DEVELOPMENT OF HSPX VACCINE AGAINST LATENT TUBERCULOSIS USING ADJUVANT-DEPENDENT T-HELPER ALTERNATING PEPTIDE STRATEGY

LEW MIN HAN

UNIVERSITI SAINS MALAYSIA

2020

DEVELOPMENT OF HSPX VACCINE AGAINST LATENT TUBERCULOSIS USING ADJUVANT-DEPENDENT T-HELPER ALTERNATING PEPTIDE STRATEGY

by/oleh

LEW MIN HAN

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

November 2020

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest gratitude to my main supervisor, Dr. Tye Gee Jun, for guiding me throughout my journey towards pursuing a Ph. D. degree in Molecular Medicine. He is a highly effective, optimistic and authentic supervisor, who has truly inspired me in many ways. Thank you for your patience and efforts to encourage me in gaining insights into the intricate network of immune system and vaccine development. He has also taught me skills to acquire independent thinking and to solve experimental problem critically. It was truly a great honour to work with him as a team player. Besides, I am thankful to my co-supervisor, Assoc. Prof. Dr. Lim Theam Soon, for his kindness to share his invaluable knowledge with me during my postgraduate study. Special thanks to Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), for providing us the necessary facilities and equipment as required for the conduct of our research work. I thank Animal Research and Service Centre (ARASC) for supplying experimental mice for my in *vivo* study, and thanks to Dr. Isma Suzyta Binti Ismail and Ms. Adilah Binti Abdul Khalil for their kind assistance. I also want to say thanks to Malaysian Institute of Pharmaceuticals and Nutraceuticals (Ipharm) for giving me the opportunity to assess their flow cytometry facilities for immunophenotyping assays. Furthermore, I thank Mybrain Kementerian Pengajian Tinggi (KPT) scholarship, Fundamental Research Grant Scheme (FRGS, 203/CIPPM/6711380) and Higher Institution Centre of Excellence (HiCoE, 311/CIPPM/4401005) grant for their financial support to my research work. I am very much thankful to my parents for their love and sacrifices in supporting me to purse a higher level of education. I also want to dedicate my gratitude to my siblings for their kindness and motivations. I would not have

reached this far without your understanding, acceptance and continuous support along this long journey. I would like to thank my colleagues, Lee Shin Yong, Sylvia Annabel Dass, Chin Ding Sheng, Jacqueline Mark Kar Kei, Matthew Wong Tze Jian, Liew HuaQiang and Ahmad Ismail Abdo, for their friendship and motivation throughout my research study. It was my pleasure to work with all of you as a team. Besides, I also like to express my thanks to all my friends and staffs at INFORMM. Thank you for participating in this part of my life, our friendship is truly meaningful to me. Lastly, I would like to send my thanks anyone of you who have been giving me support upon the completion of my postgraduate study.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

$^{-}$	Low expression/downregulation
~	Approximately
˚C	Celsius
$^{+}$	High expression/upregulation
+	Plus/with addition of
=	Equal to
>	More than
±	More or less
≤	Less than or equal to
3’	End of DNA at carbon-3 position in its sugar-ring structure
5’	End of DNA at carbon-5 position in its sugar-ring structure
A_{405nm}	Absorbance observed at wavelength 405 nm
A_{595nm}	Absorbance observed at wavelength 595 nm
A_{600nm}	Absorbance observed at wavelength 600 nm
bp	Base pair
cm	Centrimeter
g	Gram
h	Hour
kbp	Kilo base pair
kDa	Kilo Dalton
L	Liter
M	Molar
mer	Number of peptide residues

mg	Miligram
mL	Milliliter
mm	Milimeter
mM	Milimolar
N	Normality
n	Sample size
ng	Nanogram
p	*p*-value
pg	Picogram
t	Time-point
™	Trademark
V	Volt
x	Multiply
xg	Times gravity
α	Alpha
β	Beta
γ	Gamma
δ	Delta
Δ	Mutational deletion
μg	Microgram
μL	Microliter
μm	Micrometer

LIST OF ABBREVIATIONS

2B4	Signaling lymphocyte activation molecule (SLAM) family receptor, CD244
2xYT	2 x Yeast Extract Tryptone
3D	Three-dimensional
6xHis	Six residues of histidine
A2AR	Adenosine A2A receptor
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
Acr1	Alpha-crystalline protein 1
Ad35	Adenovirus serotype 35 vector
Ad5	Adenovirus serotype 5 vector
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
Ag85A	Antigen 85A complex
Ag85B	Antigen 85B complex
ANOVA	Analysis of variance
AP-1	Activator protein *1*
APC	Antigen-presenting cell
APC	Allophycocyanin
APS	Ammonium Persulfate
ARASC	Animal Research and Service Centre
B7	Peripheral membrane protein CD80/CD86
BCG	Bacillus Calmette–Guérin
BCR	B-cell receptor

BD	Beckton Dickinson company
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C57BL/6	C57 black 6 mice
cAMP	Cyclic adenosine monophosphate
CASAC	Combined adjuvant for synergistic activation of cellular immunity
CCL	CC chemokine ligand
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
CD40L	CD40 ligand, also called CD154
CD62L	L-selectin
cDNA	Complementary DNA
CFA	Complete Freund's Adjuvant
CFP-10	Culture filtrate protein 10
CFSE	Carboxyfluorescein succinimidyl ester
$CFSE^{high}$	High CFSE signals, $CFSE^{+}$
$CFSE^{low}$	Low CFSE signals, $CFSE^{-}$
CLPS	Common lymphoid progenitors
CO	Carbon monoxide
CO2	Carbon dioxide
CpG-ODN	Synthetic oligodeoxynucleotides containing unmethylated CpG motifs
CREB	cAMP response element-binding protein
cSMAC	Supra-molecular activation complex
cTECs	Cortical Thymic epithelial cells

CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL2	Chemokine ligand 2
DAB	3,3'-diaminobenzidine
DCs	Dendritic cells
DDA	Dimethyldioctadecylammonium/trehalose 6,6,9-dibehenate
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DosR	Response regulator encoded by the *Rv3133c* gene of *M. tb* strain *H37Rv*
DosS	Sensory kinase encoded by *Rv3132c* gene of *M. tb* strain *H37Rv*
DosT	Sensory kinase encoded by *Rv2027c* gene of *M. tb* strain *H37Rv*
DOTAP	Dioleoyl-3-trimethylammonium propane
DP	Double positive
DPBS	Dulbecco's phosphate-buffered saline
DPG	Polyribocytidylic acid and gelatin
dSMAC	Distal area of pSMAC
dsRNA	Double stranded RNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Program on Immunisation
ESAT-6	Early secretory antigenic target protein 6

ESX-1	ESAT-6 secretion system 1
EsxS	ESAT-6-like protein
et al.	Et alia, and others
FACS	Fluorescence-activated cell sorting
Fas	Apoptosis antigen 1 (APO-1 or APT), also known as *Fas*R
*Fas*L	Ligand for *Fas* receptor
FBS	Fetal bovine serum
Fc	Fragment crystallizable region of antibody
FcγR	Fc gamma receptor
FITC	Fluorescein isothiocyanate dye
FMO	Fluorescence Minus One
Fmoc	Fluorenylmethoxycarbonyl protecting group
FoxP3	Forkhead box protein P3
FSC	Forward scatter
fTh	Follicular T helper cell
GATA-3	Zinc finger transcription factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEV	High endothelial venules
His	Histidine
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
HspX	Heat-shock protein α-crystallin tuberculosis antigen
HspX-pRSET	HspX-containing pRSET vector

i.d.	Intradermal injection
i.p.	Intraperitoneal injection
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IEDB	Immune Epitope Database
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IgG_1	Immunoglobulin isotype class 1
IgG_{2a}	Immunoglobulin isotype class 2a
Igk	Immunoglobulin kappa chain
IL	Interleukin
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-12/23p40	Cytokine containing p40 subunit shared by IL-12 and IL-23
IL-15R	Interleukin-15 receptor
IL-2R	Interleukin-2 receptor
IL-7R	Interleukin-7 receptor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRF-3	Interferon regulatory transcription factor 3
IRF-4	Interferon regulatory factor 4
IV	Intravenous injection
JAK-STAT	Janus Kinase/Signal Transducer and Activator of Transcription.
KIR	Human killer cell immunoglobulin-like receptors, CD158
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte-activation gene 3
LC	Liquid chromatography

Lck	Lymphocyte-specific protein tyrosine kinase
LT	Lymphotoxin
LTBI	Latent tuberculosis infection
Ltd	Limited company
M. tb	*Mycobacterium tuberculosis*
M. tuberculosis	*Mycobacterium tuberculosis*
MALDI-TOF	Matrix-assisted laser desorption/ionisation time of flight
MCP-1	Monocyte chemoattractant protein-1
MDA5	Melanoma differentiation-associated protein 5
MFI	Mean florescence intensity
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class one
MHC-II	Major histocompatibility complex class two
mIFN-γ	Mouse interferon gamma
MPL	Monophosphoryl lipid A
MPP	Multi-potential progenitor
MS	Mass spectrometry
MTBC	*Mycobacterium tuberculosis* complex
mTECs	Medullary thymic epithelial cells
MVA	Modified Vaccinia Ankara
MyD88	Myeloid differentiation primary response 88
n.s	Non-significant
NaH_2PO_4	Sodium dihydrogen phosphate

$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
nark2	Probable nitrate/nitrite transporter
NC	Nitrocellulose membrane
NCBI	National Center for Biotechnology Information
NFAT	Nuclear factor of activated T-cell
NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cell
Ni-NTA	Nickel- nitrilotriacetic acid
NK	Natural killer cell
NKT	Natural killer T-cell
NLR	NOD-like receptor
NO	Nitric oxide
P1	Gated subpopulation at first level
P13K	Phosphoinositide 3-kinase
P13k/AKT	Phosphatidylinositol-3-Kinase/ Protein kinase B
P2	Gated subpopulation at second level
PAMPS	Pathogen-associated molecular patterns
PB	Pacific Blue
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with Tween detergent
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin

PE/Cy7	Tandem fluorochrome that combines phycoerythrin and a cyanine
PerCP-Cy5.5	Tandem fluorochrome that combines Peridinin-Chlorophyll-Protein with a cyanine
pfkB	Phosphofructokinase B
pH	Potential of hydrogen
PKA	Protein kinase A
PLGA	Poly(lactic-co-glycolic acid)
PMA	Phorbol myristate acetate
pMHC	Peptide-bound MHC
pMHC-I	Peptide-bound MHC class I
pMHC-II	Peptide-bound MHC class II
PMT	Photomultiplier tube
PMTV	Photomultiplier tube voltage
Poly(I:C)	Polyinosinic:polycytidylic acid
PPE	Personal protective equipment
PQC	Performance quality control
PRR	Pattern recognition receptor
pSMAC	Peripheral ring surrounding the cSMAC
pTreg	Peripheral-derived Foxp3^{+} T-regulatory cell
Pty	Proprietary
RBC	Red blood cell
rBCG	Recombinant Bacillus Calmette–Guérin
RD-1	Region of difference 1
RE	Restriction enzyme
rHspX	Recombinant heat-shock α-crystallin tuberculosis antigen

RIG-1	Retinoic acid-inducible gene I
RLR	RIG-1 like receptor
RNA	Ribonucleic acid
RORγt	Retinoic acid-related orphan receptor gamma t
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase High Performance Liquid Chromatography
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SIT	Signaling threshold-regulating transmembrane adapter protein
SOCS-1	Suppressor of cytokine signaling 1
SP	Single positive
SSC	Side scatter
STAT	Signal transducer and activator of transcription protein family
T/S	Tween-Squalene emulsion
TAP	Transporter associated with antigen processing
TB	Tuberculosis
T-bet	T-box transcription factor
Tc	Cytotoxic T-cell
Tcm	T-cell with central memory
TCR	T-cell receptor
TDB	Trehalose-6,6-dibehenate
TDR	Totally drug-resistant
Tem	T-cell with effector memory

TEMED	Tetramethylethylenediamine
TFH	T follicular helper cell
TGF-β	Tumor growth factor beta
Th	T-helper cell
Th0	Naive T-cell
Th1	T-helper type 1 cell
Th2	T-helper type 2 cell
TIM-3	T-cell immunoglobulin mucin-3
TLR	Toll-like receptor
TNFR-1	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
Tr1	Type-1 regulatory T cell
Tr35	Regulatory T-cell that produces IL-35
Treg	Regulatory T-cell
Tris-HCl	Trisaminomethane hydrochloride
tTreg	Thymus-derived $Foxp3^+$ T-regulatory cell
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UV-Vis	Ultraviolet–visible
WHO	World Health Organisation
XDR	Extensively drug-resistant
ZAP-70	Zeta-chain-associated protein kinase 70

LIST OF APPENDICES

PEMBANGUNAN VAKSIN HSPX TERHADAP TUBERKULOSIS LATEN DENGAN MENGGUNAKAN STRATEGI PEPTIDA PEMBANTU-T BERSILIH GANTI BERSANDARKAN ADJUVAN

ABSTRAK

Keimunan sel adalah satu faktor kritikal yang harus dipertimbangkan untuk pembangunan vaksin yang berkesan terhadap *Mycobacterium tuberculosis*. Hubungan antara $CD4^+$ dan $CD8^+$ limfosit T memainkan peranan yang penting untuk membunuh sel yang dijangkiti bacilli tuberkulosis. Berdasarkan prinsip tersebut, strategi vaksinasi peptida yang novel menggunakan peptida MHC untuk mengubahkan tindak balas $CD8^+$ sel T terhadap protein α-kristal antigen tuberkulosis (HspX). Adjuvan vaksin (CASAC) telah dimasukkan ke dalam rejimen vaksin kami untuk merangsangkan sinergi imuniti terhadap antigen rekombinan HspX (rHspX). Data yang diperolehi menunjukan rHspX dapat meniingkatkan imunisasi yang khusus dalam tikus C57BL/6. Antibodi IgG_1 dan IgG_{2a} dan keimunan memori $CD8^+$ sel T yang specifik telah dirangsangkan oleh adjuvan vaksin CASAC. Rejimen vaksin tersebut menguatkan tahap rembesan IFN-γ dan TNF-α dengan penindasan IL-10. Ini menunjukkan bahawa keimunan sel yang dirangsangkan adalah dikawal oleh tindak balas Th1 dominan terhadap antigen HspX. Dalam kajian vaksinasi peptida, tikus C57BL/6 disuntikkan dengan protein rHspX dan vaksin subunit yang mengandungi peptida MHC-I/MHC-II dan adjuvan vaksin CASAC. Vaksinasi peptida bersilih ganti yang novel ini merangsangkan tahap tindak balas Th1 $CD8^+$ sel T yang spesifik terhadap antigen HspX, seperti yang ditunjukkan dengan peningkatan rembesan IFN-γ dan IL-2. Ia juga menghasilkan reaksi ingatan berpusat (T_{cm}) dan efek (T_{em}) yang lebih kuat dibandingkan dengan kumpulan kajian lain. Ini membuktikan bahawa

ketahanan keimunan adalah mantap terhadap antigen HspX semasa kajian cabaran. Sel T regulator ($CD4^+$ $CD25^+$ dan $CD25^+$ $FoxP3^+$) dan sel Th1, Th2 dan Th17 dengan pelbagai fungsi mengawal tindak balas imuniti secara berterusan semasa suntikan vaksinasi peptida bergantian, tanpa menunjukkan respon maklum balas yang negatif. Selanjutnya, vaksinasi peptida bergantian tidak merangsang penuaan dan keletihan $CD8^+$ sel T khusus terhadap HspX, seperti yang ditunjukkan dengan pengekpresan yang rendah daripada KLRG1, PD1, LAG3, dan CTLA-4. Kajian kami menunjukkan bahawa vaksin peptida bergantian telah berjaya menghasilkan sel T pembunuh (CTL) yang menonjol terhadap antigen HspX, yang berkemampuan untuk menyingkir tisu yang dijangkiti dengan bakteria tuberkulosis laten. Sebagai kesimpulan, vaksinasi peptida bergantian yang unik ini berpotensi untuk mendorong tindak balas $CD8^+$ CTL yang berkesan terhadap antigen HspX. Ini adalah disebabkan manipulasi tindak balas $CD4^+$ sel-T dengan menggunakan peptida MHC-I dan MHC-II. Dengan penindasan keimunan yang minimum ini, vaksinasi peptida bergantian mungkin bermanfaat untuk digunakan sebagai imunoterapi alternatif untuk memerangi jangkitan kuman tuberkulosis terpendam di masa depan.

DEVELOPMENT OF HSPX VACCINE AGAINST LATENT TUBERCULOSIS USING ADJUVANT-DEPENDENT T-HELPER ALTERNATING PETPIDE STRATEGY

ABSTRACT

Cellular immunity is a critical factor to be considered in developing an effective vaccine against *Mycobacterium tuberculosis.* Crosstalk between $CD4^+$ and $CD8^+$ T-lymphocytes play central roles in initiating cytotoxic killing to the infected cells, contributing to a complete clearance of intracellular tuberculosis bacilli *Mycobacterium tuberculosis* during infection. Based on these principles, our study proposed a novel alternating MHC-restricted peptide vaccination strategy to improve the rise of antigen-specific $CD8^+$ T-cell response against alpha-crystalline heat-shock protein (HspX). Combined adjuvant for synergistic stimulation of cellular immunity (CASAC) adjuvant was included in the vaccine regimen to improve their immunogenicity against HspX antigen. Results indicated that recombinant HspX protein was proven to be immunogenic in C57BL/6 mice, showing an improved antigen-specific IgG_1 and IgG_{2a} antibody secretion and memory $CD8^+$ T-cell immunity under stimulation of CASAC adjuvant. Such potent adjuvant enhanced the level of IFN-γ and TNF-α secretion with reduced IL-10 suppression, demonstrating a predominant Th1-mediated immunity against HspX antigen. In peptide vaccination study, C57BL/6 mice were primed with rHspX protein and consecutively stimulated with subunit vaccines formulated with MHC-I/MHC-II peptides and CASAC adjuvant. Our novel alternating peptide vaccination significantly stimulated a substantial level of Th1-mediated $CD8^+$ T-cell response against HspX antigen, represented with elevated secretion of IFN-γ and IL-2 cytokines. It also generated a