DEVELOPMENT AND ASSESSMENT OF NOVEL VACCINATION STRATEGIES FOR HEPATITIS B VIRUS INFECTION

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To my loving family

for your boundless love, care, support and always being there for me

&

To Jean Ang

who stood by me when things looked bleak and supported me each step of the way

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Abstract

The current hepatitis B virus (HBV) vaccine is of no benefit in the treatment of patients with chronic HBV infection, and current antiviral therapies which inhibit the virus polymerase are not highly effective. The ultimate aim of this Ph.D. project was to develop and assess therapeutic vaccination strategies that induce immune responses that target virus infected hepatocytes and allow successful control of chronic HBV infection. To this end, a number of vaccination strategies were tested using the duck hepatitis B virus (DHBV) model. The DHBV model provides a versatile and reproducible experimental system for testing vaccination strategies as the outcomes of DHBV infection in ducks of different ages infected with different doses of virus have been well characterised.

In initial studies described in Chapter 3, recombinant DHBV core antigen (rDHBcAg) expressed in *E. coli*, was purified and used to immunise rabbits and mice to produce specific polyclonal and monoclonal antibodies for the detection of DHBV core antigen (DHBcAg). Immuno-staining techniques using these anti-DHBcAg-specific antibodies were then optimised. Immunoperoxidase detection of DHBcAg in duck liver sections was an essential part of this analysis as it allowed comparison with detection of DHBV surface antigen (DHBsAg) and confirmation of the percentage of DHBV-infected hepatocytes. Western Blot and immunofluorescent detection of DHBcAg were also developed and optimised and then all 3 immuno-staining techniques were used in subsequent Chapters to assess the efficacy of the different vaccination strategies.

As described in Chapter 4, duck CD40L (DuCD40L), was assessed as an immunological adjuvant in a protective DNA vaccine study. In humans, CD40L acts as a co-stimulatory molecule in the CD40-signalling pathway that is involved in the activation of antigen presenting cells (APC) and the generation of humoral and cell mediated immune (CMI)

responses. In the current studies the DuCD40L cDNA sequence was cloned using mRNA from duck peripheral blood mononuclear cells (PBMC) into the expression construct, pcDNA3, to yield pcDNA3-DuCD40L. Cells and supernatants of cells transfected with pcDNA3-DuCD40L were subsequently tested for bioactivity using *in vitro* assays. The cloning and analysis of expression of the DuCD40L was performed in our laboratory by Dr Feng Feng. The DuCD40L expression construct was then co-administered intramuscularly with DNA vaccines expressing the DHBV surface and core antigens to ducks at 14 and 28 days of age. Two weeks after the second vaccination, ducks were challenged intravenously (i.v.) with $4.5x10^{10}$ DHBV genomes. Administration of the DuCD40L expression construct and the DHBV DNA vaccines in combination resulted in a 10-fold greater anti-DHBs antibody response and a significant decrease in the number of DHBV-infected hepatocytes at day 4 post-challenge (p.c.) compared to ducks that received DHBV DNA vaccines alone. Ultimately, as expected, all of the ducks successfully cleared their DHBV infection. Nevertheless, we determined that DuCD40L enhanced humoral immune responses and lead to reductions in the percentage of DHBV-infected hepatocytes following challenge.

As described in Chapter 5, the expression of DuCD40L was further assessed in a 2^{nd} protective DNA vaccine study in 14-day-old ducks which are more susceptible to the development of persistent DHBV infection. The DuCD40L expression construct and DNA vaccines expressing the DHBV surface proteins were administered to ducks at 4 and 14 days of age. On the same day as the second vaccination, ducks were challenged i.v. with $5x10^{8}$ DHBV genomes, a dose of DHBV that is 500-times higher than the dose known to result in persistent DHBV infection. Unexpectedly, following DHBV challenge, no significant differences in the percentage of DHBV-infected hepatocytes or anti-DHBs antibody titres were observed between ducks receiving DHBV DNA vaccines with DuCD40L expression construct and ducks receiving DHBV DNA vaccines alone. At day 21 p.c., all ducks vaccinated with DHBV DNA vaccines with or without the DuCD40L expression construct

had successfully cleared their DHBV infection while five out of five vector control ducks developed persistent DHBV infection. Interestingly, two out of five ducks that received the DuCD40L expression construct alone also cleared their DHBV infection. The studies described in Chapters 4 and 5 suggest DuCD40L may enhance immune responses in ducks and that DuCD40L should be further investigated as an immunological adjuvant in future vaccine studies.

Finally as described in Chapter 6, a post-exposure vaccination study was performed that combined treatment with the Bristol-Myers Squibb nucleoside analogue, Entecavir (ETV), and "prime-boost" vaccination using DNA vaccines and recombinant fowl-pox virus (rFPV) strains that express DHBV surface or core alone. Previous "prime-boost" vaccination studies in the laboratory had used DNA vaccines and rFPV strains that expressed both DHBV surface and DHBV core in combination. We aimed to determine if DHBV surface antigen which generates neutralising anti-DHBs antibodies, or DHBV core antigen which generates non-neutralising anti-DHBc antibodies, could provide the essential epitopes in a DNA vaccine and "prime-boost" protocol to enable the resolution of DHBV infection. 14 day-old ducks were inoculated i.v with $5x10^8$ DHBV genomes and immediately treated with ETV (1.0mg/kg/day) for 14 days. At the same time, ducks received the "priming" DHBV DNA vaccines and 7 days later received the "boosting" vaccination with the rFPV-DHBV vaccines. The findings showed that protective humoral and CMI responses generated by "prime-boost" vaccination strategies with either DHBV surface or DHBV core alone blocked virus spread and replication and resulted in the targeting the destruction of infected hepatocytes and the resolution of DHBV infection. In contrast, ducks treated with ETV plus the control vectors showed restricted spread of DHBV infection in the liver during ETV treatment, but DHBV infection become widespread in four out of five ducks after ETV treatment was withdrawn. These findings indicate that DHBV surface and core antigen as measured in our studies are equally effective as components of our post-exposure "prime-boost" protocol. Since anti-

DHBc antibodies are non-neutralising this suggests that our “prime-boost” protocol may be generating effective CMI that were able to control DHBV infection.

The studies described in this thesis show that increasing levels of expression of DuCD40L may increase the efficacy of DNA vaccination protocols. Our novel vaccination strategy using ETV treatment in combination with “prime-boost” vaccination also suggests that DHBV surface and core antigen are both able to provide sufficient immunity to allow clearance of DHBV infection. Future studies are warranted to test other types of immunotherapy and antiviral agents to provide new directions for future therapeutic vaccination strategies for chronic HBV infection.

Declaration of originality and consent

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Devin TEOH 1st November 2014

Publications, presentations and awards arising from this thesis

Manuscripts published

Feng, F., **C. Q. Teoh**, Q. Qiao, D. Boyle, A.R. Jilbert (2010). "The development of persistent duck hepatitis B virus infection can be prevented using antiviral therapy combined with DNA or recombinant fowlpoxvirus vaccines." Vaccine **28**(46): 7436-7443.

Manuscripts in preparation

C.Q. Teoh, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. "Entecavir therapy combined with "prime-boost" vaccination with either duck hepatitis B virus (DHBV) surface or core are able to block virus replication and spread and target DHBV-infected hepatocytes."

C.Q. Teoh, F. Feng, Q. Qiao, B. Baradaran, A.R. Jilbert. "Cloning, expression and use of a duck CD40 ligand (CD40L) DNA construct as a molecular adjuvant for duck hepatitis B virus DNA vaccines."

C.Q. Teoh, F. Feng, A.R. Jilbert. "Purification of recombinant duck hepatitis B core antigen (rDHBcAg) particles for production of specific polyclonal and monoclonal antibodies against DHBcAg."

Oral presentations

C.Q. Teoh, F. Feng, A.R. Jilbert. Improving therapy for chronic hepatitis B infection using the duck hepatitis B virus model. Infectious Diseases Laboratories Research Seminar, IMVS, Adelaide, May, 2010.

C.Q. Teoh, F. Feng, A.R. Jilbert. Immune therapy for chronic hepatitis B infection. Australian Society of Microbiology (ASM) Student Award Night, Womens' and Childrens' Hospital, North Adelaide, November, 2010.

C.Q. Teoh, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. Entecavir therapy combined with "prime-boost" vaccination with either duck hepatitis B virus (DHBV) surface or core are able to block virus replication and spread and target DHBV-infected hepatocytes. Australian Centre for HIV and Hepatitis Virology Workshop, Novotel Barossa Valley Resort, June, 2008.

C.Q. Teoh, F. Feng, A.R. Jilbert. Immune therapy for chronic hepatitis B infection using duck model. The University of Adelaide, Ph.D. major review, Adelaide, September, 2008.

Poster presentation

C.Q. Teoh, F. Feng, Q. Qiao, A.R. Jilbert. Cloning, expression and use of a duck CD40 ligand (CD40L) DNA construct as a molecular adjuvant for duck hepatitis B virus DNA vaccines. Australian Society of Microbiology (ASM), Hobart, Tasmania, June, 2011.

C.Q. Teoh, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. Entecavir therapy combined with "prime-boost" vaccination with either duck hepatitis B virus (DHBV) surface

or core are able to block virus replication and spread and target DHBV-infected hepatocytes. 14th International Congress of Immunology (ICI), Kobe, Japan, August, 2010.

C.Q. Teoh, F. Feng, Q. Qiao, A.R. Jilbert. Cloning, expression and use of a duck CD40 ligand (CD40L) DNA construct as a molecular adjuvant for duck hepatitis B virus DNA vaccines. University of Adelaide, School of Molecular and Biomedical Science, Research Symposium, Memorial Drive, Adelaide, December, 2010.

C.Q. Teoh, F. Feng, Q. Qiao, A.R. Jilbert. Duck CD40 ligand (CD40L) DNA construct as a molecular adjuvant for duck hepatitis B virus DNA vaccines. 5th Australian Virology Group (AVG) Meeting, Lorne, Victoria, December, 2009.

C.Q. Teoh, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. Entecavir therapy combined with "prime-boost" vaccination with either duck hepatitis B virus (DHBV) surface or core are able to block virus replication and spread and target DHBV-infected hepatocytes. University of Adelaide, School of Molecular and Biomedical Science, Research Symposium, National Wine Centre, Adelaide, December, 2008.

Awards Arising

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Abbreviations

~	Approximately
℃	Degrees Celsius
µg	Microgram
µL	Microliter
1H.1	Monoclonal anti-pre-S/S antibodies
3TC	Lamivudine
AA	Amino acid
AB	Applied Biosystems
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADV	Adefovir
ALT	Alanine aminotransferase
Amp	Ampicilin
APC	Antigen presenting cells
APV	*Avipoxvirus*
ASHV	Arctic squirrel hepatitis virus
AusDHBV	Australian strain of DHBV
bp	Base pair
BSA	Bovine serum albumin
C	Core
cccDNA	Covalently closed circular DNA
CD154	CD40 ligand
CD40L	CD40 ligand
CESC	Chicken embryonic skin cells
CHB	Chronic HBV infection
CHO	Chinese hamster ovary

CMI	Cell-mediated immunity
ConA	Concanavalin A
C-ORF	Core open reading frame
CPE	Cytopathic effect
CpG	Cytosine-phosphate-guanosine
CQT-1	Polyclonal rabbit anti-DHBc antibodies
CQT-2	Polyclonal rabbit anti-DHBc antibodies
CsCl	Caesium chloride
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CTL	Cytotoxic T lymphocytes
DAB	3,3-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
dGTP	Deoxyguanosine triphosphate
DHBcAg	DHBV core antigen
DHBeAg	DHBV e antigen
DHBV	Duck hepatitis B virus
DMEM	Dulbecco's modified Eagle's minimal essential
dNTP	Deoxynucleotide triphosphates
dslDNA	Double-stranded linear virus DNA
DuCD40L	Duck CD40 ligand
DW	Distilled water
E. coli	Escherichia coli
EAA	Ethanol: Acetic Acid
EDTA	Ethylenediaminetetraacetic acid
EEV	Extracellular enveloped virus
ELISA	The enzyme-linked immunosorbent assay

ELISPOT	The enzyme-linked immunosorbent spot assay
ETV	Entecavir
FCA	Freund’s complete adjuvant
FCS	Fetal calf serum
FPV	Fowl pox virus
G	Gauge
gm	Gram
g	Centrifugal force
GSHV	Ground squirrel hepatitis virus
HBcAg	HBV core antigen
HBeAg	HBV e antigen
HBsAg	HBV surface antigen
HBSS	Hanks’ balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
hr	Hour
HRP	Horseradish peroxidase
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM-1	Intercellular Adhesion Molecule 1
IF assay	Immuno-fluorescence assay
IFN	Interferon
IFN-g	Interferon-gamma
IFN-α	Interferon-alfa
IFN-α2A	Interferon-alfa-2a

IFN-β	Interferon-beta
Ig	Immunoglobulin
IgY	Immunoglobulin Y
IL-12	Interleukin-12
IMV	Intracellular mature virus
IMVS	Institute of Medical and Veterinary Science
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITRs	Identical inverted terminal repeats
kbp	Kilo base pair
kDa	Kilo Dalton
LB	Luria broth
LMH	Chicken hepatoma cell line
MAb	Monoclonal antibodies
MAbSA	Monoclonal Antibodies SA
MHC-I	Class I histocompatibility molecules
MHC-II	Class II histocompatibility molecules
min	Minute
mL	Millilitre
MVA	Modified vaccinia Ankara
NA	Nucleot(s)ide analogues
NC membrane	Nitrocellulose membrane
NCBI	National Center for Biotechnology Information
NDS	Normal duck serum
NEB	New England Biolabs
NGS	Normal goat serum
NHMRC	National Health and Medical Research Council
NK	Natural Killer

NK-T	Natural Killer T-cell
NLS	Nuclear localization signal
NMS	Normal mouse serum
NO	Nitric Oxide
NPC	Nuclear pore complexes
NRS	Normal rabbit serum
NSS	Normal sheep serum
NTCP	Sodium taurocholate co-transporting polypeptide
O/N	Over night
OPD	O-phenylenediamine dihydrochloride
ORF	Open reading frame
p	*p*-value
p.c.	Post challenge
p.f.u	Plaque forming unit
p.i.	Post-infection
PAGE	Polyacrylamide Gel Electrophoresis
PB	Phosphate buffer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween
PCEF	Primary chicken embryonic fibroblasts
PCR	Polymerase chain reaction
PDEF	Primary duck embryonic fibroblasts
Peg	Pegylated
Pg-RNA	Pre-genomic RNA
PHA	Phytohemagglutinin M
Pol	Polymerase

P-ORF	Polymerase open reading frame
PreC	Pre-core
pre-S/S	Pre-surface
PS	Pre-surface
PRR	Pattern recognition receptors
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse transcription PCR
R39408	Polyclonal rabbit anti-DHBc antibodies
rcDNA	Relaxed circular DNA
RE	Restriction Endonuclease
rFPV	Recombinant FPV
rFPV-124	rFPV-DHBV strains expressing DHBV core
rFPV-125	rFPV-DHBV strains expressing pre-surface antigens
rFPV-DHBc	rFPV-DHBV strains expressing DHBV core
rFPV-DHBpre-S/S	rFPV-DHBV strains expressing pre-surface antigens
rFPV-DHBV	rFPV strains expressing DHBV antigens
RGHBV	Ross' goose hepatitis B virus
rHBsAg	Recombinant HBsAg
RI DNA	Replicative intermediate DNA
RT	Room temperature
S	Surface
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
Sec	Second
SGHBV	Snow goose hepatitis virus
SOC medium	Super optimal culture medium
S-ORF	Surface open reading frame

TCR	T cell receptor
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
TFV	Tenofovir
Th1	Type I
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
USA	United States of America
V	Volts
v.g.e.	Virus genome equivalents
WHO	World Health Organisation
WHV	Woodchuck hepatitis virus
WMHBV	Woolly monkey hepatitis B virus
X	X protein
X-ORF	X protein open reading frame