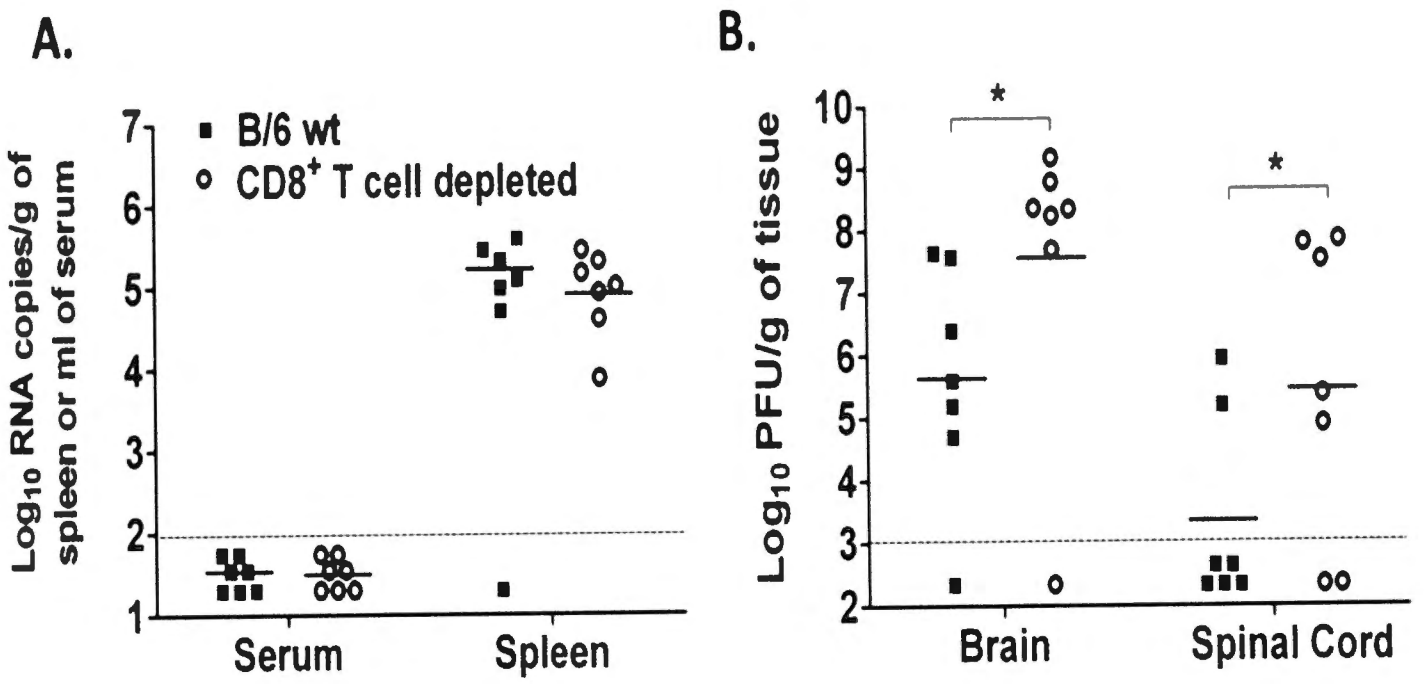


Figure 3.7 ***In vivo*** **depletion of CD8$^+$ T cells increases viral burden in brain and spinal cord.** Groups of CD8$^+$ T cell-depleted and control antibody-treated 8-week-old B/6 wt mice were infected sc with 10^3 PFU of JEV and sacrificed on day 10 pi to determine the viral load in brain and spinal cord by plaque titration. Symbols represent individual mice and data was constructed from two independent experiments. Horizontal line denotes GMT. Asterisks denote significance (* = P <0.05).

3.2.6 The protective effect of JEV-immune T cells requires both the $CD4^+$ and $CD8^+$ subpopulations.

Infection of B/6 mice with JEV elicits a vigorous cellular immune response, which is illustrated by the protective value of JEV-immune splenocyte transfer in recipient mice against JEV challenge (Table 3.3). Thus, total immune splenocytes and purified B cells protected all recipient mice against infection with 10^3 PFU JEV, sc, when transferred 1 day before challenge. However, transfer of purified JEV-immune $CD4^+$ or $CD8^+$ T cells did not provide a significant survival advantage to recipient mice challenged with JEV (Table 3.3). Interestingly, pooled transfer of both T cell subpopulations significantly reduced mortality in a group of recipient mice ($P = 0.002$; Table 3.3). These data yet again show the dominant protective role of the humoral immune response contrasting with an at best marginal contribution of $CD8^+$ T cells in recovery from primary infection with JEV.

Table 3.3 Contribution of humoral and cellular immune responses to recovery from JEV infection

Treatment [a]	Mortality (No. of deaths/total) [b]	MST (days) ± SD [c]
PBS control	70% (7/10)	12.0 ± 2.0
Naïve splenocytes (1 x 10^7 cells)	73% (22/30)	12.5 ± 2.0
Immune splenocytes (1 x 10^7 cells)	0% (0/10; $P = 0.0004$)	-
Immune B cells (5 x 10^6 cells)	0% (0/10; $P = 0.0004$)	-
Immune T cells (5 x 10^6 cells)	29% (6/21; $P = 0.0017$)	12.8 ± 1.0
Immune $CD4^+$ T cells (5 x 10^6 cells)	64% (9/14; $P = 0.99$)	12.1 ± 2.0
Immune $CD8^+$ T cells (5 x 10^6 cells)	76% (16/21; $P = 0.59$)	12.0 ± 2.3

[a] Eight-week-old donor B/6 mice were infected with 10^3 PFU of JEV, iv, or left uninfected and sacrificed 7 days later for splenocyte collection and purification of splenocyte subpopulations. Cells were transferred to 8-week-old B/6 mice and recipients infected a day later with 10^3 PFU of JEV, sc. Surviving mice were monitored for 28 days.

[b] Data are representative of 2 or 3 independent experiments. Immune splenocyte treatment groups were compared to the naïve splenocyte control group to test for statistical significance.

[c] No significant difference was noted between immune splenocyte treatment and control groups.

3.2.7 Deficiencies in the cytolytic effector pathways of lymphocytes do not exacerbate the pathogenesis of JEV. $CD8^+$ T cells exhibit direct antiviral activity by killing virus infected cells via two cytolytic effector mechanisms, the Fas-mediated death pathway and the granule exocytosis pathway, involving the delivery of perforin and granzymes into the target cell. Having already demonstrated that neither *in vivo* depletion nor transfer of virus-immune $CD8^+$ T cells significantly impacted on disease outcome, it is predicted that mice defective in Fas, perforin, or granzymes A and B would show comparable susceptibility to JEV as wt mice. This was confirmed in figure 3.8: neither mortality rate nor MST differed significantly between wt and $Fas^{-/-}$, $Perf^{-/-}$ or $GzmA/B^{-/-}$ mice tested at 8 and 12 weeks of age.

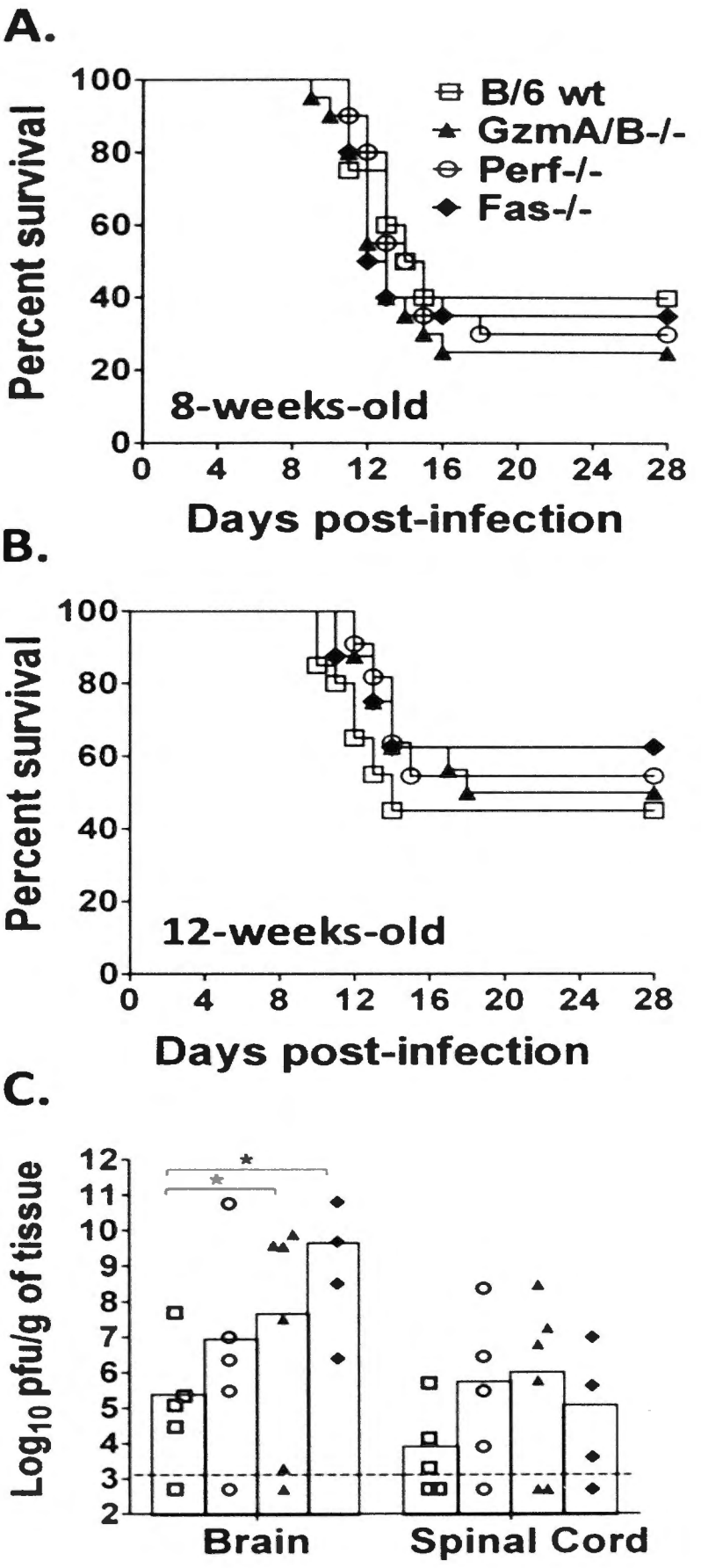


Figure 3.8 Susceptibility of mice defective in cytolytic effector pathways to infection with JEV. Groups of 8-week-old (A) and 12-week-old (B) B/6 wt, Perf$^{-/-}$, GzmA/B$^{-/-}$ and Fas$^{-/-}$ mice were challenged sc with 10^3 PFU of JEV and monitored for 28 days for mortality. Data was constructed from 3 independent experiments.

3.3 Discussion

B/6 mice showed a partial susceptibility to s.c. infection via the footpad with the prototype strain of JEV and developed a lethal encephalitis in ~60% of animals over a wide dose range of 10 – 10^5 PFU. This lack of a dose response in mortality following virus challenge by an extraneural route is a hallmark of flavivirus infection (for instance, see references (Licon Luna *et al.*, 2002; Diamond *et al.*, 2003*a*)) and is thought to reflect, at least in part, the dose dependence of induction of the innate immune responses critical in early control of virus dissemination (Monath *et al.*, 2003). Consistent with high-dose WNV and MVEV infections in B/6 mice (Licon Luna *et al.*, 2002; Wang *et al.*, 2003), the sc injection of 10^7 PFU of JEV was uniformly lethal and the MST corresponded to that of groups of mice ic inoculated with 10^2 PFU of JEV. This finding and that of the presence of multiple loci of infection in the brain supports the hematogenous route as the principal pathway of JEV entry into the CNS and implies virus content in blood as a key determinant thereof. Vero cell-grown JEV was used exclusively in this investigation. Interestingly, studies with cell culture have suggested that flavivirus propagated in mosquito cells could differentially modulate innate immune responses from virus grown in mammalian cells (Arjona *et al.*, 2007; Silva *et al.*, 2007). However, this conclusion was not supported by pathogenesis studies with mice (Daffis *et al.*, 2008; Lim *et al.*, 2010).

The humoral immune response was critically important for recovery of mice from extraneural challenge with JEV. Mice lacking B cells and the ability to produce antibody ($\mu MT^{-/-}$ mice) developed a high viremia at day 4 pi, which persisted until all animals succumbed to infection; early entry of virus into the brain with widespread dissemination in the CNS were also found. Additionally, purified immune B cell

transfer fully protected recipient wt mice from JEV challenge. The appearance of viremia and splenic tissue viral titres at day 4 pi in $\mu MT^{-/-}$ mice correlated with the time point when antibodies with neutralising activity became detectable in JEV infected wt mice. Antibodies elicited against flaviviruses clear virus by neutralization of infectivity, or indirectly by antibody-dependent cell-mediated cytotoxicity, Fc-γ-receptor-mediated clearance or complement-mediated cytotoxicity (Pierson et al., 2008). Thus, in mice lacking the ability to clear virus by antibody, JEV and WNV (Chambers *et al.*, 2008; Diamond *et al.*, 2003*a*; Diamond *et al.*, 2003*b*) produce a fulminating and lethal infection.

T cell help was not required to mount an early IgM response against JEV; however, the response was truncated in MHCII-$\alpha\beta^{-/-}$ relative to wt mice. As expected, IgG class-switching first observed in wt mice at ~8 days pi was markedly blunted in the absence of $CD4^+$ T cells. This dysfunctional antibody response largely explains the adverse outcome of JEV infection in MHCII-$\alpha\beta^{-/-}$ mice, which was characterised by uniform mortality, elevated brain viral titres and viral dissemination into the spinal cord. Moreover, 60% of MHCII-$\alpha\beta^{-/-}$ mice displayed detectable viremia late in the course of infection, when a significant disparity in neutralizing antibody titers in wt and MHCII-$\alpha\beta^{-/-}$ mice was found. Failure of effective virus clearance from extraneural tissues most likely accounted for CNS invasion in all infected MHCII-$\alpha\beta^{-/-}$ mice and, as a consequence, a 100% mortality rate. Some JEV infected MHCII-$\alpha\beta^{-/-}$ mice showed a markedly protracted time to death, which could either have been the result of a delayed entry into the CNS following the late viremia associated with waning neutralising antibody, or reduced lymphocyte infiltration into the infected brain associated with reduced immunopathological manifestations. A study on the role of $CD4^+$ T cells in WNV infection also found a truncation of IgM and blunting of IgG antibody responses

due to the absence of $CD4^+$ T cells, as well as uniform mortality with prolonged survival of some mice (Sitati and Diamond, 2006). Accordingly, the protective role of $CD4^+$ T cells by providing help for antibody responses is clearly apparent in both flavivirus models. However, a second critical function of $CD4^+$ T cells required for a sustained $CD8^+$ T cell response and, in turn, $CD8^+$ T cell-mediated clearance of virus from the CNS documented for WNV (Sitati and Diamond, 2006) was not apparent in the mouse model for Japanese encephalitis, given the failure of $CD8^+$ T cells to effectively clear JEV from the CNS and prevent a lethal disease outcome.

A striking finding from this investigation was the lack of effect of $CD8^+$ T cells on survival phenotype in the mouse model of Japanese encephalitis. I have confirmed that infection of B/6 mice with JEV elicited functional, virus-specific, $CD8^+$ T cells, illustrated by production of IFN-γ and TNF-α upon *ex vivo* stimulation of lymphocytes with a previously described H-$2D^b$ JEV NS4B peptide (Trobaugh et al., 2010). This response was comparable in magnitude to that elicited by WNV against the corresponding WNV NS4B peptide (Brien et al., 2007; Purtha et al., 2007) and followed the standard kinetics of induction of anti-flaviviral cytotoxic T cells with peak activity in the spleen at day 7 pi (Kesson et al, 1987; Purtha et al., 2007). It was also confirmed a high level of $CD8^+$ T cell infiltration into the brain in response to JEV infection and showed activation and virus-specificity of lymphocytes isolated from the infected brain. Thus, given the effective induction and functionality of anti-JEV $CD8^+$ T cells, it was surprising that transfer of this JEV-immune splenocyte population failed to provide protection against JEV in recipient mice. Similarly, *in vivo* depletion of >98% of $CD8^+$ T cells in two mouse strains (B/6 and 129), which differed in susceptibility to JEV, did not markedly impact on mortality rate following JEV challenge. However, viral burden in the CNS was significantly higher following $CD8^+$ T cell depletion than

in control mice, showing *in vivo* functionality of the response. It remains unclear, whether immunopathology associated with $CD8^+$ T cell infiltration into the brain or an insufficient magnitude of the response, per se, explain why the viral load reduction in the CNS mediated by $CD8^+$ T cells did not translate into a survival advantage. Finally, genetic deficiency in key molecules (Fas, perforin or granzymes A and B) of the two main $CD8^+$ T cell cytolytic effector pathways did not alter susceptibility of B/6 mice to JEV. Collectively, these data suggest an at best marginal contribution of $CD8^+$ T cells to recovery from infection with JEV.

This and other investigations on the immunobiology of the encephalitic flaviviruses have uncovered a conflicting role of $CD8^+$ T cells in recovery from infection. While essential for virus elimination from the CNS and survival in mouse models of West Nile encephalitis (Shrestha and Diamond, 2004; Shrestha *et al.*, 2006*a*; Wang *et al.*, 2003; Wang *et al.*, 2004), I present in this chapter that the $CD8^+$ T cell response did not markedly affect outcome of infection with JEV, whereas a disease-potentiating effect of cytotoxic T cells was documented in mice infected with MVEV (Licon Luna *et al.*, 2002). Notably, mice genetically deficient in the Fas- or perforin-dependent pathways of cytotoxicity showed greatly increased susceptibility to virulent lineage I WNV infection (Shresthaand Diamond, 2007), but did not differ from wt mice in susceptibility to infection with JEV (Fig. 8) or lineage II WNV strain, Sarafend (Wang *et al.*, 2004), and were more resistant to infection with MVEV (Licon Luna *et al.*, 2002). These findings highlight a difference in pathogenesis between closely related flaviviruses belonging to the JEV serocomplex, which most likely involves a difference in the capacity of the cellular immune response to resolve the virus infections in the CNS. It has been well documented that extraneural infection of adult mice with virulent lineage I WNV produces virus titers in brain of 10^3 – 10^5 PFU in the majority of animals

between days 6 and 10 pi and that $CD8^+$ T cell-mediated clearance of virus from the CNS allows most mice to recover (for instance, references (Shrestha and Diamond, 2004; Shrestha *et al.*, 2006*a*)). Similar immune-mediated resolution of virus infection of the CNS leading to improved disease outcome is not apparent in murine infections with JEV and MVEV, where a strong correlation between rate of CNS infection and mortality exists (this chapter and reference (Licon Luna *et al.*, 2002)). The mouse models of West Nile, Murray Valley and Japanese encephalitis reflect the human disease with these viruses, given that patients with West Nile virus meningitis and encephalitis often have less severe outcomes (Lindsay *et al.*, 2010), in contrast to patients with Japanese or Australian encephalitis who frequently suffer debilitating neurological complications (Burrow *et al.*, 1998; Solomon, 2004).

Chapter 4

Redundancy of Fas and granzymes in recovery from primary Japanese encephalitis virus infection

4.1 Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are key mediators of the host's direct antiviral functions (reviewed in (Clark and Tyler, 2009; Russel and Ley, 2002)). Naive CTL precursors require activation through the recognition of specific viral peptide determinants on an antigen-presenting cell in the context of MHC restriction elements, mostly MHC-I. Induced CTLs require, as part of the adaptive immune responses, several days of proliferation before they can exert collectively the full extent of their antiviral functions. These virus-specific CTLs are mainly of the $CD8^+$ T cell phenotype; however, the cytotoxic potential of $CD4^+$ T cells has also been recognized (reviewed in (Brown, 2010; van de Berg *et al.*, 2008)). NK cells, on the other hand, are constitutively armed with preformed cytotoxic granules (reviewed in (Chowdhury and Lieberman, 2008)), making them readily inducible, and able to rapidly respond to a viral challenge. These effector cells of the innate arm of the antiviral immune response recognize virally infected cells through a combination of activating and inhibitory receptors (reviewed in (Lanier, 2008)). CTLs and NK cells utilize identical contact-dependent mechanisms for induction of target cell apoptosis for viral clearance, viz. the Fas-FasL and granule exocytosis pathways of cytotoxicity.

Fas receptor (Fas, CD95 or Apo-1) is a transmembrane protein that is expressed on all nucleated cells (reviewed in (Kaufmann *et al.*, 2012; Lavrik and Krammer, 2012)). Ligation of Fas on virally infected cells to Fas ligand (FasL) expressed on effector cytotoxic cells initiates a classical caspase cascade, and ultimately leads to target cell death. In addition to its role in immune cell homeostasis and regulation of tumor progression (reviewed in (Bouillet and O'Reilly, 2009, Ehrenschwender and Wajant, 2009)), the Fas-FasL pathway is vital for viral load reduction and/or recovery from viral

infections, for example in protection against hepatitis B virus (HBV) (Yang *et al.*, 2009), herpes simplex type 2 (HSV-2) (Ishikawa *et al.*, 2009), and West Nile virus (WNV) infection (Shrestha and Diamond, 2007a). Moreover, it is considered as a key pathway necessary for preventing the development of chronic inflammatory disease after mouse cytomegalovirus (MCMV) infection (Fleck *et al.,* 1998). On the contrary, the Fas-FasL pathway can also contribute to adverse disease outcomes, for example after LCMV (Zajac et al., 1996), MVEV (Licon Luna *et al.,* 2002), SFV (Alsharifi *et al.*, 2006), or reovirus infections (Lopez *et al.*, 2009), and can sustain chronic hepatic inflammation caused by HCV infection (Cruise *et al.*, 2006).

The granule exocytosis pathway constitutes an effector cell release of pore-forming protein, perforin, and a family of serine proteases, predominantly granzymes (Gzm) (reviewed in (Chowdhury and Lieberman, 2008; Voskoboinik *et al.*, 2010)). To date, there are 5 known human and 11 mouse Gzm. The pro-apoptotic Gzm A and B are the most abundantly released members of this family of molecules at the site of the immunological synapse, and are consequently the most extensively studied. The role of individual molecules involved in the granule exocytosis pathway varies amongst different viral infection models. A deficiency in perforin alone results in severe disease in models of Ebola (Gupta *et al.*, 2005), LCMV (Walsh *et al.*, 1994), MCMV (Loh *et al.*, 2005; Dix et al., 2003), Theiler's virus (Rossi *et al.*, 1998), and WNV infection (Shrestha *et al.*, 2006*a*), as opposed to better outcomes in models of respiratory syncytial virus (Aung et al., 2001) and MVEV (Licon Luna *et al.*, 2002). On the other hand, Gzms seem to display functional redundancy since a deficiency in individual Gzms usually does not result to increased susceptibility, except in certain cases, for instance, ectromelia (Mullbacher *et al.*, 1999) and LCMV infection (Zajac *et al.,* 2003).

In the previous chapter, I have shown that $Fas^{-/-}$, $Perf^{-/-}$, and $GzmA/B^{-/-}$ mice did not display increased susceptibility to JEV infection, although defective clearance of virus from the CNS was observed. It remained uncertain whether immunopathology associated with leukocyte infiltration into the CNS, less than optimal killing of virus infected cells, or other factors explained why the viral load reduction in the CNS did not translate into survival advantage. Here, I re-evaluated the phenomenon with the use of mice deficient in both effector pathways of cytotoxicity. I also show that NK cells, which are competent, along with $CD8^+$ and $CD4^+$ T cells, in eliminating virus infected cells by triggering contact-dependent cell death pathways, do not significantly contribute to disease outcome in a mouse model of Japanese encephalitis.

4.2 Results

4.2.1 Combined deficiencies in Fas-FasL and granule exocytosis pathways of cytotoxicity increase the susceptibility of mice to Japanese encephalitis. In chapter 3, I showed that single cytotoxic effector pathway defects did not result in increased mortality from low dose challenge with JEV. Mice deficient in Fas, perforin, or Gzms A and B had similar survival rates relative to wt controls. Here, I extend my investigation on the contribution of T (and NK) cell-mediated cytotoxicity in the recovery from Japanese encephalitis by examining the susceptibility of mice with combined deficiencies of essential mediators of the Fas-FasL and granule exocytosis pathways. Twelve-week-old $Fas^{-/-}$, $Perf^{-/-}$xGzmA/$B^{-/-}$, $Fas^{-/-}$xGzmA/$B^{-/-}$, and wt control mice were challenged sc with 10^3 PFU of JEV, and morbidity and mortality were recorded during a 28-day-observation period. In all groups, moribund mice presented with similar clinical signs starting with generalized piloerection, paresis and rigidity, and later progressing to severe neurological signs demonstrated by postural imbalance, ataxia and generalized tonic-clonic seizures. The median survival time of mice that succumbed to infection did not differ significantly between groups (12.7 ± 1.5 days for $Fas^{-/-}$, 12.5 ± 1.8 days for $Perf^{-/-}$xGzmA/$B^{-/-}$, 12.4 ± 0.7 days for $Fas^{-/-}$ xGzmA/$B^{-/-}$, and 12.7 ± 1.6 days for wt control mice). The mortality rate of mice defective in either Fas-FasL or granule exocytosis pathways of cytotoxicity was comparable to that of wt controls: 50% for $Fas^{-/-}$, 55% for $Perf^{-/-}$xGzmA/$B^{-/-}$, and 60% for wt control mice. Interestingly, a combinatorial defect affecting both pathways of cytotoxicity resulted in a significant increase in mortality, where 90% of $Fas^{-/-}$ xGzmA/$B^{-/-}$ mice succumbed to infection (Fig. 4.1). These data indicate a redundancy of the two contact-dependent effector pathways of T or NK cell-mediated cytotoxicity in resistance to a lethal JEV infection.

4.2.2 Defective control of virus growth in the CNS of $Fas^{-/-}$xGzmA/$B^{-/-}$ mice.

Viral load in serum, and peripheral and CNS tissues of wt and $Fas^{-/-}$xGzmA/$B^{-/-}$ mice infected sc with 10^3 PFU of JEV was determined at 2-day-intervals pi (Fig. 4.2). Viremia and viral load in spleen detected by real-time RT-PCR did not differ significantly between the two groups. Peak viremia was found at day 2 pi ($5x10^4$ vs. $1x10^5$ RNA copies/ml of serum in groups of $Fas^{-/-}$xGzmA/$B^{-/-}$ and wt mice, respectively) and peak splenic viral burden on day 4 pi ($9x10^4$ vs. $7x10^4$ RNA copies/g of tissue in groups of $Fas^{-/-}$xGzmA/$B^{-/-}$ and wt mice, respectively) (Fig. 4.2A,B). However, the defect in Fas-FasL and granule exocytosis pathways significantly compromised the ability of mice to control virus growth in the CNS and/or prevent virus entry into the brain. Compared to wt, the proportion of $Fas^{-/-}$xGzmA/$B^{-/-}$ mice showing detectable virus titers in brain was markedly greater, and viral load 10- and 1000-fold higher on days 8 and 10 pi, respectively (Fig. 4.2C). In addition, dissemination of virus into the spinal cord was more evident in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice, with viral titers exceeding those in wt mice by 2 log on day 10 pi (Fig. 4.2D). These findings were supported by markedly increased detection of JEV antigen in various regions of the brain of $Fas^{-/-}$xGzmA/$B^{-/-}$ relative to wt mice (Fig. 4.3). Altogether, these data indicate that failure of control of virus growth in the CNS, but apparently not in extraneural tissues, may explain the increased susceptibility of $Fas^{-/-}$xGzmA/$B^{-/-}$ mice to infection with JEV.

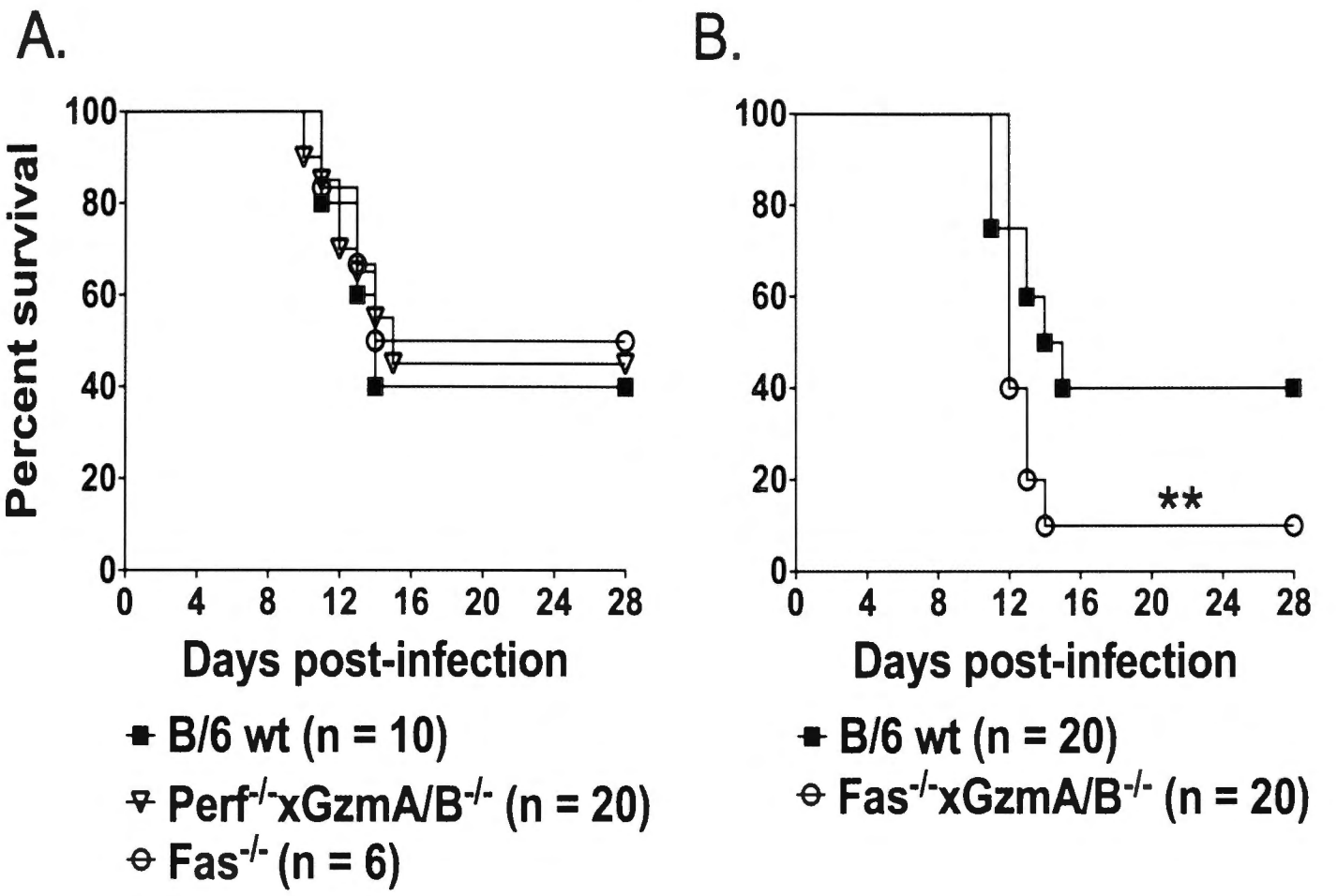


Figure 4.1 Susceptibility of $Fas^{-/-}$, $Fas^{-/-}$xGzmA/$B^{-/-}$, $Perf^{-/-}$xGzmA/$B^{-/-}$, and wild-type mice to JEV infection. Groups of 12-week-old mice were infected sc with 10^3 PFU of JEV, morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data presented were constructed from two independent experiments. Mortality in $Fas^{-/-}$, $Fas^{-/-}$xGzmA/$B^{-/-}$ and $Perf^{-/-}$xGzmA/$B^{-/-}$ mice was compared to wild-type mice to test for statistical significance using the log-rank test (**, $P < 0.01$).

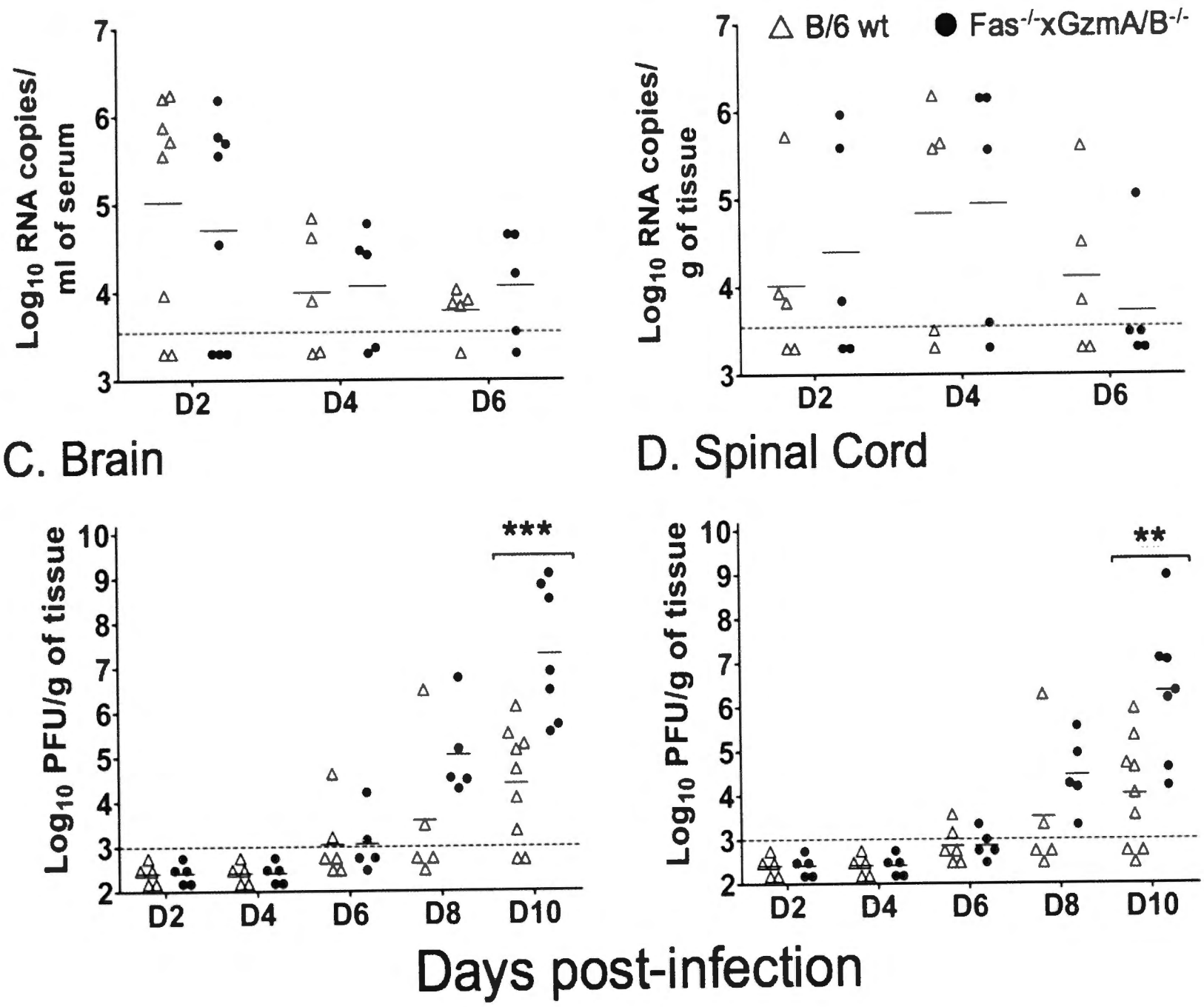


Figure 4.2 JEV burden in Fas$^{-/-}$xGzmA/B $^{-/-}$ and wild-type mice. JEV burden in serum (A), spleen (B), brain (C), and spinal cord (D) samples of Fas$^{-/-}$xGzmA/B$^{-/-}$ and wild-type after sc infection with 10^3 PFU of JEV is shown. At the indicated time points, animals were sacrificed and the viral RNA content of serum and spleen samples were measured by real-time RT-PCR, while viral content in brain and spinal cord samples were measured by plaque titration. Data shown were constructed from 2 independent experiments, each symbol represents an individual mouse, and horizontal lines indicate geometric mean titers. The lower limit of virus detection is indicated by the horizontal dotted line. Asterisks denote significant differences (**, $P < 0.01$).

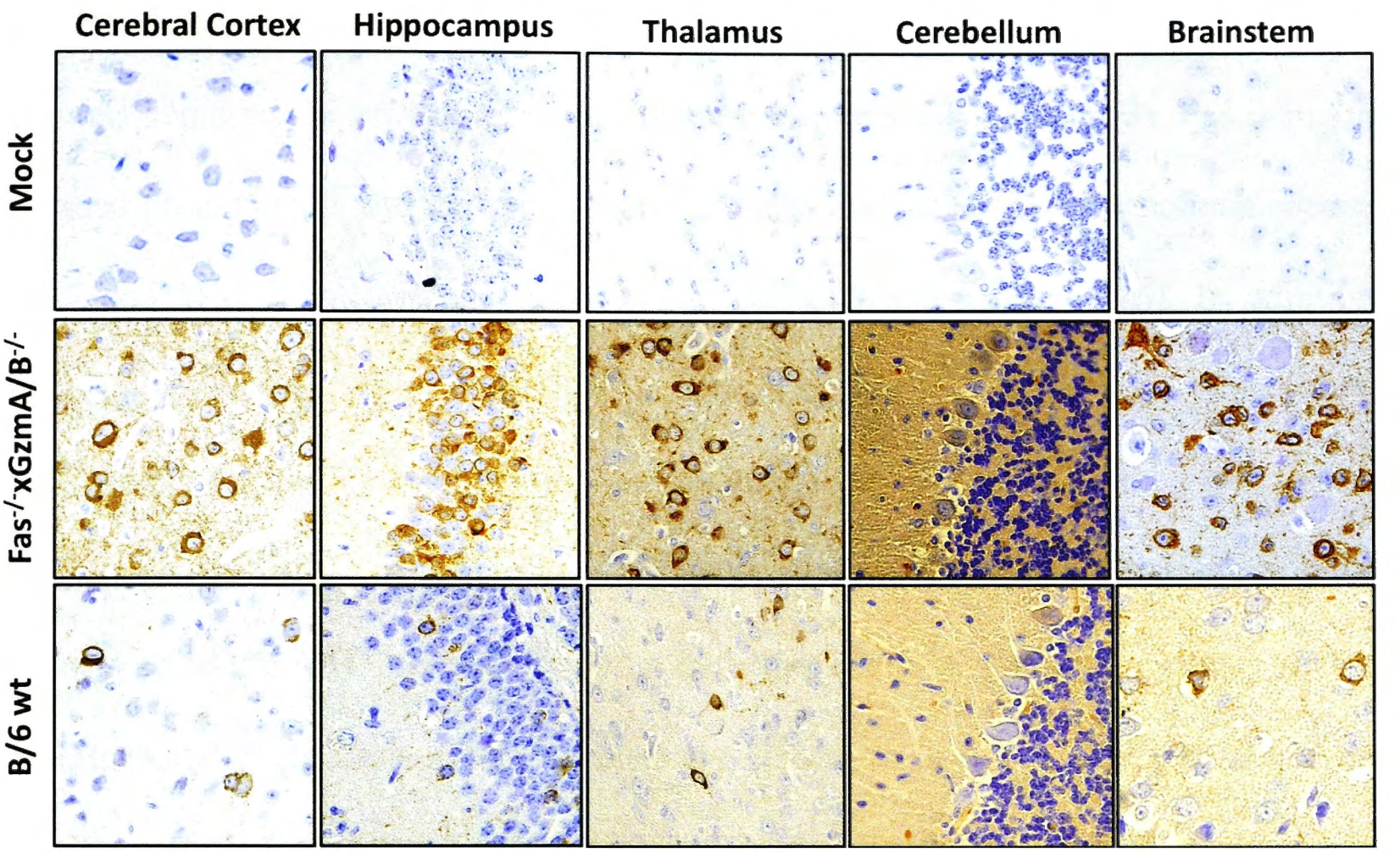


Figure 4.3 Immunohistochemistry. Brain sections of uninfected $Fas^{-/-}$xGzmA/$B^{-/-}$ mice, and $Fas^{-/-}$xGzmA/$B^{-/-}$ and wild-type mice infected for 10 days with 10^3 PFU of JEV, sc. Detection of JEV antigen by immunohistochemistry was with the use of a monoclonal antibody that recognises the flavivirus NS1 protein. Representative sections of the cerebral cortex, hippocampus, thalamus, cerebellum and brainstem are shown. Brown staining is indicative of infected cells.

4.2.3 Humoral immunity in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice. Intact humoral immunity is absolutely required for protection from Japanese encephalitis (Chapter 3). Fas is highly expressed on activated and germinal center B cells, and is essential in maintenance of both T and B cell homeostatsis (Hao *et al.*, 2008; Wang *et al.*, 1996). In addition, expression of Fas and Gzms on $CD4^+$ T cells may impact on its helper function in inducing efficient antibody production and maintenance (Desbarats et al., 1998; Gorak-Stolinska *et al.*, 2001; Maksimow et al., 2006). With this in mind, I investigated whether $Fas^{-/-}$xGzmA/$B^{-/-}$ mice exhibited a defective humoral immune response against JEV. The kinetics and magnitude of antibody responses in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice did not differ significantly from that in wt mice. Generation of anti-JEV IgM antibody was comparable in both groups, appearing first on day 4 pi, and increasing thereafter (Fig. 4.4A). Likewise, class switching to IgG1 and IgG2b isotype antibodies was similar in both groups, initially detected on day 7 pi, and increasing on day 10 pi (Fig. 4.4B). The functional activity of the anti-JEV humoral immune response measured by neutralization assay was also comparable between both groups, with mean $PRNT_{50}$ titers of 536 (range, 80 to 1280) in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice and 656 (range, 40 to 1280) in wt mice on day 10 pi (Fig. 4.4C). In summary, these data indicate that the ability of $Fas^{-/-}$xGzmA/$B^{-/-}$ mice to mount JEV-specific B cell immune responses remained unimpaired.

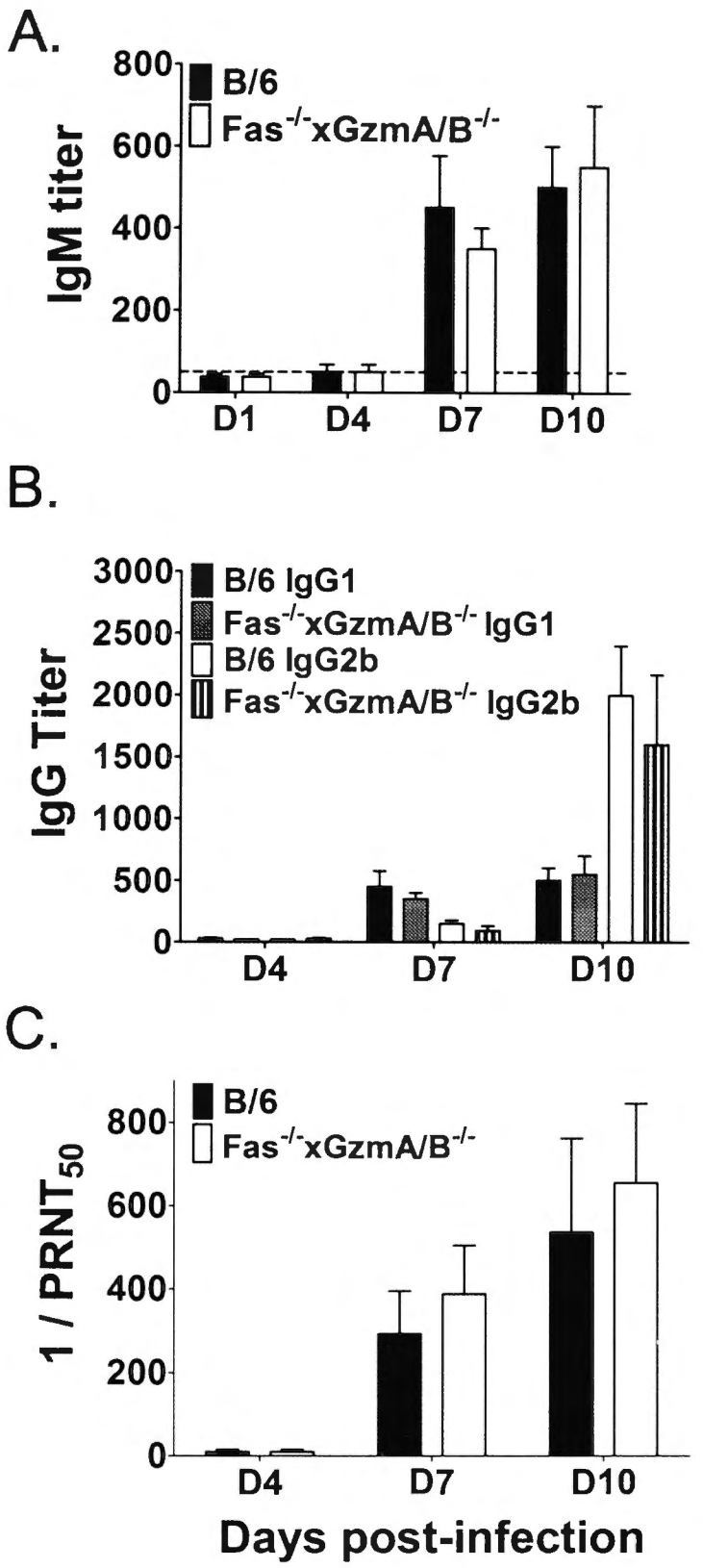


Figure 4.4 Humoral immune response in $Fas^{-/-}$xGzmA/$B^{-/-}$ and wild-type mice. Eight-week-old $Fas^{-/-}$xGzmA/$B^{-/-}$ and wild-type mice were infected sc with 10^3 PFU of JEV, and serum samples were collected at the indicated time points. Anti-JEV IgM (A) and IgG (B) isotype antibody titers were determined by ELISA. The data presented are reciprocal mean endpoint titers representative of 4 mice per time point with the SEM indicated by error bars. (C) Neutralizing antibody titers determined by plaque reduction neutralization assay. The data presented are mean $PRNT_{50}$ titers representative of 5 mice per time point with the SEM indicated by error bars.

4.2.4 Cytokine production in the absence of Fas and Gzm A and B. Besides direct cytotoxic action mediated through the Fas-FasL and granule-exocytosis pathways, the antiviral activity of NK and $CD8^+$ T cells is also mediated indirectly by the release of soluble cytokines (Mullbacher *et al.*, 2004) Therefore, I investigated the capacity of NK and $CD8^+$ T cells from $Fas^{-/-}$xGzmA/$B^{-/-}$ mice to produce IFN-γ and/or TNF-α following infection with JEV. At day 4 pi (at the peak of the NK cell response in this infection model [see Chapter 6]), the percentage of NK cells expressing IFN-γ in spleens of $Fas^{-/-}$xGzmA/$B^{-/-}$ mice was not significantly different relative to wt mice (5.2% in $Fas^{-/-}$xGzmA/$B^{-/-}$ vs. 5.5% in wt mice; Fig. 4.5A). Likewise, the $CD8^+$ T cell immune response (determined by intracellular cytokine staining following *ex vivo* stimulation of $CD8^+$ lymphocytes with a JEV NS4B protein-derived H-$2D^b$-binding peptide) in $Fas^{-/-}$xGzmA/$B^{-/-}$ was comparable to that of wt at day 7 pi (3.1% in $Fas^{-/-}$ xGzmA/$B^{-/-}$ vs. 3.5% in wt mice; Fig. 4.5B). In addition, the percentage of multifunctional $CD8^+$ T cells, characterized by expression of both IFN-γ and TNF-α in single T cells did not differ significantly between the two groups (Fig. 4.5C). Collectively, these data indicate intact production of cytokines in NK and $CD8^+$ T cells in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice.

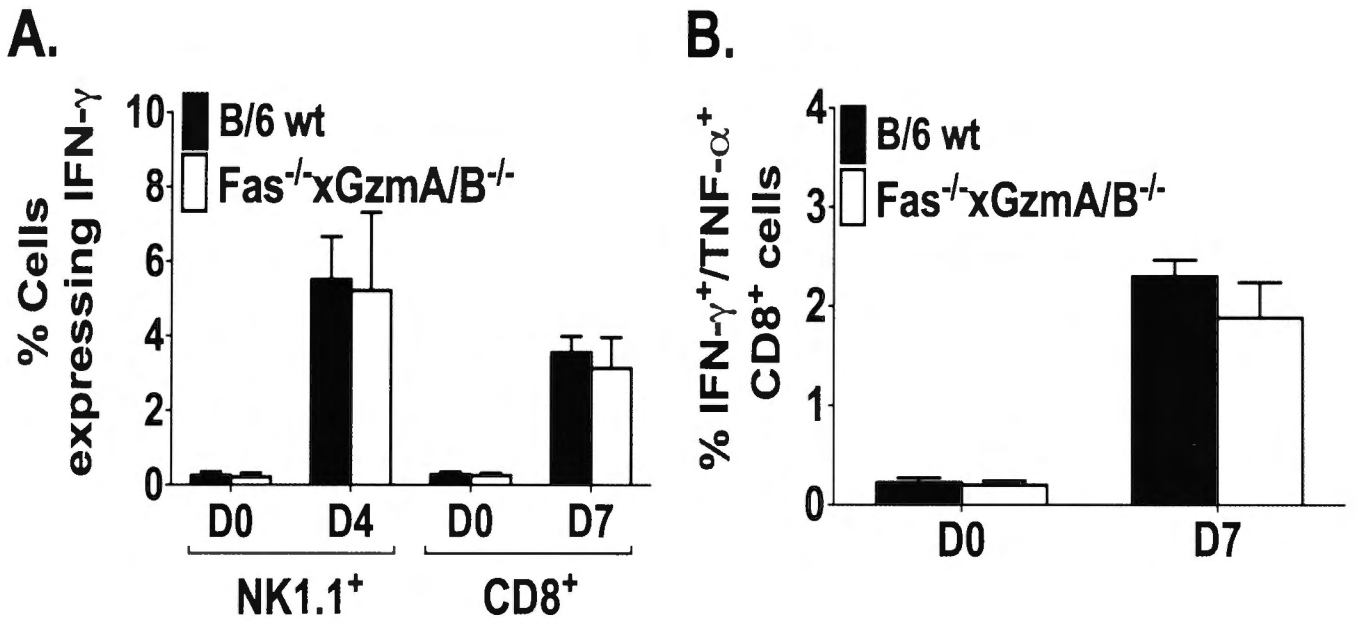


Figure 4.5 NK cell and CD8$^+$ T cell cytokine production. Eight-week-old Fas$^{-/-}$xGzmA/B$^{-/-}$ and wild-type mice were infected iv with 10^3 PFU of JEV or left uninfected. (A) Spleens were collected at indicated time points and the percentage of NK1.1$^+$ or CD8$^+$ splenocytes expressing IFN-γ were identified by flow cytometry. For CD8$^+$ T cells, splenocytes were stimulated *ex vivo* with H2-D^b-restricted JEV NS4B protein-derived peptide. Means for 4 samples ± the SEM are presented. (B) CD8$^+$ T cells expressing both IFN-γ and TNF-α at the indicated time points. Means for 4 samples ± the SEM are presented.

4.2.5 Cellular migration into the CNS. To investigate whether trafficking of leukocytes was defective in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice, leukocytes were isolated from brains of JEV infected $Fas^{-/-}$xGzmA/$B^{-/-}$ and wt mice by density gradient centrifugation and used to quantify leukocyte subpopulations by flow-cytometry. On days 7 and 10 pi, $Fas^{-/-}$xGzmA/$B^{-/-}$ mice did not show a significant difference in the numbers of NK cells, and $CD8^+$ and $CD4^+$ T cells in the brain relative to wt control mice (Fig. 4.6A-C). This result shows that deficiency of both cytolytic effector pathways did not alter the ability of NK and T cells to infiltrate the infected brain, and that the substantially higher viral burden in the CNS later in infection in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice did not result in augmented trafficking of these leukocyte subpopulations into the brain in comparison to wt mice.

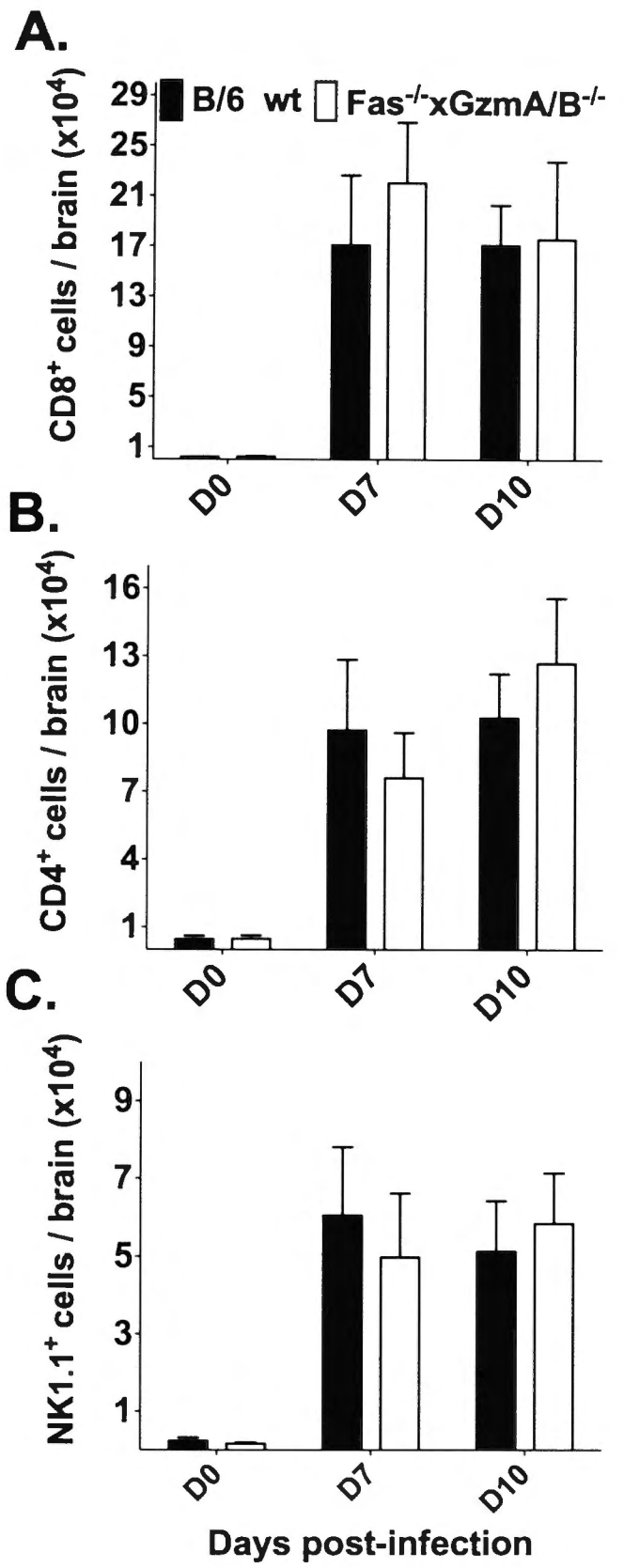


Figure 4.6 Leukocyte trafficking into the CNS. Kinetics of cell infiltration into the brain of 8-week-old $Fas^{-/-}xGzmA/B^{-/-}$ and wild-type mice infected sc with 10^3 PFU of JEV. Leukocytes were isolated by density gradient centrifugation at the indicated time points from brains, and stained for identification as $CD8^+$ T cell (A), $CD4^+$ T cell (B), $NK1.1^+$ cell (C). Data are representative of 2 independent experiments (n = 4/group), and error bars denote the SEM.

4.2.6 NK cells are dispensable for recovery from lethal JEV infection. In chapter 3, I showed that the combined transfer of immune $CD4^+$ and $CD8^+$ T cell provided partial, albeit significant, protection from lethal Japanese encephalitis. This may have been, at least in part, due to the cytotoxic potential of, and/or secretion of cytokines (IFN-γ and TNF-α) from the two lymphocyte subpopulations. Given that these antiviral mechanisms are also exerted by NK cells, it was of interest to address a putative role of NK cells in recovery from infection with JEV, using the approach of *in vivo* depletion of NK cells with an antibody. Thus, 8-week-old B6 mice were treated with anti NK1.1 antibody or mock-treated at days -1, 0, +1, +4, and +8 of sc infection with 10^3 PFU of JEV (Fig. 4.7). This protocol resulted in >98% depletion of NK cells on days 2 and 8 based on flow-cytometric staining of leukocytes using the NK1.1 antibody. The mortality rate in the group of NK cell-depleted and mock-treated mice (55% and 60%, respectively) and MST (13 ± 1.8 days and 12.8 ± 1.5 days, respectively) did not differ significantly between the two groups.

To examine whether NK cells contribute to peripheral viral clearance, I determined the viral load in serum and spleen at early time points pi. Viremia and viral load in spleens did not show a significant difference between the two groups (Fig. 4.8A,B). Lastly, clearance of virus from the CNS was not defective in the absence of NK cells, since viral titers in brain and spinal cord were not significantly different between NK cell-depleted and mock-treated mice on day 10 pi (Fig. 4.8C,D).

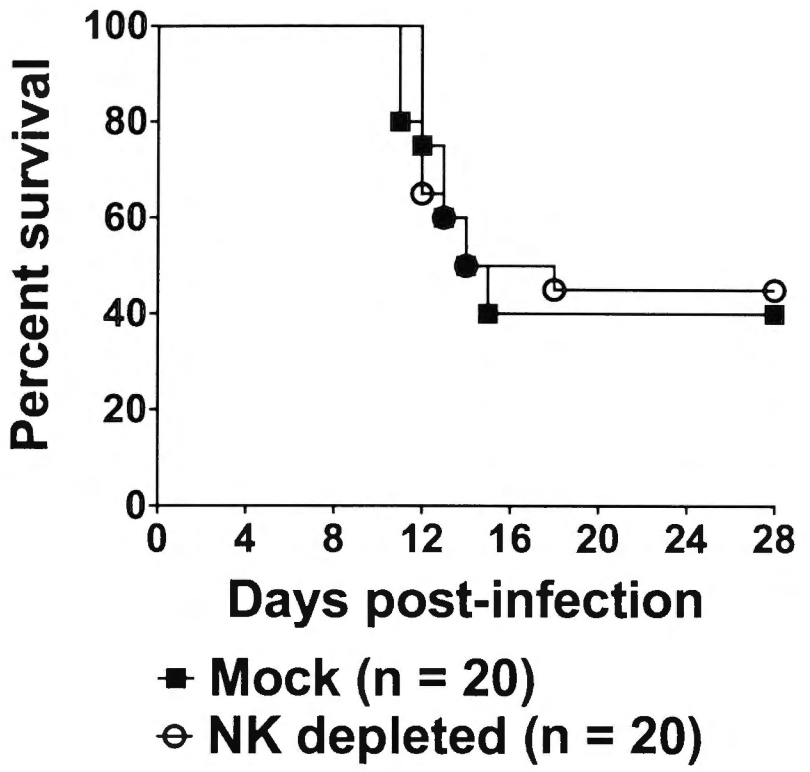


Figure 4.7 Susceptibility to JEV infection after *in vivo* NK cell depletion. Groups of 12-week-old mice were administered ip with either anti-NK1.1 antibody or PBS, and infected sc with 10^3 PFU of JEV. Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data presented were constructed from two independent experiments.

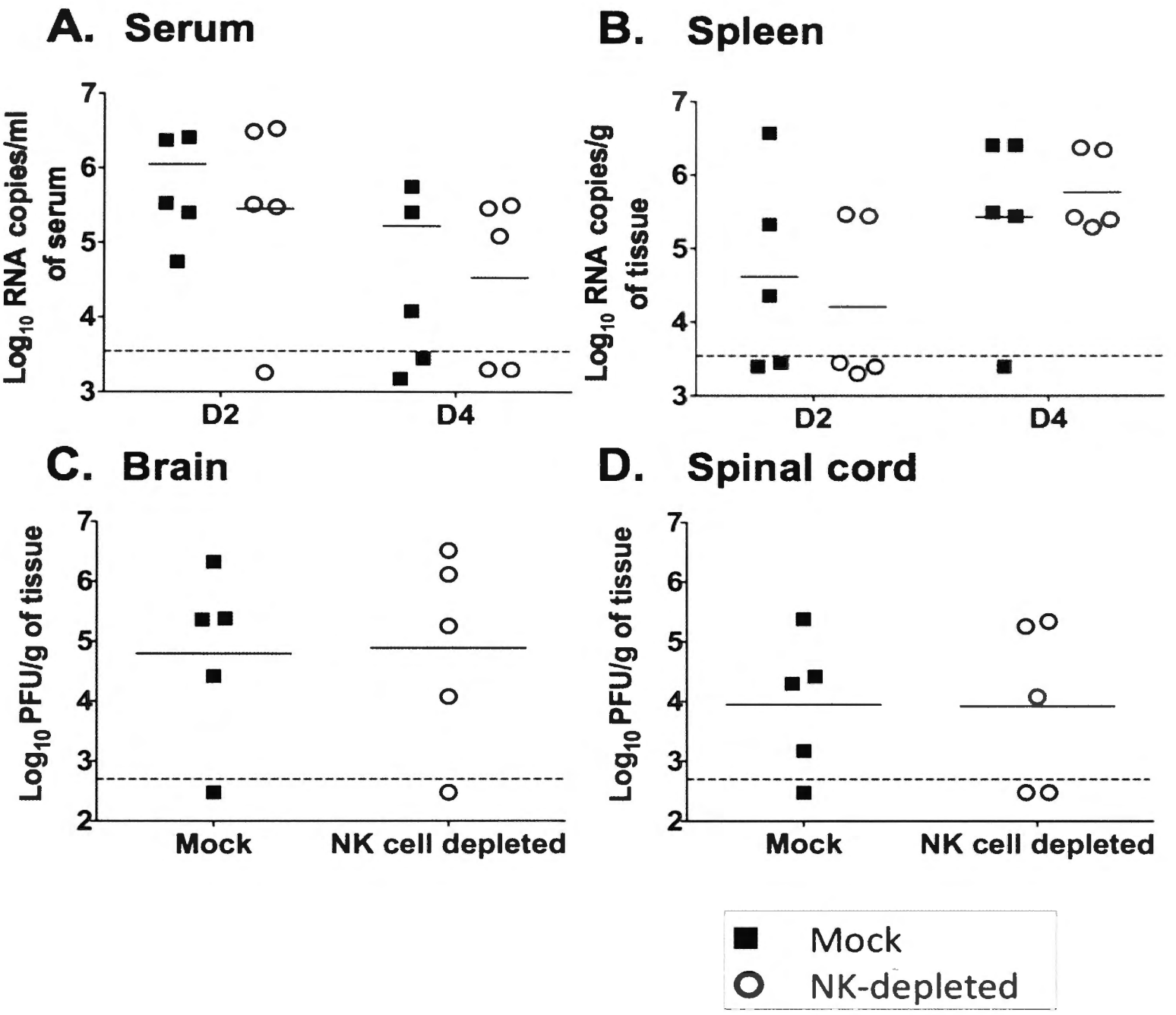


Figure 4.8 Viral burden in the absence of NK cells. JEV burden in serum (A), spleen (B), brain (C), and spinal cord (D) samples of NK cell-depleted and mock-treated B/6 wt mice after sc infection with 10^3 PFU of JEV is shown. On days 2 and 4 pi, animals were sacrificed and the viral RNA content of serum and spleen samples were measured by real-time RT-PCR, while viral content in brain and spinal cord samples on day 10 pi were measured by plaque titration. Data shown were constructed from 2 independent experiments, each symbol represents an individual mouse, and horizontal lines indicate geometric mean titers. The lower limit of virus detection is indicated by the horizontal dotted line.

4.3 Discussion

The biological significance of contact-dependent pathways of cytotoxicity in viral disease amelioration or exacerbation has become increasingly recognized over the past two decades with the availability of knockout mice with defects in key components of the pathways (reviewed in (Kaufmann *et al.*, 2012; Mullbacher *et al.*, 2003; Voskoboinik *et al.*, 2010; Wang *et al.*, 1996)). A multitude of studies using mice with single or combined deficiency in Fas, FasL, perforin, GzmA, or GzmB have revealed a lack of generalizability in terms of disease outcome between different viral models. This includes viruses that cause disease of the CNS, where one or both pathways may be absolutely required, partly beneficial, unnecessary, or detrimental to the host. For instance, an immunopathological role of the Fas-FasL and/or granule exocytosis pathways was observed in disease models of LCMV (Zajac *et al.*, 1996), MVEV (Licon Luna *et al.*, 2002), and SFV (Alsharifi *et al.*, 2006). On the other hand, one or both pathways were required for protection from Theiler's virus (Rossi *et al.*, 1998), WNV (Shrestha *et al*, 2006; Shrestha and Diamond, 2007a) and HSV-2 infection (Ishikawa *et al.*, 2009). Even viral strain differences can account for distinct disease outcomes: for instance, intact granule exocytosis plus Fas-FasL pathways of cytotoxicity were essential for recovery from infection with a virulent North-American isolate of WNV (Shrestha *et al.*, 2006*a*, Shrestha and Diamond, 2007), in contrast to a less virulent strain (WNV-Sarafend), which did not show increased disease in $Perf^{-/-}$, $Fas^{-/-}$, and FasL-deficient mice relative to wt (Wang *et al.*, 2004) Similarly, mice deficient in Fas-FasL signalling challenged with HSV-1 strain SC16 presented with defective peripheral viral clearance (Johnson et al., 2008), as opposed to Fas receptor-deficient mice challenged with HSV-1 KOS strain, which did not present with increased peripheral viral load (Pellegrini *et al.*, 2003).

Here I show that mice defective in both contact-dependent cell death pathways ($Fas^{-/-}$ x$GzmA/B^{-/-}$ mice) display increased susceptibility to extraneural challenge with a low dose of JEV. The result resembles the finding of uncontrolled lethal infection of a neurotropic strain of mouse hepatitis virus in mice lacking both functional Fas-FasL and granule exocytosis-mediated effector pathways, but not in animals with single deficiencies in either Fas or perforin (Lin *et al.*, 1997; Parra *et al*, 2000). Accordingly, both mouse models of neurotropic viral diseases showed redundancy of the two contact-dependent cytolytic mechanisms for the control of lethal viral infections of the CNS. Intriguingly, in the mouse model of Japanese encephalitis, dispensability of either Fas-FasL or granule exocytosis pathways in disease outcome measured by mortality rate (this Chapter and Chapter 3) contrasted with markedly increased viral burden in the CNS in single knockout mice defective in either Fas, perforin, or Gzms (Chapter 3). While the latter finding was explained by proposing a counter-acting balance of immunoprotection vs. immunopathology mediated by cytolytic leukocytes at the site of CNS infection, it casts doubt on an interpretation that posits solely absence of cytolytic clearance of JEV as the reason for the observed mortality rate increase in the double-deficient mice.

Other, non-cytolytic, functions of Fas and Gzms have been described that may have been beneficial in recovery from JEV, and could account for the discrepancy. Accumulating data suggest a physiological role of Fas in regulating neuronal development, growth, differentiation, and regeneration in the CNS (reviewed in (Desbarats *et al.*, 2003; Peter *et al.*, 2007; Tamm *et al.*, 2004; Zuliani *et al.*, 2006)). Accordingly, in line with proposed diverse non-apoptotic functions of Fas expression in T cell survival, proliferation and/or activation, and also liver cell regeneration, Fas may

act as a protector as well as a destroyer of cells of the CNS following infection with a pathogen or in inflammatory disorders. Fas is strongly upregulated in brains of mice infected with JEV and WNV (Gupta and Rao, 2011; Shrestha and Diamond, 2007) and disruption of this putative neuroprotective role of Fas may, at least in part, explain the increased susceptibility of $Fas^{-/-}$xGzmAxB $^{-/-}$ mice to JEV infection.

Granzymes, while residing in cytotoxic cells, are also found extracellularly, and their extracellular concentration is elevated during infection. Accordingly, it must be anticipated that the Gzms can act both intracellularly and extracellularly. Non-cytolytic activities mediated by GzmA and GzmB include diverse biological effects such as (i) stimulation of pro-inflammatory cytokines, (ii) remodeling of extracellular matrices that can result in cell death through anoikis, (iii) stimulation of type 1 IFN responses by deregulating the activity of the three-prime repair exonuclease, TREX1, and (iv) proteolytic cleavage and inactivation of viral or host factors essential for viral replication (reviewed in (Andrade, 210; Froelich *et al.*, 2009)). Regarding the latter, the direct antiviral action of GzmB is crucial in the prevention of herpes simplex virus Type 1 (HSV-1) reactivation, mechanistically through the degradation of a viral protein essential for gene expression (Knickelbein *et al.*, 2008). It is of interest that GzmB-deficient but not perforin knockout mice showed increased susceptibility to infection with WNV (strain Sarafend) (Wang *et al.*, 2004), suggesting a beneficial non-cytotoxic effect of the molecule against flavivirus infection, which was also observed in flavivirus growth experiments in human fibroblasts stably expressing GzmB (Lobigs *et al.*, 2008). The mechanism of this non-cytotoxic activity of GzmB against flaviviral infection has not been elucidated, but may involve viral or host protein cleavage by the serine protease.

Defective control of JEV infection in mice lacking functional death receptor and/or granule exocytosis pathways of cytotoxicity was reflected in reduced viral burden in brain and spinal cord at later time points pi, while viral titers in serum and spleen did not differ significantly. This suggested that the contact-dependent functions of the cellular immune response mediated predominantly by NK and T cells are not important in the control of JEV in extraneural tissues, or at early time points when relatively low viral load is detectable in serum and spleen. This observation, combined with the limited capacity of cells of the CNS to regenerate following viral or immune-mediated death, may explain the only subsidiary role of $CD8^+$ T cells in protection against Japanese encephalitis.

Other relevant immunologic factors that could account for increased susceptibility in $Fas^{-/-}$xGzmAxB $^{-/-}$ mice were excluded. First, the magnitude and functional activity of antibody responses in $Fas^{-/-}$xGzmA/B $^{-/-}$ mice remained intact, and displayed similar kinetics relative to wt. Second, NK and $CD8^+$ T cell immune responses (as measured by intracellular cytokine production) in $Fas^{-/-}$xGzmA/$B^{-/-}$ mice also displayed comparable activity relative to wt. Last, trafficking of leukocytes into the CNS remained unimpaired even with combined absence of Fas and Gzms.

The second major finding from this investigation was that NK cells were dispensable for recovery from JEV infection. While infection with JEV resulted in early activation of NK cells (measured by intracellular IFN-γ staining), depletion of NK cells did not produce increased mortality, or a difference in viral burden in serum, spleen or the CNS, relative to wt control mice. The result is consistent with the conclusion that NK cells do not provide a survival advantage in mice infected with other closely related flaviviruses belonging to the Japanese encephalitis serocomplex, viz. WNV and MVEV.

Flaviviruses are thought to have evolved an immune escape mechanism against NK cell attack involving up-regulation of MHC class I on the surface of infected cells (King and Kesson, 1988; Lobigs *et al*., 2003*a*). MHC class I can engage with NK cell inhibitory receptors and thereby down-regulate the NK cell response, which may explain why despite apparent NK cell activation, the leukocyte subpopulation did not promote a survival advantage against Japanese encephalitis.

Chapter 5

Disease-ameliorating CNS viral load reduction by Interferon-γ-producing T cells in a mouse model of Japanese encephalitis

5.1 Introduction

Interferons (IFNs) are glycoprotein cytokines important in the host defence response against diverse groups of infectious diseases (reviewed in (Macmicking, 2012; Sadler and Williams, 2008)). They are grouped into three classes based on the receptor complex through which they signal. IFN-α and IFN-β are type I IFNs, and are produced early in the course of infection. Binding of IFN-α and -β to a single (IFN-α) receptor leads to the activation of a large set of IFN-inducible genes involved in antiviral and immunoregulatory pathways. On the other hand, IFN-γ is classified as a type II IFN, and is predominantly produced by NK, NKT, and γδ T cells of the innate arm of the immune response, and $CD4^+$ Th1 and $CD8^+$ T cells of the adaptive immune response (reviewed in (Schoenborn and Wilson, 2007)). The antiviral action of IFN-γ is mediated through various mechanisms, including direct antiviral activity that overlaps with that triggered by the type I IFNs, activation and polarization of Th cell responses, enhanced major histocompatibility complex (MHC) class I antigen expression and presentation, and activation of phagocytic myeloid cells. Lastly, newly classified members of the IFN superfamily, the type III IFNs (IFN-λ1, -λ2 and -λ3), trigger similar immune responses as the type I IFNs but engage a different receptor complex, containing the molecules IFNLR1 and IL-10R2. Only limited information on the physiological role of the type III IFNs is yet available (reviewed in (Levraud *et al.*, 2007)).

Mouse models of flaviviral pathogenesis have established the critical requirement of type 1 IFN signalling in defence against disseminating infections. Mice deficient in the IFN-α receptor presented with widespread viral tropism, uncontrolled viremia, early neuroinvasion, rapid CNS viral dissemination, and complete lethality when challenged

with Japanese encephalitis virus (JEV) (Lee and Lobigs, 2002), Murray Valley encephalitis virus (MVEV) (Lobigs *et al.*, 2003*b*), and West Nile virus (Samuel and Diamond, 2005). In addition, challenge of IFN-α receptor-deficient mice with yellow fever virus (YFV) Asibi and Angola73 strains resulted in severe viscerotropic disease and complete lethality (Meier *et al.*, 2009). Although not lethal, dengue virus (DENV) infection of IFN-α receptor-deficient mice also showed rapid viral dissemination and increased viral load in comparison to type I IFN response sufficient mice (Shresta *et al.*, 2004).

In contrast, the importance of IFN-γ signalling on disease outcome after flaviviral challenge is variable. A modest increase in mortality was observed when B/6 IFN-$\gamma^{-/-}$ mice were challenged with a low dose MVEV (Lobigs *et al.*, 2003*b*), while no difference in susceptibility was observed when challenged with YFV (Liu and Chambers 2001; Meier *et al.*, 2009). Moreover, viral strain and mouse genetic background appeared to be a confounding variable influencing disease outcome: a modest increase in mortality was seen in 129 Sv IFN-$\gamma^{-/-}$ mice challenged with either DENV-1 or DENV-2 (Shresta *et al.*, 2004), however no effect was observed in an independent study where BALB/c mice deficient in IFN-γ were challenged with DENV-2 (Johnson and Roehrig, 1999); furthermore, as opposed to the inconsequential effect of an IFN-γ deficiency in challenge with WNV strains Sarafend and Kunjin (Wang Y. *et al.*, 2006), IFN-γ had an essential early antiviral role in recovery from infection with a more virulent North-American WN strain (Shrestha *et al.*, 2006*b*).

In Chapter 4, the relevance of contact-dependent mechanisms of NK and T cell-induced cytotoxicity in the pathogenesis of Japanese encephalitis was presented. Independent of these direct effector mechanisms of cell-mediated immunity, NK and T cells also exert

their protective effect by the release of the soluble factors, TNF-α and IFN-γ. In this Chapter, the role of the cytokine IFN-γ in recovery from Japanese encephalitis will be explored with the use of knockout mice deficient in production of the cytokine.

5.2 Results

5.2.1 IFN-γ is required for protection against Japanese encephalitis. Twelve-week-old IFN-$\gamma^{-/-}$ and wt control mice were challenged sc with 10^3 PFU JEV, and morbidity and mortality were observed for 28 days. Similar initial clinical signs of generalized piloerection, paresis and rigidity, invariably progressing to severe neurological signs demonstrated by postural imbalance, ataxia and generalized tonic-clonic seizures appeared in both groups. The median survival time of mice that were sacrificed when severe signs of encephalitic disease were apparent, did not differ significantly between the two groups (11.7 ± 2.3 days for IFN-$\gamma^{-/-}$ and 11.1 ± 2.1 days for wt mice. However, a deficiency in IFN-γ resulted in a significant increase in mortality rate: 86% for IFN-$\gamma^{-/-}$ versus 43% for wt mice (Fig. 5.1). This result clearly demonstrates a beneficial role of IFN-γ in the pathogenesis of Japanese encephalitis.

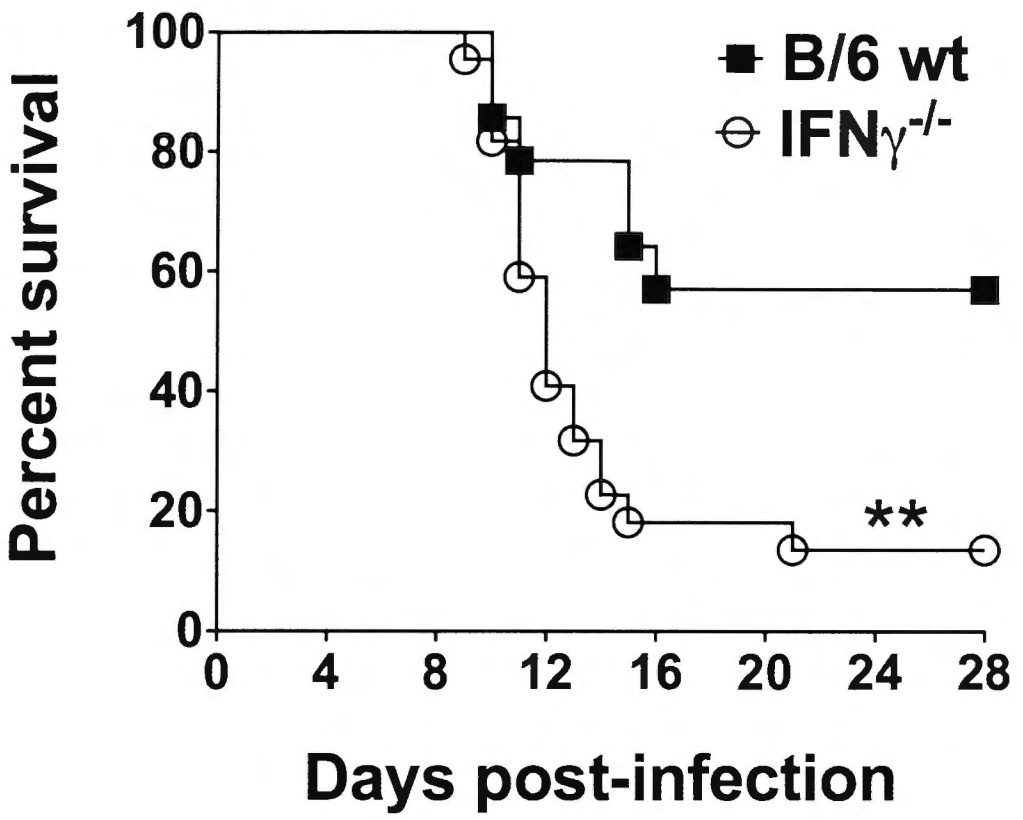


Figure 5.1 Susceptibility of IFN-$\gamma^{-/-}$ and wild-type mice to JEV infection. Groups of 12-week-old mice were infected sc with 10^3 PFU of JEV, morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data presented were constructed from two independent experiments. Mortality in groups of IFN-$\gamma^{-/-}$ mice (n = 22) was compared to that in groups of wt mice (n = 14) to test for statistical significance using the log-rank test (**, $P < 0.01$).

5.2.2 Increased viral burden in the CNS in the absence of IFN-γ. To investigate further on the mechanism of increased susceptibility to JEV infection in mice deficient in IFN-γ, viral titers in brain and spinal cord of IFN-$\gamma^{-/-}$ and wt control mice infected with 10^3 PFU of JEV, sc, were determined by plaque assay on Vero cell monolayers. A deficiency in IFN-γ resulted in increased CNS viral burden, with viral load in brains of IFN-$\gamma^{-/-}$ mice exceeding that in brains of wt mice by ~100- and ~1000-fold on days 8 and 10 pi, respectively (Fig. 5.2C). Additionally, IFN-$\gamma^{-/-}$ mice exhibited more pronounced dissemination of virus into the spinal cord, with viral titers elevated by one log and three logs on days 8 and 10 pi, respectively, relative to wt (Fig. 5.2D). Viral titers in serum and spleen were not detectable by plaque assay, and JEV RNA detection utilizing real-time RT-PCR did not show a significant difference between the groups. Peak viremia was on day 2 pi (GMT: $1.1x10^5$ and $9.4x10^4$ RNA copies/ml of serum in wt and IFN-$\gamma^{-/-}$ mice, respectively; Fig. 5.2A), and peak splenic viral burden occurred on day 4 pi (GMT: $1.2x10^5$ and $1.9x10^5$ RNA copies/g of spleen in wt and IFN-$\gamma^{-/-}$ mice, respectively; Fig. 5.2B). Together, these data indicate that IFN-γ significantly contributes to the control of virus growth in the CNS, but is not critical for clearance of virus from extraneural tissues.

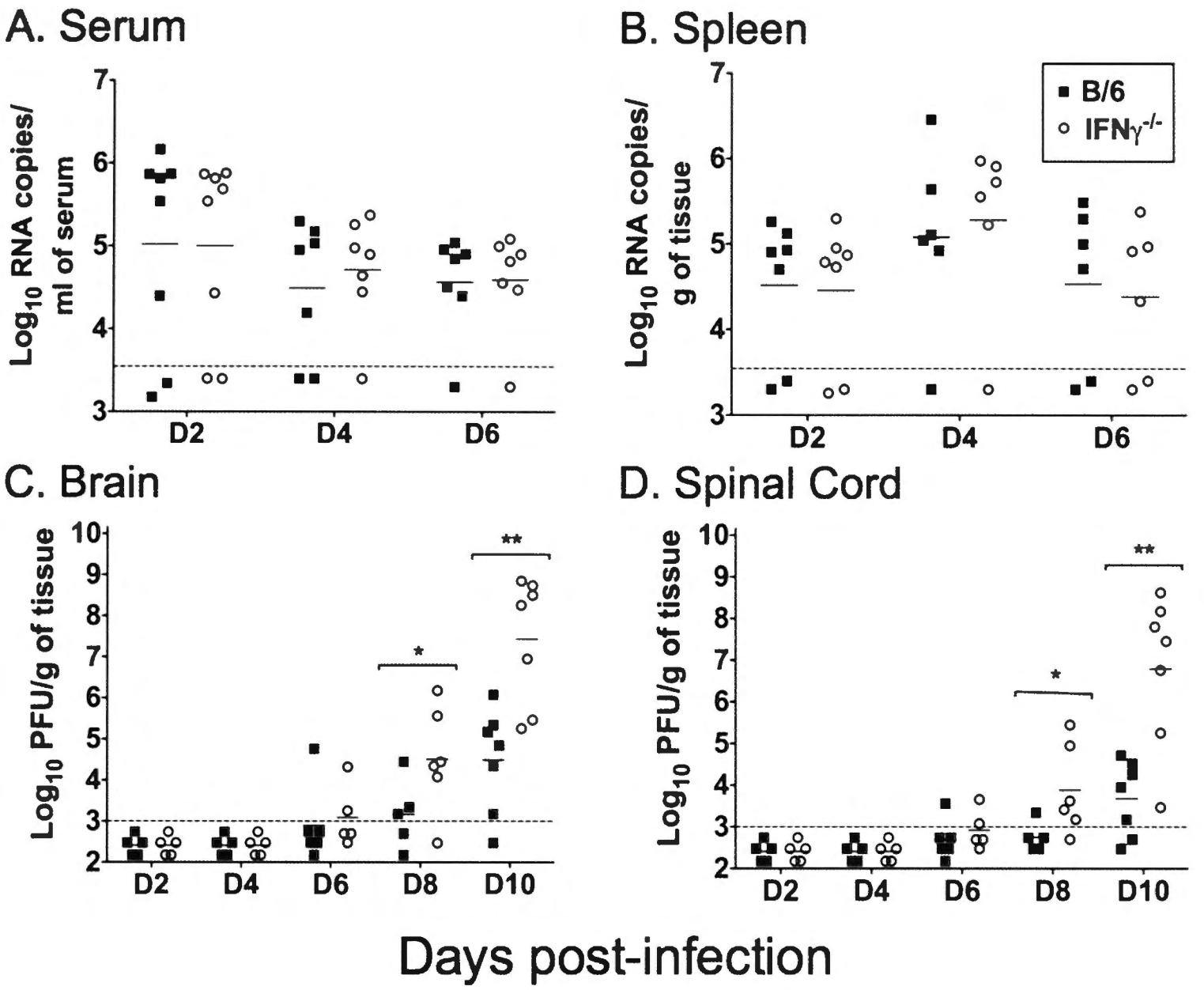


Figure 5.2 JEV burden in IFN-$\gamma^{-/-}$ and wt mice. JEV burden in serum (A), spleen (B), brain (C), and spinal cord (D) samples of IFN-$\gamma^{-/-}$ and wt mice after sc infection with 10^3 PFU of JEV is shown. At the indicated time points, animals were sacrificed, and the viral RNA content in serum and spleen samples measured by real-time RT-PCR, while viral content in brain and spinal cord samples were measured by plaque titration. Data shown were constructed from 2 independent experiments, each symbol represents an individual mouse, and geometric mean titers (GMT) are indicated by horizontal lines. The lower limit of virus detection is indicated by the horizontal dotted line. Asterisks denote significant differences (*, $P < 0.05$; **, $P < 0.01$).

5.2.3 Intact humoral immunity in the absence of IFN-γ. An optimal antibody response is an obligatory requirement for protection from lethal Japanese encephalitis (Chapter 3). Therefore, I investigated whether a deficiency in IFN-γ resulted in a poorer antibody response, and thereby causing a lethal outcome. The magnitude of anti-JEV IgM in IFN-$\gamma^{-/-}$ mice presented with similar kinetics relative to wt mice, appearing first on day 4 pi and increasing thereafter (Fig. 5.3A). Moreover, the kinetics of class switching to IgG did not differ significantly between the two groups. Anti-JEV IgG1 and IgG2b responses were similar in both groups, appearing initially on day 7 pi, and increasing on day 10 pi. (Fig. 5.3B). In both groups, the magnitude of the IgG2b response exceeded that of the IgG1 antibody isotype. The functional activity of the antibody response, as determined by an *in vitro* virus neutralization assay, also displayed similar magnitude between both groups, with mean $PRNT_{50}$ titers of 768 (range, 320 to 1280) in IFN-$\gamma^{-/-}$ mice and 652 (range, 20 to 1280) in wt mice on day 10 pi. (Fig. 5.3C). Together, these data suggest that IFN-$\gamma^{-/-}$ mice exhibit a humoral immune response after exposure to JEV that is comparable in quality and magnitude to that in wt mice.

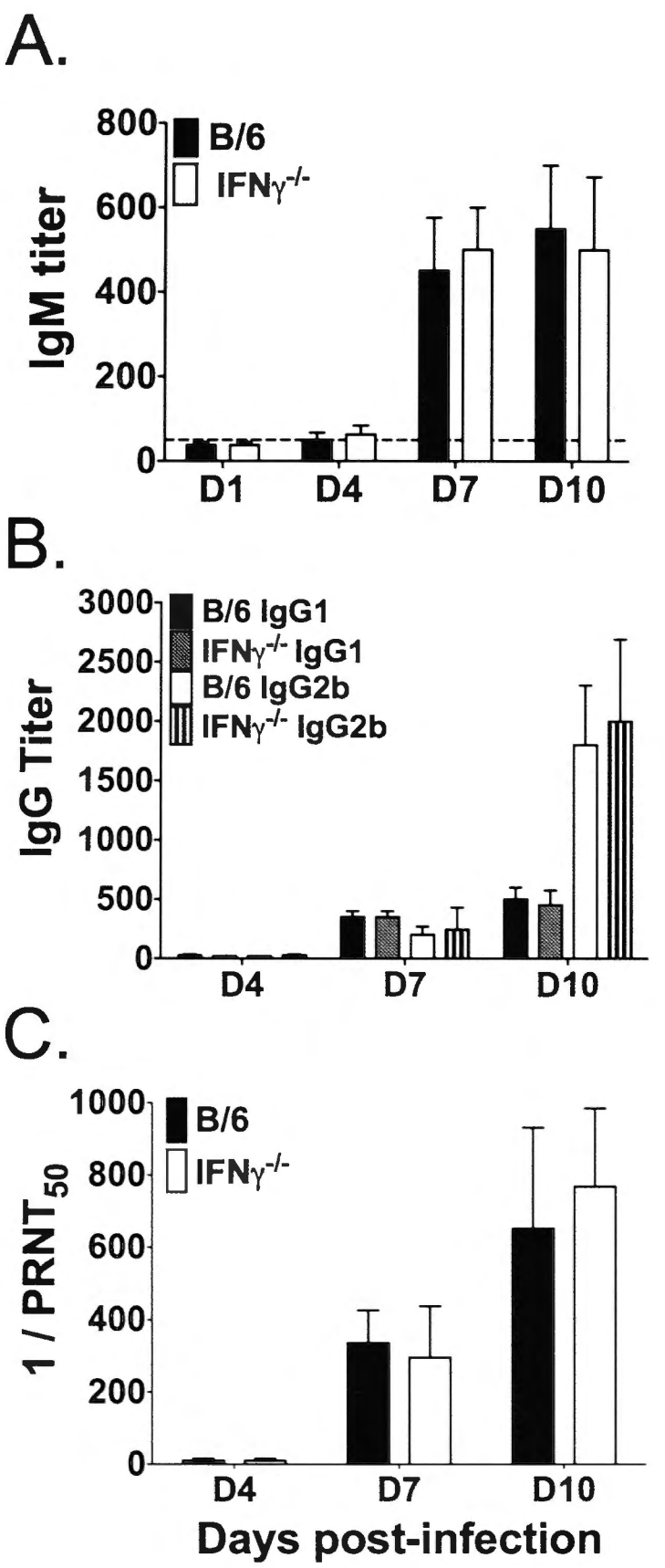


Figure 5.3 Humoral immune response in IFN-$\gamma^{-/-}$ and wt mice. Eight-week-old IFN-γ-/- and wt mice were infected sc with 10^3 PFU of JEV, and serum samples were collected at the indicated time points. Anti-JEV IgM (A) and IgG (B) isotype antibody titers were determined by ELISA. The data presented are reciprocal mean endpoint titers representative of 4 mice per time point with the SEM indicated by error bars. (C) Neutralizing antibody titers determined by plaque reduction neutralization assay. The data presented are mean $PRNT_{50}$ titers representative of 5 mice per time point with the SEM indicated by error bars.

5.2.4 Cellular Migration into the CNS. IFN-γ-mediated induction of chemokines, such as CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10, which play a role in chemotaxis of leukocytes is well documented (reviewed in (Schroder *et al.*, 2004)). To assess whether there was an impairment of trafficking of leukocytes to the site of infection in IFN-$\gamma^{-/-}$ mice, lymphocytes were isolated from brains of JEV infected IFN-$\gamma^{-/-}$ and wt mice by density gradient centrifugation, and leukocyte subpopulations quantified by flow-cytometry. On day 7 pi, IFN-$\gamma^{-/-}$ mice presented with similar numbers of NK, $CD8^+$ T, and $CD4^+$ T cells as compared to wt mice. This remained consistent on day 10 pi. Together, these data indicate that even in the absence of IFN-γ, migration of lymphocytes into the CNS after JEV infection remains intact.

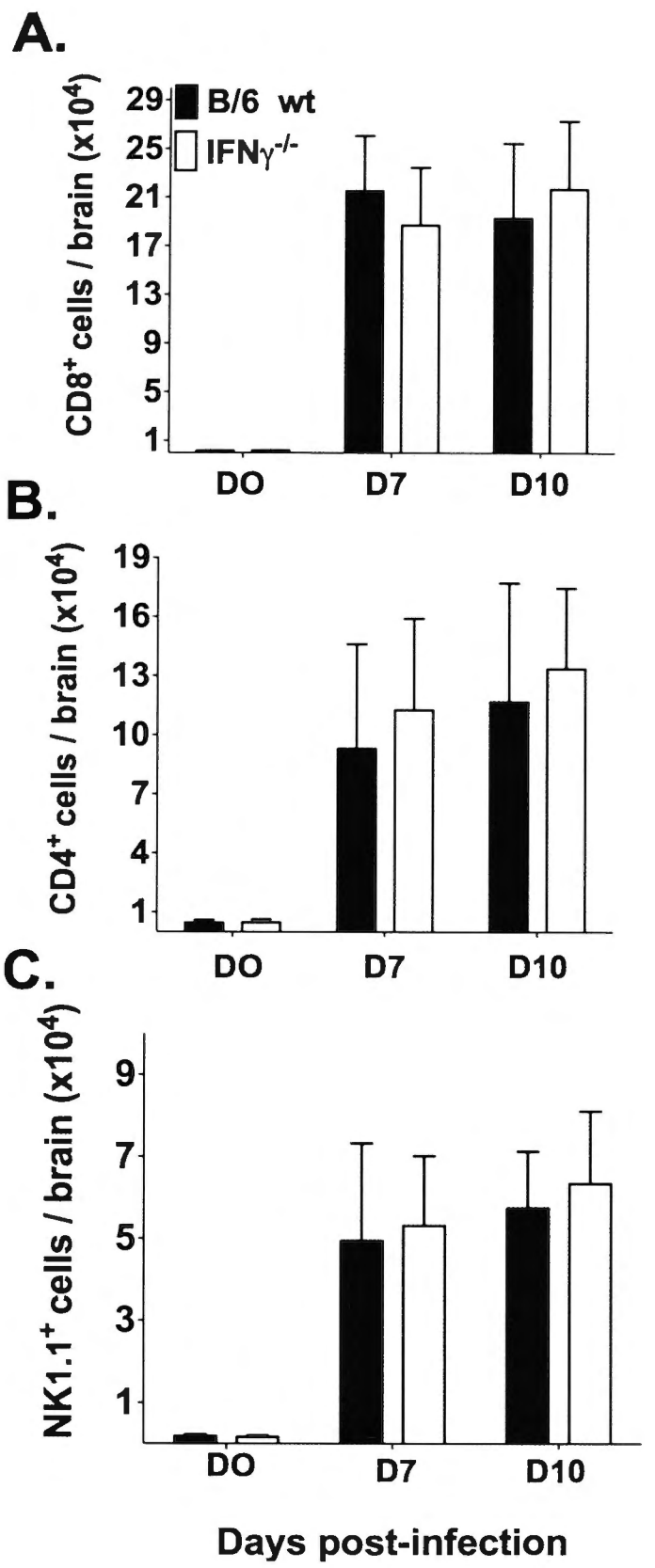


Figure 5.4 Leukocyte trafficking into the CNS. Kinetics of cell infiltration into the brain of 8-week-old IFN-$\gamma^{-/-}$ and wt mice infected sc with 10^3 PFU of JEV. Leukocytes were isolated by density gradient centrifugation from the brains of infected mice at the indicated time points, and stained for identification of $CD8^+$ T cell (A), $CD4^+$ T cell (B) and $NK1.1^+$ cell (C). Data are representative of 2 independent experiments (n = 4/group), and error bars denote the SEM.

5.2.5 IFN-γ-producing T cells are essential for viral clearance from the CNS. To confirm the protective role of IFN-γ against protection from lethal Japanese encephalitis, total splenocytes and immune T cells from JEV-infected IFN-$\gamma^{-/-}$ or wt mice were transferred to naive wt recipients that were challenged with 10^3 PFU of JEV one day after transfer (Table 5.1). Consistent with the findings in Chapter 3, total splenocyte transfer from immune wt donors completely protected recipient wt mice from lethal infection. Likewise, total splenocyte transfer from immune IFN-$\gamma^{-/-}$ mice to wt recipients resulted in a 100% survival rate. On the other hand, immune T cell transfer from wt donor mice to wt recipients conferred substantial, albeit partial, protection from lethal encephalitis relative to naive T cell transfer (mortality rate: 28% and 75%, respectively). In contrast, immune T cell transfer from IFN-$\gamma^{-/-}$ mice to wt recipients did not result in significant protection (80% and 75% mortality in immune and naïve T cell recipients, respectively).

Moreover, viral load in brain of recipients of IFN-$\gamma^{-/-}$ immune T cells was notably higher compared to that in mice that received immune T cells from wt donors (GMT: $4.8x10^5$ versus $1.2x10^3$ PFU/g of brain tissue on day 10 pi, respectively; Fig. 5.5A). Consistent with this finding, the viral load in spinal cord was also significantly higher in the former than in the latter group (GMT: $3.9x10^4$ vs. $<1x10^3$ PFU/g of spinal cord tissue, respectively; Fig. 5.5B). Taken together, these data suggest that the IFN-γ-mediated protection against lethal Japanese encephalitis is mediated, at least in part, by immune T cells, and involves partial control of virus growth in the CNS.

Table 5.1 Protective value of T cell expressed IFN-γ against lethal JEV challenge.

Treatment [a]	Mortality (No. of deaths/ total) [b]	Mean survival time (days) ± SD [c]
PBS control	67% (6/9)	12.3 ± 1.4
B/6 wt control donor mice		
Naïve wt total splenocytes (1 x 10^7 cells)	75% (15/20; *P* =0.94)	12.9 ± 1.7
Immune total splenocytes (1 x 10^7 cells)	0% (0/7; *P* =0.003)	-
Immune T cells (5 x 10^6 cells)	28% (5/18; *P* =0.01)	12.2 ± 1.3
IFN-$\gamma^{-/-}$ donor mice		
Immune total splenocytes (1 x 10^7 cells)	0% (0/5; *P* =0.01)	-
Immune T cells (5 x 10^6 cells)	80% (12/15; *P* =0.10)	12.7 ± 1.5

[a] Eight-week-old IFN-$\gamma^{-/-}$ or wt donor mice were infected with 10^3 PFU of JEV, iv, or left uninfected and sacrificed 7 days later for splenocyte collection with or without T cell purification. Cells were transferred to 8-week-old wt recipient mice that were infected one day later with 10^3 PFU of JEV into the footpad. Surviving mice were monitored for 28 days.

[b] Data are representative of 2 independent experiments. Immune splenocyte treatment groups were compared to the naive splenocyte control group to test for statistical significance.

[c] No significant difference was noted between immune splenocyte treatment and control groups.

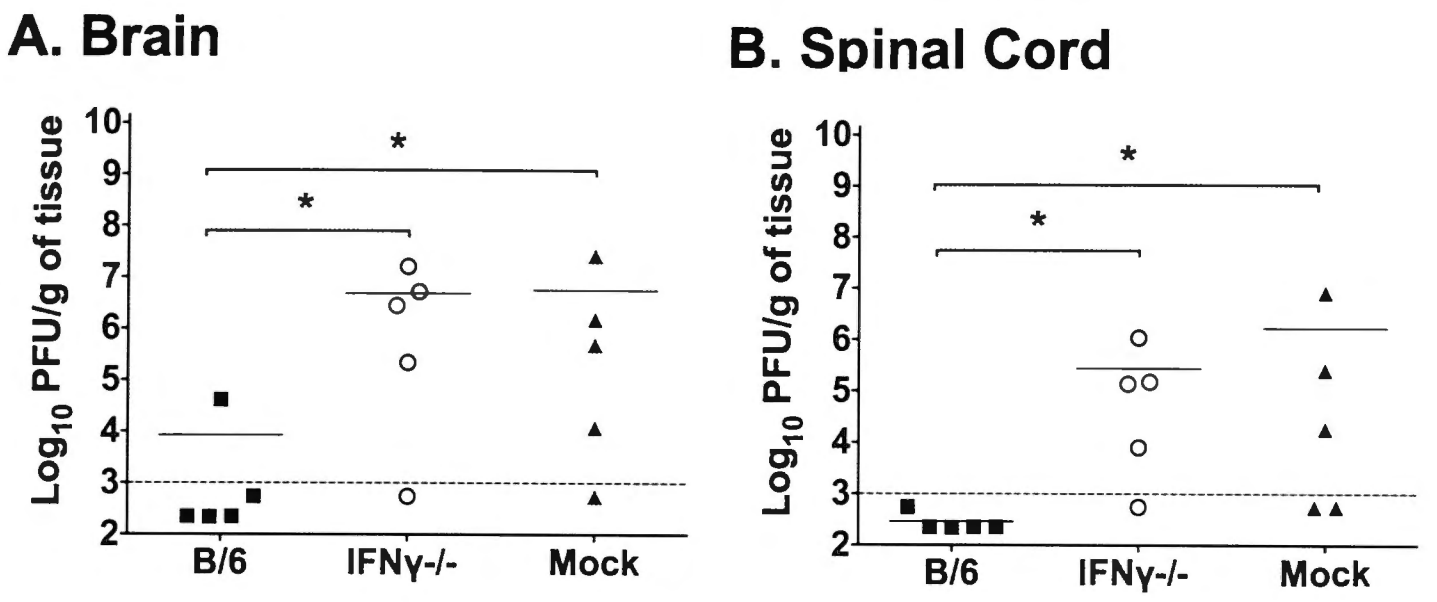


Figure 5.5 JEV burden in CNS tissue after immune splenocyte transfer.

JEV burden in brain (A), and spinal cord (B) samples in wild-type recipients of mock (naïve wt total splenocytes), IFN-$\gamma^{-/-}$, or wt immune T cells is shown. Mice were sacrificed at day 10 post-challenge with 10^3 PFU of JEV injected into the footpad, and viral content in tissues measured by plaque titration. Each symbol represents an individual mouse, and geometric mean titers are indicated by horizontal lines. The lower limit of virus detection is indicated by the horizontal dotted line. Asterisks denote significant differences (*, $P < 0.05$).

5.3 Discussion

The pleiotropic cytokine, IFN-γ, provided a substantial contribution to protection from lethal Japanese encephalitis. This was documented in the following findings: (i) low dose JEV challenge of IFN-$\gamma^{-/-}$ mice resulted in significantly increased mortality, (ii) significantly increased viral burden in brain, and (iii) more widespread dissemination of the virus into the spinal cord, relative to wt control mice; (iv) in addition, JEV-infected recipients (B/6 wt strain) of JEV immune T cells from IFN-$\gamma^{-/-}$ donor mice displayed no survival advantage relative to infected recipients of naïve T cells and a significantly higher mortality rate compared to recipients of immune T cells from IFN-γ sufficient mice, as well as (v) increased viral burden in the CNS relative to the latter. These findings are supported by an *in vitro* study that showed dose-dependent inhibition of JEV replication in IFN-γ-stimulated RAW 246.7 murine macrophages (Lin Y.L. *et al.*, 1997). They are also consistent with reports from numerous investigation on the role of IFN-γ in the control of other neurotropic viruses; for instance, Borna disease virus (BDV) (Friedl *et al.*, 2004; Hausmann *et al.* 2005), herpes simplex virus type 1 (Liu et al., 2001), measles virus (Finke *et al.*, 1995; O'Donnell *et al.*, 2012; Patterson *et al.*, 2002), mouse hepatitis virus (Bergmann *et al.*, 2003; Bergmann *et al.*, 2004; Parra *et al.*, 1999; Pearce *et al.*, 1999), lymphocytic choriomeningitis virus (Friedl *et al.*, 2004), Sindbis virus (Binder and Griffin, 2001; Burdeinick-Kerr *et al.*, 2007), and Theiler's murine encephalomyelitis virus (TMEV) (Rodriguez *et al.*, 2003) all require IFN-γ as a critical cytokine for limiting viral dissemination within the CNS.

Mechanistically, IFN-γ mediates clearance of virus (directly or indirectly) through cytolytic and non-cytolytic means (reviewed in (Chesler and Reiss, 2002; Schroder *et*

al., 2004)). Induction of cytolytic mechanisms include stimulating cell-mediated immunity by up-regulation of MHC-1 antigen processing and presentation, increasing sensitivity of target cells to apoptosis by up-regulation of Fas and TNF-α receptors, and facilitating target cell death by up-regulation of the pro-apoptotic proteins caspase-1 and IRF-1. However, given that in Japanese encephalitis the induction apoptosis in vital permanent neuronal networks would likely be counterproductive in achieving survival outcomes, the non-cytolytic mechanisms of IFN-γ action may have been of greater importance. One major non-cytolytic pathway activated by IFN-γ is through the induction of nitric oxide synthase type 1 (NOS-1) protein with consequent production of the antiviral molecule, nitric oxide (NO) (Karupiah *et al.*, 1993). As a first *in vitro* proof, Lin *et al.* (1997) have shown that the activation of the NO pathway is responsible for IFN-γ-mediated antiviral activity against JEV. The direct antiviral action of NO against JEV was corroborated in subsequent studies using various experimental approaches (Saxena *et al.*, 2000; Saxena *et al.*, 2001). Other than the NO-dependent mechanisms, IFN-γ-mediated generation of indoleamine 2,3-dioxygenase (Adams *et al.*, 2004; Mao *et al.*, 20011; Terajima and Leporati, 2005), protein kinase RNA-activated (PKR), dsRNA-specific adenosine-deaminase (ADAR), and guanylate-binding proteins (GBP) (reviewed in (Chesler and Reiss, 2002; Schroder *et al.*, 2004)) have also been implicated as antiviral proteins, although further investigations are required to confirm activity of these factors in inhibition of JEV replication.

Interestingly, other studies have shown a neuroprotective role of IFN-γ after CNS injury (Baron *et al.*, 2008; Gao *et al.*, 2000; Garg *et al.*, 2009; Imai *et al.*, 2007; Lee J. *et al.*, 2006). Glial cells and neurons express the IFN-γ receptor, and thus unsurprisingly their growth and plasticity are influenced by the IFN-γ ligand (Butovsky *et al.*, 2006; Song *et al.,* 2005; Wong *et al.*, 2004). Moreover, certain neurons express the IFN-γ ligand itself,

and when released can act in an autocrine or paracrine fashion to influence neuronal differentiation (Neumann *et al.*, 1997). Independent from its immune-mediated role in viral clearance, IFN-γ was implicated to have a neural protective effect after infection with BDV (Hausmann *et al.*, 2005) and TMEV (Rodriguez *et al.*, 2003). Given that IFN-$\gamma^{-/-}$ mice, relative to wt, are more susceptible to infection with MVEV (Lobigs *et al.*, 2003*b*) and the WNV-NY strain (Shrestha *et al.*, 2006*b*), and yet did not display viral load difference on day 8 pi, it can be speculated that IFN-γ may, in part, play an undefined neuroprotective role in flaviviral encephalitis, apart from its immune-mediated effects.

Defects in humoral immunity were not responsible for the adverse outcome observed in mice deficient in IFN-γ, since IFN-$\gamma^{-/-}$ mice displayed comparable kinetics of antibody responses and virus neutralizing titers relative to wt mice. In addition, wt recipients of total immune splenocytes, but not immune T cells, from IFN-$\gamma^{-/-}$ donor mice were completely protected from JEV challenge, thereby indicating an intact B cell response. The splenocyte transfer experiments also attributed IFN-γ-producing immune T cells as the main effector cell population responsible for the disease-ameliorating effect of IFN-γ involving the partial control of virus growth in the CNS. This protective function of IFN-γ was most likely a synergistic effect provided by both $CD8^+$ and $CD4^+$ T cell populations, since (as demonstrated in Chapter 3) only a combined transfer of both cell populations afforded protection. Here I confirmed that both cell populations efficiently trafficked into the infected brain in the absence of IFN-γ.

NK cells are also an important source of IFN-γ in viral infections, including JEV infection, which was confirmed by intracellular staining for IFN-γ in NK cells isolated from JEV infected mice (Chapter 4). It has been suggested that NK cell-expressing IFN-

γ has a contributory early role in viral clearance after DENV-2 challenge (Fagundes *et al.*, 2011). However, in the case of JEV infection, IFN-γ-producing NK cells most likely did not play a crucial role in recovery, since *in vivo* depletion of NK cells in JEV infected mice did not reduce survival rate nor impact on viral burden in CNS (Chapter 4). Finally, activated γδ T cells of the innate immune response are thought to be the main IFN-γ-producing cells responsible for early viral clearance and protection from lethal West Nile encephalitis (Shrestha et al., 2006*b*; Wang *et al.*, 2003). This is, however, unlikely the case in the mouse model of Japanese encephalitis, given that IFN-$\gamma^{-/-}$ mice did not display increased early viremia or peripheral viral load, nor shorter average survival times, relative to wt mice. Nevertheless, further research is required to confirm or discount a contribution of γδ T cells in the control of JEV infection.

Chapter 6

The Chemokine Receptor CCR5, a Therapeutic Target for HIV/AIDS Antagonists, is Critical for Recovery in a Mouse Model of Japanese Encephalitis

The findings in this Chapter has been accepted for publication:

Larena, M., Regner, M., & Lobigs, M. (2012). The chemokine receptor CCR5, a therapeutic target for HIV/AIDS antagonists, is critical for recovery in a mouse model of Japanese encephalitis. PLoS One.

6.1 Introduction

The migration of leukocytes in lymphoid organs and to sites of inflammation is coordinated by an array of chemokines that bind to specific receptors on immune cells (reviewed in reference (Allen *et al.*, 2007)). Of these, the chemokine receptor, CCR5, is expressed on natural killer (NK) cells, macrophages, and $CD4^+$ and $CD8^+$ T cells. In these cell types, it regulates chemotaxis and cell activation through interaction with the chemokine ligands CCL3, CCL4 and CCL5, which are up-regulated at the site of infection (reviewed in reference (Sorce *et al.*, 2011)). Understanding the role of CCR5 in the control of pathogen infections has important implications for human health beyond that of other chemokine ligand/receptor interactions, in view of the discovery that CCR5 is a major co-receptor for HIV-1 (reviewed in reference (Alkhatib, 2009)). Therefore, the chemokine receptor is an important target for therapeutic intervention against HIV/AIDS, and recent clinical trials investigating the efficacy of CCR5 antagonists in patients with HIV/AIDS have provided promising results (reviewed in reference (Wilkin and Gulick, 2012)). However, it has been argued that if CCR5 had a protective role against another group of pathogens, for instance in flaviviral encephalitis, it follows that a therapeutic treatment, which aims to block the receptor, could exacerbate the diseases caused by these pathogens (Lim *et al.*, 2006; Klein, 2008).

CCR5 was the first chemokine receptor recognized to play a critical role in recovery from flavivirus encephalitis in a study that showed that absence of CCR5 prevented efficient leukocyte trafficking to the brain and viral clearance in mice infected with West Nile virus (WNV) (Glass *et al.*, 2005). The important role of CCR5 in the human host response against West Nile encephalitis was supported by a retrospective cohort

study involving persons homozygous for CCR5Δ32 (Glass *et al.*, 2006), a loss-of-function mutation found in 1-2% of Caucasians (Sorce *et al.*, 2011). Compared to individuals without the mutation, persons carrying a homozygous CCR5Δ32 allele had an increased risk for symptomatic WNV infection. This finding was corroborated with a large-scale database study, which associated homozygosity for CCR5Δ32 with an increased risk of early and late clinical manifestation following WNV infection (Lim *et al.*, 2010).

CCR5Δ32 homozygosity has also been associated with severe tick-borne encephalitis symptoms (Kindberg *et al.*, 2008) caused by infection with tick-borne encephalitis virus, and a severe case of yellow fever virus-associated viscerotropic disease (Pulendran *et al.*, 2008). Tick-borne encephalitis and yellow fever viruses are also members of the *Flavivirus* genus, raising the question of generality of CCR5 as an important host factor in recovery from flaviviral infections. Confirmation of a broader link between CCR5 deficiency and augmented incidence and severity of flaviviral disease would add to the concern of potential adverse outcomes associated with CCR5 antagonist use, in view of the large number of human infections inflicted by the different pathogenic members of the *Flavivirus* genus, and their widespread global distribution (reviewed in reference (Gould and Solomon, 2008)).

In this chapter, I investigated the role and mechanism of CCR5 in recovery from infection in a mouse model of Japanese encephalitis. Japanese encephalitis virus (JEV) is closely related to WNV, and in terms of human disease incidence and severity the most important member of a serocomplex of mosquito-borne, encephalitic flaviviruses (reviewed in reference (Solomon, 2004)). It is the leading cause of viral encephalitis in Asia, annually accounting for 30,000 to 50,000 cases and ~10,000 deaths.

Approximately 3 billion people in the Asia-Pacific region are at risk of infection with JEV. Host immune factors are thought to be the dominant determinants of disease outcome in Japanese encephalitis (reviewed in (Halstead and Jacobson, 2003)), with intact type I interferon (Lee and Lobigs, 2002) and vigorous humoral immune responses (Chapter 3) essential for recovery, and $CD8^+$ T cell immunity providing a subsidiary contribution to controlling JEV infection. With the use of CCR5-deficient mice I present in this chapter that the chemokine receptor is an additional important host factor involved in reducing disease severity with Japanese encephalitis.

6.2 Results

6.2.1 CCR5 is required for protection from Japanese encephalitis. To assess the impact of CCR5 on the pathogenesis of Japanese encephalitis, mortality was recorded in 8-week-old congenic $CCR5^{-/-}$ and $CCR5^{+/+}$ wild-type mice after i.v. challenge with 10^3 PFU of JEV. Moribund mice in both groups presented with similar clinical signs starting with generalized piloerection, paresis and rigidity, invariably progressing to severe neurological signs demonstrated by postural imbalance, ataxia and generalized tonic-clonic seizures. Median survival time amongst mice that succumbed to infection did not differ between the two groups (11.7 ± 2.3 days for $CCR5^{-/-}$ and 11.1 ± 2.1 days for $CCR5^{+/+}$ mice). Nevertheless, absence of CCR5 resulted in a significant increase in mortality: 64% in $CCR5^{-/-}$ versus 28% in $CCR5^{+/+}$ mice (Fig. 6.1).

To address the mechanism for increased susceptibility of mice to JEV infection in the absence of CCR5, viral titers in $CCR5^{-/-}$ and control mice infected with 10^3 PFU, i.v., were determined by real-time RT-PCR in serum and spleen, and by plaque assay in brain and spinal cord. Both groups displayed similar viral burden in serum from day 2 to day 6 pi, with peak viremia occurring on day 2 pi (Fig. 6.2A). Similarly, viral load in spleen did not differ significantly between the two groups (Fig. 6.2B). On the other hand, viral spread into and/or clearance from the CNS was significantly affected by the absence of CCR5: compared to control mice, viral load in brains of $CCR5^{-/-}$ mice was between 10- and 10,000-fold higher on days 8 and 10 pi, respectively, with the proportion of mice showing detectable virus titers markedly greater in $CCR5^{-/-}$ mice than that in the control group (Fig. 6.2C). In addition, viral spread in the CNS was more pronounced in $CCR5^{-/-}$ mice, with viral titers in spinal cord exceeding those in $CCR5^{+/+}$ mice by four log on day 10 pi (Fig. 6.2D).

Together, these data suggest that CCR5 is critically required for recovery from JEV infection.

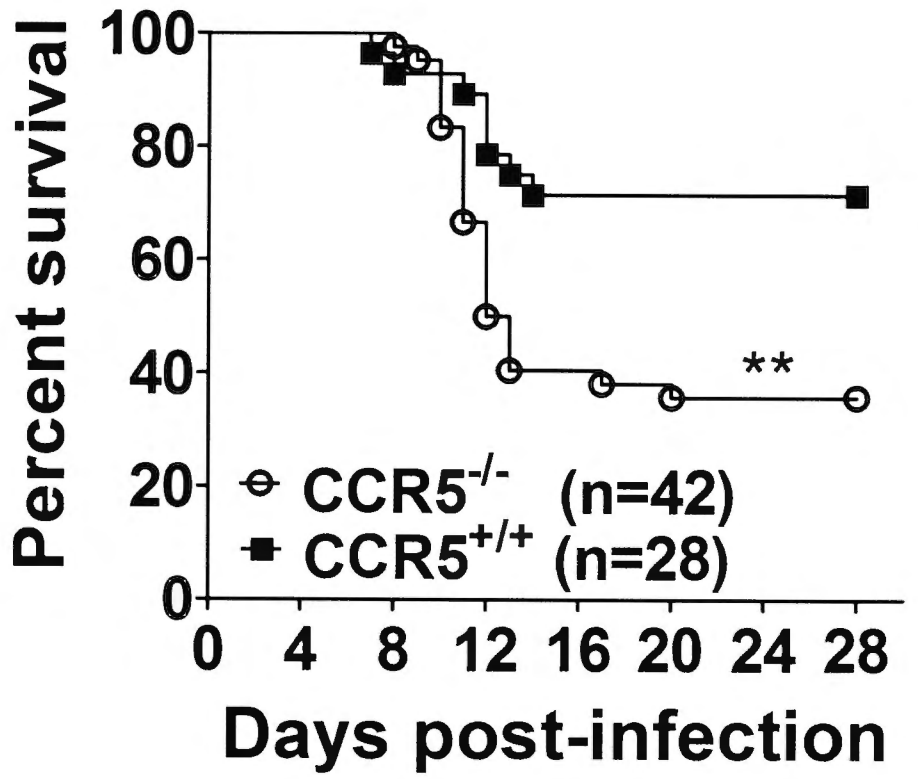


Figure 6.1 Susceptibility of CCR5$^{+/+}$ and CCR5$^{-/-}$ mice to infection with JEV. Groups of 8-week-old mice were infected iv with 10^3 PFU of JEV. Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from two independent experiments. The significance of differences in mortality between wild-type (n=28) and knockout mice (n=42) was determined by using the log-rank test (**, $P < 0.01$).

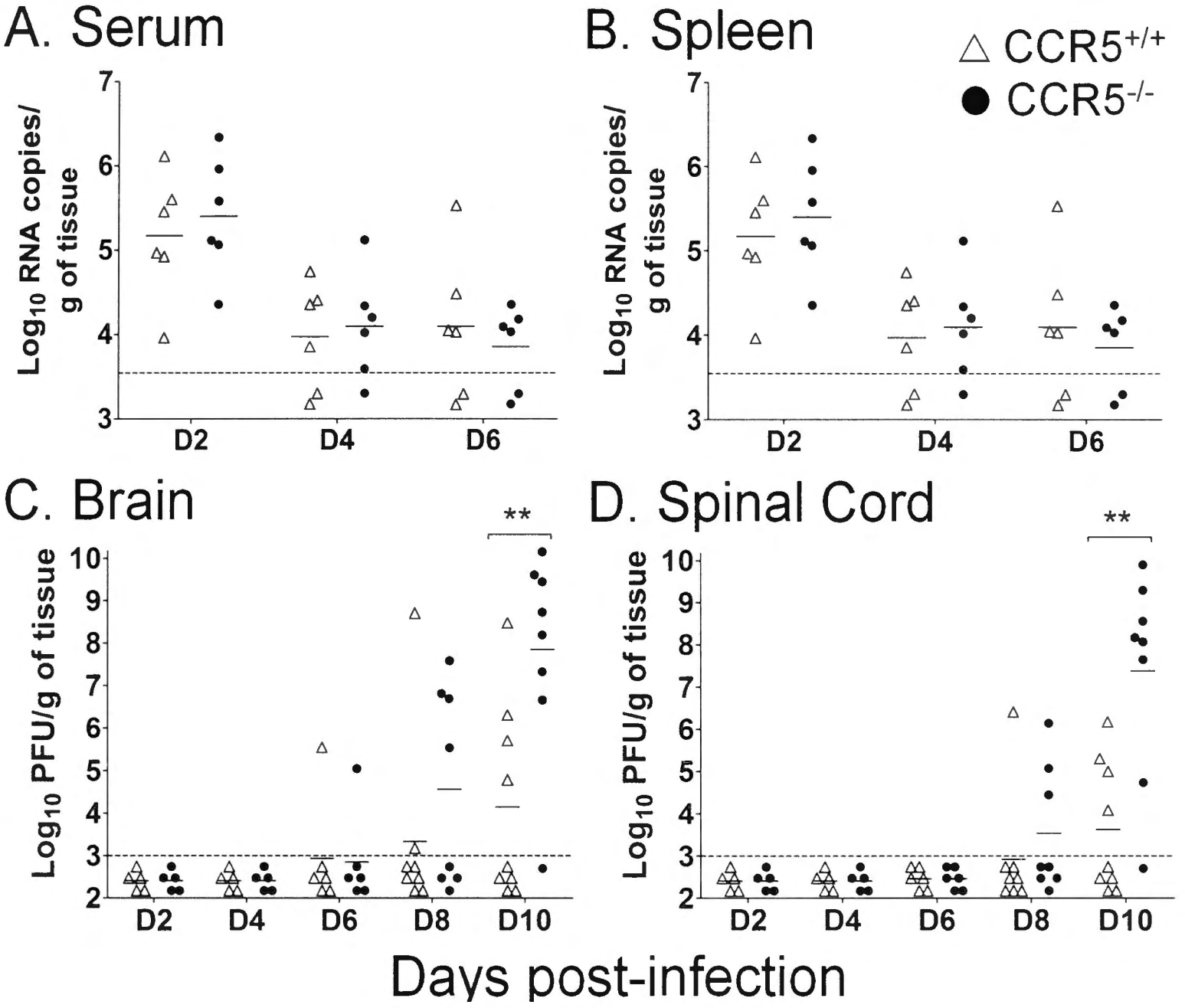


Figure 6.2 JEV burden in serum and tissue samples. JEV burden in (A) serum, (B) spleen, (C) brain and (D) spinal cord of $CCR5^{+/+}$ and $CCR5^{-/-}$ mice after iv infection with 10^3 PFU of JEV. At the indicated time points, animals were sacrificed and the viral RNA content of serum and spleen samples were measured by real-time RT-PCR, while viral content in brain and spinal cord samples were measured by plaque titration. Data shown were constructed from 2 independent experiments. Each symbol represents an individual mouse, and geometric mean titers are indicated by horizontal lines. The lower limit of virus detection is indicated by the horizontal dotted line. Asterisks denote significant differences (**, $P < 0.01$).

6.2.2 Humoral immunity in the absence of CCR5. In chapter 3, I have established a pivotal role of antibody in recovery from JEV infection. Given that CCR5 is expressed on $CD4^+$ T cells, which are required for efficient generation and maintenance of humoral immunity against JEV, I investigated whether a deficiency in CCR5 would result in poorer JEV-specific antibody responses. However, the kinetics and magnitude of the IgM response was similar in $CCR5^{-/-}$ and control mice, appearing first on day 4 pi, and peaking on day 8 pi (Fig. 6.3A). Class switching to IgG production was also comparable between both groups, initially detected on day 8 pi, and increasing on day 10 pi (Fig. 6.3B). Assessment of functional activity of the antibody responses by neutralization assay revealed no significant difference between the two groups, reaching mean $PRNT_{50}$ titers of 660 (range, 80 to 1280) in $CCR5^{-/-}$ mice and 570 (range, 160 to 1280) in $CCR5^{+/+}$ mice on day 10 pi (Fig. 6.3C).

These data indicate that a deficiency in CCR5 does not compromise the ability of mice to prime JEV-specific B cell immune responses.

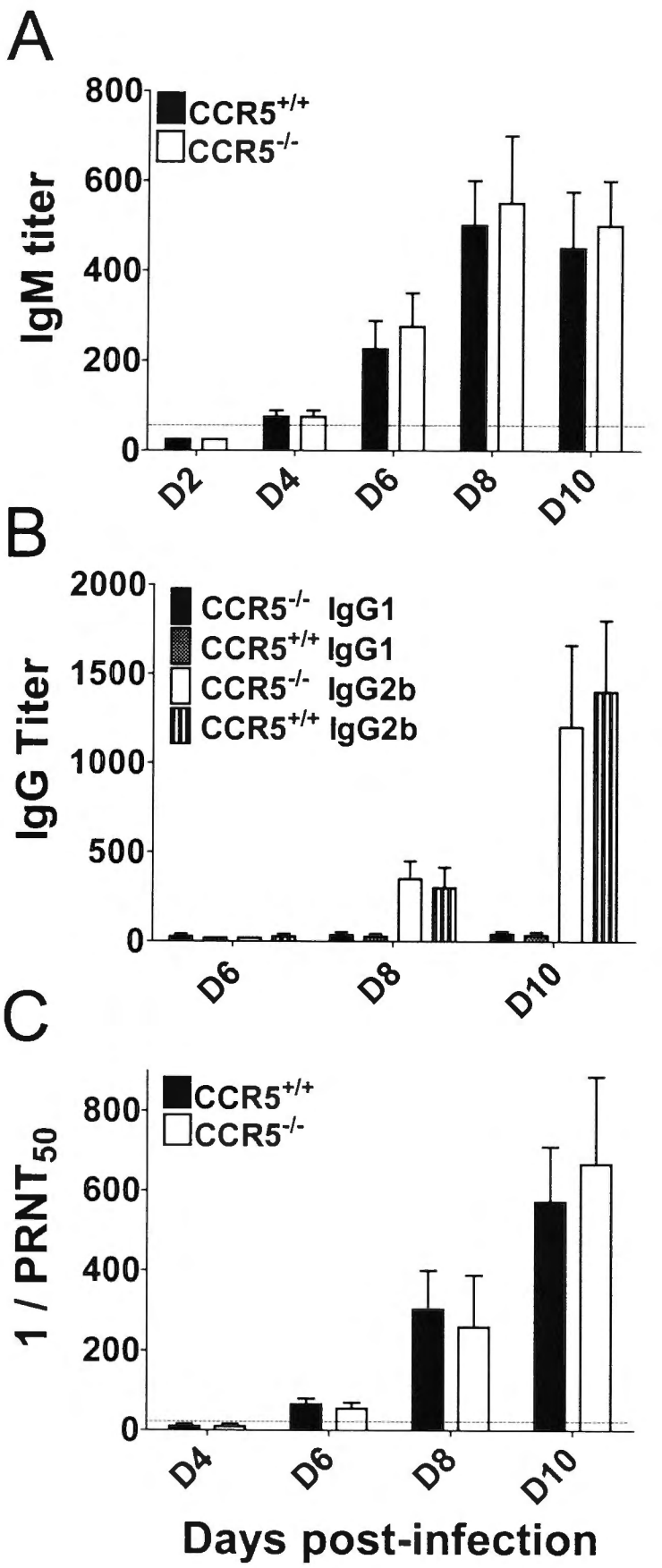


Figure 6.3 Antibody responses in $CCR5^{+/+}$ and $CCR5^{-/-}$ mice. Eight-week-old $CCR5^{+/+}$ and $CCR5^{-/-}$ mice were infected iv with 10^3 PFU of JEV, and serum samples were collected at the indicated time points. Anti-JEV IgM (A) and IgG (B) isotype antibody titers were determined by ELISA. The data presented are reciprocal mean endpoint titers representative of 4 mice per time point with the SEM indicated by error bars. (C) Neutralizing antibody titers determined by plaque reduction neutralization assay. The data presented are mean $PRNT_{50}$ titers representative of 5 mice per time point, and error bars indicate the SEM.

6.2.3 Blunted NK and $CD8^+$ T cell responses in $CCR5^{-/-}$ mice. NK cells form an important part of the cellular arm of the host's innate immunity, and function by killing virally infected cells and by release of the cytokines, IFN-γ and TNF-α. NK cell activity in JEV infected $CCR5^{-/-}$ and control mice were determined by measuring cytolytic activity against YAC-1 cells and by intracellular staining of IFN-γ. At the peak of NK cell activity (day 4 pi), lysis of YAC-1 cells was significantly reduced in $CCR5^{-/-}$ mice compared to $CCR5^{+/+}$ mice (Fig. 6.4A,B). This was substantiated by a significantly reduced number of activated, IFN-γ-expressing, NK cells in spleen of $CCR5^{-/-}$ relative to control mice at day 4 pi based on the expression of IFN-γ (Fig. 6.4C).

$CD8^+$ T cells form the dominant cytotoxic arm of the adaptive immune system. I chapter 3, I have presented a subsidiary role of $CD8^+$ T cells in recovery from JEV infection. It is hypothesized that a defective $CD8^+$ T cell response could, at least in part, account for the increase in susceptibility of $CCR5^{-/-}$ mice to JEV infection. The primary murine $CD8^+$ T cell response against JEV and other flaviviruses peaks at 7 days pi, and thereafter markedly decline (Chapter 3; Kesson *et al.*, 1987; Purtha *et al.*, 2007). Figure 6.4D demonstrates a significantly blunted JEV-immune $CD8^+$ T cell response in $CCR5^{-/-}$ relative to control mice, determined by intracellular cytokine staining following *ex vivo* stimulation of $CD8^+$ lymphocytes with a JEV NS4B protein-derived H-$2D^b$-binding peptide. Relative to $CCR5^{+/+}$ mice, $CCR5^{-/-}$ mice showed a 61% and 74% reduction in the number of IFN-γ-secreting $CD8^+$ T cells, and a 60% and 64% reduction in the number of TNF-α-secreting $CD8^+$ T cells in spleen on days 4 and 7 pi with JEV, respectively (Fig. 6.4E). This was also reflected in a significantly smaller number of multifunctional JEV-immune $CD8^+$ T cells (defined as the number of $CD8^+$ T cells expressing both IFN-γ and TNF-α following stimulation with cognate peptide), which

was reduced by 3-fold and 4-fold on days 4 and 7 pi, respectively, in the absence of CCR5 expression (Fig. 6.4F).

Collectively, these data indicate that a deficiency in CCR5 results in blunted NK and $CD8^+$ T cell responses after JEV infection.

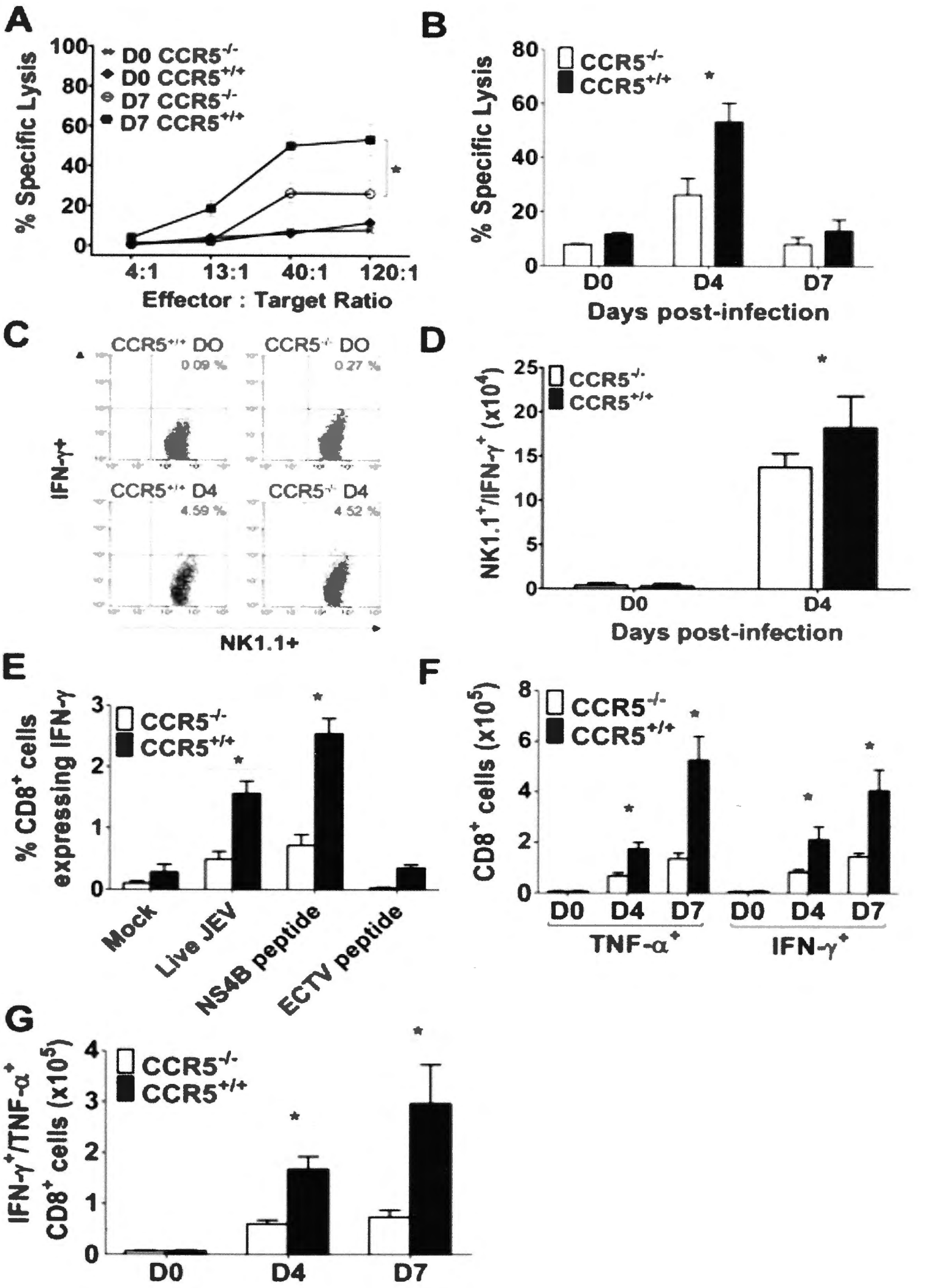


Figure 6.4 NK and CD8$^+$ T cell responses in CCR5$^{+/+}$ and CCR5$^{-/-}$ mice infected with JEV. Eight-week-old CCR5$^{+/+}$ and CCR5$^{-/-}$ mice were infected iv with 10^3 PFU of JEV or left uninfected. (A) Spleens were collected at day 4 pi and tested for NK cell cytotoxicity in a standard ^{51}Cr release assay using uninfected YAC-1 cells as targets. The percentage of NK cell lysis is plotted against an increasing e/t cell ratio. Means for 5 samples ± SEM are presented, and data are representative of 2 independent experiments. (B) NK cell cytotoxicity at indicated time points with an e/t cell ratio of

120:1. Means for 5 samples ± the SEM are presented, and data are representative of 2 independent experiments. (C) Numbers of $NK1.1^+$ stained splenocytes expressing IFN-γ were identified by flow cytometry at day 0 and 4 pi Means for 5 samples ± the SEM are presented, and data are representative of 2 independent experiments. (D) Splenocytes harvested at day 7 pi and stimulated *ex vivo* with live JEV, H-$2D^b$-restricted JEV NS4B protein-derived peptides, H-$2D^b$-restricted ECTV negative-control peptide or mock treated, and IFN-γ production in $CD8^+$ T cells was measured by flow cytometry. Means for 3 samples ± the SEM are presented, and data are representative of 2 independent experiments. (E) Kinetics of JEV immune $CD8^+$ T cell activation measured after *ex vivo* stimulation with D^b-restricted JEV NS4B peptide, showing percentage of $CD8^+$ T cells per spleen that express IFN-γ, TNF-α, or (F) both cytokines following stimulation. (G-I) Representative dot plots of cytokine production in NK and $CD8^+$ T cells at indicated time points. Asterisks denote significant differences (*, $P < 0.05$).

6.2.4 Impaired leukocyte proliferation and trafficking in CCR5$^{-/-}$ mice.

CCR5 expression drives the migration of leukocytes into the CNS after WNV infection, and thereby contributes to viral clearance and recovery from infection (Glass *et al.*, 2005). To investigate whether this is also evident in this mouse model of Japanese encephalitis, I isolated leukocytes from brains of JEV infected CCR5$^{+/+}$ and CCR5$^{-/-}$ mice by density gradient centrifugation and quantified leukocyte subpopulations by flow-cytometry. On day 7 pi, CCR5$^{-/-}$ mice showed a significant (~50%) reduction in infiltration of leukocytes into the brain relative to control mice, and this effect was found for all CCR5-expressing subpopulations, viz. NK cells, F480^{+}/CD45^{hi} macrophages, CD8^{+} T cells and CD4^{+} T cells (Fig. 6.5A-D). This difference ceased to be significant by day 10 pi. These data show that in the case of Japanese encephalitis, the absence of CCR5 resulted in delayed trafficking of leukocytes into the brain, despite the much higher virus titers in the CNS of infected CCR5$^{-/-}$ than CCR5$^{+/+}$ mice.

Given the role of CCR5 expression in augmented leukocyte trafficking into the brain, I then assessed the proportion of cells of the different leukocyte subpopulations that express the chemokine receptor using CCR5$^{+/+}$ mice that were infected with JEV. On day 7 pi, 15% of NK cells, 12% of F4/80^{+}/CD45^{hi} infiltrating macrophages, 8% of CD8^{+} T cells and 20% of CD4^{+} T cells were CCR5-positive (Fig. 6.5E). This proportion increased on day 10 pi in the case of CD8^{+} T cells (19%) and F4/80^{+}/CD45^{hi} infiltrating macrophages (16%), but decreased for NK and CD4^{+} T cells. Similar numbers of infiltrating CCR5-positive cells as a proportion of the different leukocyte subpopulations in the brain have been observed in mice with West Nile encephalitis (Glass *et al.*, 2005)

To assess whether impaired trafficking of leukocytes, *per se*, or a failure of leukocyte expansion in the periphery accounted for the reduced numbers of immune cells in the brain of infected $CCR5^{-/-}$ relative to control mice, splenic cellularity in $CCR5^{-/-}$ and $CCR5^{+/+}$ mice were quantified at various time points after JEV infection. As expected, given that spleen serves as the main immune-responsive lymphoid organ after an iv challenge, total splenocyte numbers in $CCR5^{+/+}$ mice increased following infection with JEV, with the peak expansion on day 7 pi (8×10^7 cells/spleen; Fig. 6.6A). This contrasted with a significantly lower number of splenocytes in JEV infected mice lacking CCR5 expression, although the kinetics of the response was similar between the two groups of mice (Fig. 6.6A). The deficit in cell numbers was found across the different CCR5-encoding splenocyte subpopulations, showing a reduction of 43% for NK cells, 33% for $F4/80^+$ macrophages, 34% for $CD4^+$ T cells, and 39% for $CD8^+$ T cells in $CCR5^{-/-}$ mice relative to control animals on day 7 pi (Figure 6.6B-E). The percentage reduction of T cell numbers and activation phenotype (Fig. 6.4) in spleen on day 7 pi correlated closely with the differential leukocyte migration into the brains of $CCR5^{-/-}$ and wt mice (Fig. 6.5).

Together these data imply that the impaired trafficking of leukocytes into the CNS after JEV infection in $CCR5^{-/-}$ mice may, at least in part, be a direct result of a deficit in leukocyte numbers in the periphery.

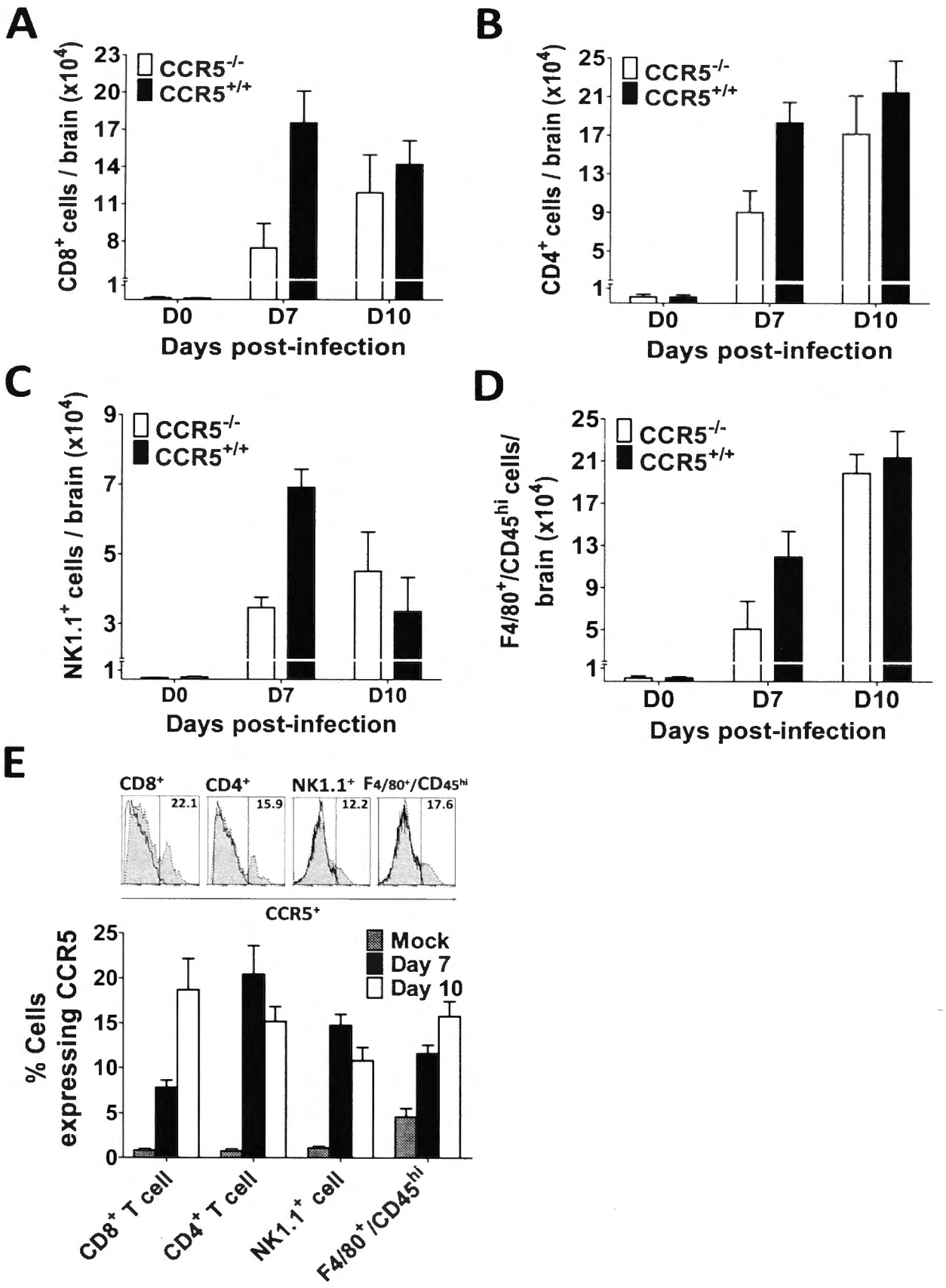


Figure 6.5 Leukocyte trafficking into the CNS. Kinetics of cell infiltration into the brain of 8-week-old $CCR5^{+/+}$ and $CCR5^{-/-}$ mice infected iv with 10^3 PFU of JEV. Leukocytes were isolated at indicated time points from the brains of infected mice and stained for identification as (A) $CD8^+$ T cell, (B) $CD4^+$ T cell, (C) $NK1.1^+$ cell and (D) $F4/80^+/CD45^{Hi}$ infiltrating macrophages. Leukocyte subpopulations were gated for CCR5 expression (E) with representative histograms on day 10 pi (F). Means are derived from 4-9 samples per time point, error bars denote the SEM, and data are representative of 2 independent experiments. Asterisks denote significant differences (*, $P < 0.05$; **, $P < 0.01$).

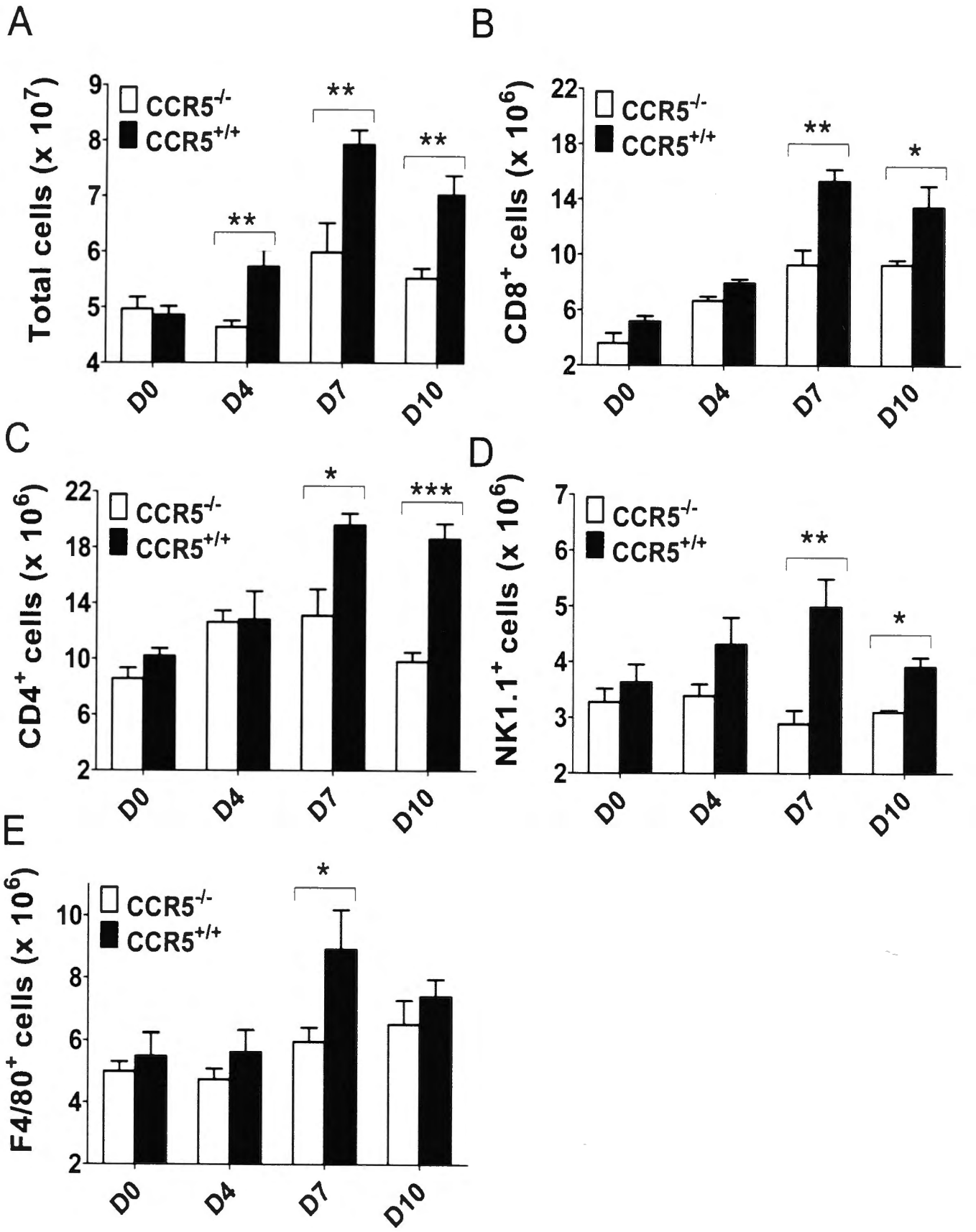


Figure 6.6 Leukocyte numbers in spleen after JEV infection. Kinetics of cell expansion in spleens of 8-week-old $CCR5^{+/+}$ and $CCR5^{-/-}$ mice infected iv with 10^3 PFU of JEV. (A) Spleens were collected at indicated time points from infected mice and total splenocyte numbers were determined. Cells were then stained and identified as $CD8^+$ T cell (B), $CD4^+$ T cell (C), $NK1.1^+$ cell (D) and $F4/80^+$ macrophages (E). Means are derived from 4-8 samples per time point, error bars denote the SEM, and data are representative of 2 independent experiments. Asterisks denote significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

6.2.5 Early transfer of CCR5-deficient splenocytes increases survival of recipient CCR5$^{-/-}$ mice challenged with JEV. The mechanistic studies on the role of CCR5 in recovery from JEV infection showed deficiencies in leukocyte trafficking to the CNS, blunted NK and CD8^{+} T cell responses, and low splenic cellularity, while a previous investigation on WNV concluded that CCR5 increased host survival predominantly by promoting leukocyte migration to the infected brain (Glass *et al.*, 2005). To test whether defective immune cell priming and expansion significantly contributed to the increased susceptibility of CCR5$^{-/-}$ mice to JEV infection, I adoptively transferred splenocytes from JEV infected CCR5$^{-/-}$ and CCR5$^{+/+}$ mice to CCR5$^{-/-}$ recipients lethally challenged with the virus. Complete protection was achieved when total immune splenocytes from either donor strain were transferred at day 1 pi with JEV (Table 6.1). In contrast, no increase in survival was observed when the transfers were performed at 3 days pi, independent of whether the donor mice were CCR5 sufficient or deficient. This suggested that following the establishment of a CNS infection, viral clearance and protection mediated by the adaptive immune responses is ineffective in the case of Japanese encephalitis. This conclusion is not limited to the CCR5-deficient recipient mice, since a similar lack of protective value against JEV of late transfer (day 3 post-challenge) of immune spleen cells was also found in wild-type B6 recipients (unpublished data).

Given the dominant role of B cells (which do not express CCR5) in recovery from Japanese encephalitis (Chapter 3), I then performed adoptive transfer experiments of B cell-depleted splenocytes from CCR5$^{-/-}$ and CCR5$^{+/+}$ donor mice to CCR5$^{-/-}$ recipients at one day after JEV infection. Interestingly, even B cell-depleted splenocytes from both donor mice provided significant, albeit not complete, protection, increasing the survival rate of recipient mice to that of untreated CCR5$^{+/+}$ mice infected with JEV (Table 6.1).

Together, these data confirm the important role of B cells in protection against Japanese encephalitis, and indicate that CCR5 expression was not required for the disease-ameliorating effect of B cell-depleted lymphocytes primed against JEV. The data also suggest that in the adoptive transfer model, control of JEV infection by immune T cells likely occurred extraneurally, since protection was seen only when the cells were provided early pi. Accordingly, migration of the lymphocytes to peripheral sites of infection did not require expression of CCR5.

Table 6.1 Adoptive transfer of immune splenocytes from CCR5$^{+/+}$ or CCR5$^{-/-}$ donor mice protect CCR5-deficient recipient mice against Japanese encephalitis.

Treatment [a]	Mortality (No. of deaths/total) [b]	MST (days) ± SD [c]
PBS control	71% (15/21)	11.4 ± 1.5
Transfer at one day post-challenge		
Naïve CCR5$^{+/+}$ splenocytes (1 x 10^7 cells)	67% (8/12; *P* =0.92)	11.3 ± 2.3
Naïve CCR5$^{-/-}$ splenocytes (1 x 10^7 cells)	78% (10/14; *P* =0.93)	11.6 ± 2.2
Immune CCR5$^{+/+}$ splenocytes (1 x 10^7 cells)	0% (0/10; *P* =0.0006)	-
Immune CCR5$^{-/-}$ splenocytes (1 x 10^7 cells)	0% (0/10; *P* =0.0006)	-
Immune CCR5$^{+/+}$ splenocytes, B cell-depleted (5 x 10^6 cells)	33% (2/9; *P* =0.019)	12.0 ± 2.8
Immune CCR5$^{-/-}$ splenocytes, B cell-depleted (5 x 10^6 cells)	20% (2/10; *P* =0.012)	11.5 ± 0.7
Transfer at three days post-challenge		
Immune CCR5$^{+/+}$ splenocytes (2 x 10^7 cells)	67% (6/9; *P* =0.57)	12.2 ± 1.9
Immune CCR5$^{-/-}$ splenocytes (2 x 10^7 cells)	80% (8/10; *P* =0.65)	11.8 ± 2.8

[a] Eight-week-old donor CCR5$^{+/+}$ or CCR5$^{-/-}$ mice were infected with 10^3 PFU of JEV, i.v., or left uninfected and sacrificed 7 days later for splenocyte collection with or without depletion of B cells. Cells were transferred to 8-week-old CCR5$^{-/-}$ recipient mice infected 3 days or 1 day earlier with 10^3 PFU of JEV i.v. Surviving mice were monitored for 28 days.

[b] Data are representative of 2 independent experiments. Immune splenocyte treatment groups were compared to the naive splenocyte control group to test for statistical significance.

[c] No significant difference was noted between immune splenocyte treatment and control groups.

6.3 Discussion

The role of the chemokine receptor CCR5 in infection is variable in terms of its impact on pathogenesis and disease outcome. An important disease-ameliorating contribution of CCR5 has been documented in settings of trypanosomiasis (Hardison *et al.*, 2006), toxoplasmosis (Khan *et al.*, 2006), influenza (Fadel *et al.*, 2008; Dawson *et al.*, 2000) parainfluenza (Kohlmeier *et al.*, 2008), West Nile encephalitis (Glass *et al.*, 2005), tick-borne encephalitis (Kindberg *et al.*, 2008), genital herpes (Thapa *et al.*, 2007), and chlamydia infection (Olive *et al.*, 2011), while no significant effect of CCR5 deficiency on the outcome of other microbial infections could be found (Zhong *et al.*, 2004; Peterson *et al.*, 2004; Nansen *et al.*, 2002; Ank *et al.*, 2005; Algood and Flynn, 2004). Mechanistically, the protective value of CCR5 has been largely attributed to its regulatory effect on leukocyte trafficking to the site of infection, although additional functions of CCR5 in the immune response involving antigen recognition (Castellino *et al.*, 2006; Ank *et al.*, 2005; Aliberti *et al.*, 2000), priming and proliferation of lymphocytes (Hickman *et al.*, 2011; Castellino *et al.*, 2006; Ank *et al.*, 2005; Aliberti *et al.*, 2000; Thapa *et al.*, 2007; Floto *et al.*, 2006; Hugues *et al.*, 2007; Nansen *et al.*, 2002), and T cell memory formation (Kohlmeier *et al.*, 2011; Kohlmeier *et al.*, 2008) have also been recognized. In contrast, a disease-potentiating effect of CCR5 has been found in experimental cerebral malaria (Belnoue *et al.*, 2003), schistosomiasis (Souza *et al.*, 2011), leishmaniasis (Sato *et al.*, 1999), cryptococcosis (Huffnagle *et al.*, 1999), herpes keratitis (Komatsu *et al.*, 2008), mouse hepatitis virus-induced multiple sclerosis (Glass and Lane, 2003), and HIV/AIDS (Salazar-Gonzalez *et al.*, 2009), mostly as a consequence of increased immunopathology, and in the case of HIV/AIDS due to usage of CCR5 as an alternative receptor for virus infection (reviewed in reference (Alkhatib, 2009)). This dichotomy in the role of CCR5 in the immune response to infection with

different pathogens prevents generalization of the impact of the chemokine receptor in disease prognosis.

In this chapter, I establish a critical role of CCR5 in recovery from Japanese encephalitis, which was reflected in increased viral burden in the CNS, but not extraneural tissues, and increased mortality in the absence of CCR5 expression. It was previously shown that type I interferon and antibody, but not T cells, are key to control of virus replication in peripheral tissues in the mouse model of Japanese encephalitis, while T cells play a role in reducing viral burden and dissemination in the CNS. CCR5-deficient mice displayed a wide-ranging debilitation of JEV-specific immune responses; these included poor NK cell activity, a suboptimal JEV-immune $CD8^+$ T cell response, low splenic cellularity, and impaired trafficking of leukocytes (NK cells, macrophages, and $CD8^+$ and $CD4^+$ T cells) to the brain. These factors most likely acted in concert to create a lethal combination of defective viral clearance from peripheral tissues and the CNS, thereby increasing the susceptibility of the host to JEV infection. In addition, recent findings suggest that CCR5, which is expressed in neurons and is up-regulated in the brain in pathological conditions, could have a direct neuro-protective function by increasing neuronal survival (reviewed in reference (Sorce *et al.*, 2011)).

Our results are consistent with the previous finding of increased susceptibility of CCR5-deficient mice in a model of West Nile encephalitis (Glass *et al.*, 2005), although mechanistic differences in the role of CCR5 in recovery from WNV and JEV infection are apparent. While a significant (≥50%) overall reduction in migration of NK cells, $CD4^+$ and $CD8^+$ T cells, and activated infiltrating macrophages to the brain of JEV and WNV infected $CCR5^{-/-}$ mice was seen in both investigations, this difference relative to $CCR5^{+/+}$ control mice narrowed between days 7 and 10 pi in the case of JEV infection,

but became more pronounced for WNV. More importantly, the WNV study found similar splenic immune responses in CCR5 sufficient and deficient mice, whereas I observed markedly reduced numbers of lymphocytes in spleens of mice lacking CCR5 expression. It is likely that the latter was a major factor contributing to the severe phenotype in JEV infected CCR5-deficient mice, since adoptive transfer of B cell-depleted immune splenocytes corrected the phenotype, independent of whether the cells were derived from $CCR5^{-/-}$ or $CCR5^{+/+}$ donor mice. Low splenocyte numbers in the absence of CCR5 may reflect restricted leukocyte proliferation secondary to poor early priming, increased cell turnover to other lymphoid organs, and/or increased T cell death. However, since the spleen is the main responsive peripheral lymphoid organ following an iv challenge, defective proliferation most likely accounts for the diminished splenocyte numbers in $CCR5^{-/-}$ mice. Moreover, other studies have also observed restricted proliferation of splenocytes, or specific subsets thereof, in the absence of CCR5, both in physiologic (Weiss *et al.*, 2011) and pathologic conditions (Ank *et al.*, 2005; Thapa *et al.*, 2007). The latter studies deal with virus challenge models with a resultant impairment in control of infection.

Concomitant with the limited number of leukocytes in spleens of CCR5-deficient mice infected with JEV, the functional activities of NK and $CD8^+$ T cell responses were blunted, while the antibody response against JEV did not significantly differ from that elicited in infected wild-type mice. This finding was consistent with several other studies that demonstrated a critical role of CCR5 expression on naïve T cells in activation of $CD8^+$ T cell responses by guiding the cells to and enhancing interaction with antigen-presenting dendritic cells in immunogen-draining lymph nodes (Aliberti *et al.*, 2000; Floto *et al.*, 2006; Hugues *et al.*, 2007; Hickman *et al.*, 2011; Castellino *et al.*, 2006). In chapter 3, I have found that co-transfer of immune $CD4^+$ and $CD8^+$ T cells, but not individual transfer of either lymphocyte subpopulations, was protective in lethal

infection with JEV. Thus, the suboptimal stimulation of virus-specific T cell responses together with their reduced migration into the CNS most likely accounted for the increased susceptibility of CCR5-deficient mice to Japanese encephalitis.

It is unclear whether the reduced NK cell activity in CCR5-deficient relative to wild-type mice contributed to the increased disease severity with Japanese encephalitis, as has been proposed in studies on herpes simplex virus in $CCR5^{-/-}$ mice (Ank *et al.*, 2005; Thapa *et al.*, 2007). Flaviviruses are in general poor inducers of NK cells, and flavivirus infection reduces the susceptibility of target cells to NK cell lysis (Momburg *et al.*, 2001; Lobigs *et al.*, 1996). The latter is thought to be a consequence of virus-induced up-regulation of MHC-I on the surface of flavivirus-infected cells (Abraham *et al.*, 2010; Kesson and King, 2001; King and Kesson, 1988; Lobigs *et al.*, 2003*a*; Mullbacher and Lobigs, 1995). Therefore, it appears that NK cell responses do not markedly enhance recovery from flavivirus infection. Consistent with this interpretation, in chapter 5, *in vivo* depletion of NK cells using monoclonal antibody anti-NK1.1 did not result in increased mortality or increased viral burden in the CNS in adult mice infected with JEV.

In conclusion, this chapter established CCR5 as a host factor against severe disease in a mouse model of Japanese encephalitis. Control and elimination of viral spread is largely dependent on rapid activation of antiviral leukocytes, and their recruitment to the sites of peripheral infection and/or into the CNS. In the absence of CCR5, an extensive immune dysfunction ensued, encompassing probable involvement of defective early priming and subsequent aberrant leukocyte activation, restricted splenocyte expansion, and limited trafficking. This finding raises possible safety concerns regarding the use of CCR5 antagonists in HIV individuals, particularly in regions where both HIV and JEV

are endemic (Erlanger *et al.*, 2009; Simon *et al.*, 2006). While the benefits of taking the drug most likely far outweigh the potential risk of enhanced susceptibility to Japanese encephalitis, mitigating the latter risk factor by instituting precautionary measures including patient education, limiting mosquito exposure, and/or vaccination may be warranted.

Chapter 7

Forward genetics approach in localizing loss-of-function mutation spontaneously generated in an inbred knockout mouse strain

7.1 Introduction

The host 'resistome' is a collection of non-redundant genes that confers resistance from challenge with pathogens (reviewed in (Beutler *et al.*, 2006, Beutler *et al.*, 2007)). While some genes are pathogen-specific, mostly provide resistance against diverse group of pathogens. The resistome defines the survival outcome with which a particular susceptible host may present after an infectious insult. The acquisition of this vital network of resistant genes allowed the selection and survival of certain species, and served as a dominant force in evolution. Identifying the list of genes catalogued under resistome is fundamental in the understanding of host-pathogen interactions.

The recent technological advancements in forward and reverse genetics enabled the discovery and dissection of viral resistance/susceptibility genes (reviewed in (Gruenheid and Gros, 2010)). Much of our understanding on the immune system is derived from studies utilizing the reverse genetics approach; where a gene of interest is knocked-out by various recombinant techniques, and subsequent recording of the observable phenotype upon exposure to a particular pathogen. Techniques used in reverse genetics would include site-directed mutagenesis, gene targeting via homologous recombination, gene silencing via RNA interference, and interference via transgenes (reviewed in (Adams and van der Weyden, 2008)). On the other hand, the classic or forward genetic approach to biological problems is unbiased by hypotheses: it begins with a phenotype of unknown origin and ends with the identification of mutation(s) that are responsible for the phenotype. The unidentified mutation can occur spontaneously or naturally, or can be randomly introduced via artificial means though radiation mutagenesis, ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis, or *Sleeping Beauty* transposon system (reviewed in (Carlson and Largaespada, 2005)).

For the past 25 years, gene mapping and positional cloning are regarded as useful experimental approaches utilized in forward genetics to successfully uncover spontaneous mutations naturally generated in inbred mouse strains (reviewed in (Gruenheid and Gros, 2010; Vidal *et al.*, 2008)). These forward genetic approaches brought about remarkable discoveries, depicting for the first time various genes with diverse immunologic roles vital for defense against microbial infections; including NK cell activity, macrophage intraphagosomal activity, pathogen sensing, intracellular signal transduction pathway intermediates, and interferon-inducible effector antiviral proteins (Table 7.1). Furthermore, the availability of the assembled sequences of the human and mouse genomes and major advances in genome sequencing technologies have made gene discovery based on the classic genetic strategy a relatively straightforward process (Nelms and Goodnow, 2001).

In this chapter, I will present a finding of a virus resistance/susceptibility gene mutation spontaneously generated in an inbred knockout mouse strain. A classic genetic approach was employed: starting with a phenotype of increased susceptibility to viral infection, subsequently localizing the gene of interest in Chromosome 1, and finally short-listing candidate genes responsible for the loss-of-function mutation. A novel mouse strain, carrying the unknown mutation, was generated, and was designated as the '***Tuara***' mice (Originally derived from a Visayan language "Tua ra!" which is literally translated in English as: "There it is". The expression is commonly used in the context of discovering an object after a long, patient, and hardwork search. It is also fitted with the acronym **T**ranslated **U**nit with **A**ntiviral and **R**egulatory **A**ctivity).

Table 7.1 Summary of resistance/susceptibility genes identified through classical or forward genetics approach

Year	Locus	Chromosome	Mouse strain	Gene (mutation)	Phenotype
1988	Mx	16	Several	Mx1 (loss of expression)	Susceptibility to influenza
1993	Bcg/Ity/Lsh	1	Several	Nramp1 (G169D)	Susceptibility to infection with BCG, S. typhimurium, L. donovani
1998	Lps	4	C3H/HeJ	Tlr4 (P714H)	LPS non-responsiveness, susceptibility to S. typhimurium infection
2001	Cmv1	6	C57BL/6J	Ly49h (presence versus absence)	Resistance to MCMV infection
2002	Flv	5	Several	Oas1b (premature STOP)	Susceptibility to West Nile virus infection
2003	Lgn1	13	A/J	Naip5	Increased L. pneumophila replication in macrophages
2003	Lps2	17	ENU-induced	TRIF/Ticam1 (1 bp deletion)	Defective response to TLR-3 and -4 ligands
2004	Char4	3	AcB55, AcB61	Pklr (I90N)	Resistance to P. chabaudi infection
2005	Cmv3	6	MA/My	Ly49p + H2k	Resistance to MCMV infection
2005	Sst1	1	C3HeB/FeJ	Ipr1(loss of expression)	Susceptibility to pulmonary TB
2005	Obl	5	ENU-induced	CD36 (premature STOP)	Reduced response to TLR-2 and 6 ligands. Susceptibility to S. aureus infection
2005	heedless	18	ENU-induced	CD14	No activation of TRIF/TRAM pathway downstream of TLR-4. Defective response to TLR-2 and 6 ligands
2006	Crtq3	11	C3H/HeJ	Irgb10 (reduced expression)	Susceptibility to C. trachomatis infection
2006	3d	19	ENU-induced	Unc93b (H412R)	Diminished response of TLR-3, -7 and -9 antigen presentation defect
2006	Ltsx1	11	Several	Nalp1b (gain of expression)	Susceptibility to anthrax lethal toxin
2007	Myls	8	BXH2	Irf8 (R294C)	Susceptibility to M. bovis, S. typhimurium and P. chabaudi infection
2007	Char9	10	A/J	Vnn1/3 (reduced expression)	Susceptibility to P. chabaudi infection

Table adapted from Grunheid and Gros (2010)

7.2 Results and Discussion

7.2.1 Spontaneous mutation in MHCII-Aα$^{-/-}$ mice resulting in enhanced susceptibility to flavivirus infection. In chapter 3, the antiviral role of adaptive immune response-effector cell populations in recovery from JEV infection was presented. For instance, MHC-II-deficient mice that lack the four classical MHC-II genes via a large deletion of the entire class II region (MHCII-Aα/β$^{-/-}$) were used as model to elucidate the role of CD4^{+} T cells in JEV pathogenesis. Infection of MHCII-Aα/β$^{-/-}$ mice, relative to B/6 wt control mice, displayed 100% mortality and comparable overall mean survival time (MST) (Fig. 3.6). Most of MHCII-Aα/β$^{-/-}$ mice succumbed to infection by day 12, while a remaining 21% had a MST of ~20 days, reflecting a failure to clear infection in the absence of CD4^{+} T help required for maintenance of the antiviral antibody response.

MHCII-Aβ$^{-/-}$ is another available MHC-II knockout mice designed via inactivation of the Aβ locus of the MHC-II complex. Challenge of MHCII-Aβ$^{-/-}$ mice with JEV similarly presented with 'delayed mortality', commencing at 12 days pi and onwards. Although, for reasons not clear, MHCII-Aβ$^{-/-}$ mice also displayed mortality rate that is comparable to B/6 wt (Fig 7.1A). MHCII-Aα$^{-/-}$ is the third alternative MHC-II-deficient mice designed via selective disruption of Aα locus of the MHC-II complex. Interestingly, sc infection of MHCII-Aα$^{-/-}$ mice with JEV presented with an unexpected disease outcome. Relative to wt, MHCII-Aα/β$^{-/-}$ and MHCII-Aβ$^{-/-}$ mice, infection of MHCII-Aα$^{-/-}$ mice resulted in a surprising phenotype of complete and rapid mortality, with MST of 8.7 ± 3 days (Fig. 7.1A). The 'early mortality' observed in MHCII-Aα$^{-/-}$ suggests an unknown spontaneous mutation generated independently of the known

MHC-II gene defect. The phenotype was more pronounced when MHCII-A$\alpha^{-/-}$ mice were infected with a naturally attenuated Australian strain of WNV, Kunjin virus (WNV_{KUN}) (Fig. 7.1B). All wt and MHCII-A$\alpha/\beta^{-/-}$ survived WNV_{KUN} challenge, while 100% MHCII-A$\alpha^{-/-}$ mice succumbed to infection with MST of 9 ± 2.0.

The investigation on the relative value of the putative viral resistance gene was then extended to a flavivirus outside of JE serocomplex. Given its importance in terms of global disease burden (reviewed in (Simmons *et al.*, 2012)), MHCII-A$\alpha^{-/-}$ and B/6 control mice were challenged intravenously (iv) with $1x10^5$ PFU of dengue virus serotype 2 (DENV-2), and mortality recorded over a 60-day observation period (Fig. 7.2A). As expected, all wt controls survived, and did not present with any signs of disease. On the contrary, 29% (2 out of 7) of MHCII-A$\alpha^{-/-}$ mice succumbed to infection, with late time of death on days 49 and 59 pi. Although not statistically significant, the data suggest that MHCII-A$\alpha^{-/-}$ mice are more susceptible with to infection with DENV-2 than wt mice.

Even more surprising is the finding that MHCII-A$\alpha^{-/-}$ mice were also susceptible to an infection with a live-attenuated vaccine, ChimeriVax-JE. ChimeriVax-JE is a recently FDA-approved vaccine against JEV, that was genetically engineered via a construct composing of genes encoding for premembrane and envelope proteins of JEV (vaccine strain SA14-14-2) and nonstructural proteins of yellow fever 17D (YF-17D) vaccine (Monath *et al.*, 2003). Subcutaneous challenge of MHCII-A$\alpha^{-/-}$ mice with $1x10^5$ PFU of ChimeriVax-JE resulted in 44% mortality, with a MST of 33.0 ± 5.4 days (Fig. 7.2B). It was shown in early preclinical studies that ChimeriVax-JE has a superior safety profile relative to its partially neuroattenuated, YF-17D vaccine. Thus, 4-week-old B/6 wt mice survived intracranial (ic) inoculation of $1x10^3$ PFU of ChimeriVax-JE, as compared to

complete lethality observed when the same dose of YF-17D was given (Guirakhoo et al., 1999, Monath et al., 2000). Similar to this finding, I have demonstrated that all B/6 wt mice survived ic challenge with $1x10^3$ PFU of ChimeriVax-JE (Fig. 7.2C). In contrast, 100% of MHCII-A$\alpha^{-/-}$ mice succumbed to the infection, with a MST of 21.4 ± 0.6 days.

Altogether, these data show that MHCII-A$\alpha^{-/-}$ mice display an extraordinarily sensitivity to flaviviral challenge. This susceptibility phenotype cannot be explained *per se* by a defect in MHC-II genes. It was hypothesized that in addition to the targeted disruption of Aα gene, a spontaneous mutation had occurred in MHCII-A$\alpha^{-/-}$ mouse strain resulting in the acquisition of an unidentified susceptibility allele for flaviviral infection.

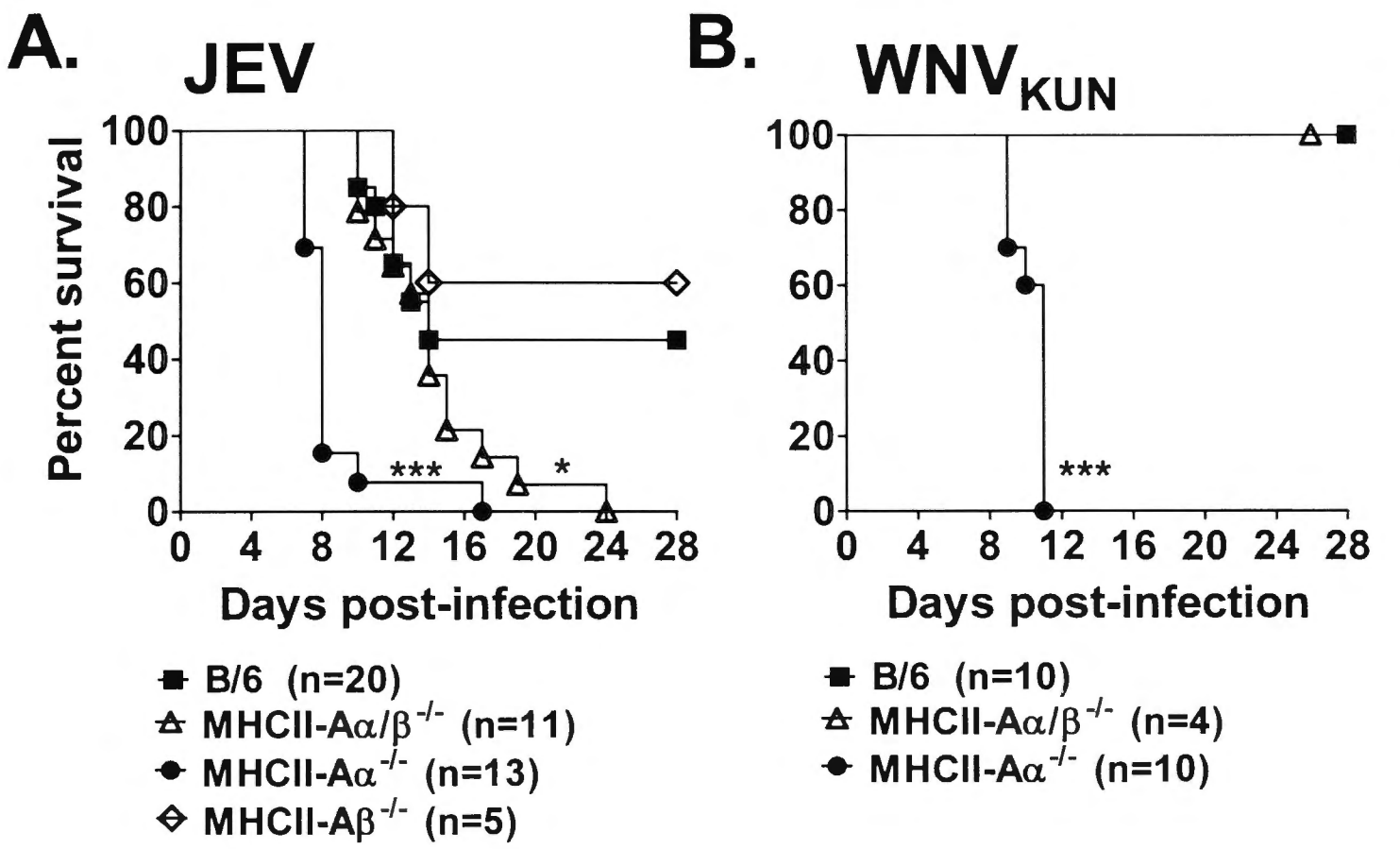


Figure 7.1 Susceptibility of MHCII-Aα/β$^{-/-}$, MHCII-Aα$^{-/-}$, MHCII-Aβ$^{-/-}$and B/6 mice to flavivirus infection. Groups of 8-week-old mice were infected sc via the footpad with 10^3 PFU of JEV (A) or WNV_{KUN} (B). Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from two independent experiments. Asterisks denote statistical significance in mortality between B/6 and various knockout mice (*, $P < 0.05$; ***, $P < 0.001$).

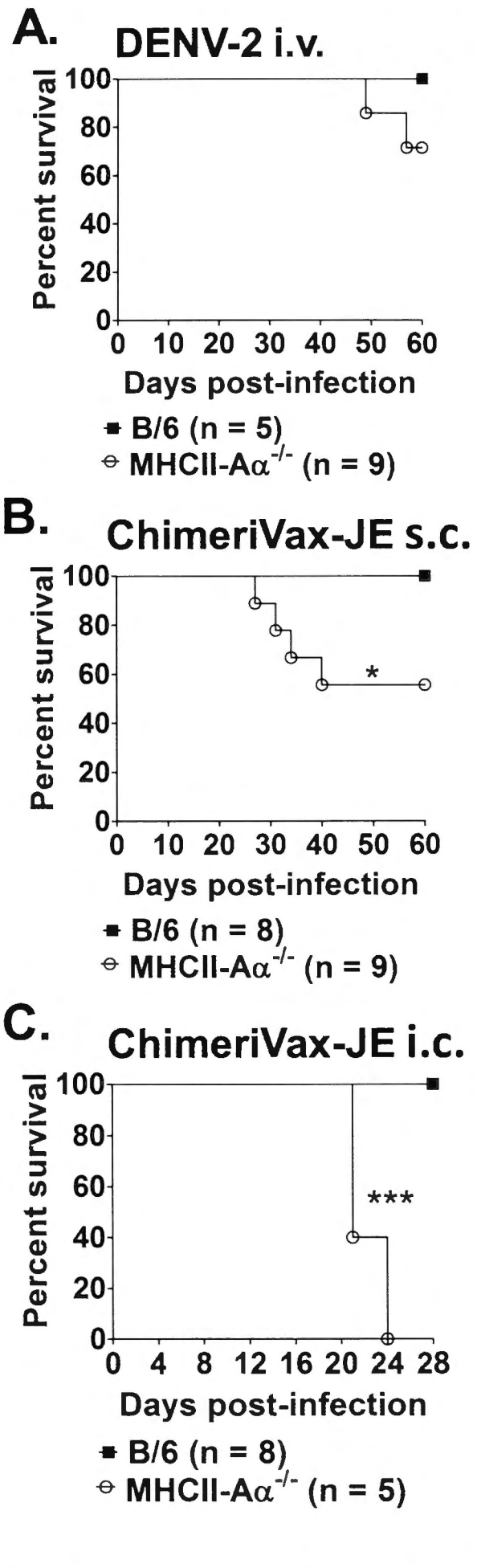


Figure 7.2 Susceptibility of MHCII-A$\alpha^{-/-}$, and B/6 wt mice to infection with DENV-2 and ChimeriVax-JE. Groups of 6-week-old mice were inoculated iv with 10^5 PFU of DENV (A) or sc with 10^5 PFU of ChimeriVax-JE (B). Morbidity and mortality were recorded daily, and surviving mice were monitored for 60 days. Groups of 4-week-old mice were inoculated ic with 10^3 PFU of ChimeriVax-JE (C), and morbidity and mortality recorded daily for a 28-day-observation period. The data shown were constructed from two independent experiments. Asterisks denote statistical significance (*, $P < 0.05$; ***, $P < 0.001$).

7.2.2 High viral burden in MHCII-Aa$^{-/-}$ mice. After sc challenge with JEV, viral load in serum and CNS were significantly higher in MHCII-Aα$^{-/-}$ mice relative to B/6 wt controls (Fig. 7.3). At day 2 pi viral load in serum of MHCII-Aα$^{-/-}$ mice was >100-fold higher than that in control mice (Fig. 7.3A). All infected MHCII-Aα$^{-/-}$ mice presented with rapid and uncontrolled virus dissemination into the CNS. At day 6 pi, viral load in brains of MHCII-Aα$^{-/-}$ mice exceeded that in control mice by ~1,000-fold (Fig. 7.3B). In addition, virus dissemination into the spinal cords of MHCII-Aα$^{-/-}$ mice appeared earlier with a significantly elevated geometric mean titer (GMT) of 2.2x10^{9} (Fig. 7.3C). This is contrasted with undetectable levels of virus in control mice. As presented in chapter 3, virus spread from brain to spinal cord of wt mice takes place between day 8 and day 10 pi in <50% of animals. It should also be noted that, in chapter 3, virus dissemination into the spinal cord of MHCII-Aα/β$^{-/-}$ mice occurred after day 8 pi (Chapter 3). Together, these data strongly suggest that in MHCII-Aα$^{-/-}$ mice, an unknown defect independent of MHC-II deficiency is responsible for the fulminant disease phenotype after JEV infection.

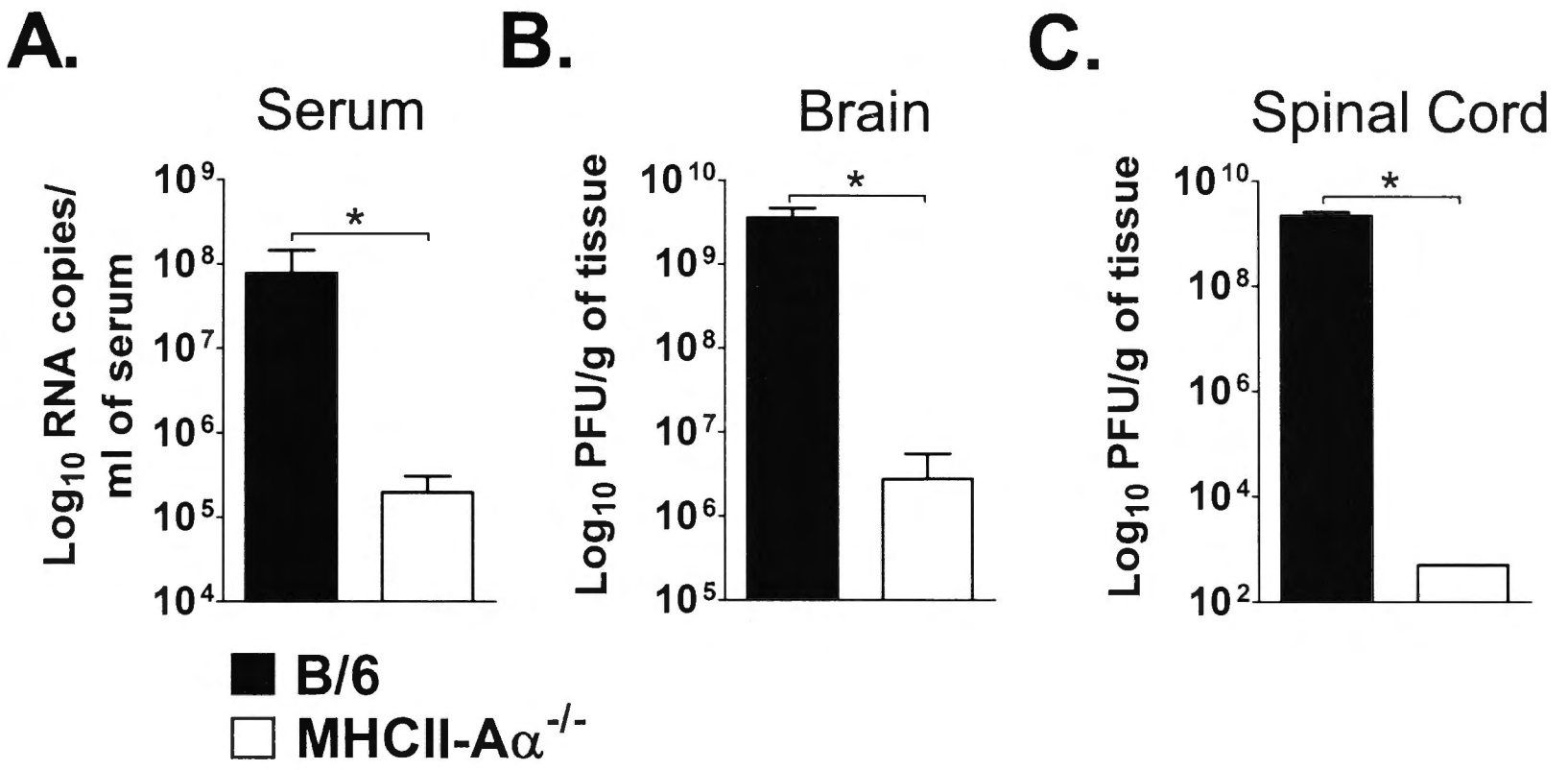


Figure 7.3 Viral burden in B/6 and MHCII-A$\alpha^{-/-}$ mice after exposure to JEV. Eight-week-old mice were challenged with 10^3 PFU of JEV injected into the footpad, bled on day 2 pi for serum collection, and sacrificed on day 6 pi for collection of CNS tissues. Viremia was measured in RNA copies/ml by qRT-PCR; viral load in tissues was measured by plaque titration in Vero cells. The lower limit of virus detection was $1x10^2$ PFU/g tissue. Asterisks denote statistical significance (*, $P < 0.05$).

7.2.3 Enhanced virus growth in primary macrophages isolated from MHCII-Aa$^{-/-}$ mice. Bone marrow-derived macrophages from mutant and B/6 wt mice were infected with JEV at multiplicity of infection (MOI) of 1, and virus yield in culture supernatant measured by plaque assay (Fig. 7.4). At 24 hour pi, virus growth in MHCII-A$\alpha^{-/-}$ macrophages was increased ~100-fold relative to that in cells from wt mice. This significant difference is similarly observed when bone marrow-derived macrophages were infected with WNV_{KUN} or YFV (Fig. 7.4). Relative to cells from wt mice, virus growth at 24 hours pi in MHCII-A$\alpha^{-/-}$ macrophages infected with WNV_{KUN} or YFV was increased by ~50-fold. This cell culture model clearly demonstrates that an innate immune factor, unrelated to $CD4^+$ T cell deficiency, is responsible for the enhanced susceptibility to viral infection.

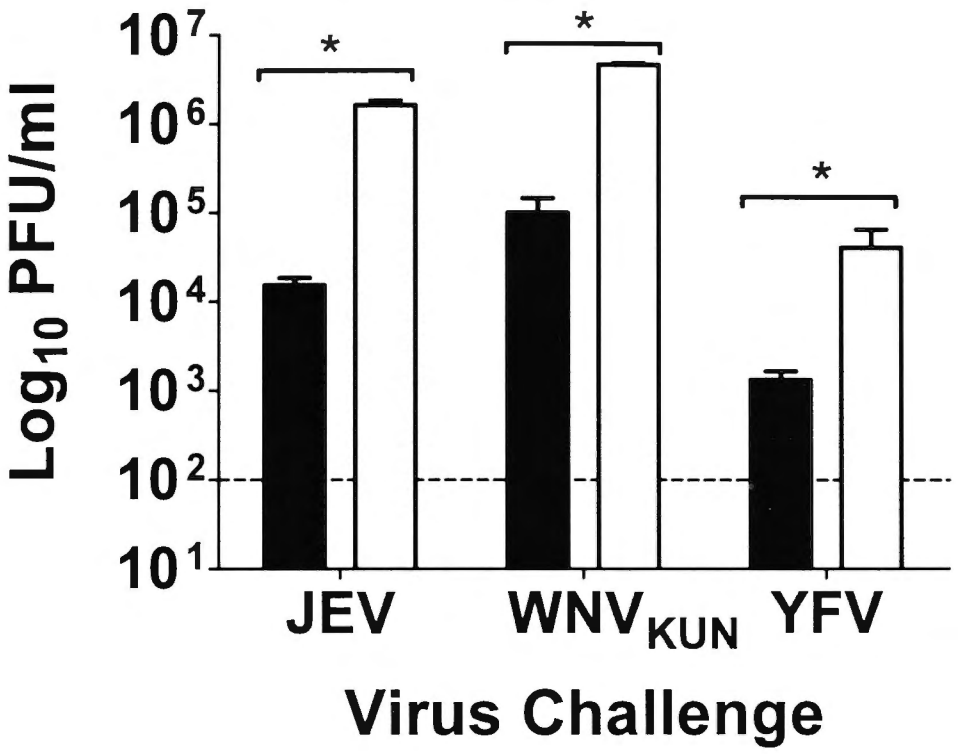


Figure 7.4 Susceptibility of B/6 and MHCII-Aα$^{-/-}$ bone marrow-derived macrophages to infection with flaviviruses. Bone marrow cells were isolated from lower extremity bones of B/6 wt and MHCII-Aα$^{-/-}$ mice as described in materials and methods, and selectively cultured for 7 days towards macrophage differentiation. Mature macrophages were then infected with JEV, WNV_{KUN}, or YFV at MOI of 1. Virus load in culture supernatants at 24 pi was determined by plaque titration in Vero cells. Asterisks denote statistical significance (*, $P < 0.05$).

7.2.4 The unknown susceptibility allele in MHCII-Aa$^{-/-}$ mice is a recessive trait with full penetrance. The genetic mapping approach enables rapid localization of a spontaneous mutation to a particular chromosomal region by co-segregation of the mutation with genetic markers interspersed throughout the genome (reviewed in (Nelms and Goodnow, 2001)). First, MHCII-A$\alpha^{-/-}$ mice were crossed with a mapping strain (B/10 mice) to produce F1 (B6xB10) progeny (Fig. 7.5). The phenotypic outcome of the progeny F1 (Filial 1 generation) mice after flaviviral challenge will define the subsequent cross necessary for genome mapping. If all F1's succumbed to infection, the mutation is expressed in a dominant fashion, and F1 progenies should be intercrossed with each other to successfully segregate and localize the gene of interest. If all F1's survived the infection, then the phenotypic expression of the mutation is due to a recessive allele, and F1 progenies should be backcrossed with the original MHCII-A$\alpha^{-/-}$ mice for a successful subsequent linkage mapping. All eight-week-old F1 mice infected sc with 10^3 PFU WNV_{KUN} survived the challenge (Table 7.2). Similarly, all MHCII-A$\alpha\beta^{-/-}$, B/6, and B/10 control mice survived the infection. In contrast, 100% of MHCII-A$\alpha^{-/-}$ mice (n = 7) succumbed to the infection with WNV_{KUN}. This indicates that the unknown susceptibility allele is a fully penetrating recessive trait.

Given that the unknown mutation is a recessive phenotype, F1 (B6xB10) mice were backcrossed to MHCII-A$\alpha^{-/-}$ mice to generate N2 (nuclear or backcross generation) (B6xB6.B10) progeny (Fig. 7.6). Fifty percent of the N2 progenies were expected to carry the unknown mutation (affected), while 50% were expected to be resistant (unaffected); also, 50% of N2 progeny were expected to be homozygous for the targeted disruption of the MHC-II *Aa* gene, and 50% heterozygous for this mutation. Progeny N2 mice were then screened phenotypically by sc challenge with WNV_{KUN}. The result displayed consistency with the expected outcome and confirmed the presence of an

unknown susceptibility gene defect independent of MHC-II (Table 7.3). The susceptibility allele segregated form the MHC-II locus in simple Mendelian pattern of inheritance: 47% of the total were affected mice with 24% carrying the MHCII-A$\alpha^{+/-}$ allele and 24% carrying the MHCII-A$\alpha^{-/-}$ allele, while 53% of the total were unaffected with 21% and 32% respectively carrying either MHCII-A$\alpha^{+/-}$ or MHCII-A$\alpha^{-/-}$ alleles.

Meiotic recombination in F1 and subsequent N2 mice generation acts as a genetic shuffle that can be used to distinguish regions of the genome that are closely linked to the susceptibility allele from regions that are unlinked. These regions were determined by linkage analysis in a last step of the mapping process by analysing the recombinant chromosomes inherited by N2 mice with a panel of single-nucleotide polymorphisms (SNPs) genetic markers that span the genome (Myajishev *et al.*, 2001; Petkov *et al.*, 2004*a*; Petkov *et al.*, 2004*b*). N2 mice were grouped as affected or unaffected for the virus susceptibility phenotype based on the outcome of infection with WNV_{KUN}. DNA from tail samples of each infected N2 mouse was isolated, stored, and subsequently pooled in affected or unaffected groups. Pooled DNA was then analysed with the use of ~90 SNPs genetic markers that can distinguish between B/6 and B/10 mice strains. Thus, SNPs closely linked to the unknown susceptibility allele will only yield the homozygous B/6 allelic fragment in N2 mutant mice, whereas analyses with unlinked markers will yield heterozygous B/6 and B/10 strain alleles. However, this initial attempt was unsuccessful. It was discovered that the original MHCII-A$\alpha^{-/-}$ mice, initially thought to be of purely B6 background, were in fact a mixture of B/6 and B/10 (data not shown). Accordingly the $B6_{B10}$ nomenclature is used for a second attempt of genome mapping.

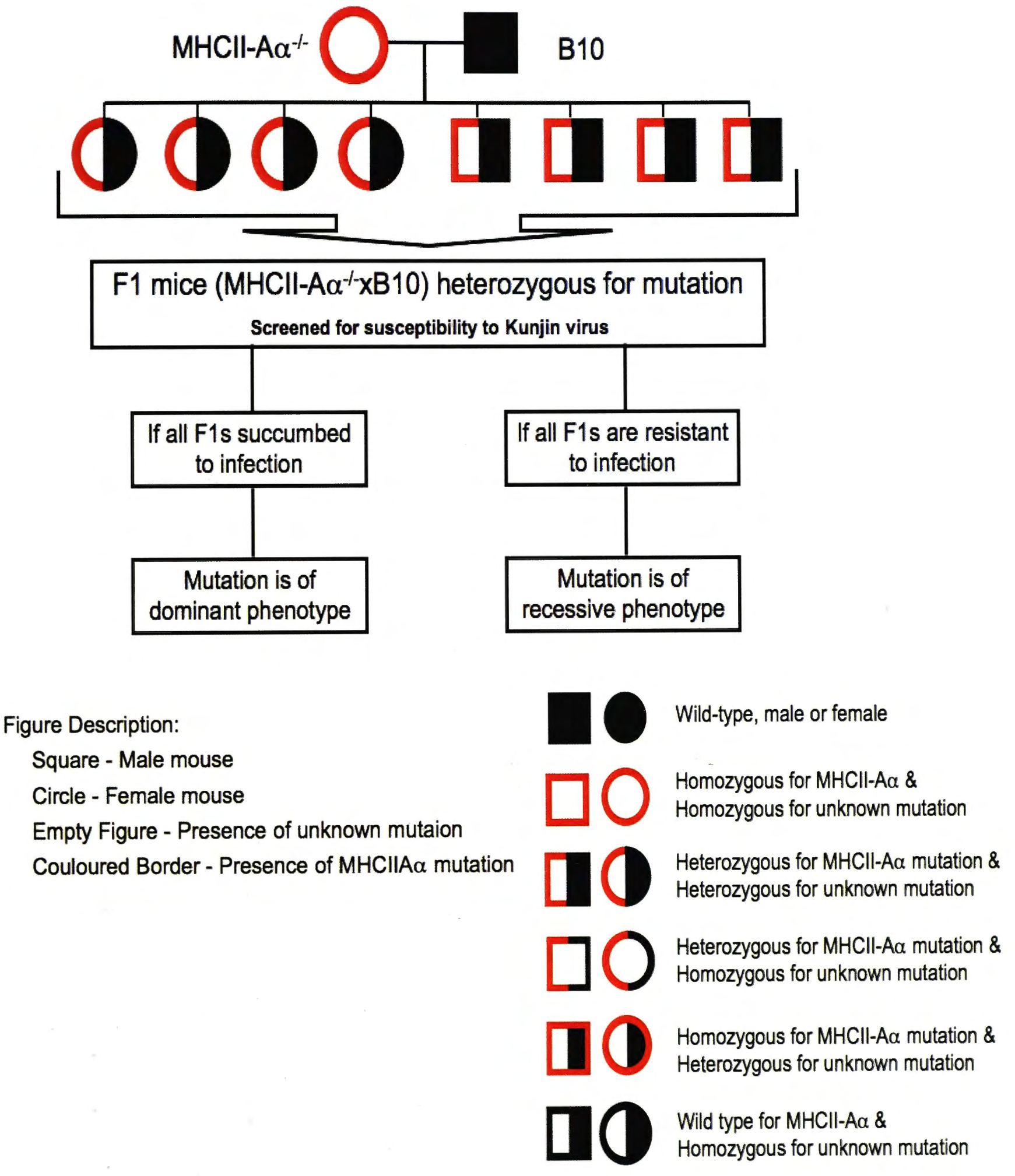


Figure 7.5 Initial mouse-cross design for genome mapping. MHCII-Aα$^{-/-}$ mice were crossed to B/10 mice, and all progenies (F1) produced are heterozygous for the MHCII-Aα defect and the unidentified mutation. Two probable outcomes were identified in the chart when F1 mice are challenged with WNV_{KUN}. If all F1's succumbed to infection, the unknown susceptibility allele is a dominant trait; whereas when all F1's survived the infection, the unknown susceptibility allele is a recessive trait.

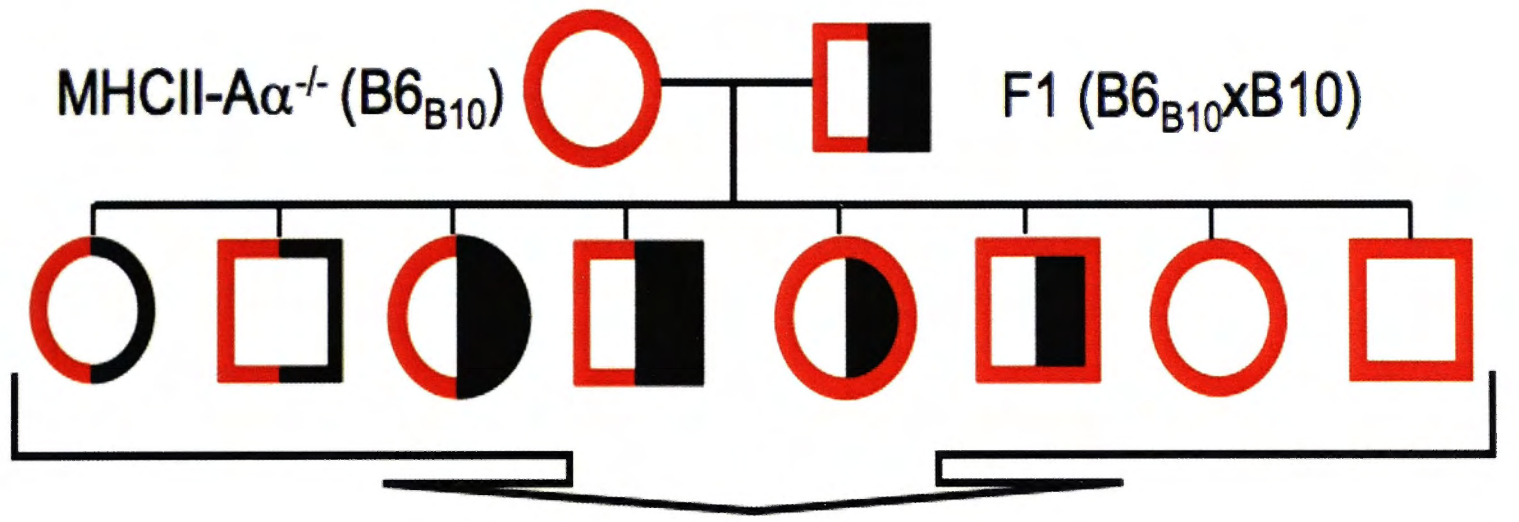


Figure 7.6 F1 x MHCII-Aα$^{-/-}$ mouse cross design for genome mapping. F1 (MHCII-Aα$^{-/-}$xB10) mice were backcrossed to MHC-II-Aα$^{-/-}$ mice, which were identified as B/6 contaminated B/10 background, hence the symbol, $B6_{B10}$. Progenies produced (N2) are expected to be: 25% homozygous for unknown mutation and homozygous for MHCII-Aα defect, 25% homozygous for unknown mutation and heterozygous for MHCII-Aα defect, 25% heterozygous for unknown mutation and homozygous for MHCII-Aα defect, 25% heterozygous for unknown mutation and heterozygous for MHCII-Aα defect.

Table 7.2 Susceptibility of F1 and various control mouse strains to WNV_{KUN} infection.

Mice[a]	Mortality[b]	MST
B/6	0% (0/5)	-
B/10	0% (0/5)	-
MHCII-A$\alpha/\beta^{-/-}$	0% (0/4)	-
MHCII-A$\alpha^{-/-}$	100% (7/7)***	10 ± 0.00
F1	0% (0/6)	-

[a] Eight-week-old mice were challenged sc with $1x10^3$ PFU sc WNV_{KUN} virus. Morbidity and mortality were recorded for 30 days.

[b] Statistical significance was computed in comparison with B/6 mice control. Asterisks denote significant difference (***, $p < 0.001$).

Table 7.3 Susceptibility of N2 mice to WNV_{KUN} infection.

MHCII genotype	Number (percentage) of affected mice	Number (percentage) of unaffected mice	Total
MHCII-A$\alpha^{+/-}$	9 (24%)	8 (21%)	17 (45%)
MHCII-A$\alpha^{-/-}$	9 (24%)	12 (32%)	21 (55%)
Total	18 (47%)	20 (53%)	38 (100%)

Thirty-eight 8-week-old N2 ($B6_{B10}xB6_{B10.}B10$) mice were challenged sc with $1x10^3$ PFU of WNV_{KUN}, and observed for 28 days to record mortality. All mice were genotyped by PCR for the MHCII-Aα mutation as described in materials and methods.

Given that the original MHCII-A$\alpha^{-/-}$ mice were in fact a mixture of B/6 and B/10 strain (earlier designated as $B6_{B10}$), a second attempt of genome mapping, using CBA mice as the mapping strain, was then performed. MHCII-A$\alpha^{-/-}$ mice were crossed with CBA mice to produce F1 progenies ($B6_{B10}$CBA) (Fig. 7.7). As expected, all F1, CBA, and B/6 control mice were resistant to challenge with $WNV_{KUN,}$ while 100% of MHCII-A$\alpha^{-/-}$ succumbed to infection (Table. 7.4). This is consistent with the phenotypic outcome observed in F1 after the initial genome mapping, again confirming that the susceptibility allele is a fully penetrant recessive trait.

Thereafter, F1 ($B6_{B10}$xCBA) mice were backcrossed to MHCII-A$\alpha^{-/-}$ ($B6_{B10}$) mice to generate N2 ($B6_{B10}$CBAx$B6_{B10}$) progeny (Fig. 7.7). Phenotypic screening of the progeny N2 mice by WNV_{KUN} challenge revealed the expected outcome, where the susceptibility allele segregated from the MHC-II locus: 54% of the total are affected mice with 29% and 21% respectively carrying either MHCII-A$\alpha^{+/-}$ or MHCII-A$\alpha^{-/-}$ alleles, whereas 46% of the total are unaffected mice with 25% each carrying either MHCII-A$\alpha^{+/-}$ or MHCII-A$\alpha^{-/-}$ alleles (Table 7.5). DNA from each infected N2 mouse was stored, and subsequently pooled in affected or unaffected groups. Pooled DNA was then analysed with the use of ~110 SNPs genetic markers that can distinguish B/6 or B/10 against CBA (Fig. 7.8). This second screen of pooled DNA from affected and unaffected mice localized the unknown virus susceptibility allele to a ~60 MB region on chromosome 1 (Fig. 7.9).

To confirm and further fine-map the location of the susceptibility allele, additionally designed SNP markers that distinguish B/6 or B/10 against CBA were used that span across the identified ~60 Mb region on chromosome 1 (Fig. 7.10). DNAs from affected and unaffected mice were screened individually, and based on the outcome of this

screen the unknown susceptibility allele was mapped and narrowed down to a 6.86 Mb region on chromosome 1 (Fig. 7.11-12). The identified region spans 93 genes on chromosome 1 (Mouse Genome Informatics browser [http://www.informatics.jax.org/]). However the region does not contain a strong candidate that can explain host resistance/susceptibility to viral infection.

A siRNA screen for human genes associated with WNV infection identified 22 host resistance factors, 21 of which were novel (Krishnan et al., 2008). Of these, 2 mouse orthologs map to chromosome 1, but to sites that are 66 and 149 MB distal to the ~7 MB region, to which the unknown susceptibility allele was mapped (Table 7.6).

The 6.86 Mb region of interest contains two genes associated with immune function: interleukin 1 receptor type 1 (IL-1R1) and interleukin-18 receptor 1 (IL-18R1) (Fig. 7.12). Most cells constitutively express IL-1R1, which plays an important role in inflammatory processeses (reviewed in (Dinarello *et al.*, 2009)). Mice devoid of IL-1R1 exhibit attenuation of inflammatory responses, which predisposes to either increased or reduced susceptibility to microbial infections. The impact of IL-1R1 deficiency in viral infection models, however, is not pronounced nor did it display with a fulminant disease phenotype. This was exemplified by infections with VACV or influenza virus in IL-1R1$^{-/-}$ mice (Belisler *et al.*, 2010; Schmitz *et al.*, 2005; Szretter *et al.*, 2007; Zhao *et al.*, 2009). Moreover, downstream signalling of IL-1 through the IL-1R1 requires the adaptor protein, MyD-88, recruited to the cytoplasmic domain for its activity. MyD88$^{-/-}$ mice infected sc with 10^3 PFU of JEV did not display a significant difference in mortality or time to death as compared to wt (Fig. 7.13). Lastly, the clinical use of IL-1 response antagonists in >100,000 patients with inflammatory diseases, for instance

rheumatoid arthritis, did not present with major adverse events such as increased susceptibility to viral infections (Dinarello *et al.*, 2009).

The receptor for IL-18 (IL-18R) is a pleiotropic cytokine belonging to the Toll/IL-1R superfamily, and is implicated in augmentation of cell-mediated immunity through interferon-γ (IFN–γ) production, enhancement of proinflammatory responses, and defense against bacterial, fungal and parasitic pathogens (Biet *et al.*, 2002). In spite of this, there is a less pronounced requirement for IL-18 signalling in recovery from viral infections. First, a defect in the IL-18R1 gene does not reflect fulminant disease phenotype observed when MHCII-Aα$^{-/-}$ mice were challenged with a flavivirus or a poxvirus (presented later). Second, IFN-γ$^{-/-}$ mice did present with 'early death' phenotype when challenged with JEV (Chapter 5), and are not susceptible to WNV_{KUN} infection (Wang Y. *et al.*, 2006). Third, Poxviruses, which are able to cause fulminant disease in MHCII-Aα$^{-/-}$ mice (as described later), encode a soluble IL-18 binding protein that blocks biological activity of IL-18 in infected mice (Reading and Smith, 2003).

It should be highlighted that the gene of interest is not in absolute certainty located in the identified ~7 Mb genomic region. Based on SNP genotyping by amplifluour technology (Fig. 7.11), some unaffected mice presented to carry the mutation (unaffected mouse number 6), while some affected mice appeared to be wild type (affected mouse number 6). It can be argued that some unaffected mice might have succumbed to WNV_{KUN} challenge, and some affected mice might have survived the infection. This is supported by the fact that based on previous experience in the lab. Occasionally, one or few B/6 wt mice succumbed to WNV_{KUN} infection (unpublished data), and my observation that a small number of MHCII-Aα$^{-/-}$ or *tuara* mice succumb

to flaviviral challenge late in the course of infection (> 20 days pi). Thus, an affected mouse could succumb to WNV_{KUN} infection beyond the 28-day-observation period, and is therefore be recorded as unaffected.

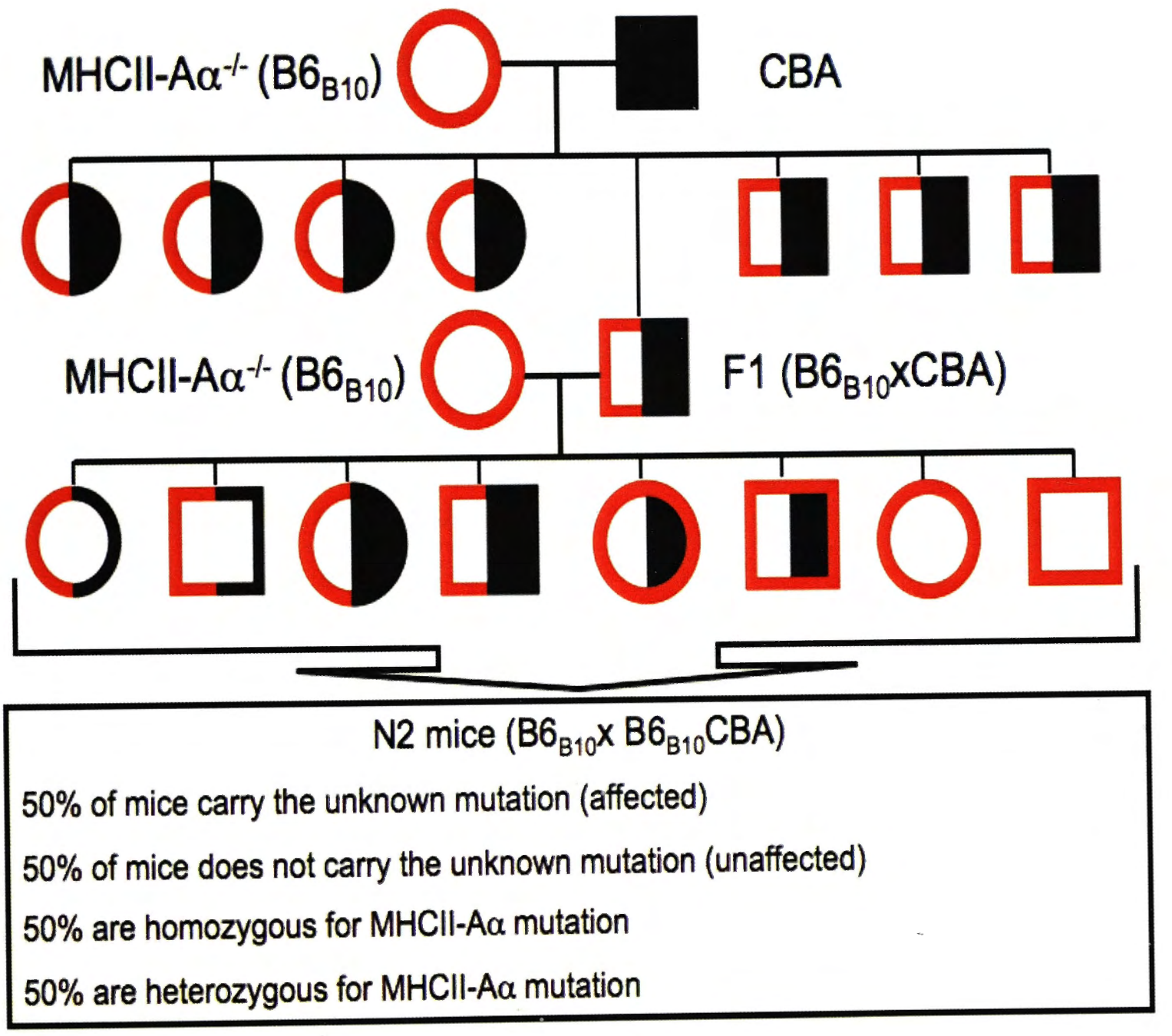


Figure 7.7 Second mouse cross design for genome mapping. MHCII-Aα$^{-/-}$ mice on $B6_{B10}$ background were crossed to CBA mice, and all progenies (F1) produced are heterozygous for MHC-II-Aα$^{-/-}$ and the unidentified mutation. All F1's survived the infection. F1 ($B6_{B10}$xB10) mice were back-crossed to MHC-II-Aα$^{-/-}$ ($B6_{B10}$) mice, and N2 ($B6_{B10}$x$B6_{B10}$.CBA) progenies produced were: 25% homozygous for unknown mutation and homozygous for MHCII-Aα defect, 25% homozygous for unknown mutation and heterozygous for MHCII-Aα defect, 25% heterozygous for unknown mutation and homozygous for MHCII-Aα defect, 25% heterozygous for unknown mutation and heterozygous for MHCII-Aα defect.

Table 7.4 Susceptibility of F1 and various control mouse strains to WNV_{KUN} infection.

Mice[a]	Mortality[b]	ATD
B/6	0% (0/5)	-
CBA	0% (0/5)	-
MHCII-A$\alpha^{-/-}$	100% (5/5)***	10.2 ± 0.45
F1	0% (0/6)	-

[a] Eight-week-old mice were challenged sc with $1x10^3$ PFU of WNV_{KUN}. Morbidity and mortality were recorded for 30 days.

[b] Statistical significance is computed in comparison with B/6 mice control. Asterisks denote significant difference (***, $p < 0.001$).

Table 7.5 Susceptibility of N2 (MHCII-A$\alpha^{-/-}$ x F1) to WNV_{KUN} infection.

MHCII genotype	Number (percentage) of affected	Number (percentage)of unaffected mice	Total
MHCII-A$\alpha^{-/-}$	7 (29%)	5 (25%)	12 (50%)
MHCII-A$\alpha^{+/-}$	6 (21%)	6 (25%)	12 (50%)
Total	13 (54%)	11 (46%)	24 (100%)

Twenty-four 8-week-old N2 mice were challenged sc with $1x10^3$ PFU of WNV_{KUN} via fp, and observed for 28 days to record mortality. All mice were genotyped by PCR for MHC-II-Aα mutation as described in materials and methods.

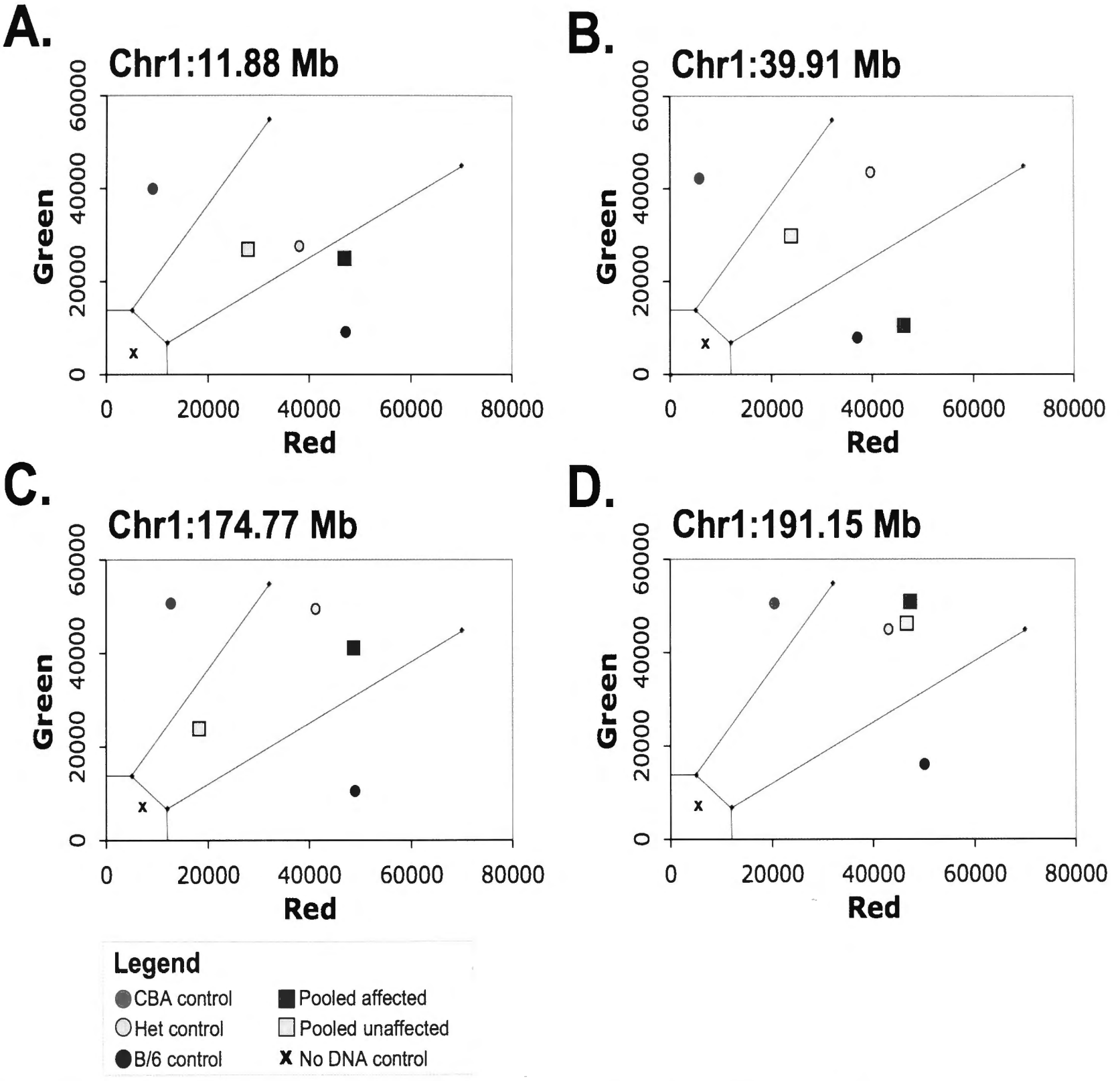


Figure 7.8 Amplifluor SNPs genotyping of pooled DNA samples. Pooled DNA from affected (red square) and unaffected (yellow square) N2 generation mice groups were analysed with the use of ~110 SNPs genetic markers that can distinguish CBA (green fluorescence) against B6 or B10 (red fluorescence) as described in materials and methods. DNA from tail samples of B/6 (red circle), CBA (green circle), and B/6xCBA (yellow circle) mice, and sample buffer without DNA (x) were used as controls. The selected example graphs represent 4 SNPs genomic markers located between 11.88 Mb and 191.15 Mb of chromosome 1, with best approximation and localization of the unknown mutation at Chr1:39.91 Mb.

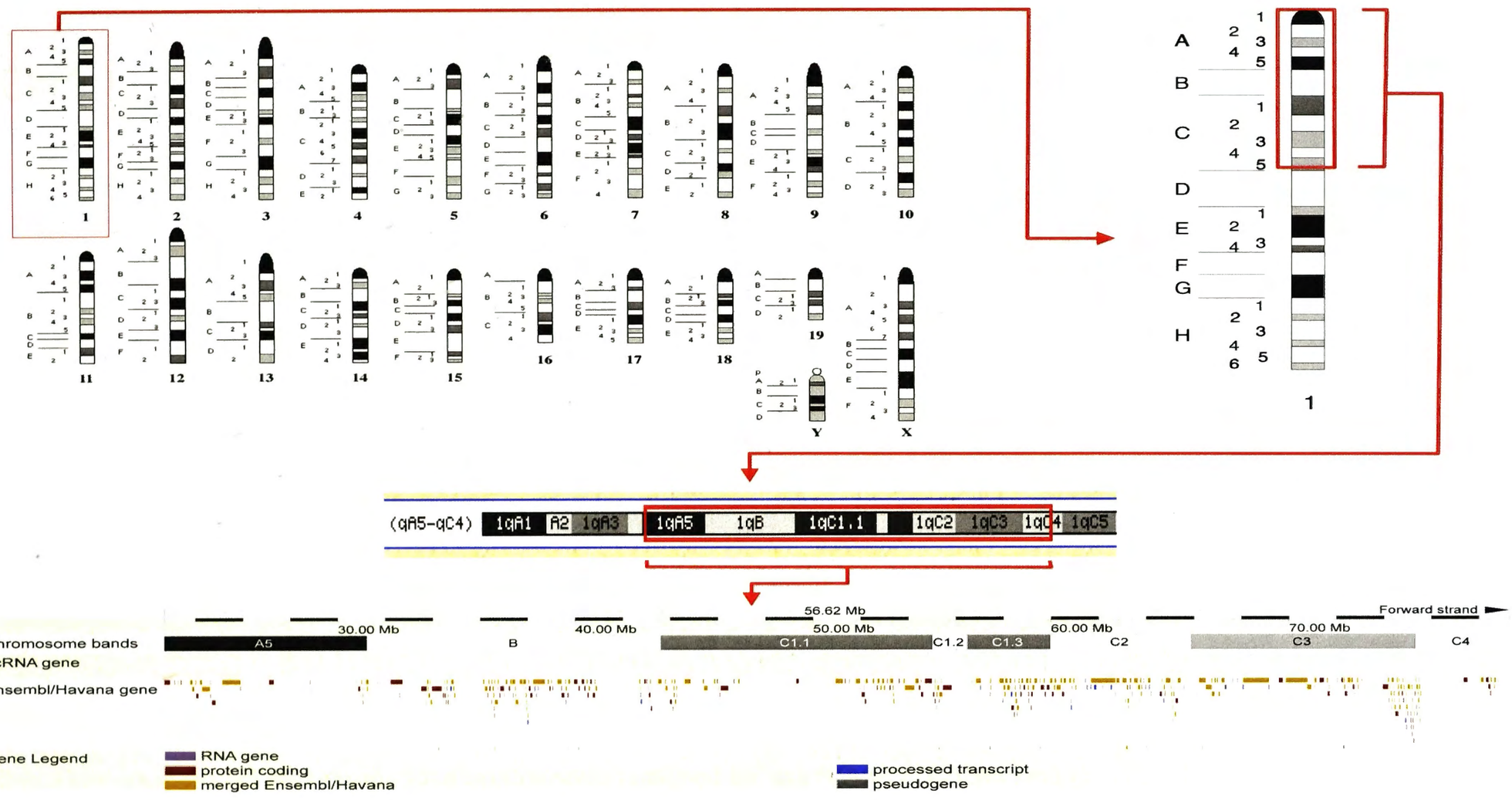


Figure 7.9 Localization of unknown mouse resistance gene. Diagrammatic representation of unidentified mouse resistance gene localized within chromosome 1 at NCBI genome build 37 positions 29.81 to 79.30 Mb. Mouse chromosome idiograms was derived from Larry.Pathology.Washington.Edu 70, a server maintained by Dr David Adler.

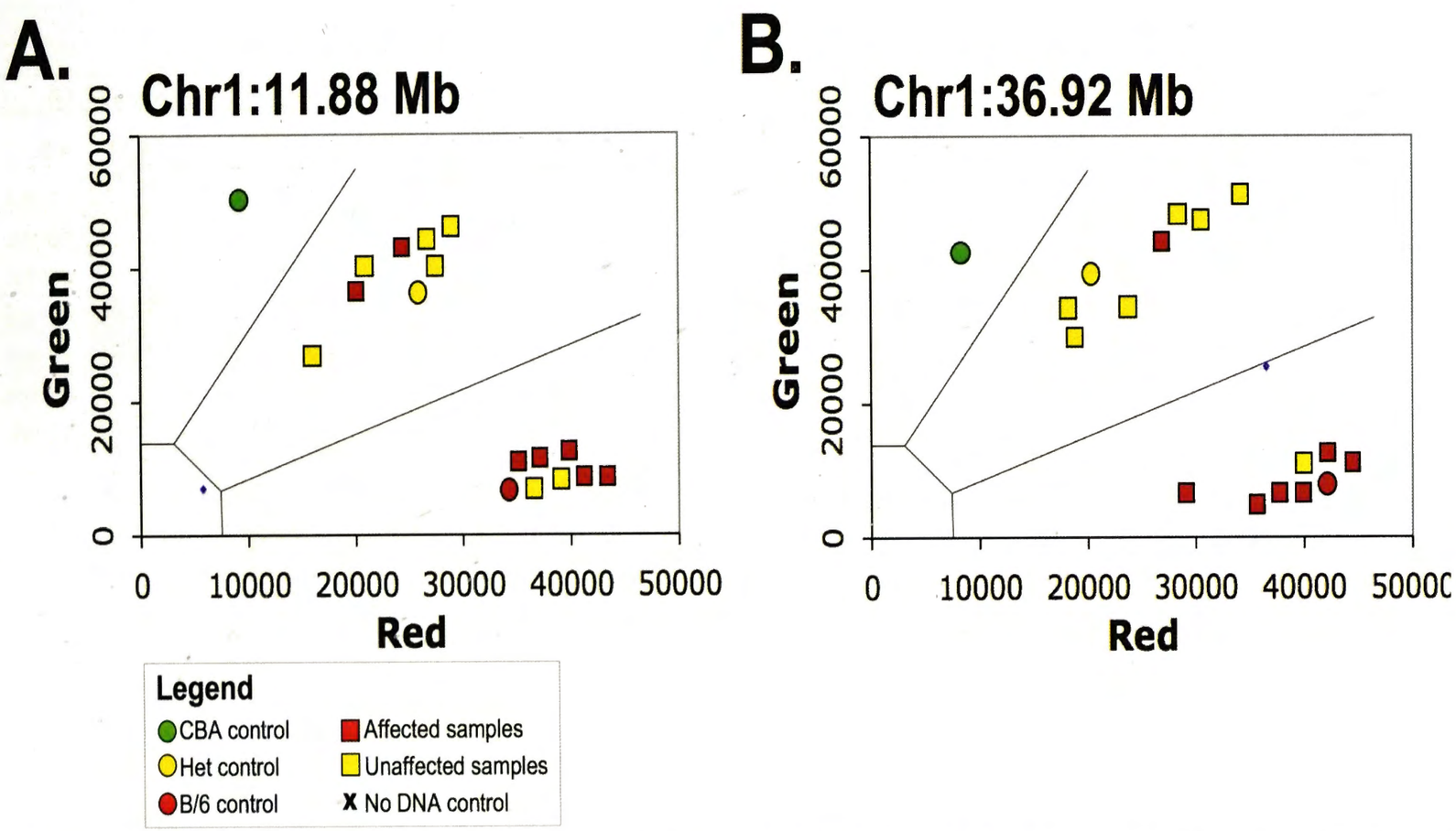


Figure 7.10 Amplifluor SNPs genotyping of individual DNA samples. Individual DNA from representative 7 affected (red squares) and 7 unaffected (yellow squares) N2 generation mice groups were analysed by amplifluor SNPs genotyping, with the use of SNPs genetic markers that can distinguish CBA (green fluorescence) against B6 or B10 (red fluorescence) as described in materials and methods. DNA from tail samples of B/6 (red circle), CBA (green circle), and B/6xCBA (yellow circle) mice, and sample buffer without DNA (x) were used as controls. The example graphs represent SNP genomic location 11.88 Mb and 36.92 Mb of chromosome 1, with best approximation and localization of unknown mutation at Chr1:36.92 Mb.

ID	rs3664960	rs3689181	rs13475788	gnf01.033.663	rs3680400	rs13459182	rs3716368	rs3683684	rs3677697	rs3684654	rs3678662	rs3720900	rs3658333
GP	**11,883,199**	**22,682,233**	**29,807,982**	**36,936,327**	**39,913,715**	**43799240**	**49,547,396**	**68,960,284**	**79,303,391**	**107,414,374**	**134,378,492**	**157,556,435**	**174,778,063**
Aff 01	Het	Het	Het	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
Aff 02	Het	Het	Het	Het	Het	Het	B6	B6	B6	B6	B6	B6	B6
Aff 03	Het	Het	B6	B6	B6	B6	B6	B6	B6	Het	Het	Het	Het
Aff 04	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	Het
Aff 05	B6	B6	B6	B6	B6	B6	B6	B6	Het	Het	Het	Het	Het
Aff 06	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het
Aff 07	B6	B6	B6	B6	B6	B6	B6	B6	Het	Het	Het	Het	Het
Aff 08	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	Het	Het
Aff 09	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
Aff 10	Het	Het	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
Aff 11	Het	Het	Het	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
Aff 12	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
Un 01	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het
Un 02	Het	Het	Het	Het	Het	Het	Het	Het	Het	B6	B6	B6	B6
Un 03	B6	B6	B6	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het
Un 04	Het	Het	Het	Het	Het	Het	Het	Het	B6	B6	B6	B6	B6
Un 05	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	B6	B6
Un 06	B6	B6	B6	B6	B6	B6	B6	B6	Het	Het	Het	Het	Het
Un 07	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het
Un 08	B6	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	B6
Un 09	Het	Het	Het	Het	Het	B6	B6	B6	B6	B6	Het	Het	Het
Un 10	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	B6
Un 11	Het	Het	Het	Het	Het	B6	B6	B6	B6	B6	B6	Het	Het
Un 12	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het	Het

Figure 7.11 Mapping of the unknown virus susceptibility allele to an ~7 Mb region on chromosome 1. Informative haplotypes corresponding to 12 affected (Aff) and 12 unaffected (Un) N2 mice are presented. SNP markers that discriminate between B6 against B10 and CBA, chromosomal location and virus susceptibility phenotypes are shown. **"Het"** (in light shade background) refers to heterozygous $B6_{B10}$ and CBA genotypes in an indicated region, while **"B6"** (in darker shade background) corresponds to homozygous $B6_{B10}$ genotype, but excludes the CBA genotype. The unknown virus susceptibility allele was mapped to a ~50 Mb region of chromosome one (NCBI Build 37.1 position 29,807,982 and 79,303,391 as enclosed by outer bold borders), and narrowed to a ~6.86 Mb region of chromosome 1 (NCBI Build 37.1 position 39,913,715 and 43,799,240 as enclosed by inner bold borders). Mice that succumbed to WNV_{KUN} infection (10^3 PFU injected into the footpad) were classified as affected, while mice that were resistant to infection were classified as unaffected.

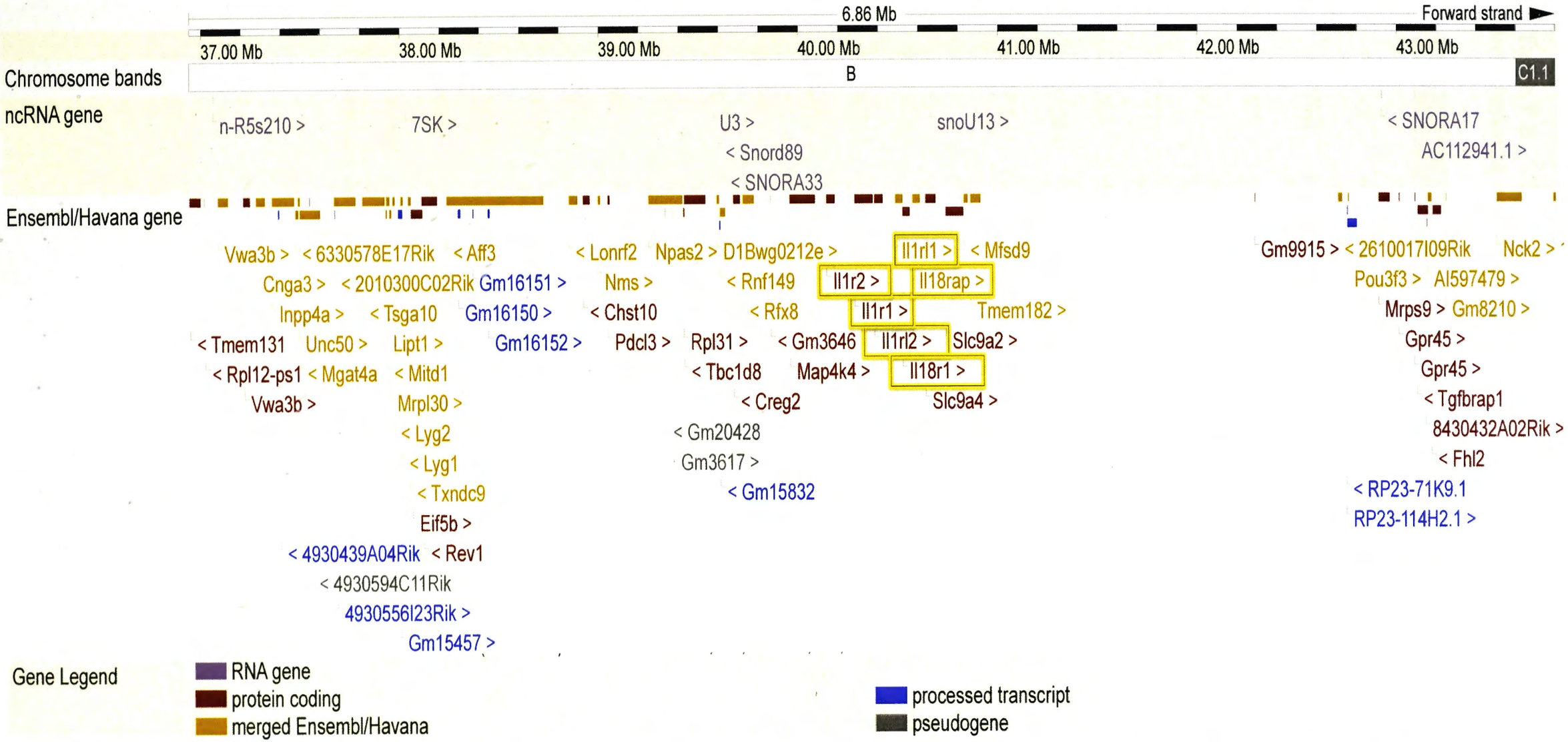


Figure 7.12 List of genes localized within NCBI mouse genome build 37.1 position 39,913,715 and 43,799,240 of chromosome 1. Summary of genes identified within the ~7 Mb localized region of interest in chromosome 1 using the Esembl genome browser. Genes with known immunologic functions were highlighted with yellow-bordered boxes.

Table 7.6 List of mouse orthologs of 22 human genes identified by RNA interference as important for resistance against WNV infection.

Gene	Gene name	NCBI reference	Mouse ortholog
ACRC	acidic repeat containing	NM_052957	no ortholog
AMELY	amelogenin, Y-linked	NM_001143	no ortholog
ANP32B	acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	NM_006401	Chr4:46463774-46485529
ATCAY	ataxia, cerebellar, Cayman type (caytaxin)	NM_033064	Chr10:80667253-80693578
DEFA3	defensin, alpha 3, neutrophil-specific	NM_005217	Chr8:22427088-22428056
DHRS10	hydroxysteroid (17-beta) dehydrogenase 14;HSD17B14	NM_016246	Chr7:52810263-52822688
DOCK4	dedicator of cytokinesis 4	NM_014705	Chr12:41172640-41573073
FAM106A	family with sequence similarity 106, member A;FAM106A	NM_024974	no ortholog
FKBP1B	FK506 binding protein 1B, 12.6 kDa	NM_004116	Chr12:4839981-4848401
IRF3	interferon regulatory factor 3	NM_001571	Chr7:52253018-52258218
KBTBD8	kelch repeat and BTB (POZ) domain containing 8	NM_032505	Chr6:95067234-95079712
KIAA0753	KIAA0753	XM_375397	Chr11:71967431-72020961
LPGAT1	lysophosphatidylglycerol acyltransferase 1	XM_375837	Chr1:193541713-193608134
MSMB	microseminoprotein, beta	NM_002443	Chr14:32955212-32971556
NFS1	NFS1 nitrogen fixation 1 homolog (S. cerevisiae)	NM_021100	Chr2:155904665-155969922
NPIP	nuclear pore complex interacting protein	NM_006985	no ortholog
NPM3	nucleophosmin/nucleoplasmin, 3	NM_006993	Chr19:45822224-45824081
SCGB2A1	secretoglobin, family 2A, member 1	NM_002407	no ortholog
SERPINB7	serpin peptidase inhibitor, clade B (ovalbumin), member 7	NM_003784	Chr1:109296232-109349266
SLC16A4	solute carrier family 16, member 4 (monocarboxylic acid transporter 5)	NM_004696	Chr3:107094148-107115033
SPTBN4	spectrin, beta, non-erythrocytic 4	NM_020971	Chr7:28141402-28232705
TMEM146	transmembrane protein 146	NM_152784	Chr17:56767566-56803879

Original data derived from Krishnan et al. (2008).

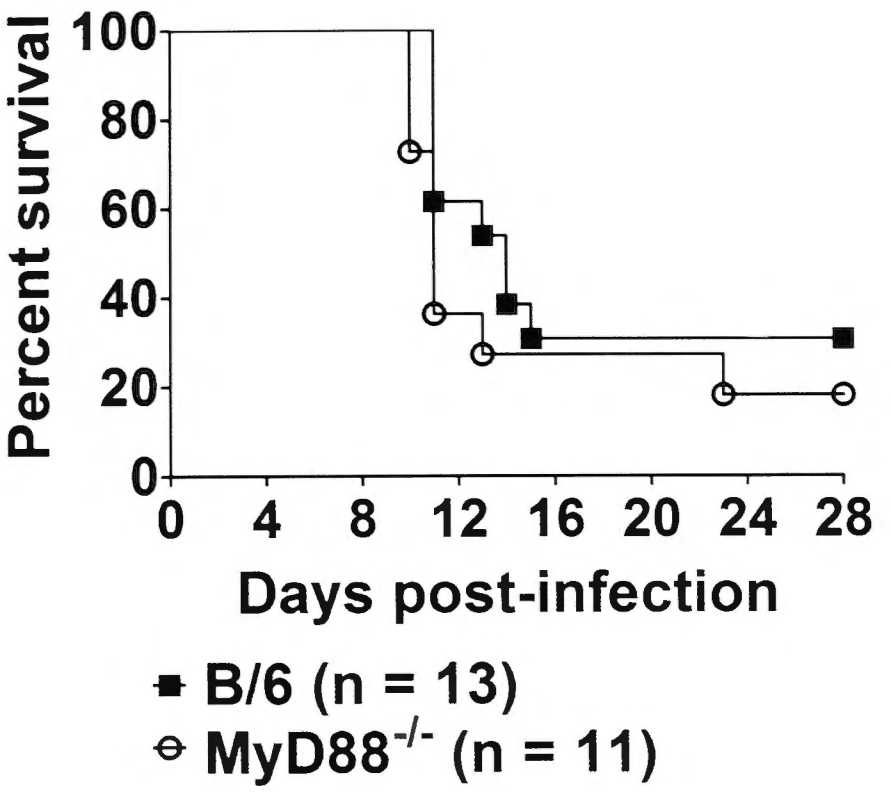
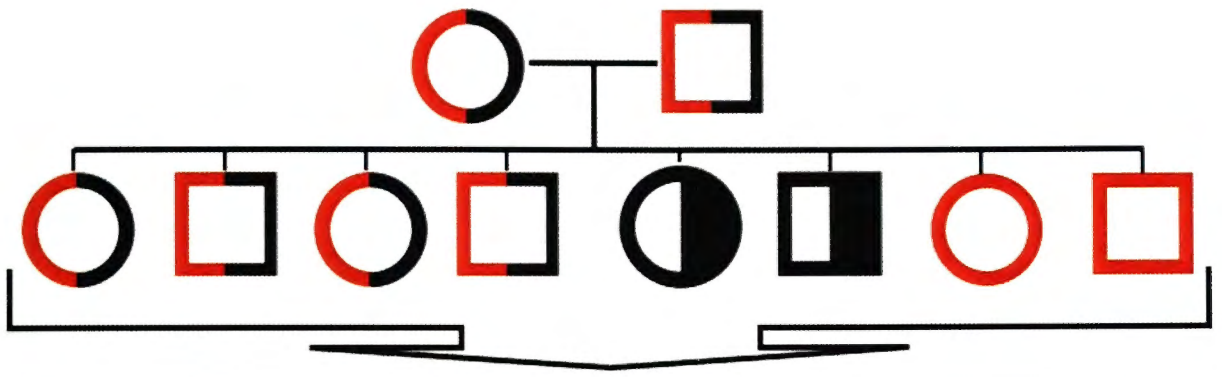


Figure 7.13 Susceptibility of B/6 wt and MyD88$^{-/-}$ mice to flavivirus infection. Groups of 12-week-old mice were infected sc via the footpad with 10^3 PFU of JEV. Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from two independent experiments.

7.2.5 The *tuara* mouse: breeding the recessive susceptibility allele to homozygocity on a background of wt MHC-II. Interpretation of the contribution of the spontaneous mutation identified in MHCII-A$\alpha^{-/-}$ mice to the phenotype of increased susceptibility to viral infection is complicated by the presence of the MHC-II defect. A new mouse strain that is homozygous for the virus susceptibility trait and wt at the MHC-II locus was then generated. Two methodologies were used to allow the segregation of the spontaneous mutation from the *Aa* gene knockout. N2 mice were screened for the MHC-II genotype by conventional PCR, and for the viral susceptibility allele by a genome screen using flanking SNPs markers at the area of interest in chromosome one. Mice that were homozygous for the virus susceptibility allele (expected "affected" phenotype) and heterozygous at the *Aa* gene locus were intercrossed to produce N3 progeny (Fig. 7.14). All progeny N3 mice were expected to be carrying the viral susceptibility allele, whereas only 25% were wt for the *Aa* gene. N3 mice that were identified by conventional PCR as wt for the *Aa* gene were intercrossed to produce N4 progeny mice. As expected, all N4 mice were homozygous for the virus susceptibility trait and wt at the MHC-II locus. These mice were established as a new strain, designated as the *tuara* mice strain. *Tuara* mice are on $B6_{B10}$xCBA genetic background (~75% $B6_{B10}$ and ~25% CBA), and future work may require that the mice be backcrossed onto the B/6 background. However, given the strong susceptibility phenotype of *tuara* mice to virus infections in comparison to B/6 and CBA wt control mice, backcrossing to B/6 was not considered necessary for functional studies of the unknown susceptibility allele in host resistance to infection. The exquisite susceptibility of *tuara* mice was confirmed when challenged with either WNV_{KUN} or JEV (Fig. 7.15A,B). All *tuara* mice succumbed to either WNV_{KUN} or JEV infection with a fulminant phenotype similar to that of MHCII-A$\alpha^{-/-}$ mice.



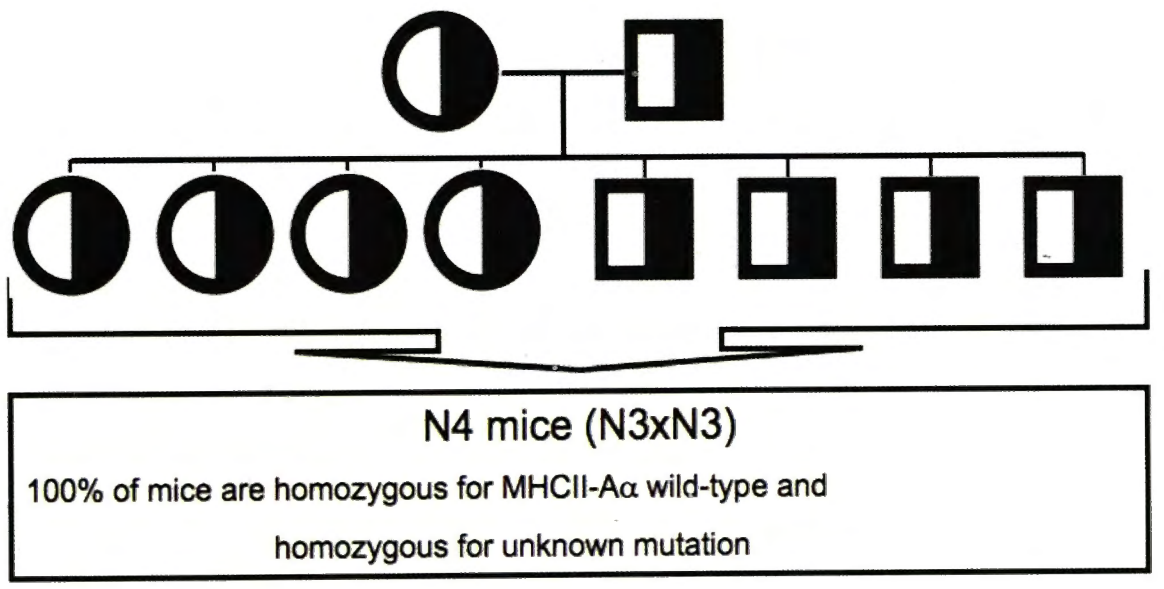


Figure 7.14 Generation of the *tuara* mouse strain. A) N2 ($B6_{B10}xB6_{B10}CBA$) mice identified as $MHCII^{+/-}$ by genotyping and carrying the unknown mutation based on SNP marker detection were crossed. All N3 progenies carry the unknown mutation: 50% homozygous for unknown mutation and heterozygous for MHCII-Aα defect, 25% homozygous for unknown mutation and homozygous for MHCII-Aα defect, 25% homozygous for unknown mutation and wt for MHCII-Aα. B) N3 mice that were homozygous for the unknown mutation and wild-wild type for MHCII-Aα were intercrossed to produce N4 progenies that are 100% homozygous for the unknown mutation and wild-type for MHCII-Aα, which are designated as the *tuara* mouse strain.

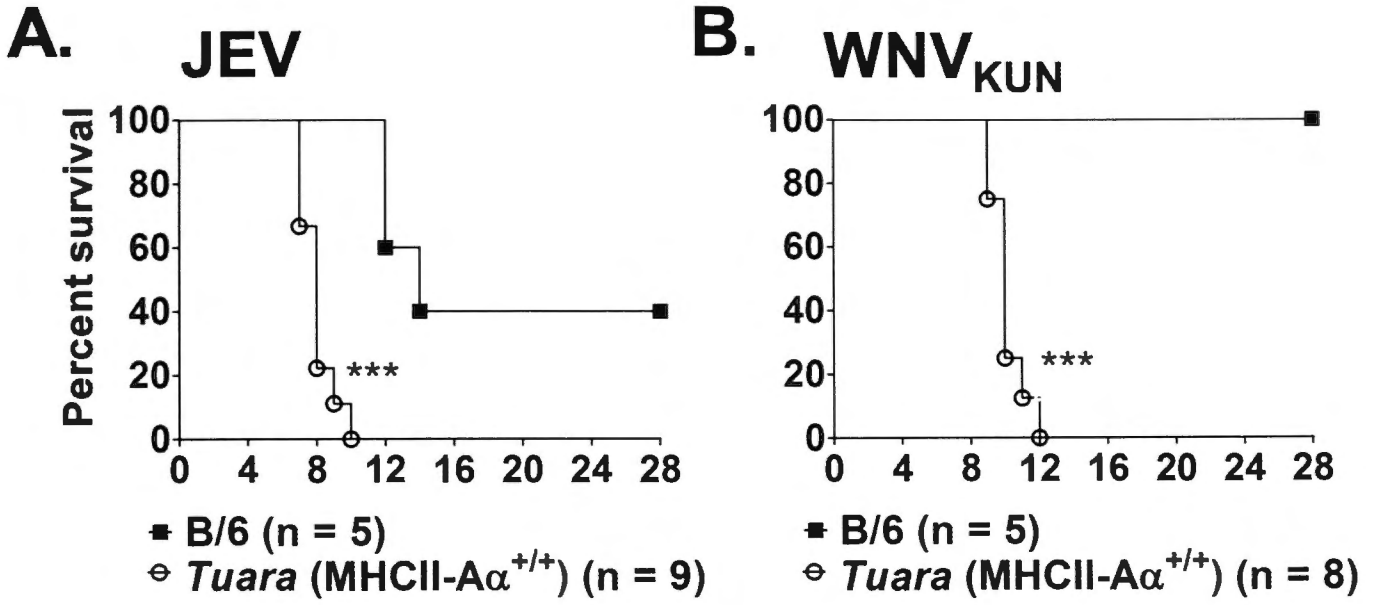


Figure 7.15 Susceptibility of *tuara* mice to challenge with JEV and WNV_{KUN}. Groups of 8-week-old mice were infected sc with 10^3 PFU of JEV (A) or WNV_{KUN} (B). Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from two independent experiments. Asterisks denote statistical significance (***, $P < 0.001$).

7.2.6 High viral burden and widespread viral tropism in *tuara* mice. *Tuara* and B/6 wt mice were infected sc with $1x10^3$ PFU of JEV, and serum and various tissues were collected at 2-day-intervals to determine viral load by plaque titration in Vero cells. Viral load in serum and spleens of B/6 wt mice were undetectable, whereas *tuara* mice displayed persistent and significantly elevated viremia (Fig. 7.15A), and persistently detectable virus in spleens with GMT of $> 10^5$ PFU/g of tissue all throughout the recorded time points (Fig. 7.16B). Also, all infected *tuara* mice presented with rapid and uncontrolled virus dissemination into the CNS. Virus was initially detectable in brains of *tuara* mice on day 4 pi, and peaked at day 7 pi with the GMT ~5 log higher than that in wt mice (Fig. 7.16C). In contrast to undetectable virus in spinal cords of wt mice, virus dissemination into the spinal cords of *tuara* mice appeared already on day 4 pi, and was significantly elevated on day 7 (Fig. 7.16D). Moreover, *tuara* mice displayed widespread viral tropism. Various peripheral tissues including liver, kidney, muscle, and lungs on day 7 pi had detectable virus with GMT levels exceeding $> 5x10^3$ PFU/g of tissue (Fig. 7.16E). This is in contrast to undetectable levels in the case of wt control group. Altogether, these data indicate that even in the absence of the MHC-II defect, *tuara* mice presented with a severe disease phenotype characterized by persistent viremia, widespread viral tropism, and widespread dissemination of the virus into the CNS.

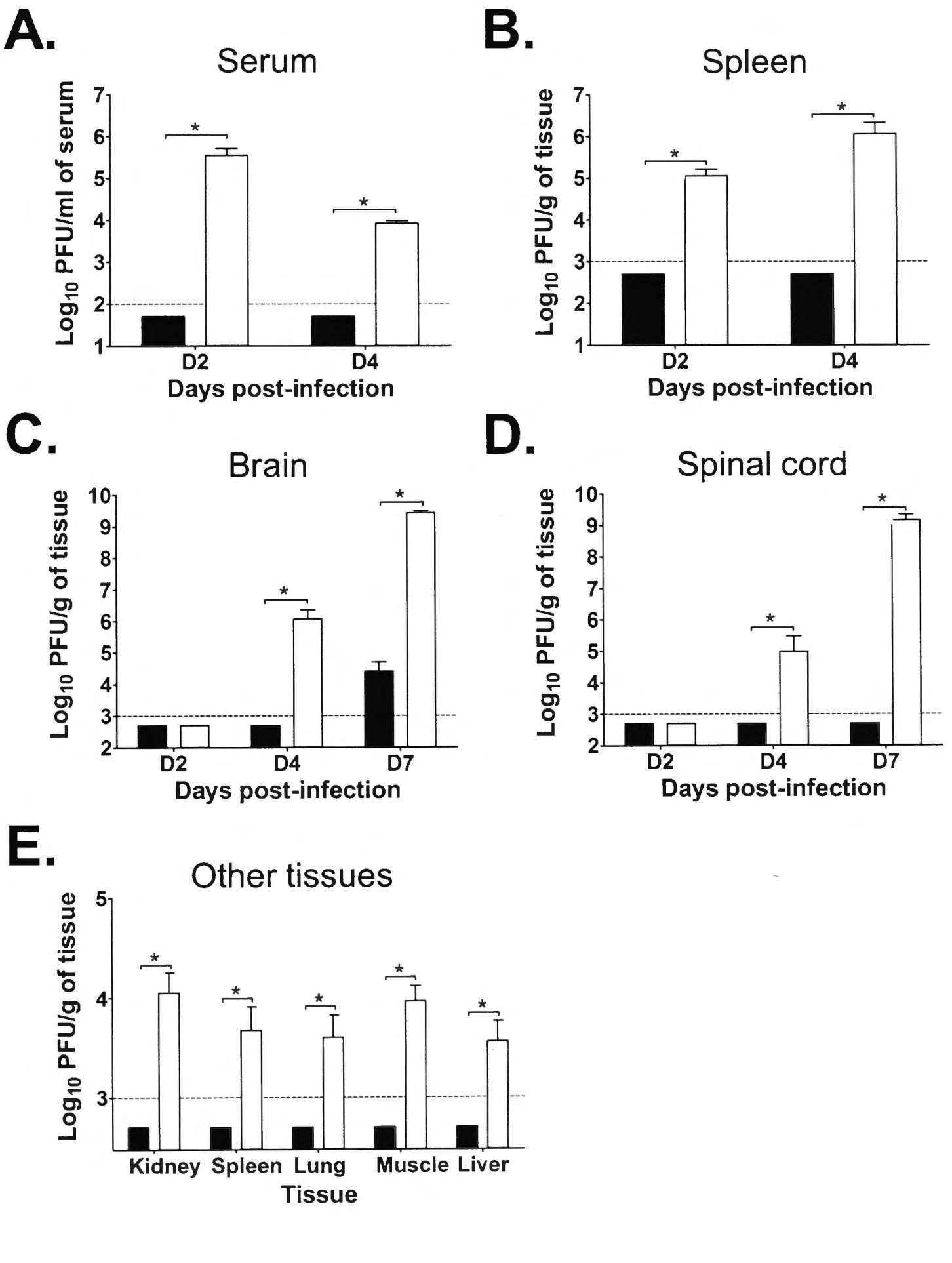


Figure 7.16 High viral burden and widespread viral tropism in *tuara* mice. Eight-week-old mice were challenged with 10^3 PFU of JEV injected into the footpad, and sacrificed at indicated time points for serum and tissue collection. Viral load in (A) serum (B) spleen (C) brain and (D) spinal cord and (E) other tissues (D7 pi only) was measured by plaque titration in Vero cells. Asterisks denote statistical significance (*, $P < 0.05$).

7.2.7 Intact adaptive immune responses in *tuara* mice. Given the rapid, fulminating infection observed in *tuara* mice after exposure to JEV, the putative mutation most likely results from disablement of a critical innate virus resistance gene, and does not involve the adaptive immune responses. This is supported by the finding that, *ex vivo*, JEV infection of bone marrow-derived macrophages from *tuara* mice resulted in significantly elevated viral load in culture supernatants as compared to wt mice (~2 logs difference) (Fig. 7.17). Moreover, antibody and $CD8^+$ T cell immune responses in *tuara* mice remains intact, and were comparable to those in wt mice. The serum anti-JEV IgM response displayed similar kinetics between both mice groups, was first detectable on D4 pi, and increased to similar levels on D7 pi (Fig. 7.18A). As expected, anti-JEV IgG1 and IgG2b were comparable but only marginally detectable on day 7 pi in both mice groups (Fig. 7.18C). The functional activity of humoral response, as measured by 50% plaque reduction neutralization assay, also displayed similar titers between *tuara* and B/6 control mice on D7 pi (Fig. 7.18C). IFN-γ expression of JEV NS4B peptides-specific CD8+ T cells was also comparable between *tuara* and B/6 control mice on D7 pi (Fig. 7.18D).

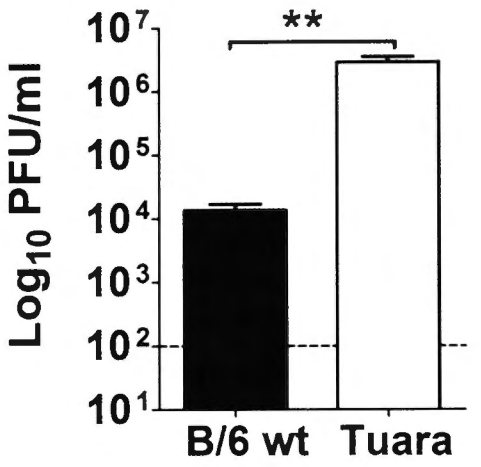


Figure 7.17 Susceptibility of B/6 and *tuara* bone marrow-derived macrophages to infection with JEV. Bone marrow cells were isolated from lower extremity bones of B/6 wt and *tuara* mice as described in materials and methods, and selectively cultured for 7 days towards macrophage differentiation. Mature bone marrow-derived macrophages were then infected with JEV at MOI of 1. Virus load in culture supernatants at 24 pi was later determined by plaque titration in Vero cells. Asterisks denote statistical significance (**, $P < 0.01$).

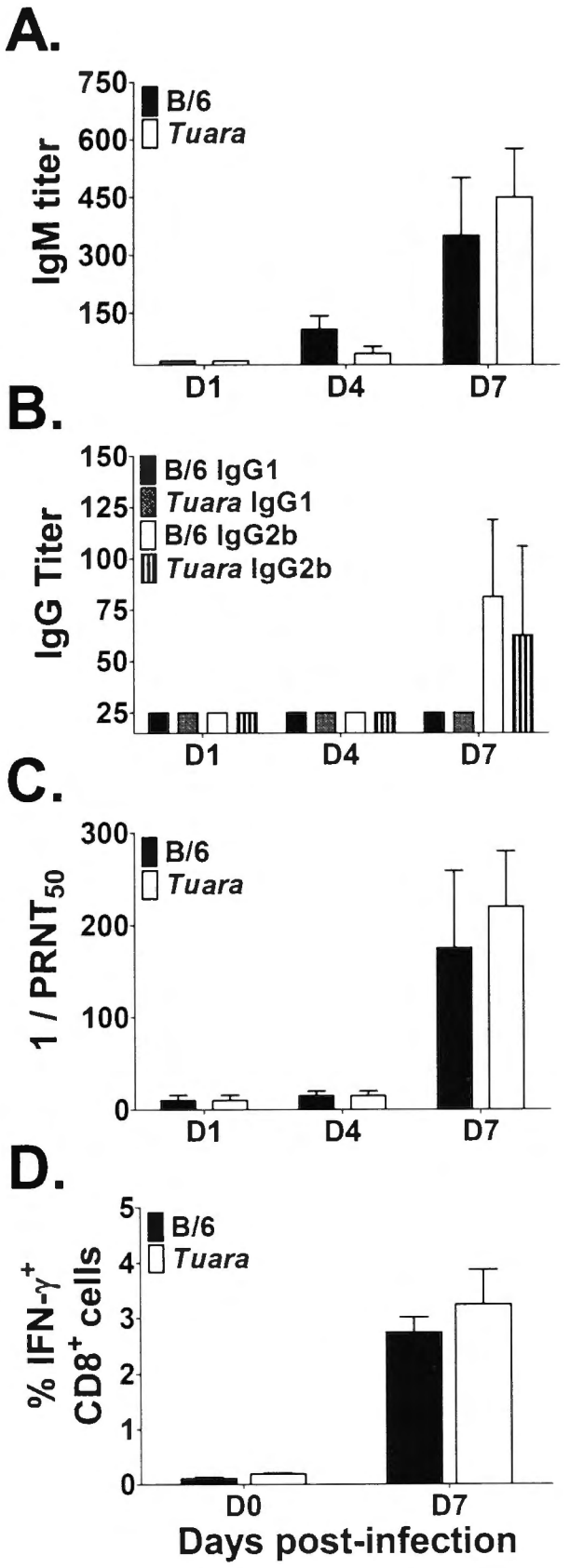


Figure 7.18 Antibody and CD8$^+$ T cell responses in JEV-infected B/6 and *tuara* mice. Eight-week-old *tuara* and wt mice were infected sc with 10^3 PFU of JEV, and serum samples and splenocytes were collected at the indicated time points. Anti-JEV IgM (A) and IgG (B) isotype antibody titers were determined by ELISA. The data presented are reciprocal mean endpoint titers representative of 4 mice per time point with the SEM indicated by error bars. (C) Neutralizing antibody titers determined by plaque reduction neutralization assay. The data presented are mean $PRNT_{50}$ titers representative of 4 mice per time point with the SEM indicated by error bars. (D) Splenocytes harvested at day 0 and 7 pi and stimulated *ex vivo* with H-2D^b-restricted JEV NS4B protein-derived peptides, and IFN-γ production in CD8$^+$ T cells was measured by flow cytometry. Data represent means of 4 mice samples per time point with the SEM indicated by error bars.

7.2.8 Susceptibility of *tuara* mice to DNA viruses. To investigate if the unknown mutation conferring increased susceptibility to flaviviral infection has a broader role in the defense against diverse groups of viruses, *tuara* and control mice (B/6 and CBA) were challenged with a DNA virus. As expected B/6 and CBA mice challenged iv with $1x10^5$ PFU of vaccinia virus-Western Reserve strain ($VACV_{WR}$) did not show mortality nor did they present with any signs of disease. In sharp contrast, $VACV_{WR}$ infection of *tuara* mice resulted in a fulminant disease phenotype, with 100% mortality and MST of 4.4 ± 0.5 days (Fig. 7.19).

It should be noted that an earlier study by Chaudhri *et al* (2006) have showed that Ectromelia virus (ECTV) challenge in MHCII-$A\alpha^{-/-}$ mice used in this work, displayed 100% mortality. However, >90% of mice succumbed to infection only after 15 days pi. A similar challenge with ECTV in the same mouse strain during my time, in contrast, revealed a fulminant disease phenotype of complete and rapid lethality, with all mice dying by day 5 pi (data not shown). This suggests that the spontaneous mutation in the MHCII-$A\alpha^{-/-}$ mice occurred during the past ~8 years.

These findings strongly suggest that the susceptibility allele is pivotal in the genetic resistance to a wide range of viral infections, with a possibility of an even broader role in host resistance against bacterial and other microbial pathogens.

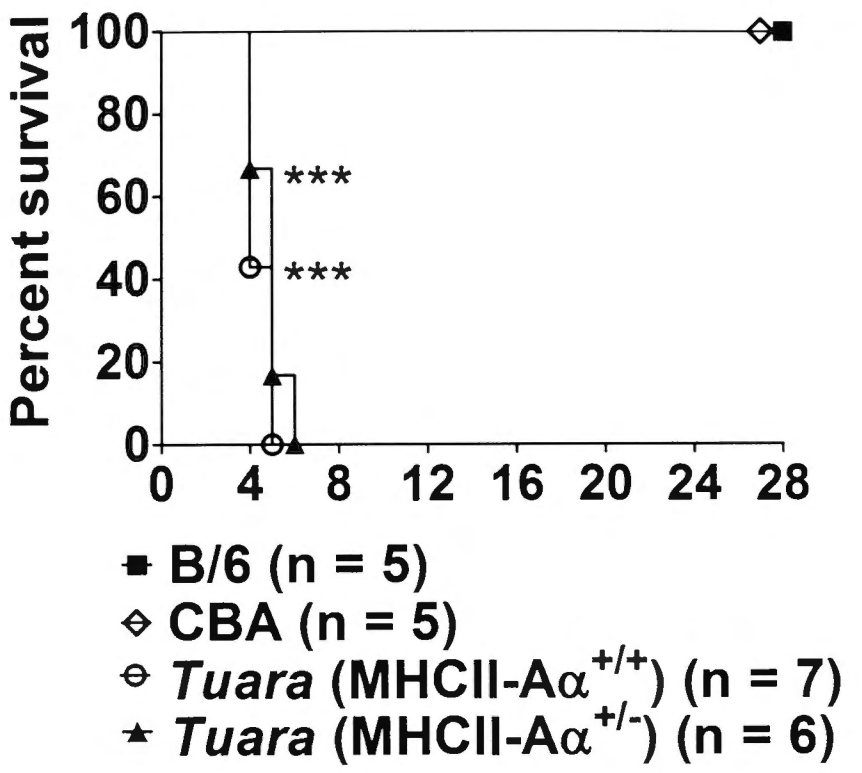


Figure 7.19 Susceptibility of *tuara* mice to a DNA virus. Groups of 8-week-old mice were infected ip with 10^6 PFU of $VACV_{WR}$. Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from two independent experiments. Asterisks denote statistical significance (***, $P < 0.001$).

7.2.9 The unknown susceptibility allele display partial inactivation of the interferon (IFN) antiviral pathway. One viral infection model that could perhaps explain the fulminant disease phenotype observed in *tuara* mice is a defect in the type 1 IFN pathway. IFN-α-$R^{-/-}$ mice infected with WNV_{NY99} (Samuel and Diamond, 2005), WNV_{KUN} (Daffis *et al.*, 2011), JEV (Lobigs *et al.*, 2009), or VACV (van den Broek *et al.*, 1995) display rapid and complete lethality. However, it should be noted that, compared to *tuara* mice, the MST is shorter when IFN-α-$R^{-/-}$ mice were challenged with WNV_{KUN} (9 ± 2 days in *tuara* mice vs. 5 ± 0.4 in IFN-α-$R^{-/-}$ mice) (Daffis *et al.*, 2011; Lobigs *et al.*, 2009). A possible contamination with an in-house IFNR-α-$R^{-/-}$ mouse strain to MHCII-Aα$^{-/-}$ mice was also ruled out, since genotyping of MHCII-Aα$^{-/-}$ revealed that they were wt for the type 1 interferon receptor (data not shown). While no known components of the IFN pathway map to the ~7 MB chromosomal region putatively encompassing the susceptibility allele, it remained important to verify an intact type I IFN response in the *tuara* mouse.

ELISA determination of interferon production in JEV-infected bone marrow-derived macrophages from *tuara* and B/6 control mice was performed (Fig. 7.20). Macrophages from *tuara* mice secreted high levels of IFN-α following infection with JEV for 24 hours (>400 pg/ml), which exceeded IFN-α levels in infected B/6 control cultures (Fig. 7.20A). In addition, serum IFN-α levels during 48 hours of JEV infection were comparable in *tuara* and B/6 control mice (Fig. 7.20B). To determine the integrity of downstream IFN signalling and antiviral activity, bone marrow-derived macrophages from *tuara* and B/6 control mice were treated for 16 hrs with titrated concentrations of IFN-α, and later infected with JEV at MOI of 1. Virus growth inhibition was subsequently determined by plaque titration in Vero cells. Pre-treatment of bone marrow-derived macrophages from *tuara* and B/6 control mice with 250 or 100 IU IFN-

α completely abolished growth of JEV, while untreated cells showed > 2 logs difference in virus yield at 24 h pi in *tuara* mouse-derived cells in comparison to that in a control culture at 24 h pi (Fig. 7.21). However, pre-treatment of bone marrow-derived macrophages from *tuara* mice with 1 IU of IFN-α only resulted in marginal inhibition of virus growth as opposed to almost complete inhibition observed in B/6 mouse-derived cells. The difference is even more pronounced (> 2 logs difference) when bone marrow-derived macrophages were treated with much lower concentration of IFN-α at 0.1 IU.

Altogether, these data suggest that the susceptibility allele impacts on type I IFN signalling and antiviral responses. The spontaneous mutation may involve a complete disruption of an IFN-inducible antiviral effector gene or a partial dysfunction of a key intermediate gene involved in signal transduction for IFN responses.

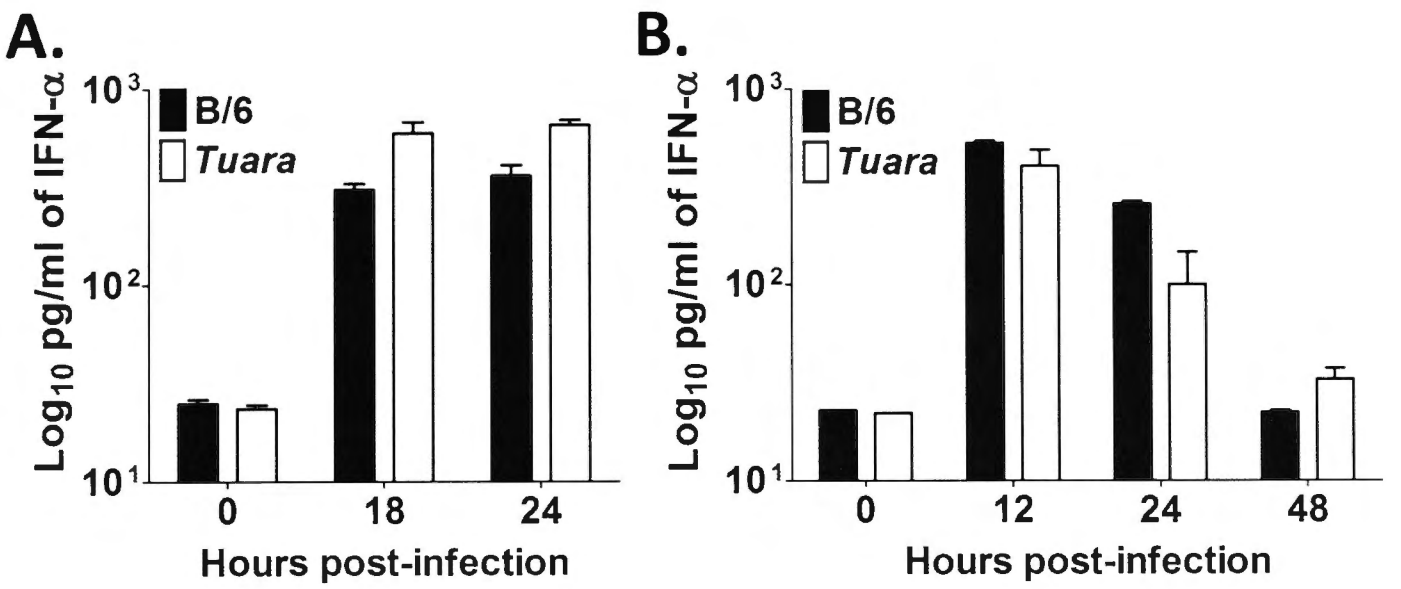


Figure 7.20 IFN-α production in JEV-infected *tuara* and B/6 mice. Bone marrow cells were isolated from lower extremity bones of B/6 wt (black bar) and *tuara* (white bar) mice as described in materials and methods, and selectively cultured for 7 days towards macrophage differentiation (A). Mature macrophages were infected with JEV at MOI of 1. IFN-α levels in culture supernatants at indicated time points were determined. (B) IFN-α production in serum in JEV-infected B/6 wt and *tuara* mice at indicated time points. Each bar graph represents 3 biological samples

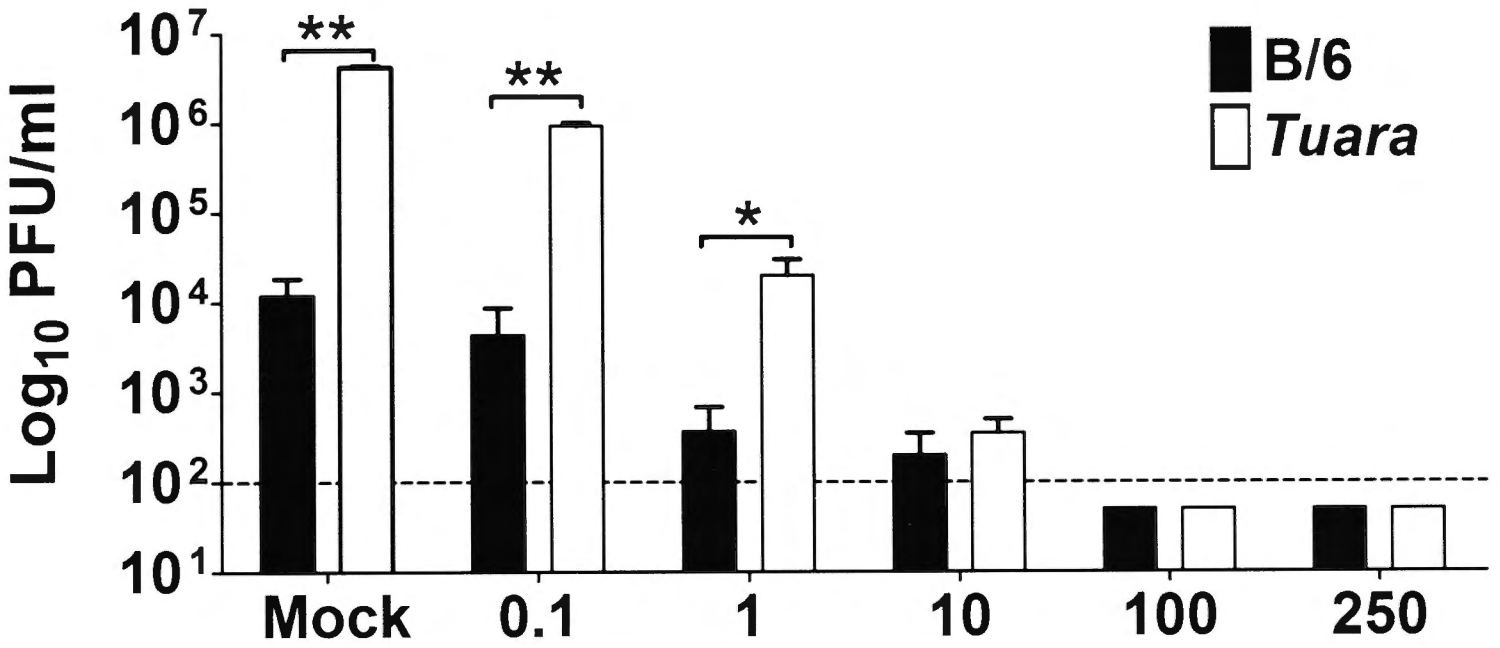


Figure 7.21 Inhibition of JEV replication after IFN-α stimulation of bone marrow-derived macrophages from B/6 wt and *tuara* mice. Bone marrow cells were isolated from lower extremity bones of B/6 wt (black bar) and *tuara* (white bar) mice as described in materials and methods, and selectively cultured for 7 days towards macrophage differentiation. Mature macrophages were treated with 250, 100, 10, 1, 0.1 IU of IFN-α or mock-treated with tissue culture media for 16 hrs. Macrophages were then infected for 24 hrs with JEV at MOI of 1. Viral load in culture supernatants were measured by plaque titration in Vero cells. Each bar graph represents 3-6 samples from two independent experiments. Asterisks denote statistical significance (*, $P < 0.05$; **, $P < 0.01$).

7.2.10 Customized sequence capture and re-sequencing identified candidate mutations responsible for the fulminant disease phenotype. At this point, the candidate gene was successfully segregated from the defective MHC-II gene in a new mouse strain, *tuara*. In summary, *tuara* mice are homozygous for the virus susceptibility trait and wt at the MHC-II locus, and when challenged with JEV, displayed a fulminant disease phenotype characterized by i.) complete and rapid mortality, ii.) early neuroinvasion, iii.) widespread virus dissemination into the CNS iv.) widespread viral tropism. In addition, the fulminant disease phenotype observed in *tuara* mice is most likely attributed to a mutation of a critical innate immune response gene, given that *tuara* mice presented with i.) intact antibody and ii.) CD8+ T cell immune responses, and that bone marrow derived-macrophages from *tuara* mice displayed increased susceptibility to JEV infection. Another significant finding was that the unidentified gene was not limited to conferring resistance against flaviviral infection, but also provided defence against infection with a DNA virus.

It should also be highlighted that the original MHCII-A$\alpha^{-/-}$ mice and/or bone marrow-derived macrophages from MHCII-A$\alpha^{-/-}$ mice displayed susceptibility to a low virlunce flaviviruses, including WNV_{KUN}, DENV-2, and YFV. A more surprising discovery was the finding that ic challenge in parent MHCII-A$\alpha^{-/-}$ mice strain with a highly attenuated live vaccine, ChimeriVax-JE, resulted in complete lethality. This is remarkable, especially when mice deficient in the type 1 interferon receptor (Lobigs *et al.*, 2009) or B cells (Chapter 8) tolerated vaccination with ChimeriVax-JE. Accordingly, I hypothesized that a combination of crucial innate immune response factor plus deficiency in antibody production (as a result of the $CD4^+$ T cell deficiency in MHCII-A$\alpha^{-/-}$ mice) has contributed to the lethal phenotype of MHCII-A$\alpha^{-/-}$ mice in ChimeriVax-JE infections. This was later supported, given that 100% of B cell

sufficient and antibody -competent *tuara* mice, survived ic challenge with ChimeriVax-JE (data not shown).

To elucidate the remarkable findings described in this chapter, a forward genetics approach was employed: starting with an observable phenotype of increased susceptibility to viral infection to localization of the susceptibility allele responsible for the phenotype. In this hypothesis-free driven exercise, preliminary data was obtained with the gene of interest identified and narrowed within a ~7 Mb region of chromosome 1 at reference positions 36.94 to 43.80 Mb (NCBI mouse genome Build 37.1). However, no identifiable gene involved in IFN pathway signalling is located within the region. To identify the unknown mutation, two options were available: i.) Targeted exome re-sequencing or ii.) customized sequence capture and re-sequencing. The former is a robust target-enrichment strategy designed to selectively sequence only the exonic regions of the genome, and is based on the assumption that the mutation is confined to the coding regions (Bamshad *et al.*, 2011). A significant limitation includes the exclusion of sequencing non-coding regions that may be responsible for the fulminant disease phenotype observed in *tuara* mice. Although uncommon, genome wide association studies have correlated diseases to particular SNVs in the non-coding regions of the genome, including introns and intergenic regions (reviewed in (Manolio, 2010)). On the other hand, customized sequence capture using the Nimblegen hard-array and re-sequencing using Illumina HiSeq system, selectively sequences a defined region of interest (from 5 to 50 Mb) initially narrowed by chromosomal linkage analysis (Sulonen *et al.*, 2011). An advantage of customized sequence capture is that it sequences all genomic regions including exons, introns, UTRs, and intergenic regions. Additionally, the high depth coverage (more than 50 times) provided by the subsequent second generation sequencing (Illumina HiSeq) gives the greater likelihood of detecting

large insertions or deletions. Hence, a decision to proceed with customized sequence capture and re-sequencing was made.

However, as earlier discussed, it should be highlighted that the gene of interest is not at full certainty located in the initially identified 7 Mb genomic region. Given that there were variants outside of the expected outcome in the individual SNP genotyping of the N2 mice during genome mapping, a customized sequence capture array was designed to include a wide genomic area (from 29 Mb to 79 Mb of chromosome 1) to provide a greater degree of confidence in detecting the mutation.

Pure quality genomic DNA samples (> 500μg/sample, concentration of > 1μg/μl) from tails of three *tuara* mice were prepared by the Animal Phenomics Facility (APF). Customized sequence capture bait/primer design was provided by the Nimblegen bioinformatics team. Sequence capture by Nimblegen hard array hybridization and re-sequencing by Illumina HiSeq systems were performed by the next generation sequencing team of the Australian Genomics Resource Facility (AGRF) in Melbourne, Australia. Bioinformatics teams from AGRF and APF independently provided analysis of the raw sequence data generated from Illumina HiSeq re-sequencing. The analysis revealed that, due to reasons not yet clear, only 2 out of 3 samples were successful during the sequence capture step. The analysed sequence data, provided by the 2 samples that were successfully captured and sequenced, revealed 3 candidate protein-coding gene mutations that can be responsible for the observed fulminant disease phenotype: Prss40 (Protease, Serine, 40), Cyp20a1 (cytochrome P450, family 20, subfamily a, polypeptide 1), and STAT1 (Signal Transducers and Activators of Transcription) (Table 7.7-8).

The Prss40 gene is localized at position 34,600,820-34,617,788 bp of chromosome 1. The protein product is known to have proteolytic action for an undefined cellular function (Mouse Genome Informatics). It is largely expressed in testicular tissue, and limitedly expressed in all other tissues (Fig. 7.22). The mutation identified is a G>A transition at genomic coordinate 34,616,619 of chromosome 1, and results in arginine to tryptophan substitution at amino acid position 79 of the Prss40 protein. The second candidate gene, Cyp20a1, is localized at position 60,400,167-60,444,904 bp of chromosome 1. As a cytochrome P450 enzyme, it is involved in oxidation-reduction biological processes for yet undefined cellular function (Mouse Genome Informatics). Notably, it is highly expressed in cells of monocytic lineage (Fig 7.23). The mutation is a 6-nucleotide deletion at a splice site region of the gene at genomic coordinates 60,409,744 to 60,409,749 of chromosome 1. Lastly, the STAT1 gene, is localized at position 52,176,282-52,218,704 bp of chromosome 1. It has a protein product that is involved in diverse cellular processes with well-defined functions (Mouse Genome Informatics). Most importantly, it is a key intermediate protein important for type 1 IFN signalling (reviewed in (Sadler *et al.*, 2008)). The G>T transversion at reference position 52,179,547 bp of STAT1 gene results in a nonsynonymous mutation, with a Tryptophan to Leucine substitution at amino acid position 37 of the STAT1 protein. It has been previously shown that $STAT1^{-/-}$ mice displayed significantly increased susceptibility to infection with diverse group of viruses (Muller *et al.*, 1994) Although *tuara* mice presented with less severe disease outcome relative to $STAT1^{-/-}$ mice in terms of survival times, it may be that the STAT1 mutation in *tuara* mice only resulted in partial dysfunction of STAT1 protein. Treatment with lower concentrations IFN-α of bone-marrow-derived macrophages from *tuara* mice, relative to B/6 wt mice, only resulted in partial antiviral activity, supporting a probable partial dysfunction of the STAT1 protein. Given its predominant role in antiviral signalling pathways, it is most

likely that the susceptibility allele is a result of partial dysfunction of the STAT1 protein.

Further experiments are needed to confirm STAT1, and rule out Prss40 and Cyp20a1, as the causal gene mutation for the fulminant disease phenotype. Thus, the initial comprehensive work done in this chapter leads to a likely identification and confirmation of a partial loss-of-function STAT1 mutation with profound biological consequences. The STAT1 mutation in *tuara* mice is a distinct mutation when compared to the 10 characterized $STAT1^{-/-}$ mice (3 chemically induced and 7 targeted mutations) listed on the Mouse Genome Informatics database, given the unique locus of the mutation.(Table 7.9). In terms of clinical relevance, particular mutations in the human STAT1 gene had been associated with increased susceptibility to microbial infections and, at certain times, autoimmunity (Figure 7.24 and Table 7.10). Since the initial discovery of human STAT1 mutation in 2001 (Dupuis *et al.*, 2001), an increasing number had been identified for the past years, and was recently classified by Boisson-Dupuis *et al.*, (2012) into 4 subclasses: i.) autosomal recessive with partial STAT1 deficiency, ii.) autosomal recessive with complete STAT1 deficiency, iii.) autosomal dominant with loss-of-function STAT1 deficiency, and iv.) autosomal dominant with gain-of-function STAT1 disorder (Table 7.10).

Lastly, this chapter also highlights the issue of frequency of spontaneous mutations generated in inbred wt or knockout mouse strains. With 30,000 mouse structural genes and average mutation frequency per gamete per locus of 10^{-5}, it is though that the mutation rate in an inbred mouse is 0.3 per gamete (Bailey, 1978). Subline divergence of inbred mouse strains due to spontaneous mutations has been reported in multiple studies (Acton *et al.*, 1973; Crusio *et al*, 1991; Grewal, 1962, Keightley, 1998;

Whitmore and Whitmore, 1985; van Abeleen and Hughes, 1986). The advantage of the mutation discovered in this chapter is that the phenotype is patently observable. Problems can arise when a spontaneous mutation generated produces a subtle phenotypic effect, which can affect the observational outcome, and bring about unwarranted and incorrect conclusions derived from experimental findings. This may, in part, explain reported variability in outcomes or published results produced by different laboratories, using a similar wt or knockout mouse strain maintained in their respective facilities. Certain recommendations were put forward to avoid subline divergence, and one is regular restocking of mouse colonies from common international frozen embryo source (Bailey, 1978). As full genome sequencing becomes substantially less expensive, it may be best that, in the future, complete genome sequences of all mouse strains used in any study be performed before publication.

Table 7.7 List of candidate Indel mutations

Mutation	Start-coordinate	End-coordinate	Variant Length	Gene	Gene desccription
Deletion					
	60409744	**60409749**	**-6**	**CYP20A1**	**cytochrome P450, family 20, subfamily A, polypeptide 1 [Source:MGI Symbol;Acc:MGI:1925201]**
	74606950	74606952	-3	PLCD4	phospholipase C, delta 4 [Source:MGI Symbol;Acc:MGI:107469]
	40081149	40081150	-4	MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4 [Source:MGI Symbol;Acc:MGI:1349394]
	90108571	90108572	-2	UGT1A5	UDP glucuronosyltransferase 1 family, polypeptide A5 [Source:MGI Symbol;Acc:MGI:3032634]
Insertion					
	90163100	90163100	+1	HJURP	Holliday junction recognition protein [Source:MGI Symbol;Acc:MGI:2685821]

Note: Highlighted gene indicates mutation identified in 2 out of 2 samples.

Table 7.8 List of candidate SNV mutations

SNP genotype	Gene coordinate	Reference base	Variant base	Gene	Gene description
Homozygous					
	34616691	**G**	**A**	**PRSS40**	**protease, serine, 40 [Source:MGI Symbol;Acc:MGI:1270857]**
	52179547	**G**	**T**	**STAT1**	**signal transducer and activator of transcription 1 [Source:MGI Symbol;Acc:MGI: 103063]**
Heterozygous					
	151677280	G	T	PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent) [Source:MGI Symbol;Acc:MGI:1195256]
	171966128	C	T	DDR2	discoidin domain receptor family, member 2 [Source:MGI Symbol;Acc:MGI:1345277]

Note: Highlighted gene indicates mutation identified in 2 out of 2 samples.

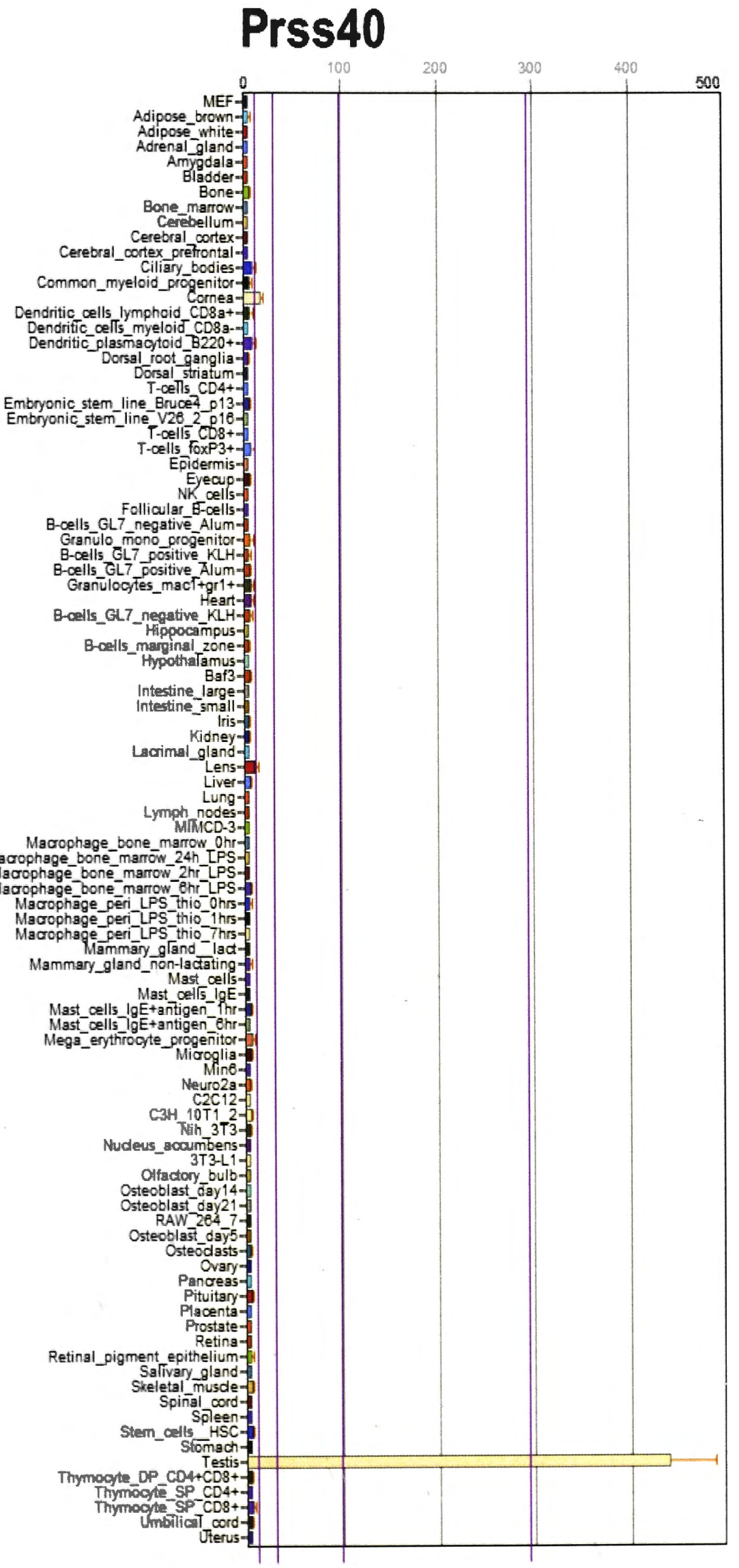


Figure 7.22 Expression profile of Prss40 gene. High-throughput gene expression profiling of Prss40 gene using the gene annotation portal www. bioGPS.org.

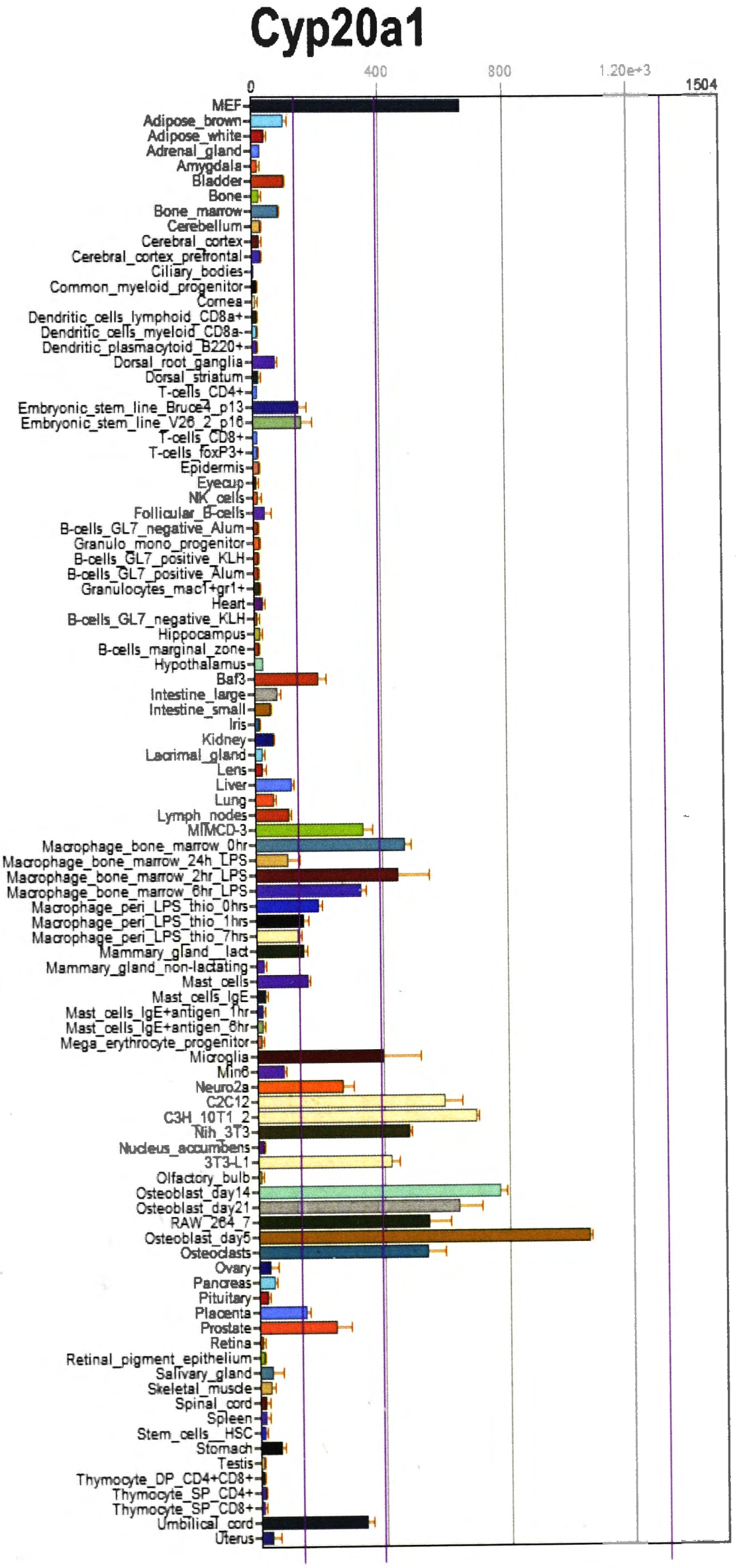


Figure 7.23 Expression profile of Cyp20a1 gene. High-throughput gene expression profiling of Cyp20a1 gene using the gene annotation portal www. bioGPS.org.

Table 7.9 List of STAT1 knockout mice

Mouse	Mutation description	Reference
Chemically-induced (ENU)		
Stat1^{tae}	T-to-A transversion at nucleotide position 2263, M654K	Yates *et al.*, 2006
Stat1^{m1Btlr}	T-to-A transversion in exon 11, V319E	Crozat *et al.*, 2006
Stat1^{m2Btlr}	T-to-A transversion at nucleotide position 31788; frameshift occurs after codon 544 that introduces a premature stop codon following codon 561	Whitley *et al.*, 2008
Targeted Knockout		
Stat1$^{tm1.1Mmul}$	A loxP site was inserted upstream of exon 6. A floxed neo cassette was inserted downstream of exon 10. Cre-mediated recombination removed the neo cassette and left exons 6 through 10 floxed.	Wallner *et al.*, 2012
Stat1$^{tm1.2Mmul}$	Cre-mediated recombination removed exons 6 through 10 and the neo cassette.	Wallner *et al.*, 2012
Stat1^{tm1Div}	A neomycin resistance cassette replaced 5.7 kb of sequence, including 3 exons and a portion of a fourth encoding amino acids 221-365	Durbin *et al.*, 1996
Stat1^{tm1Mam}	A targeting vector was designed to place a loxP flanked neomycin resistance gene upstream of the first untranslated exon followed by a loxP site downstream of the first translated exon. The neo cassette was removed by germ-line cre-mediated resombination leaving loxP sites flanking the first two untranslated exons and the first translated exon.	Klover *et al.*, 2010
Stat1^{tm1Rds}	A neomycin resistance cassette replaced the first three coding exons and 0.7kb of 5' upstream flanking sequence.	Meraz *et al.*, 1996
Stat1^{tm1Tdec}	Exon 22 and 23a were replaced with a segment containing exon 22, a floxed neo cassette and exon 23 containing a nucleotide substitution that results in an amino acid substitution of an alanine for a serine at position 727 (S272A), a site within the transactivation domain. The neo cassette was removed by germ-line, cre-mediated recombination.	Varinou *et al.*, 2003
Stat1^{tm1Uvin}	Nucleotide substitution(s) results in the amino acid substitution of glutamine for glutamic acid at position 705 (E705Q) that abolishes SUMO conjugation.	Begitt *et al.*, 2007

Knockout mouse list and mutation description adapted from Mouse Genome Informatics website (www.informatics.jax.org).

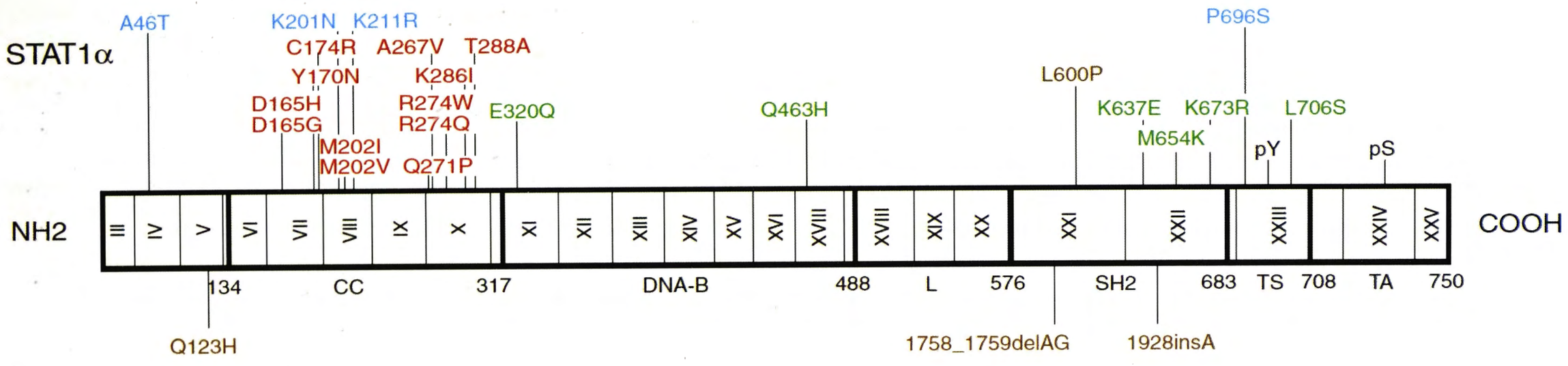


Figure 7.24 Identified pathogenic mutations in human STAT1 gene. Schematic diagram of human STAT1 gene with representative regions coding for domains III-XXV of the protein, or regions coding for the coiled-coil domain (CC), DNA-binding domain (DNA-B), linker domain (L), SH2 domain (SH2), tail segment domain (TS), and transactivator domain (TA), with their amino-acid boundaries demarcated by bold lines. Morbid mutations are represented by coloured text: green (autosomal dominant with partial STAT1 deficiency), brown (autosomal recessive with complete STAT1 deficiency), blue (autosomal recessive with partial STAT1 deficiency) and red (autosomal dominant with gain-of-activity of STAT1). Figure Adapted from Boisson-Dupuis *et al.*, (2012).

Table 7.10 Classification of STAT1 mutation in humans

Type	Affected domain	Main cellular phenotype	Main clinical phenotype	References
Autosomal recessive with complete STAT1 deficiency	N-terminal domain SH2 domain	No STAT1-dependent response to IFN-γ, IFN-α/β, IFN-l and IL-27	Lethal intracellular bacteria (mainly mycobacteria) and viral (mainly herpes) diseases	Dupuis *et al.*, 2003 Chapgier *et al.*, 2006*a* Vairo *et al.*, 2011
Autosomal recessive with partial STAT1 deficiency	N-terminal domain Coiled-coil domain Tail segment domain	Impaired STAT1-dependent response to IFN-γ, IFN-α/β, IFN-l and IL-27	Curable intracellular bacteria (mainly mycobacteria) and viral (mainly herpes) diseases	Chapgier *et al.*, 2009*b* Kong *et al.*, 2010 Kistensen *et al.*, 2011
Autosomal dominant with loss-of-function STAT1 deficiency	DNA-binding domain SH2 binding domain Tail segment domain	Impaired STAT1-dependent response to IFN-γ and IL-27	Mycobacterial diseases To date, incomplete clinical penetrance is oberved	Chapgier *et al.*, 2006*a* Dupuis *et al.*, 2001 Tsumura *et al.*, 2012 Sampaio *et al.*, 2012 Hirata *et al.*, in preparation
Autosomal dominant with gain-of-function STAT1 disorder	Coiled-coil domain	Enhanced STAT1-dependent response to at least IFN-γ, IFN-α/β, IL-27, IL-6 and IL-21. Impaired IL-17 T-cell immunity	Chronic mucocutaneous caindidiasis and autoimmunity	Liu *et al.*, 2011 van de Veerdnok *et al.*, 2011 Smeekens *et al.*, 2011

IFN – Inteferon; IL – Interleukin. Table adapted, with some modifications, from Boisson-Dupuis *et al.*, (2012).

Chapter 8

Protective role of memory B cells in vaccination against JEV and related flaviviruses

8.1 Introduction

Vaccination is the most cost-effective intervention to prevent Japanese encephalitis (JE) (Ding *et al.*, 2003; Siraprapasiri *et al.*, 2006; Surratdecha *et al.*, 2006). The public health impact of incorporating JE vaccines into the national immunization program in Japan cannot be underestimated; since the introduction of formalin-inactivated mouse brain-derived JE vaccine (globally marketed by Sanofi-Pasteur as JE-VAX), the annual incidence of JE cases dropped from more than 1,000 per year in the late 1960's, to less than 100 from 1982 to 1991, and then to less than 10 from 1992 to 2004 (Matsunaga *et al.*, 1999; Arai *et al.*, 2008). However, due to perceived issues of safety, the Japanese authorities have recently recommended the suspension of its use (reviewed in (Halstead and Thomas, 2011)). Hence, the production of JE-VAX, the only internationally licensed JE vaccine at that time, was ceased in 2005. The policy was also implemented in anticipation of the replacement of JE-VAX with new-generation JE vaccines, such as, ChimeriVax-JE and inactivated Vero-cell culture derived (ccJE) vaccines.

ChimeriVax-JE is a recently licensed live-attenuated JE vaccine, that was designed via a construct composed of genes encoding for envelope and premembrane proteins of JEV SA14-14-4 strain and non-structural proteins of Yellow Fever 17D (Chambers *et al.*, 1999; Guy *et al.*, 2012). A series of studies revealed that ChimeriVax-JE is a promising vaccine with an impressive safety and efficacy profile (Chokephaibulkit *et al.*, 2010; Monath *et al.*, 2002; Monath *et al.*, 2003; Nasveld *et al.*, 2010; Torresi *et al.*, 2010). On the other hand, Advax-adjuvanted ccJE (JE-ADVAX™) is another promising new-generation JE vaccine, which was shown to elicit robust humoral immune response against JEV, with antibody titres comparable to that induced with ChimeriVax-JE, and

superior to that elicited with non-adjuvanted JE-VAX or alum-adjuvanted JEV vaccine (Jespect; Novartis) (Lobigs *et al.*, 2010).

In spite of increasingly available list of licensed and candidate JE vaccines, a profound understanding on the immunological correlates underlying vaccine-mediated protection remains not completely defined (reviewed in (Lobigs and Diamond, 2012)). A robust humoral immune response was deemed crucial in vaccine-mediated homologous protection against JE. Neutralizing antibodies against flaviviral E protein produced after immunization provided complete protection from lethal encephalitis, whereas $CD8^+$ T cells was documented to be less substantial, and at best provided only limited protective contribution (Konishi *et al.*, 2003; Konishi *et al.*, 1998*b*; Pan *et al.*, 2003). Furthermore, the cross-protective efficacy of flaviviral vaccines, such as Advax-adjuvanted ccJE, prM/E protein chimeric flaviviruses, and prM/E protein-based DNA vaccination are thougth to largely depend on competent humoral immune responses (Bosco-Lauth *et al.*, 2011; Lobigs *et al.*, 2009; Lobigs *et al.*, 2003*c*, Lobigs *et al.*, 2011).

The earlier chapters delved on the basic immunobiology of Japanese encephalitis, in the aim of using the insights in defining the immunological correlates of protection of rationally designed JE vaccines. Notably, a robust humoral immune response is critically important against primary infection with JEV (Chapter 3). While $CD4^+$ T cells provide essential help in enhancing antibody responses, $CD8^+$ T cells are, at best, marginal in providing contribution in disease amelioration. In this chapter, I extend my investigation on establishing the mechanism of vaccine-mediated protection against homologous and heterologous flavivirus infections.

8.2 Results and Discussion

8.2.1 Absolute requirement for B cells in vaccine-mediated immune response. B cells but not $CD8^+$ T cells are instrumental in recovery from primary infection with JEV (Chapter 3). Here I investigated whether a similar pivotal role of B cells exists in vaccine-mediated protection against JE. Groups of 8-week-old $\mu MT^{-/-}$ mice were immunized subcutaeneously (sc) with $1x10^5$ PFU of ChimeriVax-JE or 2 doses, given 2 weeks apart, of 1 μg of ccJE + Advax (Table 8.1). Another mice group, that did not receive any immunization, served as control. A month after completion of vaccine regimen, mice were challenged via the footpad (fp) with 10^3 PFU of JEV. All immunized and control mice succumbed to disease on or before D12 post-infection (pi). This data clearly demonstrate the absolute requirement for antibodies in vaccine-mediated homologous protection against JE.

To determine the role of $CD4^+$ T cells in vaccine-mediated immune responses, groups of 8-week-old MHCII-$A\alpha\beta^{-/-}$ mice were immunized sc with $1x10^5$ PFU of ChimeriVax-JE, or left unimmunised (Table 8.1). A month after immunization, mice were challenged via the fp with 10^3 PFU of JEV. Control mice displayed complete mortality with mean survival time (MST) of 12.2 ± 0.4 days. On the other hand 60% of immunized succumbed to infection, with delayed MST of 14.2 ± 3.2 days. Although the difference was not statistically significant, this data, together with the findings in Chapter 3, clearly reflect the essential helper function of $CD4^+$ T cells in inducing protective humoral immune responses after a primary infection or immunization.

Table 8.1 Contribution of vaccine-mediated humoral and $CD4^+$ T cell immune responses against challenge with JEV.

Mice and Immunogen[a]	Mortality (no. of deaths/total)	MST ± SD, days	*P value*[b]
$\mu MT^{-/-}$			
Naive	100% (4/4)	11.8 ± 1.0	
ChimerVax-JE	100% (4/4)	11.0 ± 0.0	0.21
ccJE 1μg + Advax	100% (5/5)	11.2 ± 1.1	0.44
MHCII-$A\alpha\beta^{-/-}$			
Naive	100% (5/5)	12.2 ± 0.4	
ChimerVax-JE	67% (4/6)	14.2 ± 3.2	0.29

[a] Eight-week-old $\mu MT^{-/-}$ or MHCII-$A\alpha\beta^{-/-}$ mice were immunized sc with $1x10^5$ of ChimeriVax JE or 2 doses, given 2 weeks apart, of 1 μg of ccJE + Advax. Naïve control mice did not received any immunization. A month after completion of vaccination schedule, mice were challenged via the fp with 10^3 PFU of JEV.

[b] Statistical significance was computed in comparison with naive control.

8.2.2 $\beta 2m^{-/-}$ mice are highly susceptible to JEV infection and provide a model for evaluating B cell memory responses following vaccination. For the purpose of evaluating memory B cell responses after vaccination, I established a mouse model characterized by deficiency in $CD8^+$ T cells and persistent antibody, thereby uncovering protective B cell immunity following vaccination. $\beta 2m^{-/-}$ mice exhibit a congenital deficiency in $CD8^+$ T cells due to a defect in MHC-I assembly, cell surface transport and antigen presentation (Koller *et al.*, 1990). In addition, the mice also lack long-lived IgG as a result of a deficiency of neonatal Fc receptor (FcRn) (Brambell, 1970; Junghans and Anderson, 1996; Ghetie et al., 1997; Suzuki et al., 2010). FcRn has a MHC-I-like molecular structure, with β2m protein incorporated as the light chain (Simister and Mostov, 1989; West and Bjorkman, 2000). Along with other proteins, IgG is taken up by various cells in the body by fluid-phase endocytosis, is transported to an endosomal compartment, and, at low pH, is bounded to FcRn with high affinity (reviewed in (Junghans, 1997; Ghetie and Ward, 2000)). The strong binding of IgG with FcRn, diverts and prevents its catabolic degradation within lysosomes, and recycles it back extracellularly upon neutral-pH induced dissociation with FcRn (reviewed in (Rojas and Apodaca, 2002)).

To test the impact of β2m deficiency on susceptibility to JEV infection, 8-week-old $\beta 2m^{-/-}$ and B/6 wt control mice were inoculated iv with 10^3 PFU of JEV (Fig. 8.1A). Relative to wt control, $\beta 2m^{-/-}$ mice displayed significantly increased mortality (100% vs. 75%, respectively), and shorter MST (12.0 ± 2.3 vs. 13 ± 1.4 days, respectively). Subcutaneous challenge of 8 or 12-week-old $\beta 2m^{-/-}$ mice with 10^3 PFU of JEV similarly presented with increased mortality (95% and 94%, respectively) in comparison to wt controls (65% and 55%, respectively) (Fig. 8.1B,C).

To determine whether the increased mortality rates observed in $\beta 2m^{-/-}$ mice were attributed to defective viral clearance, groups of 8-week-old $\beta 2m^{-/-}$ and B/6 wt mice were inoculated with 10^3 PFU of JEV, and sacrificed at 2-day-interval time points for serum and tissue collection. Viral load was determined in serum and spleens by real time RT-PCR, and in brains and spinal cords by plaque titration in Vero cells. Serum viral load in $\beta 2m^{-/-}$ mice presented with similar kinetics and magnitude relative to wt mice (Fig. 8.2A). Peak viremia appeared on D2 post-infection (pi), with geometric mean titres (GMT) decreasing afterwards. Likewise, viral load in spleen of $\beta 2m^{-/-}$ mice was similar to that of wt controls, with peak titres on D4 pi (Fig. 8.2B). On the contrary, viral load in brains and viral dissemination into the spinal cord were significantly elevated in $\beta 2m^{-/-}$ mice than wt controls. Virus in brains of $\beta 2m^{-/-}$ mice was initially detected on day 6 pi, and relative to wt mice, GMT was ~100-fold and ~1,000-fold higher on days 8 and 10 pi, respectively (Fig. 8.2C). Furthermore, viral dissemination into the spinal cord was more pronounced in $\beta 2m^{-/-}$ mice relative to wt mice (~ 3 logs GMT difference on D10 pi) (Fig. 8.2D).

As earlier described, $\beta 2m^{-/-}$ mice exhibited short-lived IgG isotype antibodies brought about by absence of β2m protein-dependent protection of serum IgG from catabolism (reviewed in (Rojas and Apodaca, 2002)). To determine whether this phenomenon is similarly observed in JEV-infected $\beta 2m^{-/-}$ mice, serum antibody titres were determined by ELISA in comparison to wt controls. The kinetics and magnitude of anti-JEV IgM antibodies was similar in both groups, initially detected on D4 pi, and peaked on D8 pi (Fig. 8.3A). Anti-JEV IgG1 and IgG2b antibodies in wt mice were initially detected on D8 pi, and increased on D10 pi (Fig. 8.3B). This is in contrast to what was observed in $\beta 2m^{-/-}$ mice, where IgG1 and IgG2b levels were significantly lower on D10 pi, and were threefold and twofold lower than the levels found in wt controls. In spite of less than

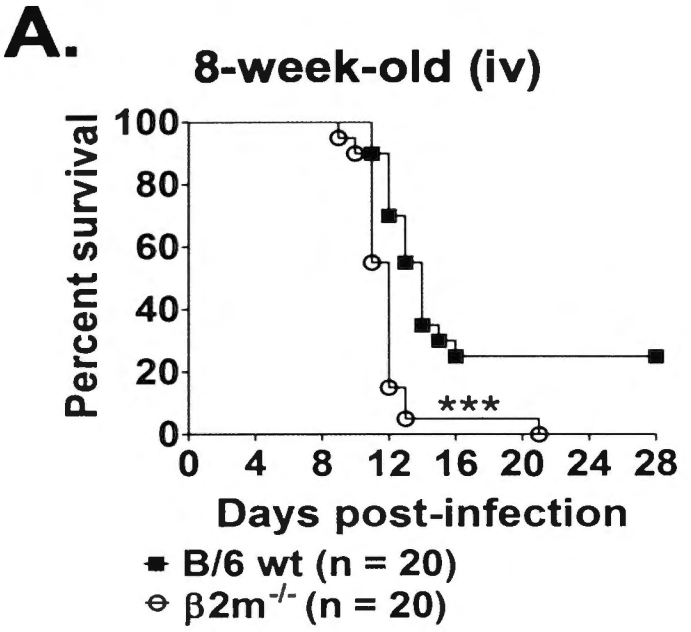
optimal IgG responses in $\beta 2m^{-/-}$, the functional activity of antibodies, as measured by 50% plaque reduction and neutralization assay ($PRNT_{50}$), was comparable to wt controls (Fig. 8.3C). However, it should be noted that, in addition to direct neutralization measured by *in vitro* assays, alternative *in vivo* mechanisms of viral clearance by antibodies such as Fc-portion interaction with complement and Fc receptors on cells of monocytic lineage should not be discounted (Chung *et al.*, 2005; Hofmeister *et al.*, 2011; Oliphant *et al.*, 2005; Oliphant *et al.*, 2006). Fc-mediated clearance of viruses has been suggested to play critical protective role in human infections with WNV (Hofmeister et al., 2022).

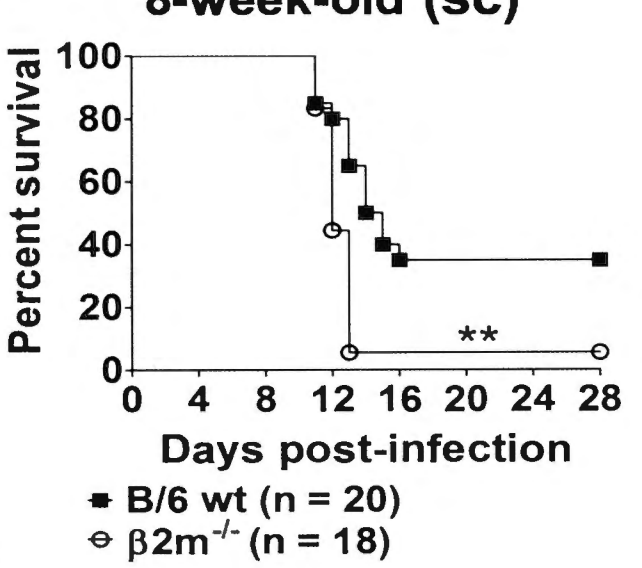
In addition to blunted IgG antibody responses, $\beta 2m^{-/-}$ mice were also documented to possess dysfunctional NK cell responses (Alsharifi *et al.*, 1996; Muller *et al.*, 1995; Tay *et al.*, 1995). To investigate whether NK cell response remains intact after exposure to JEV, $\beta 2m^{-/-}$ and B/6 wt control mice were inoculated iv with 10^3 PFU of JEV, splenocytes isolated at indicated time points, and NK cell responses were measured by chromium release assay (Fig. 8.3D). On D3 pi, NK cell activity in $\beta 2m^{-/-}$ was approximately threefold lower to that of B/6 wt mice. This was reversed on D7 pi, where NK cell activity in $\beta 2m^{-/-}$ mice increased, and was higher than in wt mice. This data represented a similar observation found in previous studies, where $\beta 2m^{-/-}$ mice exhibited delayed NK cell responses after viral infection. However, given that NK cell depletion did not alter susceptibility of mice to JEV infection (Chapter 5), dysfunctional NK cell responses may be of little probative value in increasing the susceptibility of $\beta 2m^{-/-}$ to challenge with JEV.

A less explored factor, that may explain the increased susceptibility to JEV infection, is the failure to induce an optimal IFN-γ response in $\beta 2m^{-/-}$ mice. Previous studies have

suggested the role of β2m protein in the efficient production of IFN-γ (Antonelli *et al.*, 1988; Klingel *et al.*, 2003). This is especially important, given the data I presented in chapter 5 that IFN-γ plays an essential protective role against lethal JE. However, my initial investigation revealed comparable kinetics and magnitude in serum IFN-γ levels in both JEV-infected B/6 wt and $\beta2m^{-/-}$ mice (data not shown). Lastly, another unexplored factor is the documented presence of hereditary hemochromatosis in $\beta2m^{-/-}$ mice. Hepatic iron overload in $\beta2m^{-/-}$ mice is well documented, and is attributed to the absence of β2m-interacting protein important for iron homeostasis (Feder *et al.*, 1997; Santos *et al.*, 1996; Santos *et al.*, 2000, Rothenberg and Voland, 1996; Waheed *et al.*, 2002). It can be speculated that a slow but progressive liver disease (Rodrigues *et al.*, 2006; Khan *et al.*, 2007) can impact on the distinct immunologic functions of the liver. Hence, an impairment on hepatic metabolism of Vitamin D, and hepatic synthesis of Vitamin D binding protein, C-reactive protein, and all complement proteins can be expected (reviewed in (Baeke *et al.*, 2010; Crisp, 2009; Gao *et al.*, 2008)). The latter of which is known to be critical for protection against lethal West Nile encephalitis (Mehlhop *et al.*, 2005).

In summary, $\beta2m^{-/-}$ mice displayed high mortality, high brain viral load, and widespread dissemination of virus into the CNS. The exquisite susceptibility of $\beta2m^{-/-}$ mice to JEV challenge is not only attributed to $CD8^+$ T cell deficiency, but also credited to myriad of factors contributing to severe immunodeficiency. As such, $\beta2m^{-/-}$ mice were identified as a suitable stringent challenge model for preclinical evaluation of vaccines against JEV.





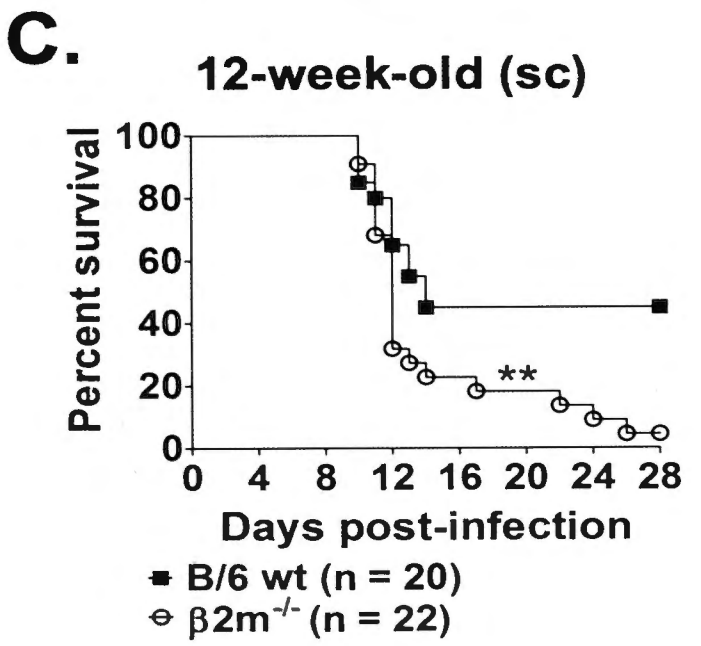


Figure 8.1 Susceptibility of β2m$^{-/-}$ mice to low-dose challenge with JEV. Intravenous inoculation of 8-week-old (A) or sc inoculation of 8-week-old (B) and 12-week-old (C) β2m$^{-/-}$ and B/6 wt control mice with 10^3 PFU of JEV. Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from three independent experiments. Asterisks denote statistical significance (**, $P < 0.01$; ***, $P < 0.001$).

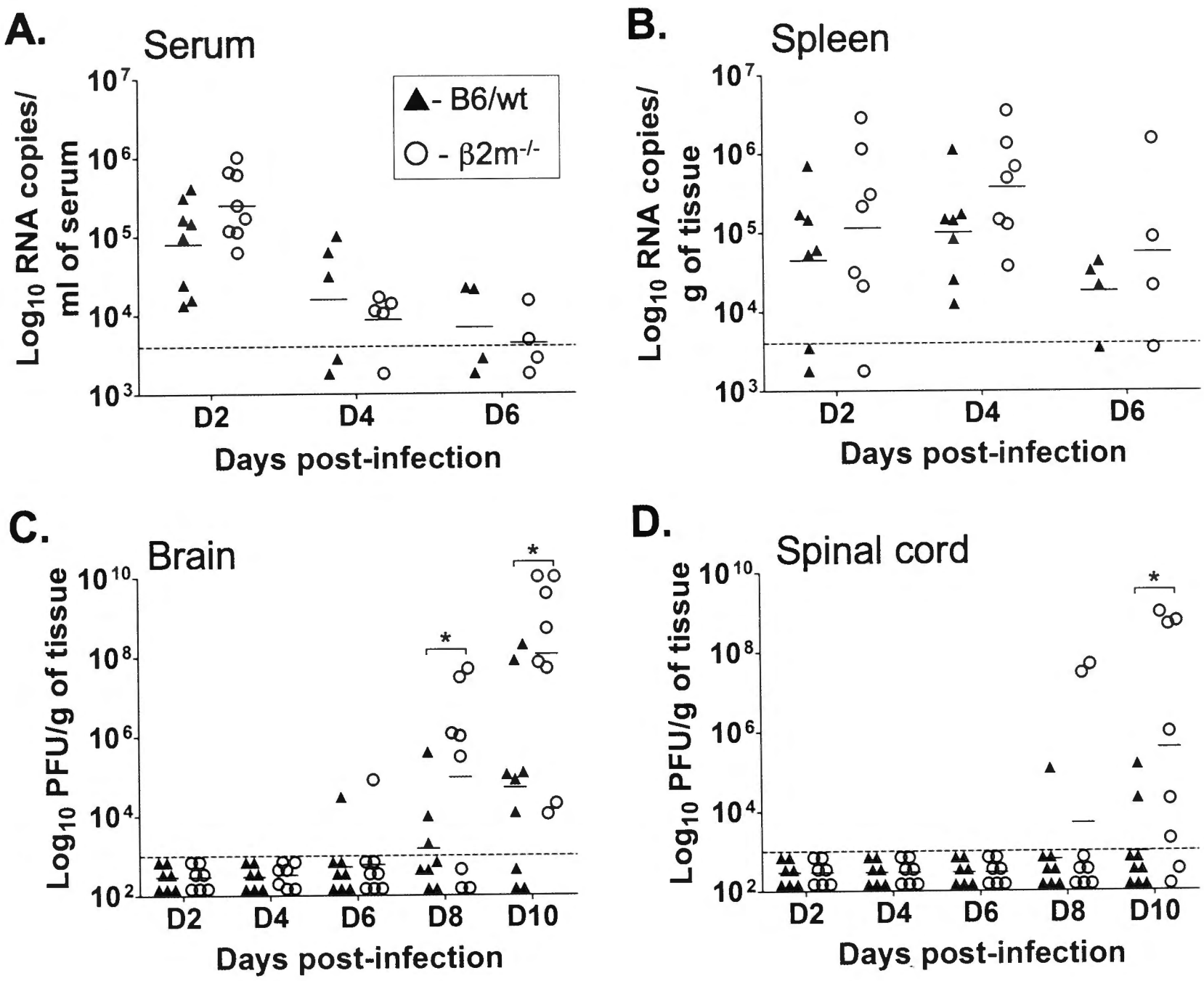


Figure 8.2 Viral burdens in B/6 wt and β2m$^{-/-}$ mice after exposure to JEV.

Eight-week-old mice were challenged sc with 10^3 PFU of JEV, and sacrificed at 2-day interval time points for serum and tissue collection. Viremia was measured in RNA copies/ml by qRT-PCR; viral load in brain and spinal cord was measured by plaque titration in Vero cells. The lower limit of virus detection was $1x10^3$ PFU/g of tissue, and is indicated by a dotted line. The data shown were constructed from two independent experiments. Asterisks denote statistical significance (*, $P < 0.05$).

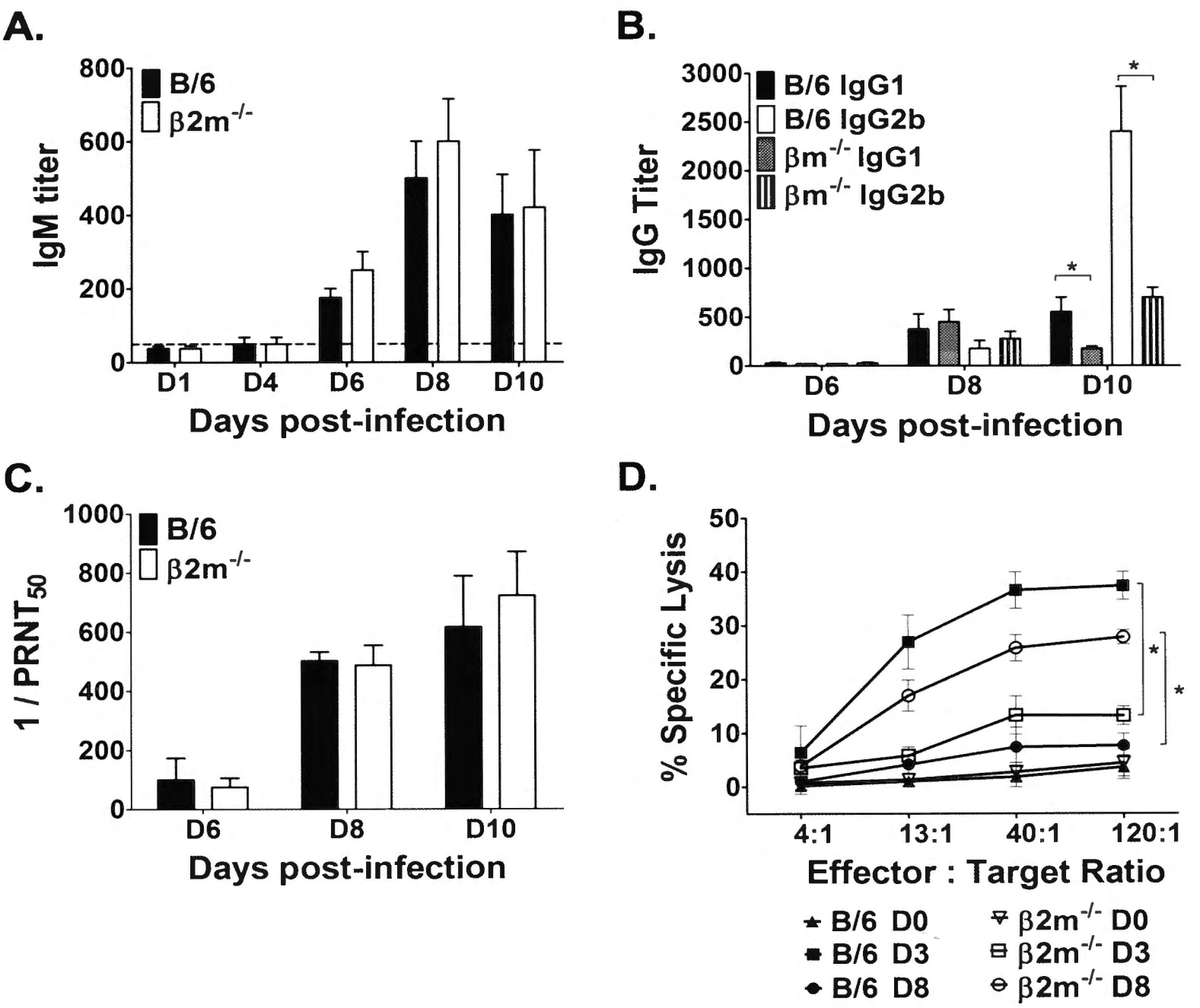


Figure 8.3 Antibody and NK cell responses in JEV-infected B/6 wt and β2m$^{-/-}$ mice. Eight-week-old B/6 wt and β2m$^{-/-}$ mice were infected sc with 10^3 PFU of JEV, and sacrificed at indicated time points for serum and tissue collection. Anti-JEV IgM (A) or IgG (B) isotype antibody titres were determined by ELISA. The data presented are reciprocal mean endpoint titres representative of 4-5 mice per time point with the SEM indicated by error bars. (C) Neutralizing antibody titres were determined by plaque reduction neutralization assay. The data presented are mean $PRNT_{50}$ titres representative of 4-5 mice per time point with the SEM indicated by error bars. (D) Splenocytes harvested at indicated time points, and NK cell activity determined by chromium release assay as described in materials and methods. Data represent means of 4 mice samples per time point with the SEM indicated by error bars. Asterisks denote statistical significance (*, $P < 0.05$).

8.2.3 Inactivated JE vaccines protect β2m$^{-/-}$ mice from sc challenge with JEV.

Given the exquisite susceptibility of β2m$^{-/-}$ mice to challenge with JEV, I have used it as a stringent challenge model to evaluate the protective value of JE vaccines. Two Vero cell-grown inactivated JE vaccines (JESPECT®, which is adjuvanted with alum, and JE-ADVAX™, which contains the polysaccheride adjuvant, inulin, in two different formulations either as standard or as supermix) and ChimeriVax-JE were used to immunise groups of β2m$^{-/-}$ mice. The immunisation schedule was with 2 doses given at 2 weeks apart, with the following treatment groups: PBS alone (as negative control), low (0.05 μg) and high (0.5 μg) doses Jespect (as positive control), low and high doses of ccJE alone, low and high doses of ccJE adjuvanted with Advax supermix, and low dose ccJE adjuvanted with Advax (Table 8.2). Another group of mice received sc 1x10^5 PFU of ChimeriVax-JE. A month after completion of immunization schedule, mice were challenged sc with 10^3 PFU of JEV, and mortality recorded over a 28-day-observation period. As expected, control β2m$^{-/-}$ mice immunized with PBS alone displayed high mortality rate after JEV challenge (87%) (Table 8.2). In contrast, control β2m$^{-/-}$ mice groups immunized with either low or high dose Jespect vaccine displayed complete resistance from lethal Japanese encephalitis. Also, 100% survival rate was observed in mice receiving ChimeriVax-JE, and in mice groups receiving high dose ccJE adjuvanted with either Advax supermix or Advax. Even in the absence of adjuvant, immunization with high dose ccJE alone similarly provided significant protection from JEV infection (mortality of 1 out of 10), while immunization with low dose ccJE alone only provided limited protection (mortality rate of 53%). The efficacy of low dose ccJE vaccine was significantly improved when it was adjuvanted with either Advax supermix or Advax (mortality of 0% and 10%, respectively (Table 8.2).

It is well documented that humoral immunity is crucial for vaccine-mediated protection against lethal Japanese encephalitis (reviewed in (Lobigs and Diamond, 2012)). As a standard for clinical efficacy for JE vaccines, the established correlate of protection is neutralizing titres of at least 1/10 of serum dilution. However, as earlier presented, $\beta 2m^{-/-}$ mice exhibited dysfunctional IgG antibody responses after live JEV infection. JEV-specific IgG responses in $\beta 2m^{-/-}$ and B/6 wt mice immunized with low-dose ccJE + Advax or low-dose ccJE alone were then determined. IgG1 and IgG2b antibody responses in all mice groups were initially detected at low levels on D8 post-vaccination (Table 8.3). On D18 pi, B/6 wt mice immunized with ccJE + Advax expectedly presented with higher IgG1 and IgG2b antibody responses relative to B/6 wt mice immunized with ccJE only, and this difference was maintained until D28 pi. $\beta 2m^{-/-}$ mice immunized with ccJE alone presented with low to undetectable titres on days 18 and 28 pi. Interestingly, $\beta 2m^{-/-}$ mice immunized with ccJE + Advax similarly presented with markedly low IgG antibody responses on days 18 and 30 pi, with titres ranging from undetectable to 200. This consistently reflected a dysfunctional IgG response uniquely found in mice with deficiency in β2m protein.

Measurement of pre-challenge neutralizing titres revealed that $\beta 2m^{-/-}$ mice immunized with ccJE + Advax was markedly low compared to its counterpart B/6 controls (mean $PRNT_{50}$ titre of <10 vs. 116, respectively) (Table 8.4). This finding is paradoxical to the notion that pre-challenge neutralizing titres serve as the main correlate of protection against lethal Japanese encephalitis, since almost all $\beta 2m^{-/-}$ mice immunized with ccJE + Advax survived the challenge with JEV. It was then speculated that in spite of failure to maintain detectable levels of post-immunization neutralizing IgG antibodies, $\beta 2m^{-/-}$ mice are still capable of memory B cell development, that are readily inducible to produce protective antibodies in time of viral challenge. Interestingly, on days 5 and 10

pi, β2m$^{-/-}$ mice immunized with ccJE + Advax presented with neutralizing titres higher than β2m$^{-/-}$ mice immunized with ccJE alone (D5 pi: 38 vs. 14, respectively; D10 pi: 672 vs. 385, respectively). Indeed, post-infection neutralizing titre levels in immunized β2m$^{-/-}$ mice correlated well with the outcome observed in earlier mice challenge experiments. This data reflected that β2m$^{-/-}$ mice are capable of inducing memory immune responses vital for protection against JEV infection.

The phenomenon of vaccine-mediated protection in β2m$^{-/-}$ might help explain the disparity in established vaccine efficacy and clinical effectiveness of mouse brain-derived formalin-inactivated JE vaccine, JE-VAX. It has been noted that the annual incidence of JE in Japan was significantly declining in the past decades, despite more than 50% of a representative cohort population aged 20-59 years old had undetectable post-immunization neutralizing antibodies (Kitano, 1993; Konishi and Suzuki, 2002). A three-dose vaccination regimen of JE-VAX afforded an overall time-limited efficacy of 91% for only up to two years (Hoke *et al.*, 1988), and yet based on crude evaluation of clinical effectiveness, vaccinated individuals had 2,000 to 80,000 times greater ratio of subclinical to clinical infections than unvaccinated population (Konishi and Suzuki, 2002). It is hypothesized that majority of the individuals exposed to JEV were protected by vaccination received during childhood. The discrepancy in vaccine efficacy and clinical effectiveness might be explained by rapidly induced anamnestic antibody response in vaccinated individuals with undetectable levels of pre-exposure neutralizing antibodies. This essentially reflected immunized β2m$^{-/-}$ mice, which were primed for the development of durable memory B cells that are readily and rapidly inducible to produce neutralizing antibodies at the time of JEV challenge. This phenomemon was similarly observed in a previous study, where wild-type mice immunized with a plasmid vaccine encoding prM and E genes of JEV displayed low to undetectable levels of post-

immunization neutralizing titres (Konishi *et al.*, 1999). However, upon live JEV challenge, mice rapidly induced neutralizing antibodies, and were completely protected.

In summary, this section confirmed the suitability of $\beta 2m^{-/-}$ mice as a stringent challenge model for evaluating protective value of JE vaccines. This novel mice model have demonstrated that memory B cells, induced by either ChimeriVax-JE or JE-ADVAX™ immunization, were adequate and capable to protect mice from lethal JE. This significant protection was afforded in the absence of $CD8^+$ T cells and long-lived IgG response.

Table 8.2 Protective value of Advax-adjuvanted ccJE vaccine.

Immunogen[a]	Mortality (no. of deaths/total)	MST ± SD, days	*P value*[b]	*P value*[c]
PBS	87% (13/15)	16.2 ± 4.9		
ChimeriVax-JE	0% (0/5)	-	0.0029	
JE-spect 0.5 μg	0% (0/5)	-	0.0029	
JE-spect 0.05 μg	0% (0/8)	-	0.0029	
ccJE 0.5 μg	6% (1/16)	16	0.0029	
ccJE 0.5 μg + Advax supermix	0% (0/10)	-	0.0029	
ccJE 0.05 μg	53% (8/15)	17.9 ± 5.8	0.03	
ccJE 0.05 μg + Advax supermix	0% (0/10)	-	<0.0001	0.007
ccJE 0.05 μg + Advax	10% (1/10)	13	0.0004	0.04

[a] Eight-week-old $\beta 2m^{-/-}$ mice were immunized with 2 doses of PBS (control) or indicated vaccine formulations. A month after completion of vaccination schedule, mice were challenged sc via footpad with 10^3 PFU of JEV.

[b] Statistical significance was computed in comparison with PBS only control.

[c] Statistical significance was computed in comparison with 0.05 μg ccJE only vaccine formulation.

Table 8.3 JEV-specific IgG isotype antibody responses after Advax-adjuvanted ccJE immunization.

Mouse strain and Immunogen[a]	IgG1[b]			IgG2b[b]		
	D8	D18	D28	D8	D18	D28
B/6 wt						
ccJE 0.05 μg	<2.0 (<2.0-2.0)	2.5 (2.0-2.9)	2.4 (2.0-2.6)	<2.0 (<2.0-2.0)	2.3 (2.0-2.6)	2.1 (<2.0-2.6)
ccJE 0.05 μg + Advax	2.4 (2.0-2.6)	>4.1 (>4.1)	>4.1 (3.8->4.1)	2.3 (2.0-2.6)	>4.1 (>4.1)	>4.1 (>4.1)
$\beta 2m^{-/-}$						
ccJE 0.05 μg	<2.0 (<2.0-2.3)	2.1 (<2.0-2.3)	<2.0 (<2.0)	<2.0 (<2.0-2.3)	<2.0 (<2.0-2.0)	<2.0 (<2.0-2.0)
ccJE 0.05 μg + Advax	<2.0 (<2.0-2.0)	2.2 (<2.0-2.3)	2.0 (<2.0-2.3)	2.2 (<2.0-2.3)	2.0 (<2.0-2.3)	<2.0 (<2.0)

[a] Groups of 8-week-old B/6 wt or $\beta 2m^{-/-}$ mice were immunized with 2 doses, given 2 week apart, with 0.05 ccJE + Advax or 0.05 ccJE alone. Sera were collected on days 8, 18, and 28 post-immunization for IgG1 and IgG2b isotype antibody determination.

[b] ELISA endpoint titres of individual test sera were determined as described in the Materials and Methods section; mean titres (n = 4/ group) and range are given.

Table 8.4 Post-immunization and post-challenge JEV-specific neutralizing antibody titres against JEV

Mouse strain and Immunogen[a]	Day post-immunization[b]		Day post-challenge[b]	
	D8	D28	D5	D10
B/6 wt				
PBS	-	-	21 (<10-40)	388 (20-640)
ccJE 0.05 μg	<10 (<10-10)	<10 (<10-10)	27 (<10-80)	424 (40-640)
ccJE 0.05 μg + Advax	32 (10-80)	116 (10-320)	232 (40-640)	736 (160->1280)
$\beta 2m^{-/-}$				
PBS	-	-	10 (<10-20)	320 (<10-1280)
ccJE 0.05 μg	<10 (<10-10)	<10 (<10)	14 (<10-40)	385 (<10-640)
ccJE 0.05 μg + Advax	16 (<10-40)	<10 (<10-10)	38 (10-80)	672 (80-1280)

[a] Groups of 8-week-old B/6 wt or $\beta 2m^{-/-}$ mice immunized with 2 doses of 0.05 ccJE + Advax, 0.05 ccJE alone, or PBS control, given 2 weeks apart. Serum was collected at days 8 and 28 post-immunization, and days 5 and 10 post-infection with 10^3 PFU of JEV.

[b] Plaque reduction neutralization against JEV was determined as described in the Materials and Methods section; mean titres (n = 4/ group) and range are given.

8.2.4 JE-ADVAX™ elicits immune B cells that efficiently protect against lethal JEV challenge. Given that $\beta 2m^{-/-}$ mice are deficient in $CD8^+$ T cells, it remains to be determined whether B cells or $CD4^+$ T cells are the main cell effector population responsible for protection provided by Advax-adjuvanted ccJE vaccine. Eight-week-old $\beta 2m^{-/-}$ donor mice were immunized with 2 doses of 0.5 μg ccJE + Advax, and a month later after completion of vaccination schedule, were sacrificed for splenocyte isolation (Fig. 8.5). $CD4^+$ T cells were purified via depletion of B cells using magnetic beads, while B cells were purified via complement-mediated lysis of $CD4^+$ T cells. Purified immune $CD4^+$ T or B cells were then injected iv into 10 to 12-week-old $\beta 2m^{-/-}$ mice. A day after transfer, recipient and naive control mice ($\beta 2m^{-/-}$ mice group receiving naive total splenocytes) were challenged sc with 10^3 PFU of JEV. Relative to naive control group, immune $CD4^+$ T cell-recipient mice displayed decreased, but not statistically significant, mortality rates (67% vs. 89%, respectively), and longer MST (13.3 ± 2.0 vs. 11.9 ± 1.4 days, respectively). On the other hand, mice recipients of immune B cells presented with significantly lower mortality rate relative to naive control group (10% vs. 89%, respectively). The substantial protection observed in immune B cell-recipient mice was correlated with higher serum antibody and neutralizing titres on days 5 and 10 pi (Table 8.5). On day 5 pi, immune B cell-recipient mice presented with $PRNT_{50}$ titres of 56 (range: 20-80), levels that are notably higher than what was observed in $CD4^+$ T cell recipient-mice [26 (range: 10-40)] or naive control group [<10 (range: <10-20)]. The difference was more pronounced on day 10 pi, where immune B cell-recipient mice displayed higher $PRNT_{50}$ titres of 832 (range: 320-1280), compared to 528 (80-1280) in $CD4^+$ T cell recipient-mice or 452 (20-640) in naive control group.

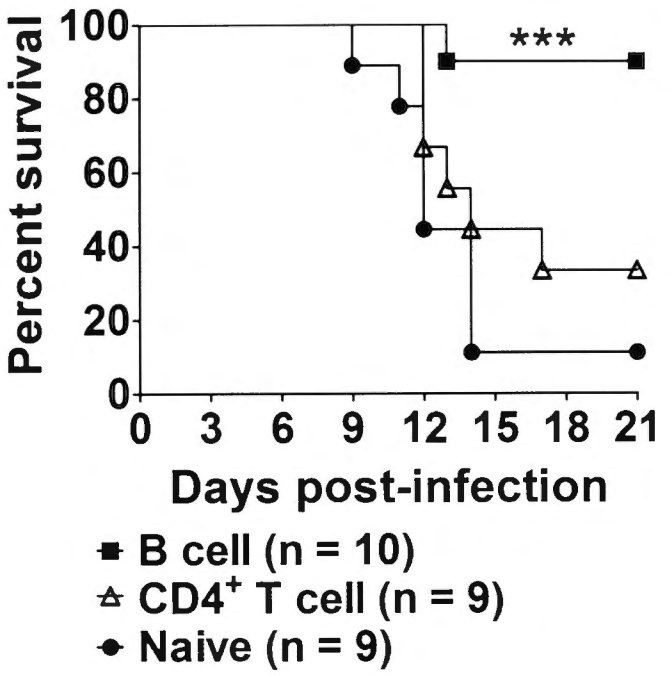


Figure 8.5 Immune B cells mediate protection of Advax-adjuvanted ccJE against homologous challenge with JEV. Eight-week-old $\beta 2m^{-/-}$ donor mice were immunized with two doses of 0.5 μg of ccJE + Advax, delivered 2 weeks apart, $1x10^7$ B cells and $CD4^+$ T cells purified at 1 month after completion of the vaccination schedule, and adoptively transferred ($1x10^7$ cells/mouse) into 8-week-old $\beta 2m^{-/-}$ mice recipients. Another group of $\beta 2m^{-/-}$ mice received naive total splenocytes, and served as control. At one-day post-transfer, mice were challenged sc with 10^3 PFU of JEV. Mice were monitored twice daily for morbidity and mortality over a 21-day-observation period. Asterisks denote statistical significance (***, $P < 0.001$).

Table 8.5 Post-challenge serum antibody and $PRNT_{50}$ titres in $\beta 2m^{-/-}$ recipient mice groups.

Mouse strain and Immunogen[a]	Mean Ab titre [log_{10}] (SD)[b]		Mean $PRNT_{50}$ titre (range)[c]	
	D5	D10	D5	D10
Naive	2.4 (2.0-2.3)	2.5 (2.0-2.9)	<10 (<10 – 20)	452 (20-640)
B cell	>4.1 (3.8->4.1)	>4.1(3.2->4.1)	56 (20-80)	832 (320-1280)
$CD4^+$ T cell	2.8 (2.0-3.2)	3.8(3.2->4.1)	26 (<10-40)	528 (80-1280)

[a] Groups of 10 to 12-week-old $\beta 2m^{-/-}$ mice recipients of immune B cell or $CD4^+$ T cells (from $\beta 2m^{-/-}$ donor mice vaccinated with 2 doses of 0.5 μg ccJE + Advax, given 2 weeks apart). Naive control mice group received naive total splenocytes.

[b] ELISA endpoint titres of individual test sera were determined as described in the Materials and Methods section; mean titres (n = 4/group) and range are given.

[c] Plaque reduction neutralization by individual sera against JEV was determined as described in the Materials and Methods section; mean titres (n = 4/group) and range are given.

8.2.5 Durability of protection with JE-ADVAX™ To determine whether the protective value of Advax-adjuvanted ccJE vaccine is long-lived, groups of 6-week-old $\beta 2m^{-/-}$ were immunized with 2 doses of 0.05 μg ccJE + Advax or with PBS (control) (Fig. 8.6). Six months after completion of the vaccination schedule, mice were inoculated sc with 10^3 PFU of JEV, and mortality recorded over a 28-day-observation period. Relative to PBS control group, $\beta 2m^{-/-}$ mice immunized with low dose ccJE + Advax exhibited complete protection against JEV. As expected, the pre-challenge antibody ELISA and $PRNT_{50}$ titers were undetectable in both groups (Table 8.6). However, on day 5 pi, antibody and neutralizing titers were threefold and fourfold higher in low dose ccJE + Advax-immunized mice than in control group. This indicates that although $\beta 2m^{-/-}$ mice are unable to maintain long-lived antibody levels due to defects in antibody metabolism (reviewed in (Rojas and Apodaca, 2002)), these are able to produce durable B cell memory that is readily responsive upon homologous challenge even at 6 months post-immunization.

To support the finding that ccJE + Advax immunization induces the development of long-lived protective memory B cell response, an immune B cell splenocyte transfer experiment was done. Eight-week-old B/6 wt donor mice were immunized with two doses of 0.5 μg of ccJE + Advax, delivered 2 weeks apart (Fig. 8.7A). Splenocytes were isolated at 3 months post-completion of vaccination schedule, and B cell were purified via complement-mediated lysis of $CD8^+$ and $CD4^+$ T cells. Naive B cells purified from unvaccinated mice served as control. Immune and naive B cells were then transferred iv into B/6 w/t recipient mice. A day after transfer, recipient mice were challenged sc with 10^3 PFU of JEV, and mortality was recorded over 28-day-observation period. Mice group receiving immune B cells displayed significantly improved outcome relative to control mice group (mortality rate of 20% vs. 80%, respectively). A second experiment

was done, wherein immune B cells were collected and purified from donor mice immunized for 6 months with the same vaccine regimen (Fig. 8.7B). Again, naive B cells purified from unvaccinated mice served as control. Similarly, immune B cell-recipient mice group displayed significantly lower mortality rate relative to naïve B cell-recipient mice group (40% vs. 90%, respectively). Relative to control group, the improved survival outcome in mice recipient of immune B cells was correlated with higher serum antibody and neutralizing titres on D5 pi (Table 8.7). Mice recipient of immune B cells isolated from 3 months post-vaccinated donor mice had sixfold and eightfold higher antibody and $PRNT_{50}$ titres, respectively; while mice recipient of immune B cells isolated from 6 months post-vaccinated donor mice had threefold and fourfold higher antibody and $PRNT_{50}$ titres, respectively.

Altogether, these data strongly suggest that immunization of mice with Advax-adjuvanted ccJE confers long-lasting B cell-mediated immunity that is substantially protective against homologous challenge with JEV.

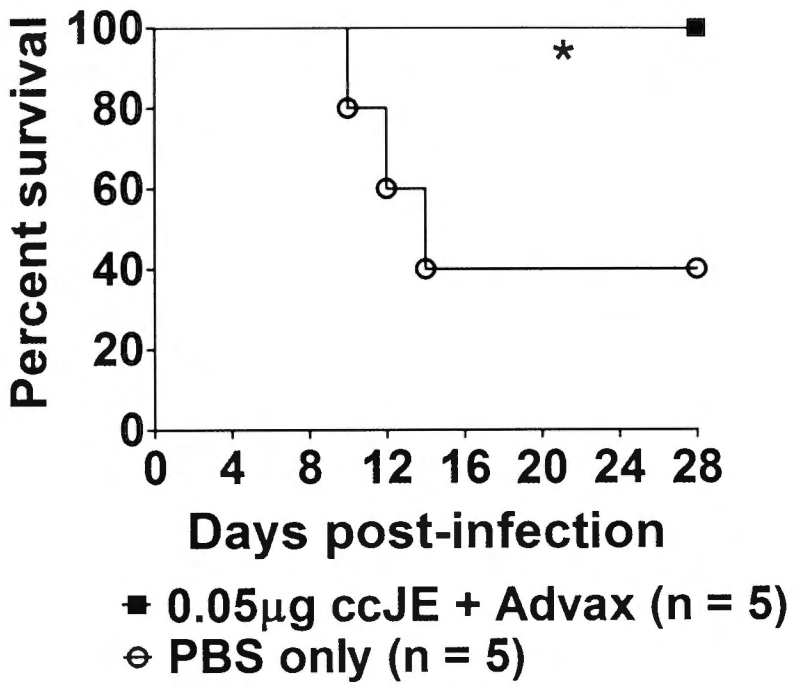


Figure 8.6 Low dose Advax-adjuvanted ccJE vaccine exerts long-term protection against challenge with JEV. Six-week-old $\beta 2m^{-/-}$ mice were immunized with two doses of 0.05 μg of ccJE + Advax or PBS control at 2 weeks apart. Six months after the last dose, mice were challenged sc with 10^3 PFU of JEV. Mice were monitored twice daily for morbidity and mortality over a 28-day-observation period. Asterisks denote statistical significance (*, $P < 0.05$).

Table 8.6 Post-challenge serum antibody and $PRNT_{50}$ titres in $\beta 2m^{-/-}$ mice.

Treatment[a]	Mean Ab titre [log_{10}] (SD)[b]		Mean $PRNT_{50}$ titre (range)[c]	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
PBS	< 2 (< 2)	2.5(2.0-2.9)	< 10 (<10)	28 (10-80)
0.05 μg ccJE + Advax	< 2 (< 2)	3.1(2.3-3.5)	< 10 (<10)	108 (20-320)

[a] Groups of 8-week-old $\beta 2m^{-/-}$ immunized with PBS control or 2 doses of 0.05 μg ccJE + Advax, given 2 weeks apart, and challenged with 6 months later 10^3 PFU of JEV. Sera were collected at day 5 pi for serologic examination.

[b] ELISA endpoint titres of individual test sera were determined as described in the Materials and Methods section; mean titres (n = 4/group) and SD are given.

[c] Plaque reduction neutralization by individual sera against JEV was determined as described in the Materials and Methods section; mean titres (n = 4/ group) and range are given.

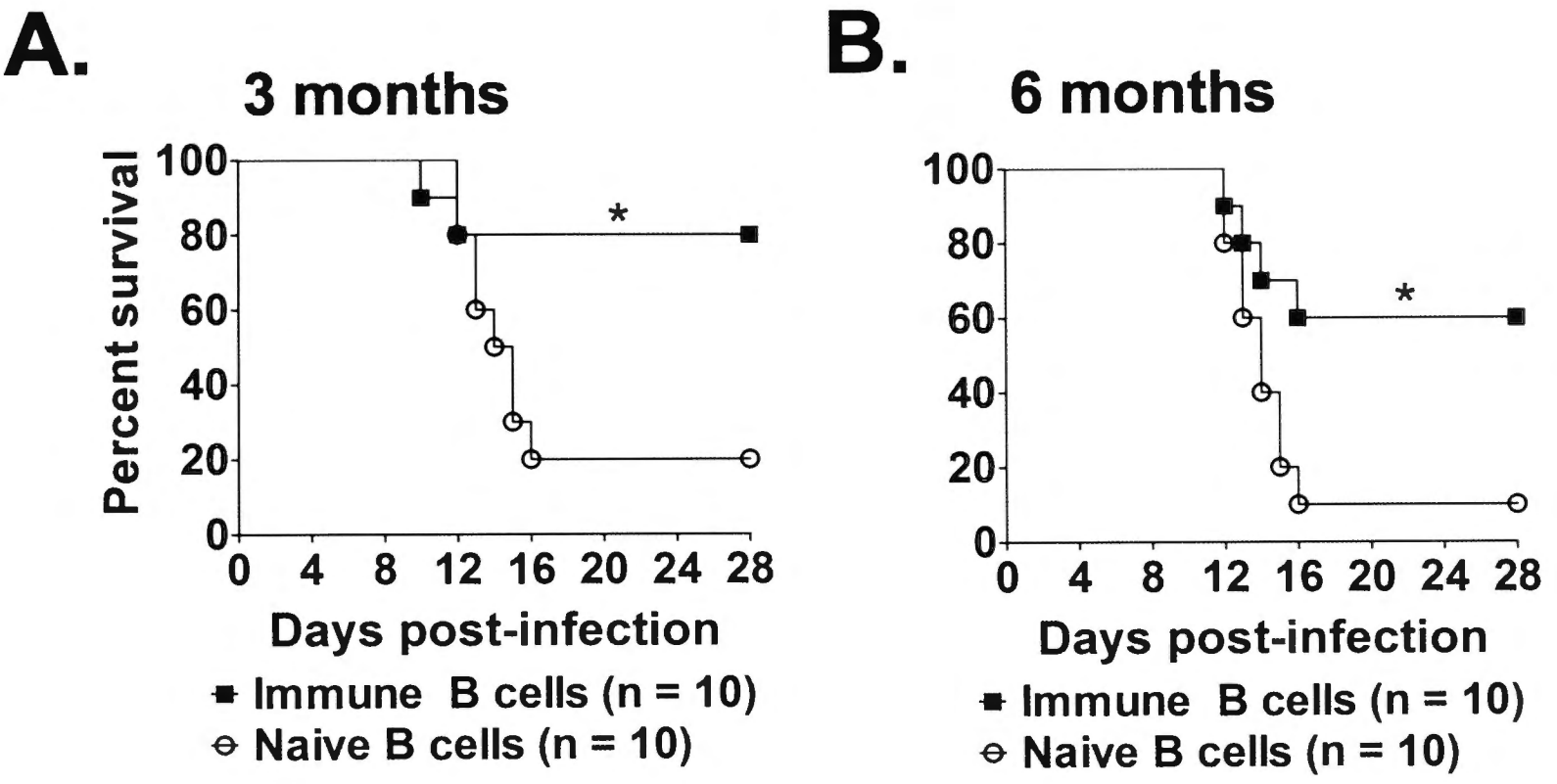


Figure 8.7 Advax-adjuvanted ccJE vaccine induces durable and protective B cell response. Eight-week-old B/6 wt donor mice were immunized with two doses of 0.5 μg of ccJE + Advax, delivered 2 weeks apart, B cells purified at 3 (A) or 6 (B) months after completion of the vaccination schedule, and adoptively transferred ($1x10^7$ cells/mouse) into 8-week-old B/6 mice recipients. A control group of B/6 mice received naive B cells purified from unvaccinated mice. A day after transfer, mice were challenged sc with 10^3 PFU of JEV. Mice were monitored twice daily for morbidity and mortality over a 28-day-observation period. Asterisks denote statistical significance (*, $P < 0.05$).

Table 8.7 Post-challenge serum antibody and $PRNT_{50}$ titres in B/6 wt recipient mice groups.

Treatment[a]	Mean Ab titre [log_{10}] (range)[b]	Mean $PRNT_{50}$ titre (range)[c]
3 months		
Naive B cell	2.4 (<2.0–2.6)	12 (<10–20)
Immune B cell	3.2 (2.0–3.5)	98 (10–320)
6 months		
Naive B cell	2.7 (2.0–2.9)	21 (<10–40)
Immune B cell	3.0 (2.6–3.5)	84 (20–160)

[a] Groups of 8-week-old B/6 mice recipients of naive or immune B cells (mice groups were derived from Figure 8.7). Immune B cells were from B/6 wt donor mice immunized (2 doses of 0.5 μg ccJE + Advax, given 2 weeks apart) for 3 or 6 months, and naive B cells were from unvaccinated B/6 wt donor mice. A day after transfer, all recipient mice were challenged sc with $1x10^3$ PFU of JEV, and sera collected at D5 pi for serology.

[b] ELISA endpoint titres of individual test sera were determined as described in the Materials and Methods section; mean titres (n = 4/group) and range are given.

[c] Plaque reduction neutralization by individual sera against JEV was determined as described in the Materials and Methods section; mean titres (n = 4/ group) and range are given.

8.2.6 Cross-protection against highly virulent WNV_{NY99} challenge with JE-ADVAX™ in the absence of CD8+ T cells.

Previous studies have shown that vaccination against encephalitic flaviviruses can be associated with either cross-protective immunity or disease-potentiating immunity (reviewed in (Lobigs and Diamond, 2012)). Enhancement of disease post-vaccination was attributed to an antibody-dependent phenomenon, a mechanism similarly hypothesized to be the basis of severe dengue hemorrhagic fever after secondary dengue virus infection (reviewed in (Murphy et al., 2011)). This is especially important, given that antigenically closely related flaviviruses under JE-serocomplex can co-exist within a particular geographical area, for instance, WNV and JEV in India, or WNV and MVEV in the Australasian region (reviewed in (Mackenzie *et al.*, 2004; van de Hurk *et al.*, 2009)). Establishing the feasibility of a broadly cross-protective flaviviral vaccine is also important given that, up to this date, there is no currently available WNV vaccine, and the likelihood of developing a vaccine against low endemicity MVEV is seen as economically unviable.

To further extend the investigation on whether Advax-adjuvanted ccJE vaccine exhibits cross-protective value against a closely-related flavivirus, 8-week-old $\beta2m^{-/-}$ mice were immunized with two doses of 0.5 μg ccJE + Advax vaccine preparation, and challenged sc with $1x10^3$ PFU of WNV_{NY99} a month after completion of vaccination schedule (Fig. 8.8). All $\beta2m^{-/-}$ control mice immunized with PBS only succumbed to WNV_{NY99} challenge, with MST of 11.5 ± 1.4 days. In contrast, $\beta2m^{-/-}$ mice immunized with Advax-adjuvanted ccJE had significantly lower mortality rate (15%) and longer MST (12.5 ± 0.7 days). This data clearly demonstrate that Advax-adjuvanted ccJE vaccine provides substantial cross-protection against lethal West Nile encephalitis.

To determine the cell population mainly responsible for cross-protection of Advax-adjuvanted ccJE vaccine against WNV_{NY99} infection, an immune splenocyte transfer experiment was done (Fig. 8.9). Eight-week-old B/6 wt donor mice were immunized with 2 doses of 0.5 μg ccJE + Advax vaccine preparation, and sacrificed 3 weeks later after the last vaccine dose for splenocyte isolation. $CD4^+$ T cells were purified by B cell depletion via magnetic beads and subsequent $CD8^+$ T cell depletion via complement-mediated lysis, while B cells were purified via complement-mediated lysis of both $CD4^+$ and $CD8^+$ T cells. Purified immune $CD4^+$ T or B cells were then injected iv into 8-week-old B/6 mice. A day after transfer, recipient and naive control mice were challenged sc with 10^4 PFU of WNV_{NY99}. Relative to the naive control group, immune $CD4^+$ T cell recipient mice group displayed lower, but not significantly different, mortality rates (33% and 50%, respectively). On the contrary, immune B cell recipient mice group displayed significantly improved outcome after WNV_{NY99} infection, with a 13% mortality rate. This data clearly demonstrate that memory B cells play a predominant role in Advax-adjuvanted ccJE vaccine-mediated heterologous protection against WNV_{NY99} infection.

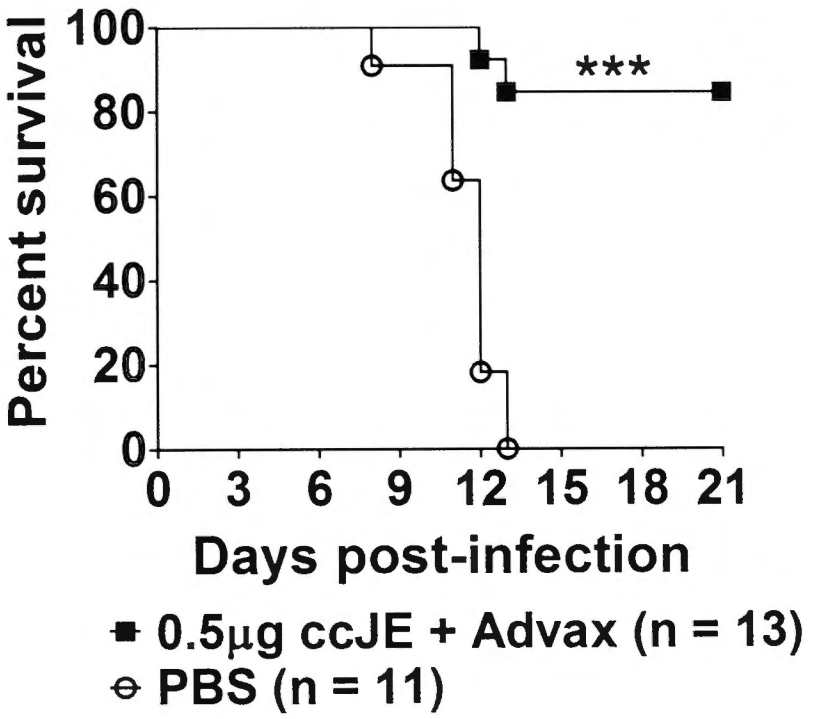


Figure 8.8 Cross-protective value of Advax-adjuvanted ccJE vaccine against challenge with WNV_{NY99}. Groups of 8-week-old $\beta 2m^{-/-}$ mice were immunized with two doses of 0.5 μg of ccJE + Advax or PBS control, delivered 2 weeks apart. Six weeks after completion of the vaccination schedule, mice were inoculated sc with 10^3 PFU of WNV_{NY99}. Morbidity and mortality were recorded daily, and surviving mice were monitored for 21 days. The data shown were constructed from two independent experiments. Viral challenge was performed by Dr Natalie Prow at a PC3 facility in University of Queensland. Asterisks denote statistical significance (***, $P < 0.001$).

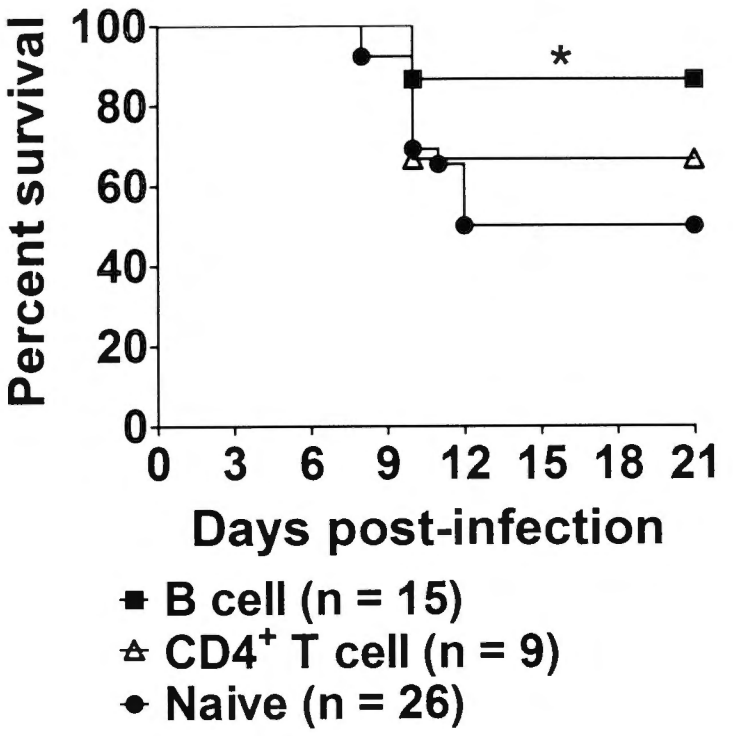


Figure 8.9 Advax-adjuvanted ccJE vaccine provides B cell-mediated cross-protection against WNV_{NY99} challenge. Eight-week-old B6/wt donor mice were immunized with two doses of 0.5 μg of ccJE + Advax, delivered 2 weeks apart, B cells and $CD4^+$ T cells purified at 3 weeks after completion of the vaccination schedule, and adoptively transferred ($1x10^7$ cells/mouse) into 6-week-old B/6 wt mice recipients. Another group of B/6 wt mice did not received any cells, and served as naive control. A day after splenocyte transfer, mice were challenged sc with 10^4 PFU of WNV_{NY99}. Mice were monitored twice daily for morbidity and mortality over a 21-day-observation period. Experiment was performed, together with Dr Natalie Prow, at a PC3 facility in University of Queensland. Asterisks denote statistical significance (*, $P < 0.05$).

8.2.7 Cross-protection with MVE. Previously, Licon Luna *et al.* (2002) demonstrated that mice deficient in $CD8^+$ T cell effector pathways are less susceptible to challenge with MVEV. In support of the aforementioned finding, $\beta 2m^{-/-}$ mice had better survival outcome relative to B/6 wt control when challenged with MVEV (unpublished data). Given the relative resistance to MVEV challenge (mortality rate of < 30%), $\beta 2m^{-/-}$ mice is not a suitable model for a vaccine protection experiment. Alternatively, to address the question whether Advax-adjuvanted ccJE vaccine also exerts cross-protection against MVEV challenge, an immune B cell transfer to weanling mice was performed (Fig. 8.10). Weanling mice are highly susceptible to MVEV, and provide a standard virulence model. Eight-week-old B/6 wt donor mice were immunized with 2 doses of 0.5 μg ccJE + Advax. Six weeks after the last dose, donor mice were sacrificed, splenocytes were isolated, and immune B cells were purified via complement-mediated lysis of $CD4^+$ and $CD8^+$ T cells. Purified immune and naive B cells (isolated from unvaccinated mice) were then injected ip into 4-week-old B/6 mice. A day after transfer, recipient mice were challenged iv with 10^5 PFU of MVEV. Relative to naive B cell recipient control mice group, immune B cell recipient mice displayed lower mortality rate (40% vs. 80%, respectively), and longer MST (12.8 ± 1.5 vs. 11.3 ± 1.3 days, respectively). This data demonstrate that memory B cell cells, induced by Advax-adjuvanted ccJE vaccine, provides significant cross-protection against Murray Valley encephalitis.

As a supplementary contribution to an earlier publication (Lobigs *et al.*, 2009), I also investigated on the cross-protective value of ChimeriVax-JE against MVEV, with the use of another stringent mice model. Groups of 6-week-old IFN-$\alpha R^{-/-}$ mice were immunized ip with $1x10^5$ PFU of ChimeriVax-JE, or left unimmunised (Table 8.8). A month later after immunization, mice were inoculated iv or via the fp with $1x10^2$ PFU

of MVEV. All unimmunised mice succumbed to the disease, with MST delayed by ~1 day in mice challenged via the fp in comparison to that following iv challenge. All immunized mice were completely protected from infection. Next, I investigated on the mechanism of cross-protection of ChimeriVax JE against MVEV, through splenocyte transfer experiments. ChimeriVax-JE immune splenocytes were transferred to naive mice with or without the additional transfer of immune serum (Table 8.8). Recipient mice were challenged with MVEV (10^2 PFU) by the iv or sc route. Transfer of ChimeriVax-JE immune splenocytes (1×10^7 or 5×10^7 cells) did not provide protection from lethal challenge by either of the two routes but significantly prolonged survival by 1 to 2 days in the recipient groups relative to untreated mice (Table 8.8). Naive splenocyte transfer did not alter MST and mortality. Immune serum transfer (300 µl total given at intervals pre- and post-challenge [Table 8.8]) also failed to protect animals from lethal MVEV infection but delayed the MST by ~3 days in comparison to that seen in the untreated controls. $PRNT_{50}$ titers of pooled immune sera used in the transfer experiments were in the ranges of 320 to 640 against JEV and 20 to 40 against MVEV. Mice were partially protected against lethal MVEV challenge following transfer of immune serum plus splenocytes, demonstrating the partial contribution of cell-mediated immunity elicited with ChimeriVax-JE in heterotypic protection. This was at least in part mediated by T cells, given that B-cell-depleted splenocytes maintained some protective value when transferred in the presence of immune serum, although this effect was marginal.

Altogether, this final section further supports the feasibility of using cross-protective vaccines against closely-related flaviviruses. Both Advax-adjuvanted ccJE and ChimeriVax-JE provided significant cross-protection against challenge with MVEV, and this protection is largely mediated by a durable humoral immune response.

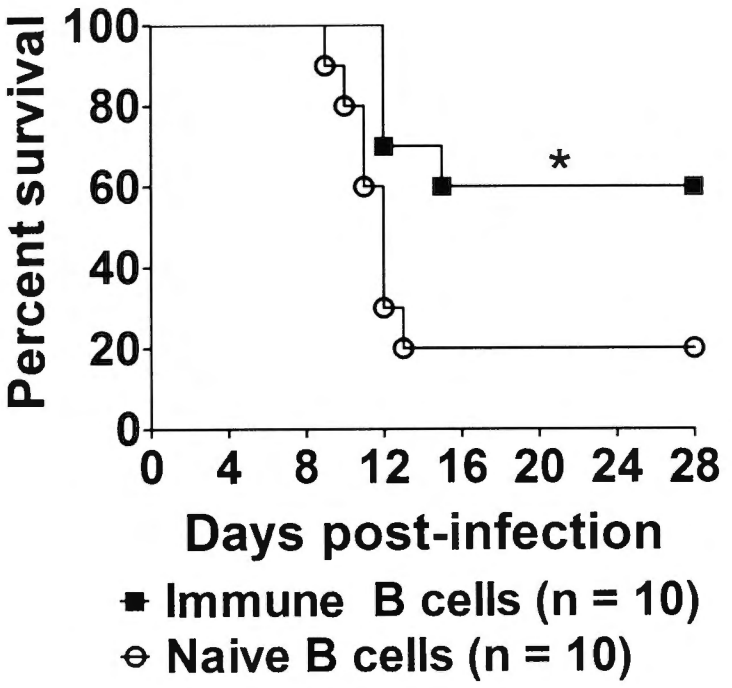


Figure 8.10 Advax-adjuvanted ccJE vaccine exerts cross-protection against challenge with MVEV. Eight-week-old B/6 wt donor mice were immunized with two doses of 0.5 μg of ccJE + Advax, delivered 2 weeks apart. B cells were purified at 6 weeks after completion of the vaccination schedule, and adoptively transferred ip ($1x10^7$ cells/mouse) into 4-week-old B/6 mice recipients. A control group of B/6 mice received naive B cells isolated from unvaccinated mice. A day after transfer, mice were challenged iv with 10^5 PFU of MVEV. Mice were monitored twice daily for morbidity and mortality over a 28-day-observation period. Asterisks denote statistical significance (*, $P < 0.05$).

Table 8.8 Contribution of immune responses to heterotypic protection by ChimeriVax-JE against MVEV

Challenge route and treatment[a]	No. of survivors/total	ATD, days (*P*)[b]
iv		
None	0/8	5.9
Immunization (10^5 PFU ChimeriVax-JE)	5/5	NA
Immune serum (300 μl)[c]	0/4	9.5 (0.003)
Immune splenocytes (1×10^7 cells)	0/8	7.4 (0.0004)
Immune splenocytes (5×10^7 cells)	0/6	7.3 (0.001)
sc		
None	0/5	6.8
Immunization (10^5 PFU ChimeriVax-JE)	11/11	NA
Immune serum (300 μl)[d]	0/6	9.7 (0.006)
Naive splenocytes (2×10^7 cells)	0/10	6.8
Immune splenocytes (2×10^7 cells)	0/8	7.8 (0.002)
Immune serum (300 μl)[d] plus splenocytes (2×10^7 cells)	4/10	12.8 (0.0006)
Immune serum (300 μl)[d] plus T cells (1×10^7 cells)	2/6	9.0 (0.002)
Immune serum (300 μl)[d] plus T cells (5×10^6 cells)	1/5	7.8 (0.01)

[a] Six-week-old IFN-α-$R^{-/-}$ mice were immunized with 10^5 PFU of ChimeriVax-JE ip or left untreated. These donor mice were sacrificed 4 weeks later, and serum and spleen cells were harvested and transferred to 10-week-old IFN-α-$R^{-/-}$ recipients, which were challenged with 10^2 PFU MVEV iv or by injection into the fp. Age-matched naive and ChimeriVax-JE-immunized mice were included as controls.

[b] The ATD was analyzed for significance using the Wilcoxon signed-rank test. For mice challenged iv, the ATD was compared to that of the naive group. For mice challenged sc, the effect of serum transfer only on ATD was compared to that of the naïve group, while the effect of splenocyte transfer with or without immune serum on ATD was compared to that of naïve splenocyte recipient mice. NA, not applicable.

[c] Immune serum transfer schedule: 150 μl on day 1 pre-challenge and 150 μl on day 1 post-challenge.

[d] Immune serum transfer schedule: 100 μl on day 1 pre-challenge and 50 μl on days 0, 1, 3, and 5 post-challenge.

Chapter 9

Concluding Remarks

A sound understanding on the immunobiology of infectious diseases is essential. Fundamental discoveries can generate basic insights that can aid in designing rational vaccines and novel therapies. Japanese encephalitis (JE) is the leading cause of viral encephalitis in Asia. In terms of incidence and severity of encephalitis in human populations, Japanese encephalitis virus (JEV) is the most important member of the JE serocomplex, which includes Murray Valley encephalitis virus (MVEV) and West Nile virus (WNV). In spite of that, the immunological correlates for recovery from primary JEV infection remains poorly defined, in contrast to a more detailed understanding with regards to the pathogenesis of West Nile encephalitis.

In this work, I have established a pathogenesis model for JE. C57BL/6 wild-type (wt) mice, inoculated subcutaneously (sc) via the footpad with low-dose virus JEV, displayed fatal encephalitis in ~60%, and viral burden in the central nervous system (CNS) (Chapter 3). The disease closely resembles human viral encephalitis, which is characterized by seizures, ataxia, postural imbalance, and at times, unilateral hind limb paralysis. Wt mice induces a robust immune response to the sc challenge with JEV (Figure 9.1), characterized by systemic type I interferon response within the first 48 hours, NK cell activity that peaks at ~D3-4 post-infection (pi), JEV-specific IgM response that is initially detectable on D4, and peaks on D8 pi, JEV-specific IgG response that is initially detectable on D8 pi and increases thereafter, gradual splenocyte expansion of $CD4^+$ and $CD8^+$ T cells, extensive $CD8^+$ T cell activation (judged by upregulation of surface markers CD69 and CD25 and cytokine production after stimulation with a JEV NS4B protein-derived H-$2D^b$-binding peptide) peaking on ~D7 pi, and CCR5-mediated trafficking of leukocytes into the CNS (Chapters 3-7).

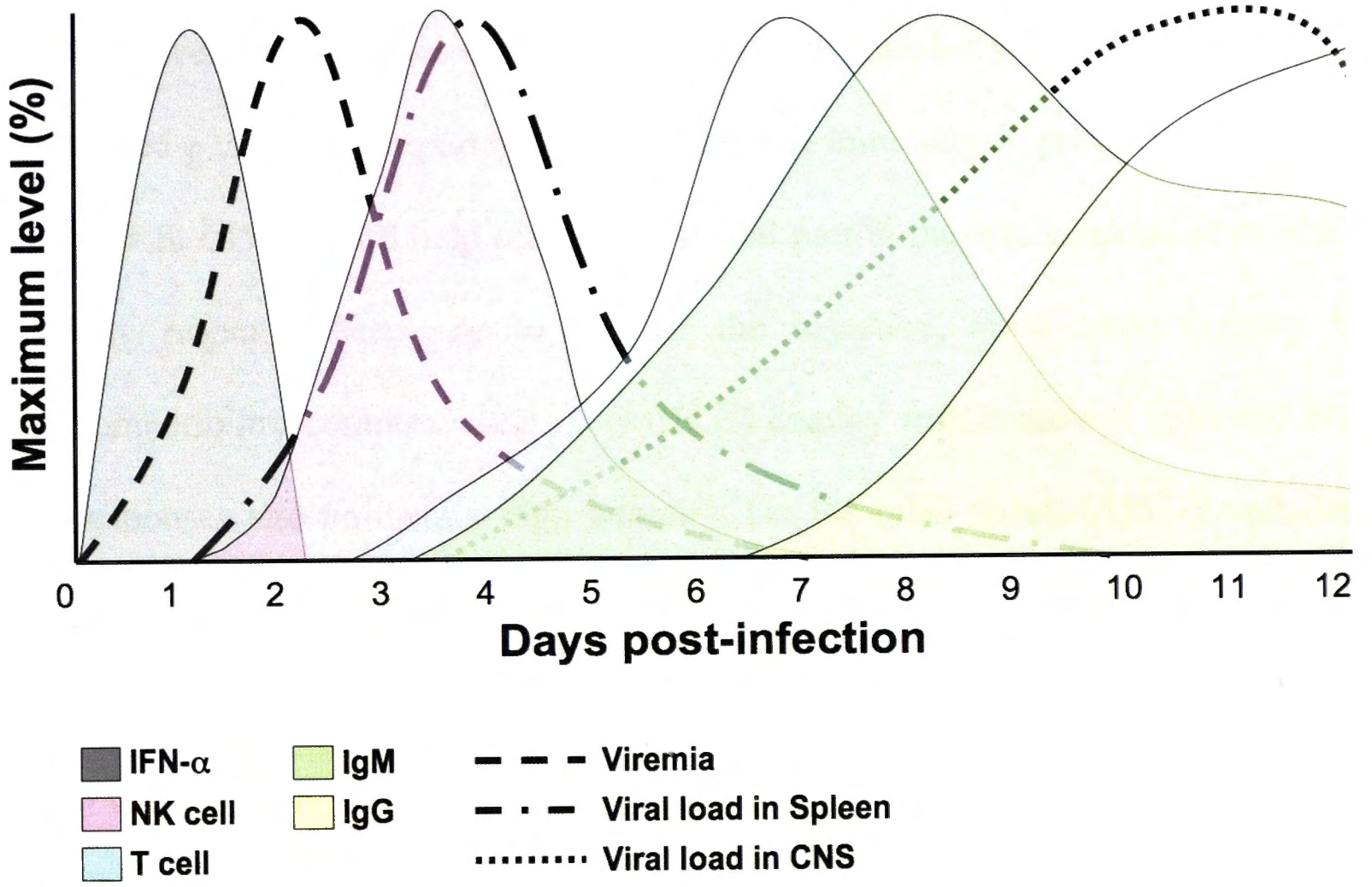


Figure 9.1 Kinetics of immune response after primary JEV infection. Approximate graphical representation of immune response kinetics (shaded areas under solid lines) and viral dissemination (non-solid lines) in wt mice after sc infection with low-dose JEV. Data is extrapolated from the following: IFN-α - serum levels determined by ELISA, NK cell – cytotoxic assay and intracellular staining of IFN-γ, T cell – T cell expansion and intracellular cytokine staining by FACS, Anti-JEV IgM – serum levels determined by ELISA, and Anti-JEV IgG - serum levels determined by ELISA, serum and spleen viral load – real time RT-PCR, CNS viral load – plaque titration in Vero cells.

I have demonstrated, by *in vivo* depletion of NK cells in wt mice, that this innate cytolytic effector cell population is dispensable for survival, and control of JEV infection in the periphery and in the CNS (Summarized in Table 9.1). Using mice lacking B cells ($\mu MT^{-/-}$ mice) and immune B cell transfer to wild-type mice, I have established a critically important role for humoral immunity in preventing virus spread to the CNS. $CD4^+$ T cell help plays an essential part in the maintenance of an effective antibody response necessary to combat the infection, since mice lacking major histocompatibility complex class II (MHCII) display with truncated IgM and blunted IgG responses and uniformly high lethality. On the other hand, $CD8^+$ T cells have a limited role in recovery from primary infection with JEV. Only a combined transfer of immune $CD4^+$ T and $CD8^+$ T cells, and not the transfer of immune $CD8^+$ T cell alone, afford protection in JEV-challenged recipient wt mice. Using *in vivo* depletion of $CD8^+$ T cells in wt mice, an increase viral burden in the CNS is observed, however no significant effect is seen on the survival phenotype. It remains unclear whether immunopathology associated with $CD8^+$ T cell infiltration of the brain or an insufficient magnitude of the response, *per se*, explains why the viral load reduction in the CNS mediated by $CD8^+$ T cells do not translate into a survival advantage. Together, these data suggest, at best, a marginal contribution of $CD8^+$ T cells in the pathogenesis of JE.

The relative importance of effector pathways of cytotoxicity in protection from lethal JE was examined using knockout mice models (Chapters 3-5). Mice lacking individual key effector molecules (Fas receptor, perforin, or granzymes) of cytolytic pathways display increased CNS JEV burden, however, present with no impact on survival rate. Only mice defective in both granule exocytosis and Fas-Fas ligand pathways display increased susceptibility to JEV infection. The two cell contact-dependent cytotoxic effector mechanisms act within the CNS to reduce disease severity, and indicate a

redundancy in control JEV infection (Chapter 4). The pleiotropic cytokine, IFN-γ, is critical for protection from severe JE (Chapter 5), through a mechanism that involves suppression of virus growth in the CNS. T cells trafficking into the CNS are the main source of IFN-γ, which promotes non-cytolytic clearance of virus from the CNS.

In Chapter 6, I have extended the investigation on a key chemokine receptor documented as a critical host antiviral factor against JE. The chemokine receptor CCR5 play a role in activation and chemotaxis of leukocytes after JEV infection. Inoculation with JEV in mice deficient in CCR5 significantly displays increased mortality and viral burden in the CNS. Humoral immune responses, which are essential in recovery from JEV infection, are of similar magnitude in CCR5 sufficient and deficient mice. However, absence of CCR5 results in a multifaceted deficiency of cellular immune responses characterized by reduced natural killer and $CD8^+$ T cell activity, low splenic cellularity, and impaired trafficking of leukocytes to the brain. These findings are consistent with a mechanism by which CCR5 expression enhances lymphocyte activation, and thereby promotes host survival in JE.

Table 9.1 Factors affecting outcome of JEV infection

Factor	Outcome of JEV infection
Age	↑ disease severity in younger animals
Route of infection	intracranial and intranasal = high mortality extraneural = ↓ disease severity
Mouse strain background	impacts on disease outcome 129 is more resistant than C57Bl/6
Innate Immunity	
Natural Killer cells	absence = no effect on disease severity
Macrophages	susceptible to JEV infection
MyD88	absence = no effect on disease severity
Interferon-α receptor	absence = ↑ disease severity
Adaptive Immunity	
Cell effectors	
B cells	absence = ↑ disease severity
CD4+ T cells	absence = ↑ disease severity
CD8+ T cells	absence = ↑ CNS viral burden without impact on mortality rate
Cytolytic Pathways	
Perforin	absence = no effect on disease severity
Granzyme A/B	absence = no effect on disease severity
Fas	absence = no effect on disease severity
Perforin + Granzyme A/B	absence = no effect on disease severity
Fas + GzmA/B	absence = ↑ disease severity
Other Cytokines and Chemokines	
CCR5	absence = ↑ disease severity
Interferon-γ	absence = ↑ disease severity

(↑) increased, (↓) decreased

The above findings highlight some differences in the pathogenesis of JE and closely related encephalitic flaviviruses. First, $CD8^+$ T cells display variable and conflicting roles in the pathogenesis of flaviviral encephalitis. Whereas the $CD8^+$ T cell response is required for virus elimination from the CNS and survival in mouse models of West Nile encephalitis (Shrestha and Diamond, 2004; Shrestha et al., 2006*a*; Wang *et al.*, 2003; Wang *et al.*, 2004), a disease-potentiating effect of cytotoxic T cells was documented in mice infected with MVEV (Licon Luna et al., 2002). In this work, I have presented that $CD8^+$ T cells are at best, subsidiary in providing protective contribution against lethal JE. Second, mice with genetic deficiency in either FasL-Fas or perforin-dependent pathway of cytotoxicity showed greatly increased susceptibility to virulent lineage I WNV infection (Shrestha and Diamond, 2007; Shrestha *et al.*, 2006*a*) but did not differ from wt mice in susceptibility to infection with JEV (Chapter 3) or lineage II WNV strain Sarafend (Wang *et al.*, 2004) and were more resistant to infection with MVEV (Licon Luna *et al.*, 2002). Only a combined deficiency in FasL-Fas and perforin-dependent pathways, increases the susceptibility of mice to severe JE, indicating a redundancy in contact-dependent pathways of cytotoxicity. Third, the mechanism of increased susceptibility to WNV infection in IFN-$\gamma^{-/-}$ mice was mainly attributed to failure of early peripheral viral clearance by a potent IFN-γ-producing γδ T cells (Shrestha *et al.*, 2006*b*), whereas in the case of JE, the morbid phenotype in IFN-$\gamma^{-/-}$ mice is largely attributed to absence of IFN-γ-producing T cell-mediated non-cytolytic clearance of virus from the CNS.

Another extended investigation is the establishment of a novel immunodeficient mouse strain, *Tuara*. Remarkably, the loss-of-function mutation, conferring enhanced susceptibility to flavivirus infections, is a spontaneously generated defect found in an inbred knockout mouse strain, MHCII-A$\alpha^{-/-}$ mice. After series of experiments, the gene

of interest have been segregated from the MHCII defect to produce the *Tuara* mouse, for which, after customized sequence capture and re-sequencing, is identified to carry candidate mutations within ~50 Mb region of Chromosome 1, with the G>T transversion at mouse genome reference position 52,179,547 bp of STAT1 gene as the most likely causal mutation. This is a unique STAT1 mutation, with a likely profound biologic consequence of clinical relevance. This finding can provide valuable additional insights, in relation to the increasing diverse list of STAT1 deficiencies in humans (reviewed in (Boisson-Dupuis *et al.*, 2012)).

Finally in Chapter 8, in application of the insights gained from the experiments on the basic immunobiology of JE, I have investigated on the mechanism of protection of new generation JE vaccines (live attenuated ChimeriVax-JE and Advax-adjuvanted inactivated Vero cell-derived JE vaccines) against homologous or heterologous flaviviral challenge. Using a $\beta 2m^{-/-}$ mice challenge model, the crucial role of long-term memory B cells in vaccine-mediated protection against JEV and WNV_{NY99} have been established. The significant level of protection has been provided in the absence of $CD8^+$ T cells or long-term vaccine-induced neutralizing antibodies. Using splenocyte transfer experiments in IFN-α-$R^{-/-}$ or weanling wt mice, humoral immunity is identified as the key survival determinant in JE vaccine-mediated cross-protection against Murray valley encephalitis. Altogether, it can be concluded that humoral immunity plays the most critical role in both primary and vaccine-mediated immune responses against infection with JEV or related flaviviruses.

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