Study of two types of protein-protein interactions : i, a high affinity antigen-antibody complex and ii, a transient complex between an oncoprotein and a methyltransferase

Tambe, Anagha

2018

Tambe, A. (2018). Study of two types of protein-protein interactions : i, a high affinity antigen-antibody complex and ii, a transient complex between an oncoprotein and a methyltransferase. Master's thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/74114>

<https://doi.org/10.32657/10356/74114>

STUDY OF TWO TYPES OF PROTEIN-PROTEIN INTERACTIONS:

I, A HIGH AFFINITY ANTIGEN-ANTIBODY COMPLEX AND

**II, A TRANSIENT COMPLEX BETWEEN AN ONCOPROTEIN AND A
METHYLTRANSFERASE**

ANAGHA TAMBE

SCHOOL OF BIOLOGICAL SCIENCES

2018

**STUDY OF TWO TYPES OF PROTEIN-PROTEIN INTERACTIONS:
I, A HIGH AFFINITY ANTIGEN-ANTIBODY COMPLEX AND
II, A TRANSIENT COMPLEX BETWEEN AN ONCOPROTEIN AND A
METHYLTRANSFERASE**

By

ANAGHA TAMBE

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to

The Nanyang Technological University in partial fulfilment of

The requirement for the degree of Master of Science

2018

ACKNOWLEDGEMENTS

This journey of pursuing the degree of M.Sc. involved kind co-operation and collaboration at each step. I am amazed at how my moderate thesis required assistance of so many persons at various stages. These embroidered interactions contributed to my thesis, are my most treasured and cherished memories, which I will always hold dear to my heart for my entire life.

Most respectable of course is my M.Sc. Supervisor Prof. Julien Lescar. I am indebted to him for this opportunity and the guidance which would affect my entire professional life. Thanks for helping in creating model and refinement of my crystal structure. I must have done some very good deeds in past to get him as my mentor. I take this opportunity to thank him for everything.

My heartfelt thanks to my current Employer Dr. Jianzhu Chen for offering me timely much needed support and motivation to complete my M.Sc.

During the course of this research, I got to work with very motivating collaborators. Prof. Su I-Hsin from NTU in Vav-Ezh2 project, Dr. Paula Hamond and Dr. Jianzu of Vaccine Group at SMART, Prof. Shee Mei Lok from Duke-NUS, Dr. CongBao from NTU for NMR and Prof. Susana Geifman Shochat from NTU for SPR. Working with these experts in the particular field was absolute pleasure and enriching experience. I would also like to thank Nandini, Ishin, Sharif, Michelle, Hoe Jue, Jiaqi, Li Yan and Jediael for their valuable cooperation.

I am extremely obliged to Dr. Shawn from IMCB for his kind help of plasmid.

My efforts were also guided by my Thesis advisory committee members. I am grateful to Prof. Par Norlund, Prof. Daniela Rhodes, Prof. Gao, Prof. Leiw Ding Xiang and Prof. Chandra for their timely advice.

Thank you, Prof. Konstantin Pervusion for a great motivation and guidance in NMR.

I am very much obliged to all my past and present lab colleagues for guiding me in my experiments. I would like to thank Abbass, Boon Chong, Angeline, David,

Thong Sock-yu, Aksconditiona, Xinlei, Chong Wai, Yee Hwa, Ramya, Edmond, Jianqing, Chong, Tiong Han, Jia Huan, Swati and our sweet Grace. I enjoyed being introduced to Computational Modeling by Boon Chong. I am grateful to Boon Chong also for his valuable comments on my thesis. I would like to thank Abbass for his kind co-operation to get my crystals ☺ I also got kind co-operation from our neighboring groups from Prof. Dahai, Prof. WuBin, Prof. Daniela Rhodes and Prof. Gao.

I found my absolute home at SMART (Singapore-MIT Alliance in Research and Technology) and would like to thank Hooi Lim for her help in understanding a Chinese thesis and accessibility for the same. Yuko for solid moral support, Weijian and Marvin, Mallica for warm friendship and co-operation. My thanks to Katell for encouragement.

I am grateful to Michelle Turvey for her able guidance in animal experiments. Shareef and Divakara for collaboration.

I would like to thank Akshita and Prabha and Serap for advising me on my thesis. All my friends in SBS, Biopolis and in Singapore made me feel at home. I enjoyed great scientific talks with my friend Ufuk. I would like to thank all my friends for making these years an enjoyable experience.

I am indebted to all my family members for their strong support. Lastly, I offer this effort to the ultimate divine power in Universe.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
SUMMARY	xvi
POSTER PRESENTATION	xvii
ABBREVIATIONS	xviii
PREFACE	xx
PART I A STRONG INTERACTION BETWEEN FLAVIVIRAL ENVELOPE ANTIGEN AND CROSS-PROTECTIVE MONOCLONAL ANTIBODY	xxii
Part I INTRODUCTION	 1
1.1 Flaviviridae.....	2
1.1.1 Flaviviruses and flavivirus infections.....	2
1.2 Global Incidence	3
1.2.1. Dengue virus Global Spread.....	3
1.2.2 West Nile Virus Global Incidence.....	7
1.2.3 Epidemiology	7
1.2.4 Zika Virus Global Emergency.....	8
1.3 Flavivirus Life cycle.....	10
1.4 Flavivirus RNA replication	10
1.5 Structure of Flavivirus.....	10
1.5.1 Structure OF West Nile Virus	11
1.5.2 Structure of Zika Virus.....	11
1.5.3 <i>Flavivirus</i> Structure Dynamics.....	12
1.6 <i>Flavivirus</i> Structural Proteins.....	15
1.7 Domain III of the Envelope protein (ED III)	17
1.7.1 Interaction with host cell surface receptors	19
1.8 Low pH induced endosomal fusion by flavivirus	20
1.9 Pathogenesis of Flaviviruses	21
1.9.1 Pathogenesis of dengue virus manifestations	21
1.9.2 Pathogenesis of West Nile Virus	22
1.9.3 Pathogenesis of Zika.....	23
1.10 Antibody Dependent Enhancement.....	25
1.11 Anti-flaviviral antibodies	28

1.11.1 Potent Cross-Protective Antibodies and the Mechanism of Neutralization	28
1.11.1.1 Ab 9F12:	28
1.11.2 Antibodies that inhibit fusion of the virus after receptor binding:	29
1.11.2.1 Ab 3E31 (PDB ID: 4BZ1) [89].....	29
1.11.2.2 Antibody 513 - Best example of engineered antibody.....	29
1.11.3 Antibody Neutralization by Forcing Pre-mature fusion:.....	30
1.11.3.1 Ab2D73 (PDB id: 4bz2) [34, 92].....	30
1.12 Scope of Engineered monoclonal Antibodies:	30
1.12.1 Design Strategy for Engineered antibody.....	31
1.12.2 Biotechnological Instruments Available to Artificially Generate Immune-Protection through Antibody:	32
1.12.3 Modification of Antibody motifs for better Adaptation to Immunotherapy.....	32
1.12.3.1 Single Chain Fv (scFv)	32
1.12.3.2 Humanization of Antibodies	34
1.13 Vaccine.....	35
1.13.1 Stages of Development of Vaccine	36
1.13.1.1 Pre-clinical development	36
1.13.1.2 Clinical development	36
1.13.1.3 SMART Vaccines	37
1.13.1.4 Flaviviral Vaccination.....	38
1.13.1.4 Dengue vaccine.....	39
1.13.2 The status of Dengue vaccines	39
1.13.3 Vaccine Delivery by Microneedle Patch.....	40
Part I AIMS And OBJECTIVES	43
Consensus ED III.....	44
Consensus ED III as vaccine candidate.....	44
Crystal Structure of Consensus EDIII:	45
m366.6	45
Zika virus Neutralization by m366.6.....	45
Part I MATERIALS AND METHODS	46

Materials and Methods	47
2.1 Over-expression of ED III.....	47
2.2 Lysis of Cells.....	48
2.3 Re-solubilizing the Inclusion Body	49
2.4 Refolding of EDIII	50
2.4.1 Rapid dilution	50
2.4.2 Dialysis	51
2.5 Gel Filtration Chromatography	52
2.6 Complex Formation and Detection	55
2.7 Crystallization Trials for Dengue and West Nile EDIIIs in Complex with scFv m366.6:.....	55
2.8 Consensus Envelope Domain III.....	56
2.8.2 Expression and Purification of soluble Consensus domain III.....	56
2.8.2.1 Periplasmic Expression.....	56
2.8.2.2 Lysis of the Harvested Cell Pellet.....	57
2.8.3 Two-Step Purification of the Consensus Protein.....	58
2.8.4 Crystallization of Consensus EDIII:.....	58
2.8.5 Micro-seeding of Consensus EDIII protein crystal	59
2.8.6 Consensus EDIII Crystal Diffraction	59
2.8.7 Complex Formation and Detection	60
2.8.8 Crystallization Trials	60
2.9 Surface Plasmon Resonance.....	60
2.9.1 Interaction Kinetics of Consensus EDIII with mAb513.....	60
2.9.2 Interaction Kinetics of Consensus EDIII (EDIIIC) with scFv m366.6: 61	
2.10 Plaque reduction neutralization (50%) test (PRNT50%) of Ab m366.6 for Zika	61
2.11 Micro Needle Assembly.....	62
2.11.1 Encapsulation of EDIII Consensus protein onto the Microneedles.....	62
2.11.2 Analysis of release kinetics of consensus ED III from the Microneedles:	63
2.11.3 Detection of Consensus EDIII in Mouse Ear:	63
2.11.3.1 The ear Consensus EDIII preparation:.....	63

2.11.3.2 Protocol:	64
2.11.3.3 The ELISA for Detection of Consensus EDIII from Mouse Ear:	64
Part I RESULTS AND DISCUSSION	65
3.1 Consensus Domain III:	66
3.1.1 Periplasmic Expression of Consensus EDIII in Soluble Form:	66
3.1.2 Sub-cloning of Consensus EDIII and Expression in periplasm of E.coli:	66
3.1.3 Purification of Consensus Domain III	66
3.1.4 Size-Exclusion Chromatography of Consensus ED III	66
3.1.5 Small-scale Complex formation of Consensus EDIII with m366.6	66
3.2 Crystallization of Consensus EDIII protein:	69
3.2.1 Micro-Seeding of Consensus EDIII crystal:	70
3.2.2 Crystallization and Seeding Concept in Brief:	72
3.3 Crystal Structure of Consensus ED III	75
3.3.1 Brief Introduction to Protein crystallization and associated terms	75
3.3.2 Symmetry	76
3.3.3 Lattice directions:	77
3.3.4 Molecular Replacement:	79
3.3.5 R and R _{Free} Factor	79
3.3.6 CC _{1/2}	81
3.3.7 X-ray Data Collection:	82
3.4 Refinement of the structure model:	85
3.5 The Quality Check of the Structure:	85
3.5.1 Functional Efficiency of Consensus EDIII as Potential Vaccine Candidate:	86
3.5.1.1 Vis 513 [88] epitope Alignment:	87
3.5.1.2 4E11 [69] epitope Alignment:	87
3.5.1.3 Ab 2D73 epitope Alignment:	87
3.5.1.4 Ab 3E31 epitope Alignment:	87
3.6 Therapeutic Anti-EDIII Monoclonal antibodies Epitopes and Significance of Consensus EDIII:	88

3.6.1 Neutralization Mechanism Similarity Features of The successful monoclonal Antibodies:.....	88
3.6.2 Stable B-molecule of E-protein at three-fold axis:.....	88
3.6.3 Three-fold axis – stoichiometry of ab binding	89
3.6.4 Importance of Lysine 310 epitope	90
3.6.5 Importance of target site between residues 323 to 327	90
3.6.6 Target site ensures lysosomal clearance.....	90
3.6.7 Scope for scFv mediated Immunotherapy	90
3.6.8 Brief Introduction of Ab 513 Neutralizing Efficiency for Dengue serotype four:	91
3.7 Surface Plasmon Resonance (SPR) of Consensus ED III with mAb 513	92
3.7.1 Small Scale Analysis of Complex formation on Analytical Superdex 75 5/150.....	94
3.7.2 Complex of m366.6 with DEN2TEDIII.....	94
3.7.3 Complex of m366.6 with WNVEDIII on Superdex 75 10/300.....	95
3.7.4 Complex of m366.6 with DENV1EDIII on Superdex 75 16/60	95
3.8 Zika Virus is not neutralized by m366.6	97
3.9 Surface Plasmon Resonance (SPR) of Consensus EDIII with scFv m366.699	
3.10 Crystal of complex of Consensus envelope domain III and scFv m366.6:....	
.....	100
3.10.1 Micro-seeding of Complex micro-needles:	101
3.11 Consensus EDIII A Potential Vaccine Candidate:	103
3.11.1 Thermostability of Consensus EDIII:.....	103
3.11.2 Electrostatic Solvent Accessible Surface Comparison of Consensus EDIII:.....	104
3.12 Assembly of Consensus EDIII in Micro Needle	106
3.12.1 Successful Encapsulation and Release of Consensus Domain III from Dissolving Type Microneedle	106
3.12.2 Detection of Fluorescent labeled EDIIIC in Skin:	110
3.12.3 Functional Viability of Delivered EDIIIC in Skin tissue:	112
3.12.4 Dengue Vaccine Perspectives:	113
3.12.5 Vaccination through Skin:	114

Conclusions	115
Future Work	117
Part II: TRANSIENT INTERACTION BETWEEN GTP-EXCHANGER VAV1 AND HISTONE METHYLTRANSFERASE EZH2	119
Part II ABSTRACT	120
Part II INTRODUCTION	122
4.1 Vav Proteins	123
4.1.1 Vav proteins have a modular multi-domain organization	125
4.1.2 Cooperative dynamics of Vav1 physiology	126
4.2 Ezh2 (Enhancer of Zeste Homologue) Protein	127
4.2.1 Ezh2 in Cellular Transformation	128
4.2.2 Treatment is to target Ezh2 interaction and not Ezh2	129
4.3 Vav1CH domain and Ezh2 interactions	129
Part II AIMS AND OBJECTIVES	131
Part II MATERIALS AND METHODS	133
5.1 Vav1	134
5.1.1 Vav1-CADPZ	134
5.1.1.1 Construct Vav1-CADPZ	134
5.1.1.2 Expression of Vav-1 CADPZ	134
5.1.1.3 Lysis of Cells	134
5.1.1.4 Two-Step Purification of the Vav-1 CADPZ	134
5.1.1.5 Tev protease digestion	135
5.1.1.6 Size-exclusion Chromatography	135
5.1.2 Vav1 1-172 mouse Construct	135
5.1.3 Construct Vav1 (1-187) and Vav1 (1-390)	136
5.2 Ezh2	136
5.2.1 Ezh2 with 6x His-tag	136
5.2.2 Expression and Purification of Ezh2 (201-255 mouse)	136
5.2.3 Ezh2 Double Mutant:	137
5.3 Site directed mutagenesis was performed using Phusion Flash High-Fidelity PCR master mix with following Primers:	137

5.4 Expression and Purification of Ezh2 (Residues 201-255 Mouse) Double mutant (Ezh2M)	137
5.5 Circular Dichroism of Ezh2 (res 201-252).....	138
5.5.1 Co-Crystallization of Vav1 (mouse 1-172) and wild type Ezh2 (mouse 201-255):	138
5.6 Nuclear Magnetic Resonance	138
5.7 Vav1 and EZh2 interaction Computational Model	139
Part II RESULTS AND DISCUSSION	142
6.1 Brief Introduction:.....	143
6.2 Vav1 Expression and Purification.....	144
6.2.1 Vav1	144
6.2.1.1 Vav1 (MBP-His-CADPZ- human)	144
6.2.2 GST-Vav1 (1-172 mouse) Purification	145
6.3 Ezh2 (res. 201-252 mouse).....	146
6.3.1 Ezh2 (201-255 mouse) Secondary Structure by Circular Dichroism (CD)	146
6.3.2 NMR Results	147
6.3.3 NOE Plots.....	150
6.3.4 GST-Vav1 (mouse 1-172) and Wild Type Ezh2 (mouse 201-255) Co-crystallization:	155
6.3.5 Computational model of Vav1 and Ezh2 Interaction	156
6.3.6 Ezh2 Double Mutant (K234, 245R) Expression and Purification:.....	160
Part II CONCLUSIONS	162
Part II ONGOING WORK	164
EPILOGUE	166
APPENDIX	168
Appendix I: Reported Envelope domain III residues and their significance	169
Appendix II: Comparison of Envelope domain III Epitopes of Ab 513, 4E11 1A1D2 3E31 and 2D73	170
Appendix III: Flaviviridae Phylogenetic Tree	172
Appendix IV: Modular Structure of Vav1.....	173
REFERENCES	174

TABLE OF FIGURES

Fig. 1.1 Emergence of Dengue fever and associated complication like DHF after 1960.....	3
Fig. 1.2 Global Spread of Denuge by serotype. Image replicated from[11].....	6
Fig. 1.3 Global spread of mosquito-borne Zika virus. [16]	8
Fig. 1.4 Per Objective resource requirements for Zika. Image replicated from [22]	9
Fig. 1.5 Geographic spread of Zika Virus as per WHO.Image replicated from [11]	9
Fig.1.6 <i>Flavivirus</i> Structure	12
Fig.1.7 <i>Flaviviral</i> Structural Dynamics	14
Fig 1.8: Envelop protein Dynamics and Participations of EDIII.....	16
Fig 1.9 Ribbon diagrams of several flavivirus ED3 proteins.....	18
Fig.1.10 Schematic overview of the DENV membrane fusion process.	20
Fig.1.11 Overview of the DENV Envelope Protein	21
Fig. 1.12 Antibody-Dependent Enhancement of dengue infection.....	27
Fig. 1.13 9F12 epitopes [1]	29
Fig. 1.14 Basic structure of an IgG Ab molecule.	33
Fig. 1.15 Core values and multicriteria decision making for vaccine development	37
Fig. 1.16 Comparison of Hypodermic syringe and Micro needle[111]	41
Fig. 2.1 Expression Profile for Inclusion body of Dengue Serotype 1 on 18% SDS-PAGE:	49
Fig. 2.2 Ni-Affinity and Size Exclusion Profile of ED III of DEN 2 TSV 01, WNV and DENV 1.....	54
Fig. 3.1 Consensus Domain III constructs alignment and Cloning	67
Fig. 3.2 Purification Profile of Consensus ED III.....	68
Fig. 3.3 The Needle Crystal of Consensus EDIII	69
Fig. 3.4 The Optimized Plate Crystal of Consensus EDIII.....	70
Fig. 3.5 The Micro-seeding of Consensus EDIII crystal	72
Fig. 3.6 Protein Crystallization Phase Diagram.....	73

Fig. 3.7 The X-ray Diffraction Pattern of Consensus EDIII crystal	82
Fig. 3.8 Unit Cell of EDIIIC	84
Fig. 3.9: Consensus EDIII motif comparison with All Four Serotypes	86
Fig. 3.10 SPR results for Analysis of Affinity between Consensus ED III Ab51393	
Fig. 3.13 Large Scale purification of ED IIIs in Complex with m366.6	96
Fig. 3.15 PRNT assay for testing m366.6 neutralization of Zika Virus	98
Fig. 3.16: SPR Results for Consensus EDIII interaction with scFv m366.6	99
Fig. 3.17 Micro-needle crystal of complex of Consensus EDIII with scFv m366.6	
.....	101
Fig. 3.18 Micro-seeding Condition of EDIIIC in complex with scFv m366.6....	102
Fig. 3.19 Micro-seeding Condition 2 of EDIIIC in complex with scFv m366.6.	102
Fig. 3.20: Electrostatic Solvent Accessible Surface of Consensus EDIII comparison	
with All Four Serotypes	104
Fig. 3.21 Layer-By-Layer Micro-Needle Assembly	107
Fig. 3.22 Consensus EDIII Encapsulation Assembly on Microneedle	108
Fig. 3.23 Determination of Encapsulation Amount on Microneedle.....	109
Fig. 3.24 Release Kinetics of Consensus EDIII from Microneedle assembly	110
Fig 3.25 Fluorescent Visualization of EDIIIC protein in layer-by-layer (LbL) films	
on microneedles and Delivery in Mouse Ear Skin.....	111
3.26: Efficiency of Microneedle to Lodge EDIIIC in Mouse Ear through Skin by	
ELISA	112
Fig. 4.1 The cardinal regulatory pathways in which Vav is involved as an organizer	
of the cytoskeleton. <i>Image Replicated From [168]</i>	124
Fig. 4.2 C-terminal Src homology domains presumably direct cellular localization	
<i>[Image Modified from [173]]</i>	126
Fig. 4.3 Rapid cell migration requires coordinated action of VAV1 and Ezh2 along	
with other cellular partners (Image replicated from[182])	128
Fig. 4.4 PRC2 complex structure with EZH2	130
Fig. 5.1 Structure Motifs selected for Hex Dock Prediction of interaction	139
Fig.5.2 Conjugate Gradient Minimization for Sample Preparation in MD	
Simulation	140
Fig. 6.1 Comparative Model of Vav1-Ezh2 Interaction	143

Fig. 6.2 Vav1 (CADPZ) purification profile	144
Fig. 6.3 Purification profile of GST-Vav1-1-172	145
Fig. 6.4 Ezh2 wild type construct two-step purification and CD spectrum of the Construct	146
Fig. 6.5 ¹ H- ¹⁵ N HSQC Backbone Assignments of Wild Type Ezh2 Mouse (res.201-255)	147
Fig. 6.6 Secondary structural analysis of EZH2.	149
Fig. 6.7 Sequential NOE Plot.....	150
Fig. 6.8 NOE i+4 of α Helix	151
Fig. 6.9 NOE Strip showing Helix Turn	152
Fig. 6.10 3D Structure of Ezh2	153
Fig. 6.11 Aligned 3D structures of Ezh2 NMR structure obtained with Published Ezh2 in PRC2 complex[2].	154
Fig. 6.12 GST-Vav1 (mouse 1-172) and Wild Type Ezh2 (mouse 201-255).....	156
Co-crystallization Condition.....	156
Fig. 6.13 MD Simulation of Vav1 and Ezh2	158
Fig. 6.14 Molecular Dynamic Simulation Analysis of affinity interaction of Wild type and Double mutant (L234R and L245R) Ezh2 with CH domain of Vav1 over time scale	159
Fig. 6.15: Ezh2 Double Mutant Purification Profile.....	160
Fig. AP VI: Vav1 Auto inhibition.....	173

LIST OF TABLES

Table 2.1: Details of domain III expression conditions	47
Table 2.2: Summary of biochemical characteristics of recombinant domain IIIs (ED III) used in this study	50
Table 2.3: Published Crystallization Conditions of ED III-Antibody Complexes.....	57
Table 3.1: X-ray Diffraction Data Processing and Refinement Parameters.....	83
Table 3.2: Zika ED III Sequence Identity Matrix with other ED IIIs.....	99
Table AP I: Summary of reported significances of Envelop domain III	169
Table AP II: Epitopes of Ab 513, 4E11 and 1A1D2 at a glance	171-172

SUMMARY

Protein-protein interactions together constitute the complex fabric of biochemical circuitry that drives the physiological functions. This tinkering network is controlled in part by the nature of affinity of these interactions. Some interactions demonstrate strong affinity and most are of transient nature with weak affinity.

In this work we describe two such interactions. One interaction is between a broad-spectrum antibody (m366.6) with neutralization capacity for all four Dengue serotypes. This is a very strong interaction with K_D value in nanomolar range. The antigen of this antibody is situated in third last domain of Dengue envelope (ED III). We tried to analyze the complex of this interaction with crystallography with the aim of exploring the atomic structure to facilitate engineering better antibody for passive immunotherapy and / or better antigen as a potential candidate for vaccine against flavivirus. With this perspective we also analysed a consensus domain III engineered to have optimum similarity with all four Dengue serotype ED IIIs. It being thermostable we tested it further as a potential vaccine candidate through a novel mode of delivery by Micro-needles. We tested its affinity for cross-protective antibody Ab 513 by SPR. Lastly, we also checked ability of m366.6 to neutralize Zika virus. We also analyzed the atomic structure of Consensus EDIII by X-ray crystallography to analyze its conserved fold.

The second interaction is of transient type of $0.1\mu\text{M}$ K_D between two proteins Vav1, a GDP/GTP exchange factor and Ezh2, a polycomb group Histone methyltransferase. Reports indicate involvement of this interaction in mobilization of hematopoietic cells by influencing the adhesion dynamics through integrin pathway. This is implicated in transformation of cells. Abolishing this interaction could be a mode of treatment for such cellular transformation. We in our work tried to identify the residues involved in this interaction NMR. Currently, we have solved individual structure of the Ezh2 construct by NMR and co-crystallization.

POSTER PRESENTATION

Title: A Monoclonal Antibody Neutralizing All Four Dengue Virus Serotypes:

Implications for Therapy and Prevention

At Sigapore-Lubek Symposium on 30th September 2013

ABBREVIATIONS

LB	Luria Bertani broth
mAb	monoclonal antibody
mM	Millimolar concentration
μ M	Micromolar concentration
Ni-NTA	Nickel- Nitrilotriacetic acid
OD ₆₀₀	Optical density at 600nm wavelength
ORF	Open reading frame
PDB	Protein data bank
RNA	Ribonucleic acids
rpm	Rotations per minute
scFv	Single chain fragment variable
SDS-PAGE	Sodium dodecyle sulfate-polyacrylamide gel electrophoresis
sE	Soluble ectodomain (of E protein)
V _H	Variable heavy chain of the immunoglobulin molecule
V _L	Variable light chain of the immunoglobulin molecule
WNV	West Nile virus
FPLC	Fast protein liquid chromatography
ADE	Antibody dependent enhancements (of infection)
ATCC	American type culture collection
CDR	Complementarity determining region (of antibody)

Cryo EM	Cryo-electron microscopy
DC-SIGN	Dendritic cell ICAM3-grabbing non-integrin
DENV	Dengue virus
DENV (1-4)	Dengue virus serotypes 1-4
DENV2	Dengue serotype 2 genotype TSV01
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
DTT	Dithiothreitol
E	Envelope glycoprotein
ED III	Envelope domain III
FDA	Food and Drug Administration (of USA)
ICAM	Inter cellular adhesion molecules
IgC	Immunoglobulin constant domain
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
kDa	Kilodalton
LAMP1 -	Lysosomal-associated membrane protein 1

PREFACE

Protein-protein interactions form the central network of biochemical interplay of physiological systems. They are of various affinities depending upon the final function achieved. In this thesis, we have analyzed two types of protein-protein interactions. One is a very strong interaction between a broad-spectrum antibody, which is capable of neutralizing all four Dengue serotypes and the other one is of transient type between two proteins Vav1 and Ezh2.

Part I of this thesis describes our work aimed at understanding the strong interaction between a cross-protective antibody m366.6 with its antigen on a dengue envelope. This is an antibody generated through naïve yeast display library with human scaffold; making it a very promising antibody for passive immunotherapy. During the course of this thesis, we also studied another antibody 513 of mouse origin that was carefully engineered to demonstrate better affinity for Dengue serotype IV as compared to its parent antibody. We characterized its interaction with a consensus epitope of the antigen, so that all four serotype epitopes are immunologically recognized with comparable affinities. As antigen-antibody interactions are predominantly of the highest affinity; the performance of the antibody is at its best when it can bind with its antigen very avidly. We found that the interaction of the engineered antibody 513 was very strong with the engineered common epitope of all four serotypes. Therefore, we further tested this common epitope as potential candidate of vaccine with a novel mode of delivery by Microneedles and by crystallography structure determination.

In Part II of this thesis we examined a transient interaction weak affinity with K_D low μM range. This is an interaction between a GDP/GTP exchange factor, Vav1 of Vav family of signal transducing proteins and a polycomb group (PRC2) Histone Methyltransferase Ezh2. Vav1 is reported to recruit Ezh2 to interact with Talin leading to modification in adhesion dynamics of the cells of especially hematopoietic lineage. This interaction was reported to influence cell transformation. Therefore we attempted to define the interacting residues by NMR and crystallography.

**PART I A STRONG INTERACTION BETWEEN
FLAVIVIRAL ENVELOPE ANTIGEN AND
CROSS-PROTECTIVE MONOCLONAL
ANTIBODY**

Part I INTRODUCTION

1.1 Flaviviridae

A large family of positive strand RNA viruses, Flaviviridae, consists of three genera: hepaciviruses, pestiviruses and the *flaviviruses* [12] (Refer Appendix VIII). The Flaviviridae comprise many medically important viruses including HCV, YFV, WNV, and DENV.

1.1.1 Flaviviruses and flavivirus infections

The flavivirus genus consists of nearly 80 viruses, which depend on arthropod vector to infect humans. Phylogenetic and epidemiological analyses indicate that more virulent genotypes are now replacing those with lower epidemiological impact[13]. Flaviviruses are responsible for a variety of diseases accompanied by fever, and occasionally encephalitis (WNV) and hemorrhagic fever (DENV). Flavivirus infections of regional endemic concern are Murray Valley encephalitis (MVE), Kyasanaur Forest Disease (KFD), tick-borne encephalitis (TBE), St. Louis encephalitis (SLE), and West Nile (WN) encephalitis. The infections of major global concern encompass the dengue infection and related hemorrhagic complications, along with Yellow Fever and Japanese Encephalitis. Dense urbanization, increased means of easy transportation coupled with difficulty in mosquito control measures have contributed to the re-emergence of flaviviruses such as DENV in South-America (e.g. Brazil).

Neurotropic flaviviruses can produce severe fatal central nervous system diseases with serious sequelae. The first WNV isolate that was responsible for New York City Outbreak in 1999, was first reported in western hemisphere [14]. The West Nile virus found in U.S. is endemic in Canada, parts of Central and South America and the Caribbean and is continuously expanding [15].

Fig. 1.1 Emergence of Dengue fever and associated complication like DHF after 1960

1.2 Global Incidence

1.2.1. Dengue virus Global Spread

Dengue virus, a member of *Flavivirus* genus, is being termed as the most important mosquito-borne viral disease in the world due to its global impact, rate of spread and number of people getting infected by at least one of its serotypes. It is estimated that almost half of world population lives in dengue-endemic countries thereby underscoring its impact on global health and economy. Its emergence across the world from 1960 is alarming (Fig.1.1). The various estimates about cases of infection further point to the lack of systemic study about global impact of dengue virus. WHO estimates of 50 to 100 million cases is significantly different from modeling done in 2012; where 390 million cases were estimated. The difference however, was on account of the later study that included both apparent and unapparent cases. Using only apparent cases with 95% percentile statistical significance, the number ranges from 67 million to 136 million; which maps well but on the higher end of estimates of WHO study [16]. Again, these numbers are at variance with another study carried out by Shepard et al [17]. This study suggests a range of 23.6 million to 121.9 million for 95% Confidence Interval (*cf. interval*). It

further estimated, from Global Burden of Disease Study 2013, the total annual global average cost of dengue in 2013 to be \$8.9 billion; which, for 95% *cf. interval*, ranges from \$3.7 billion to \$19.7 billion.

There are four phylogenetically and antigenically distinct dengue viruses (Serotype I-IV). These serotypes present 60-70% similarity in their outer structure. Humoral immunity developed at the first encounter of Dengue virus confers protection for life time for that particular serotype. However, because of the similarity in the surface of four serotypes, Antibody dependent enhancement of the disease is observed upon encounter of different serotype of Dengue. Hence, impact studies for dengue virus spread should include host and virus genetics as well as sequential and/or concurrent circulation patterns.

Serotype I and II were first discovered in 1943-44; while serotype III-IV were first reported in 1953. All four types were first reported in Asia-Pacific region. Their spread followed the spread of its principal mosquito vector *Aedes aegypti* globally. The following figures (Fig. 1.2 (a)-(d)) [18] demonstrate global spread of dengue virus over last 70 years based on its four known serotypes. Rapid urbanization and ever increasing global transportation further exacerbated the situation. Till 1980s most regions reported only one or two types of dengue virus, after which incidences of infection for more serotypes in one geographic area increased. This can be attributed to the fact that more cost-effective and less labor-intensive type-specific test were developed. Moreover, existence of fifth serotype is also suggested though it is not yet found. With global organizations speculating further urbanization especially in emerging market regions, it is imperative that thorough scientific analysis of yet neglected dengue virus is carried out [18].

Fig 1.2 (a) Spread of Serotype – I

Fig 1.2 (b) Spread of Serotype – II

Fig 1.2 (c) Spread of Serotype – III

Fig 1.2 (d) Spread of Serotype - IV

Fig. 1.2 Global Spread of Dengue by serotype. Image replicated from[11]

1.2.2 West Nile Virus Global Incidence

A member of genus *Flavivirus* of the family Flaviviridae, West Nile Virus (WNV) is a zoonotic arbovirus [19]. It spreads in a sylvatic cycle and primarily involves arthropod vectors and avian hosts. Mosquitos of genus *Culex* are identified as predominant vectors [20]. Humans and horses are considered dead-end hosts [19]. Migratory birds are thought to be primary mode by which WNV is transmitted globally. Even though *Culex* mosquitos are primary transmitters; other genera have also been found to be vectors. For example, *Cx. pipiens*, *Cx. univittatus*, and *Cx. Antennatus* are primary vectors in Europe and Africa while in India, species of the *Cx. vishnui* complex have been primary transmitters. Non-vector transmission routes generally identified are; oral transmission in birds and cats, mosquito bites, blood transfusion, transplantation, intrauterine transmission and organ donation in humans [21].

In humans, it was first isolated in the West Nile province of Uganda in 1937 from the blood of a woman suffering from a mild febrile illness. However, till early 1990's, it wasn't considered as major risk to human health. The Romanian outbreak of WNV in 1996, included predominantly neuroinvasive cases, that were followed by huge epidemics and equine epizootics in North America in late 1990s; brought forth concerns regarding public health due to WNV [20]. Conclusive data regarding global spread of WNV is not readily available. However, Center for Disease Control in United States of America publishes annual data on WNV cases since 1999. According to the data, in USA, during span of 16 years (i.e. 1999-2015) 43,397 cases of WNV infection were reported, of which, 4% resulted in deaths.

1.2.3 Epidemiology

Of the two distinct lineages revealed phylogenetically; Lineage I is found to be commonly associated with humans and horses. The virus is successful to conserve itself genetically. Single common amino acid change in envelope gene (V159A) is common to most strains found since 2002 in North America and old world strains.

1.2.4 Zika Virus Global Emergency

Zika virus (ZIKV) emulates DENV in its choice of primary vector, *Aedes aegypti*. In addition, *Aedes albopictus* has also been found to transmit the Zika virus. Its clinical presentation and epidemiology and transmission cycle are similar to chikungunya and dengue, although the illness is generally milder.

First identified in monkey in 1947 and in human in 1952 in Uganda, Zika virus has spread globally. First reports from Pacific region locate to Yap Island in 2007 and French Polynesia in 2013.

Situation of global health emergency has arisen from the contemporary rise in Zika incidences (eg. Brazil, November 2015) and related cases of microcephaly, Guillan-Barré syndrome (GBS) and congenital malformations [22].

As per Zika situation report published by WHO[23] on July 27, 2016; Arboviral transmission of Zika is reported in territories that include 67 countries since 2007.

Fig. 1.3 Global spread of mosquito-borne Zika virus.

Cumulative number of countries, territories and areas by WHO reporting mosquito-borne Zika virus transmission in years (2007–2014), and monthly from 1 January 2015 to 27 July 2016
Replicated from [23]

Of these, 50 countries and territories in 2015, reported outbreak for the first time. Four countries have been classified as facing possible endemic transmission in 2016. Region of Caicos (UK and Northern Ireland) reported mosquito born Zika infection for the first time in the week of July 27, 2016. Remarkably, congenital or arboviral Zika infection leading to central nervous system (CNS) malformations and microcephaly are reported in 14 countries or territories. Additionally, occurrences of GBS and/or confirmed Zika infection associated GBS, have been reported in 15 countries in various territories.

Fig. 1.4 Per Objective resource requirements for Zika. Image replicated from [22]

The WHO expects Zika to spread throughout the American continent, but scientific fraternity fears that Asian countries too could experience major outbreaks.

Fig. 1.5 Geographic spread of Zika Virus as per WHO. Image replicated from [11]

1.3 Flavivirus Life cycle

The molecular biology of flaviviruses is best understood in the context of the viral life cycle [24]. Flaviviruses replicate in a variety of cells of insect, mammal, or avian origin suggesting concomitant multitude of receptors. It is established that E protein initially interacts with highly sulfated heparan sulfate (HSHS) residues omnipresent in a wide variety of cells. Natural flaviviral transmission in hosts like insect and vertebrates indicates its ability to engage the conserved cell receptor or fast adaptation to different receptors. A short hydrophobic segment (amino acids 306-314) in domain III of dengue E protein is thought to be responsible for virus receptor mediated invasion [25]. This hydrophobic motif and amino acid 306 of JEV were reported to contribute to the interaction domain III with cell surface glycosaminoglycans. The arrival of WN/KUN viruses inside host cells is thought to be occurring via receptor-mediated internalization in coated pit vesicles [26]. The perinuclear region of the endoplasmic reticulum (ER) of vacuolated and hypertrophic cells are identified as the site of viral replication [27].

1.4 Flavivirus RNA replication

Flavivirus follows similar lifecycle patterns inside the cells. It starts with the arthropod vector sucking in a blood meal while injecting the virus into the host system. Viral proteins are translated using the input template RNA resulting in a polyprotein, which later gets cleaved into individual structural and nonstructural proteins. Progeny virions are then assembled from structural proteins and viral RNA and are exported through the Golgi compartment and secreted.

1.5 Structure of Flavivirus

The structure of Dengue was solved using cryoelectron microscopy and then fitting the known structure of E glycoprotein into the electron density map. It was first solved at resolution 24 Å [9] and then at 12 Å-14 Å [28, 29] and most recently the structures are solved at much higher resolutions at different temperatures. 3.1 Å - 6

Å at 28°C (Mosquito body temperature) and 13 Å-14 Å at 37°C (Human body temperature) [4, 30].

Concentric layers of density are observed in a cross-sectional view indicating multilayer composition of the virion. The outer most layer which corresponds to the E and M trans-membrane proteins shows the highest electron density. A 35-40 Å thick lipid bilayer appears non-spherical with the trans-membrane components of the M and E (Fig.1.7 A). In the cryo-EM reconstruction of Dengue virus it seems to have smooth outer shell and the E protein dimers appear arranged in a “herringbone” pattern (Fig. 1.6 and 1.7 Ci).

1.5.1 Structure OF West Nile Virus

The genome consists of approximately 11000 bp in length of a single stranded RNA of positive polarity. It consists of 5′ untranslated region (UTR), a single long open reading frame and a 3′ untranslated region [20]. Structure of WNV is revealed to be icosahedral with 50 nm diameter (Fig. 1.6 and 1.7). The outer layer consists of viral envelope and membrane proteins embedded in lipid bilayer; while nucleocapsid core with multiple copies of capsid protein and genomic RNA are found inside the envelope. During viral entry E protein is thought to react with one or more surface receptors where DC-SIGN, alphaVbeta3 integrin and laminin-binding protein are amongst the most probable ones. Post binding, clathrin-mediated endocytosis takes up virus and viral fusion with cellular membranes results as a consequence of conformational change in E protein in acidified endosome. The viral positive strand RNA gets translated into a polyprotein comprising of seven non-structural proteins and three Structural proteins (Capsid protein (C), Membrane precursor (prM), and the Envelope (E) protein) [20].

1.5.2 Structure of Zika Virus

Structure of the Zika virion particle was solved with three-dimensional cryo-EM technology to near atomic resolution of 3.8 Å [31]. Overall structure is very similar to other flaviviruses. The M protein is covered by E protein layer. The E & M proteins are arranged in an icosahedral symmetry with 60 repeating units (Fig. 1.6). Individual repeat unit contains three E-protein molecules (A, B and C) [31]. The

Zika E protein is composed of approximately 500 amino acids. The N terminus has the characteristic three domains I, II and III similar to other flaviviruses with predominantly β sheet fold (Fig.1.6).

Fig.1.6 Flavivirus Structure

On the left is icosahedral Virion. The triangle represents the asymmetric unit of the structure consisting the E-protein monomers labeled as A, B and C. The numbers represent the 2, 3 and 5 fold symmetry of the icosahedron. On the right is the structure of one E-protein dimer. Domain 1, 2 and 3 are rendered in red, yellow and blue colours respectively. Image replicated from [31].

1.5.3 Flavivirus Structure Dynamics

Flaviviruses exhibit collage of biophysical and biochemical structure variations during their life cycle with pre-mature structure, mature structure, Pre-fusion hoisting of trimer as explained in Fig.1.10. At all these stages, the virus surface proteins are having different biochemical niche along with their physical position dynamics. The homodimers lying on the mature viral surface pose a very different antigenic environment when compared with the trimeric protrusion. Therefore varied epitopes are displayed or exposed at individual structural condition to the immune system.

The most dynamic region, which undergoes extensive structural rearrangement, is the Three-fold axis of the viral icosahedron (Fig. 1.8 A, D and E) [7, 9, 32, 33].

Dengue virus is observed to respond to temperature variation during its life cycle through different hosts, at 28⁰C in mosquito and at 37⁰C in humans [4]. The viral particle displays a thermal expansion which is also termed as Viral Breathing at

37°C [30]. This is effected by alienation of E-protein monomers A, B and C (Fig 1.8A) to rearrange themselves with different adjoining partners to form trimmers, resulting in hoisting of EDII (Fig.1.10). This resembles Pre-fusion intermediate of the virus in Endosomal environment (Fig. 1.7C and 1.8 D)[9, 30] [7]. Analysis of this dynamics is of utmost importance because most strategically significant epitopes of broadly neutralizing epitopes are at the hinge of this movement on EDIII [34]. These epitopes represent most functionally relevant conserved amino acid residues at this position. The concern is the lack of exposure of these epitopes on a mature virus. These epitopes assume all the more critical value from perspective of vaccine formulation. Reason being, these conserved epitopes are mostly the favored linear peptides displayed by Antigen representing cells like Dendritic cells, macrophages or platelets[35] and subsequently T-cells are activated to generate both Th1 and Th2 arms of the T-cell dependent acquired immune response. T-cell independent humoral immune response is a desirable outcome considering the scope for possibility of artificial improvisations. However, it is observed that the T-cell dependent peptide presentation and clonal expansion of previously present memory B-cells dominates the response on field [36]. Therefore, the proactive approach to design a vaccine is to tweak or positively direct this clonally expanded existent anti-sera to the desired epitope destination or improving its strength, avidity and eventually the efficiency using non-immuno-dominant conserved functionally relevant epitopes. Another potentially promising intervention option is to generate efficient broadly neutralizing antibodies as immunotherapy.

Both these goals call for extensive structural understanding of significant epitopes and their characteristic exposure pattern at different stages of structural arrangements and molecular or atomic level detail knowledge of the paratopes of the Cross-protective potent antibodies and their neutralization mechanism. The following chapters would be specifically addressing some of these aspects.

Fig.1.7 Flaviviral Structural Dynamics

All the figures are replicated from the source mentioned. A. Cryo-EM image cross-section of DENV. Cyan: Envelop and Membrane protein layer (235-249 Å), Glycosylation sites are shown in blue, Red: RNA core, Yellow: Capsid [4]. B. Immature virus spiked structure, The E-protein domains I, red; II, yellow; III, blue. The Fusion tip on DII is covered by PrM indicated in cyan [6]. C. Dynamics at 3-Fold Axis on a $T=3$ Icosahedron at 37°C and lower pH. Expansion of particle from (i) Mature virus (ii) Fusion-intermediate, Arrows indicate direction of E-monomer movement (iii) Fusion conformation. Color code as in B [9]. D. Black arrows indicate immature virus with spikes and White arrows indicate smooth mature viral particles [4]. E. Furin mediated trimming of Pre-mature virion particle (with E proteins arranged in vertical dimer with prM) to mature virion particle devoid of pr protein arranged in a horizontal E-protein dimer [11]. All the figures are replicated from the source mentioned as on the next page along with the legend of this Infographic

1.6 *Flavivirus* Structural Proteins

Falvivirus has three structural proteins: capsid that encloses the positive-strand genome, precursor membrane protein (prM) and envelope (E) (Fig. 1.7), last two being components of virion envelop structure. Antibodies (Abs) to prM are generally poorly neutralizing but potent enhancers of infection [37], whereas Abs against E show more potent neutralizing activity [25, 38, 39].

E (Fig. 1.8, Fig.1.11) is composed of three domains (EDI, EDII and EDIII) [40]. ED I and ED II are situated at the membrane-proximal N terminus of the protein in form of discontinuous fold; ED II contains the fusion loop. Flaviviral cross-reactive Abs targeting the highly conserved fusion loop [41, 42] confer these antibodies low avidity and concomitant weak neutralization, because the hydrophobic fusion loop is not exposed to infectious virions [42]. ED III is followed at the stem anchor region by two helices named H1 and H2; the C-terminus of which runs through the membrane via trans-membrane segments called TM1 and TM2 (Fig.1.8B). A common feature of class II fusion protein is low pH converts them from a homodimeric native state into a trimeric fusogenic complex.

Fig 1.8: Envelop protein Dynamics and Participations of EDIII

All figures are replicated from the mentioned source. A. E protein dynamics at 3-fold axis cryoEM maps (i) E-proteins at 28°C, A, B and C molecules of each monomer of E-proteins and A⁰, B⁰ and C⁰ are corresponding monomers from opposite direction in each of the dimer arranged at 3-fold axis. The red triangle indicates the 3-fold axis where the molecules alienate at thermal expansion as in (ii) Thermal expansion of E-protein molecules at 37°C.[4] B. Stem anchor region and E-protein cartoon, TM1 and TM2 the transmembrane domains and CS, a conserved sequence are α -helices adapted from [8]. C. Pre- and post-fusion conformations of E-protein. (i) At neutral pH the E-protein in horizontal dimer on mature virus in pre-fusion state (ii) At endosomal conditions the protein domains rearrange to form the fusion-trimer. The C-terminal shifts closer to the fusion loop at the top of DII (yellow) [10]. Continued...

C. Schematic of rearrangements associated with the (i) dimer to (ii) trimer fusogenic complex leading to alienation of each E-protein at three-fold axis. (iii) Yellow cylinders represent E-protein and the Fusion loop is indicated as Green extension from the cylinders. Below is a top view of the Dimer to trimer rearrangement in pre-fusion intermediate and above is a side view of this arrangement at the three-fold axis showing the hoisting of fusion loop towards host cell membrane. Note the shifting and rotation of E-protein domains [7]. E. The rearrangement presented in D above is represented as electron density in form of wireframe density map with domain II is represented in yellow, the domain III is represented in Blue and domain I represented with red color. (i) view from top of the particle (ii) View from sides of the rearrangement at 3-fold axis hoisting the DII tip harboring the fusion loop at the three fold axis [7].

1.7 Domain III of the Envelope protein (ED III)

Among the three domains of the E protein, the C-terminal domain III, with a predominantly β -strand secondary structure, can fold independently. ED III is continuous and comprises residues 296-400 of the E protein sequence (DENV1 numbering). Its fold is compact and homologous to the constant region of immunoglobulin (IgC-like) (Fig 1.9) and stabilized by a disulfide bond between residues Cys302 and Cys333. The structures of recombinant ED III domains have been solved by NMR methods for YFV, JEV, WNV, DENV2 and DENV4 [43-46]. The structure of the isolated ED III domain is close to its structure in the crystals of the E protein. In spite of the serotype specific variability in ED III, nine β strands are commonly found in these subunit proteins in flavivirus group. β 1- β 4 strands appear almost similar in all the flaviviruses. β 3 is absent in the NMR structures of JEV and WNV, structures with exception of WNV, wherein both the NMR and X-ray crystal structure indicate that they are slightly longer compared to its peers.

Conversely, $\beta 5$, $\beta 6$ and $\beta 7$ exhibit huge variability. This region of the protein is

Fig 1.9 Ribbon diagrams of several flavivirus ED3 proteins

Image modified [45]

more variable and dynamic than the rest of the ED III subunit. The flaviviral ED IIIs have similar secondary structure and fold with distinct charge distribution on surface representative of serotype or strain difference. EDIII of DENV2 has significantly more negative charges at the C-terminus compared to DENV3 and 4, while DENV3 EDIII displays more negative charges near the N-terminus of the protein. DENV4 ED III is distinct with larger uncharged surface area. The distinct electrostatic charge on the surfaces of EDIIIs is predicted to influence the concomitant antigenic variability and other biological properties of the viruses [45]. This further substantiates the hypothesis of sylvatic origin of human dengue infection. Wang et al contemplate evolution of dengue virus adapting to newer host receptors reaching human territory from its initial sylvatic cycles in *Aedes* mosquitoes and monkeys [47]. The same study on analyzing the E sequences

identified 9 amino acid differences, of which five (K304R, M342V, I335T, I364V and A382V) are in the ED III, attributing to the emergence of DENV4 as human virus from a sylvatic cycle. Also, changes in cell receptor-binding properties of ED III contributed to evolution of DENV as human pathogen. This may be a probable hint of emergence of newer subtypes or even serotypes by further host-viral interactions, both by modified environmental and host factors. Functionally, the EDIII is directly involved in receptor binding [48, 49] by virtue of its structure resembling an IgC like fold and its position on the whole virus; where the former projects above the convex surface allowing access to potential receptor molecules and neutralizing antibodies. Also, the ED III plays a major role in providing the necessary ‘horse power’ required for the fusion machinery. The ED III acts as viral agent to engage primary or secondary cell receptors, including heparin sulfates and ribosomal protein SA. Consistently, recombinant ED III domains inhibit infectivity [41] of the cognate virus for DENV1, DENV2, WNV and JEV and some authors have proposed to use ED III as a basis for therapeutic molecules. Cell tropism and virulence is found to be dictated by mutations in the EDIII domains. of YFV and DEN V2 affect their cell tropism and virulence [50, 51].

1.7.1 Interaction with host cell surface receptors

West Nile virus binds to Toll like receptor-3 (TLR-3) [52]. Several primary receptors and low affinity co-receptors of cellular attachment molecules have been proposed for DENV including the highly sulfated heparan sulfate [53], the dendritic cell-specific ICAM3-grabbing-non-integrin (DC-SIGN) on immature dendritic cells [54], the L-SIGN [55], the mannose binding receptor on macrophages [56] and the laminin-binding protein [57, 58]. Several other cell surface putative receptors have been proposed for flavivirus binding [59]. In addition, opsonization with immunoglobulins enhances virus particle binding and infection of cells expressing immunoglobulin Fc receptors [60]. Despite this variety of host cell surface receptors identified for entry, the only one viral component that was consistently shown to bind directly to cellular receptors is the IgC-like domain III of the Envelope protein (E protein) [25, 49, 61, 62].

1.8 Low pH induced endosomal fusion by flavivirus

Upon binding to the receptors, virions enter the host cells through the clathrin coated pits via receptor mediated endocytosis [63]. Once brought into the prelysosomal endocytic compartment, extensive structural rearrangement of the E protein homodimer occurs to form a homotrimer and this leads to baring the fusion loop at the domain II raising it perpendicular to the virus surface. The multiple events leading to the fusion of the virus through the fusion tip with the endosomal membrane are well characterized and summarized [32, 33, 64-67] (Fig.1.10).

Fig.1.10 Schematic overview of the DENV membrane fusion process.

(A) Envelope protein homodimer before fusion (B) Dissociation of homodimer at reduced pH in endosome (C) Ahairpin-like structure of Envelope protein trimer. (D) Hemifusion intermediate. (E)The viral RNA released through fusion pore into host cytoplasm. Image Modified from [42] [68]

Formation of trimer contacts spread from, the fusion loops at the trimer tip to domain I at the base. Domain III shifts and rotates, folding the C terminus of sE back towards the fusion loop. The length of the interdomain linker facilitates

independent rotation of each domain III, aiding the required spontaneous-symmetry break.

Envelope Domain III undergoes the most significant transition from dimer-to-trimer. It rotates by about 70° , and its centre of mass shifts by $36\text{\AA}$ towards domain II. This folding-over brings the C terminus of domain III (residue 395) $39\text{\AA}$ closer to the fusion loop (Fig. 1.8, C) [33].

Fig.1.11 Overview of the DENV Envelope Protein

(A) *DENV-2 sE dimer. (DI, red; DII, yellow; DIII, blue). The fusion loop is colored in green. The arrows indicate the view zoomed in (B).* (B) *Close-up showing DIII from one subunit in the sE dimer and its orientation with respect to the viral membrane in the mature virion.* (C) *The mature DENV-2 virion (PDB accession number 1THD). View down an icosahedral 3-fold axis (central gray triangle). Icosahedral 5- and 2-fold axes of the particle are marked with pentagons and ovals, respectively Image modified [69]*

1.9 Pathogenesis of Flaviviruses

1.9.1 Pathogenesis of dengue virus manifestations

Dengue virus infection in humans manifests into symptoms ranging from mild febrile illness to severe and fatal hemorrhagic disease. Clinical diagnostic features are almost similar for all four serotypes which may vary on the scale of severity. The severity varies with immune status which in turn depends on heredity and aging and on the basis of whether it is primary or secondary infection and serotype involved. The incubation period varies from 3 days to 14 days [70].

Classic dengue fever primarily affects the age group of >15 years and adults. Hemorrhagic manifestations are not uncommon in Dengue fever patients but mostly limits itself and not fatal. The characteristic manifestations of Dengue Hemorrhagic Fever (DHF) are plasma leakage, thrombocytopenia and haemoconcentration. A severe and fatal form of the disease is Dengue Shock Syndrome (DSS). Rashes erupt all over the body and on the face accompanied by prolonged shock with a 40% mortality rate [71].

There still is severe lack of knowledge about DSS and DHF and the mostly accepted hypothesized cause is the antibody-dependent enhancement (ADE) which implies that patients encountering a successive infection with heterologous DENV serotype run higher risk of experiencing DSS and DHF. Pre-existing heterologous antibody recognizes the infecting virus and forms an antigen-antibody complex; approaches antigen representing cells like leukocytes e.g. Macrophages and gets internalized upon binding to Fc receptors. The antibody being heterogeneous, fails to neutralize virus leaving it capable of replicating inside the macrophage. This boosts replication of DENV in cells resulting into a flared up hyperimmune inflammatory response [72].

1.9.2 Pathogenesis of West Nile Virus

Mosquitoes harboring WNV are inject the virus into various species of vertebrates. The commonly reported estimates are from Serologic surveillance data gathered during the New York epidemic and demonstrated that out of five infected individuals, only one developed fever. The most severe disease outcome was severe neurological complication developed in 1 out of 150 infected individuals [14]. Year 2007 outbreak reported 64% cases of fever with milder symptoms whereas 36% cases neurological complications like encephalitis or meningitis. Incubation period of WNV was 2 to 14 days in humans with symptoms like fever and headaches. Viremia lasted only for first few days of infection. WNV pathogenesis and the innate and adaptive host immune responses in detail have been discussed by Gea-Banacloche, J., et al and Samuel, M.A. and M.S. Diamond [10, 73].

1.9.3 Pathogenesis of Zika

Zika virus has close similarity to Dengue virus. Its common symptoms are conjunctivitis (red, sore eyes), mild fever, joint pain, headache, and rash, manifested in approximately 20% of infected cases.

Unavailability of vaccine or drug treatment forces patients to rest and increase their fluid intake as the only methods of recovery. Of more concern, is the impact on babies developing in the womb and the subsequent increase in microcephaly.

Microcephaly is a condition that is noted by an abnormally small head of newly born baby due to inadequate development of their brains. If the brain development is severely obstructed, which results in the brain that is incapable of regulating functions vital to life; then such conditions can lead to death of infant. Such children even if they do survive, may face development delays and intellectual disability.

Since 22 October 2015, more than 4,700 cases have been reported. Amongst these 404 cases are confirmed; while 3,670 cases are still being investigated. WHO strongly suspect the link with Zika virus, however the research has not yet confirmed the link. Virus has been found in the brain, placenta and amniotic fluid of some babies who have died. Viral envelope protein binding to specific receptors like AXL, DC-SIGN, TIM-1, and Tyro3 leads to particle internalization by the receptor bearing cells. This switches on the transcriptional activation of RIG-I, OAS2 stimulated by Interferon, Toll-like receptor 3 (TLR3), MX1, MDA5, ISG15 and beta interferon gets triggered [74].

Primary victims of infection are cutaneous antigen-presenting cells (Skin fibroblasts, epidermal keratinocytes, and skin dendritic cells). Another initial important target for Zika virus are the immature dendritic cells.

Scientist belonging to the Carlos Chagas Institute of Paraná Fiocruz demonstrated crossing of placenta by Zika virus during pregnancy, on the basis of experiments on viral proteins found placental cells. Presence of FcRn receptor on the placenta seems to assist the viral uptake and transport. Preexisting antibodies of dengue or YF due to epitopes of these viruses, can bind with Zika and enhance replication of

virus, its transfer through placenta or infect cell of placenta. Some reports support this assumption.

Conservation of envelope Domain II B cell epitope, a primary culprit to non-neutralizing cross reaction for dengue, explains the cross reactivity with dengue and YF [75]. Recent Brazilian dengue isolates are distinct from Domain III of Zika. This domain is supposed to be the main target of most neutralizing antibodies. The virology and immunology of Zika infection in the human host should be clearly understood so that strategies to manage Zika infection and spread can be delineated. The key questions to be answered are:

1. Duration and levels of viremia
2. Natural immune response.
3. Role of naturally produced antibodies, especially the non-neutralizing ones in enhancement of the disease.
4. Role of the pre-existing antibodies elicited by other flaviviruses or flaviviral vaccines.

Currently no over the counter drug is available for Zika. The target population being pregnant women and fetuses makes it almost urgent but more time-consuming affair to devise any particular treatment. It turns out, when clinical testing for a medical countermeasure (MCM) is conducted; pregnant women are typically tested last.

Carefully evaluated safety, effectiveness and risk/reward ratio in addition to extensive time investment coupled with great caution; characterize design strategy of an over-the-counter prescribed vaccine for Zika infection for the distribution to the general population. This especially applies to a vaccine that is being developed for protection against a virus with apparent association with both neurologic teratogenic effects and neurologic autoimmune disease (GBS). Additionally, if the virus belongs to a genus known to enhance antibody-mediated infection, the stakes are raised further [76-78].

Outbreak of Zika, on the Yap Island in 2007 further underscores the aforementioned statement. During the outbreak, 73% of the residents contracted Zika virus infection only within four months. This suggests the criticality of the time element in vaccine development, to prevent Zika from being endemic in susceptible regions.

Monoclonal antibodies therapies are reviewed towards arboviral infections to assess their potential as treatment option. In the wake of ADE, they need to be carefully designed and vigilantly distributed.

1.10 Antibody Dependent Enhancement

Antibodies produced against Dengue virus play two opposite roles. The antibody generated against the primary infection of a particular serotype provides lifelong protection against that serotype. But, the same antibody recognizes the second heterologous serotype upon secondary infection due to the 60-70 % similarity in antigenic surface of all four serotypes. This leads to binding of this antibody to the second serotype virus without complete neutralization owing to the subtle difference in the morphology. However, these non-neutralizing antibodies bring the virus to Fc receptor bearing cells by attaching to the Fc-receptor. The virus being capable of infection invades the Fc-receptor bearing cells. This results into increase in viremia and related severe complications [79]. This phenomenon is termed as Antigen Dependent Enhancement (ADE).

ADE is a new type of Gell-Coombs immunopathology.

1. Type I - IgE-mediated immediate hypersensitivity;
2. Type II - antibody-mediated acute immune complex disease;
3. Type III - IgG-mediated complement-dependent foreign antigen immune complex disease;
4. Type IV - cell-mediated immune and autoimmune diseases; and
5. Type V - IgG immune complex enhancement of microbial infection in Fc-receptor (FcR)-bearing cells.

In case of dengue, the ADE phenomenon was attributed to enhanced growth of DENV infection in primary monocytes and macrophages in the presence of nonneutralizing enhancing dengue antibodies. However, in case of humans, studies of FcR-bearing cells show that sub-neutralizing monoclonal dengue antibodies may form infectious immune complexes. In vivo, the activity is dependent on availability of a virion surface antigen at subneutralizing antibody concentration, to get

attached. In practice, antibodies directed at surface epitopes that are not involved in virus entry efficiently, produce ADE. Many studies have shown that cross-linking of IgG immune complexes with Fc γ receptors increases intracellular infection. This contributes to exacerbation of disease severity by mechanism of intrinsic ADE (iADE) [76].

As per research by Guzman et al [80], antibodies to prM are highly cross-reactive across serotypes and are major component of response. Further, these antibodies have low neutralization capacity leading to ADE. Hence, according to the authors, partial cleavage of prM masks a neutralizing antigen density evading neutralizing antibodies, further facilitating ADE by antibody to prM. Reports [81] of fully immature dengue particles becoming highly infectious when interacting with prM antibodies further strengthen the hypothesis.

Immature particles (Fig.1.12) are ineffective because of their inability to efficiently engage the cell surface receptors, but Anti-prM antibodies, which are in abundance offer assistance to these inefficient virions in binding. Efficient intracellular processing of prM to M by furin activity within the target cell enables the immature virion to fuse with endosomal membrane (Fig.1.7 E, 1.12). The prM antibodies facilitate efficient binding and cell entry by immature particles into Fc-R-expressing cells. Thus, pointing some of the possible underlying reasons to ADE.

Fig. 1.12 Antibody-Dependent Enhancement of dengue infection.

Image replicated from [82]

1.11 Anti-flaviviral antibodies

The human antibody response to DENV infection favors predominantly antibodies to prM and followed by domain I and II (DI/II) of the envelope (E) glycoprotein [37, 75, 83]. Thus the antibody responses are dominated by weak neutralizers, directed to the fusion loop and prM in humans. Overall, the IgG response to domain III appears variable and comprises only a small fraction of the whole antibody response in humans [37, 84-86]. However the most desirable potent cross-protective neutralizing antibodies are found to target Envelope Domain III. There are several reasons why the predominant humoral response of humans is weak towards EDIII. In humans, there is very less contribution from D (of VDJ recombination) gene in affinity maturation of B cells in the acute phase of secondary infection. Previously available clone of memory B cells are expanded rather than naive new B cell population. This clonal anti-serum is generally found to have a signature of residues ARD(Y)₅GMDC[87] and is found to target an immunodominant epitope on the EDIII at residues 309-320 [34]. This region on mature Dengue virus surface is mostly hidden.

Therefore, the humoral immune response of humans needs to be modulated to generate antibodies towards non-immunodominant but neutralizing epitopes by proper vaccine or passive immunization should be considered with strategically designed antibodies towards desired epitopes [88]. To achieve this, currently published cross-protective antibodies, should be analyzed for their mechanisms which is discussed as follows:

1.11.1 Potent Cross-Protective Antibodies and the Mechanism of Neutralization

1.11.1.1 Ab 9F12:

Inhibits Entry of Virus by blocking the receptor binding of the virus Ravikumar et al. [1] reported a cross-protective antibody which was raised against DENV2 TSV01 in mouse model. This antibody potently neutralized all dengue serotypes and West Nile virus. This antibody targets at A strand of the virus which is immunodominant region. It also targets the BC loop (Fig.1.13).

Fig. 1.13 9F12 epitopes [1]

The epitopes are marked on EDIII motif. Each Beta strand is labeled with an Alphabet. Epitopes in blue are on A-strand, Epitope on BC loop in Black. Image modified from [1]

1.11.2 Antibodies that inhibit fusion of the virus after receptor binding:

1.11.2.1 Ab 3E31 (PDB ID: 4BZ1) [89]

This antibody generated in mouse framework of antibody was found to neutralize all four serotypes by inhibiting fusion of virus after receptor binding, presumably by impeding the folding of EDIII at the hinge region for the fusion. This antibody does target the immunodominant epitope on A [34] strand along with B-strand epitopes and E strand epitopes (Strand description as per fig.1.13).

1.11.2.2 Antibody 513 - Best example of engineered antibody

Tharakaraman et al [90] used structural – physicochemical statistical approach to generate a derivative antibody called 4E5A from the parent mouse cross-reactive antibody 4E11 directed to the EDIII epitope. This derivative was aimed at improved affinity, especially towards DENV-4, which remained moderate (100nM K_D) compared to other serotypes in spite of the modifications. Robinson et al [88] further used this scaffold of 4E5A to engineer a better version called Ab513 by employing the epitope-prototype interface (EPC). They developed a framework to compute the inter residue atomic interactions of the Complementarity Determining Residue (CDRs) of antibody and adjoining epitope residue. This was represented in a 2D graph format as the epitope-paratope connectivity network. Favorable contact points between antibody and antigen were extensively interrogated by EPC network analysis.

CDR-H1 of 4E5A was subjected to extensive epitope-paratope interrogation as it was believed to be contributing to subtle changes to the binding interface. This interrogation was an atomic level comparison of two antibodies i.e 4E5A and 4E11.

Ab 513 exhibited strong promise of immunotherapy due to the following attributes:

- Ab513 demonstrated strong in vitro neutralization of DENV-1 to -4.
- Ab513 successfully neutralized all tested challenge viruses.
- Ab513 exhibits similar or better potency than EDE mAbs [91] of subclass 1 and comparable activity to those of subclass 2, which are sensitive to glycosylation state of DENV.
- Ab513 triggered very low levels of ADE as compared to control antibody against all DENV serotypes.
- Ab513 neutralized all four DENV serotypes in the presence of uptake, with DENV-immune complexes trafficked to the LAMP-1 compartment. A single dose of Ab513 was able to effectively neutralize the virus and prevent migration of virus to the CNS.

Ab513 conferred speedy recovery of human platelet levels to virally challenged humice upon single dose of Ab513. Also ab513 demonstrated significant reduction in blood levels of INF- γ and IL-10 in DENV 2 challenge as compared to control antibody [88].

1.11.3 Antibody Neutralization by Forcing Pre-mature fusion:

1.11.3.1 Ab2D73 (PDB id: 4bz2) [34, 92]

This antibody was found to force the pre-mature fusion of the virus and fix the virus in this position at the time of receptor binding. This way it could neutralize all four Dengue virus serotypes.

1.12 Scope of Engineered monoclonal Antibodies:

Ab 513 poses an excellent example of biotechnologically engineered Antibody. These engineered antibodies exhibit vast scope to aid the natural immune response to overcome the challenges posed by ever-evolving pathogens. Particularly in case

of Dengue the immune system handles different but similar viral serotypes. Acquired immunity plays very important role. T-cell mediated response is instrumental but depends on processed antigens and thus have limitations. But humoral immunity is more important because it can target unprocessed epitopes directly on the intruding virus. This, in turn, gives opportunity to modulate this response by designing better paratopes for immunotherapy and epitopes for better immunization. Design of efficient acquired Immune Response towards *Flaviviruses* can be facilitated by detailed understanding of efficiently neutralizing Antibodies and their preferred epitopes, which can be followed by careful tinkering of epitope-paratope repertoire through biotechnological engineering.

1.12.1 Design Strategy for Engineered antibody

In general, the non-complicated primary *Flaviviral* infections are non-symptomatic and not fatal because of the efficient innate and humoral immune response. Antibodies efficiently capture the invading viruses and restrict dissemination of the infection in body system even in cases of encephalitis. Antibodies are believed to protect against flavivirus infection through multiple mechanisms, which include inhibition of viral fusion, complement-mediated lysis of virus or infected cells, Fc- γ -receptor-dependent viral clearance, direct neutralization of receptor binding, and antibody-dependent cytotoxicity of infected cells [93].

On the basis of the current limited knowledge of the natural antibody repertoire, some attributes can be assigned for the most desired antibody. Most of these attributes would target the problem of antibody mediated disease enhancement. The most prioritized attribute obviously is that the antibody should be cross-protective. It should have a very high affinity towards its epitope to compete with the previously present non-neutralizing but cross-reactive antibodies (mostly Anti-prM antibodies). This can be achieved by choosing an appropriate epitope.

Choice of epitope is the most important factor in generating a potent cross-protective antibody. Reports from research till today suggest that the epitope should be conserved, accessible on virion surface at all infectious stages and should be present in abundant amount on the virus to allow greater occupancy for the antibody

to generate huge complexes to engage the Fc γ RIIB [94]. This epitope should be able to elicit robust response from CD8+T-cell mediated immune response. Finally, the strategically designed therapeutic response to flaviviral challenge would have a multi-faceted and simultaneous action approach with inclusion of more than one antibody with different but additive modes of action with a supportive hand of robustly designed vaccination.

1.12.2 Biotechnological Instruments Available to Artificially Generate Immune-Protection through Antibody:

The use of hybridoma technology [95] has facilitated the generation and selection of highly specific antibodies from immune B cells for both diagnostic and therapeutic purposes. Passive immunotherapy with monoclonal antibodies is very attractive option towards the treatment of dengue. Reports of monoclonal antibodies capable of rapid and substantial reduction of viral titer in numerous instances like: influenza and HIV further support the hope

Panning of B cells isolated from challenged mice or from infected patients had been instrumental in identifying various Dengue-neutralizing antibodies including cross-protective ones [83, 95-99].

Methods to characterize the antibodies for their anti-viral, physicochemical and structural properties range from the old classical plaque reduction neutralization tests (PRNT) to a variety of newer innovative tools like the yeast surface display of a variety of epitopes. An extensively characterized potential neutralizing antibody becomes a prospective candidate for development as therapy.

1.12.3 Modification of Antibody motifs for better Adaptation to Immunotherapy

1.12.3.1 Single Chain Fv (scFv)

Immunoglobulins are big proteins, which the immune system, generates to combat pathogens. There are five classes of Immunoglobulins namely – IgA, IgD, IgE, IgG and IgM – of which IgG is one of the most focused as they are most commonly found in human blood (Fig.1.14) [100].

Two heavy and two light protein chains compose its structure. Disulphide bonds that depend on antibody species and antibody class, hold together two halves of antibody in the hinge region. Two Fab fragments, with each one comprising V_L , V_H , C_L and C_{H1} domains, compose the antibody. Antibody Fv fragment is even smaller than Fab fragment. It consists of only V_L and V_H domains. Recombinant synthesis is the only method to obtain it reliably. Only relatively weak non-covalent interactions held Fv fragment together. CDR3 loop between antibodies has difference in sequences that impacts the V_L - V_H interaction stability and affects the stability of Fv fragment. Linker peptide attachment between domains V_L and V_H is the solution to overcome this problem. This activity results in scFv fragment[101]. The length of the peptide linkers vary from 10 to 25 amino acids. Most of the amino acids forming the linker are hydrophilic in nature with prominent among them is decapentapeptide $(Gly_4Ser)_3$. Linker length is the determining factor for multimer formation. Here, linkers with shorter lengths create favorable conditions for dimers and trimers. The length of linker in such cases varies from 0 to 12 amino acids. Even though both - V_H - linker - V_L and V_L - linker - V_H - orientation are found in variable regions being connected; former is found more commonly.

Fig. 1.14 Basic structure of an IgG Ab molecule.

Heavy chains are shaded in blue and light chains are shaded yellow with the lined regions representing the variable domains. Fab is the antigen binding region harboring the Fv region with specificity for the Antigen. Image Replicated from [100].

Moreover, these orientations have probable impact on stability, efficiency of expression as well as binding activity of antigen.

Complementarity Determining Regions (CDR) constitute the antigen (Ag)-binding site of an Ab, three each from the variable heavy- and variable light domains supported by the framework (FR) regions, determines antibody specificity [102].

1.12.3.2 Humanization of Antibodies

Administration of therapeutic monoclonal antibodies (mAbs) [103-105] of xenogeneic origin, poses a risk of attack by host immune reactions in human patients. This is detrimental in terms of efficacy of the drug. Most worrisome consequence is the onset of systemic inflammatory response syndrome.⁴ mAbs with sequence closer or typical of human doesn't elicit such immune reaction. This is avoided by humanizing the non-human antibodies, mostly from rodent origin by replacing its parts by their counterparts, from naturally occurring human antibody while preserving its original antigen recognizing features. This is achieved by two major engineering strategies.

First is, humanization, which can be called as “discard all but complementarity-determining regions (CDRs). In this, only the antigen binding parts are retained from the original non-human antibody by inserting CDRs from the original version on to the canonical β -sheet scaffold of human variable region. This results into an engineered antibody with Fc (Immunoglobulin constant region) with selectively the CDRs from the non-human antigen binder.

The second strategy, chimerization, involves planting the non-human antibody variable regions on the Fc region of a human antibody. This process offers to choose the functions of the human IgG subtype and corresponding Fc effector which are best matched to the expected drug ability of the derived antibody. The resulting chimeric antibody is no more antigenic with significant xenogeneic content. The

highly conserved and modular structure of antibodies facilitates both the strategies; chimerization and humanization.

The initial step of humanization is the categorization of the antibody's variable region sequences into framework and CDR.

Orientation Fingerprint is the sharing of VH-VL orientation by variants in spite of their unique sequence. Humanization involves selecting the humanization variants to preserve the VH-VL orientation fingerprint from the nonhuman version. This ensures an improved humanization with highly successful preservation of original antigen-binding properties.

1.13 Vaccine

In this thesis, we have contemplated on the perspective of EDIII as a possible vaccine candidate for Dengue. Before going into the details regarding vaccine for Dengue, I would like to introduce vaccine with a broader perspective briefly.

A vaccine is a product developed using biological preparations to impart immunity from a disease which may be permanent, long lasting or short term. It can be administered through injections, by mouth or by aerosol.

An agent, resembling killed, weakened or partial form of disease causing agent which may be a microbe or a toxin, constitute major component of a vaccine. Vaccine leverages on the ability of body to keep memory of previous pathogen attack in the form of antibodies developed to prevent future attack of the same pathogen. By simulating pathogen attack environment, vaccines allow immunizing system of the body to develop more antibodies and improve immunity against the attack [106].

According to Global Vaccine Action Plan 2011-2020 of WHO, if vaccines were 100% efficient and 100% immunization is carried out then especially in developing countries, approx.14% of dying children can be saved. It also emphasizes that - Haemophilus influenzae serotype b, measles, neonatal tetanus and pertussis – are the four diseases which account for more than 98% of deaths which can be prevented by vaccination.

1.13.1 Stages of Development of Vaccine [107]

Vaccine development is very time consuming and costly end result obtained after years of scientific research, clinical trials and statistical on ground analyses of safety and efficacy.

The process can be broadly classified into two steps.

1.13.1.1 Pre-clinical development

At this stage extensive research is undertaken to discover desirable vaccine candidate in various labs and in experimental animals. The aims and objectives of the study include:

Selection of candidate antigens, defining the vaccine concept, and in vitro and in vivo evaluation of vaccine efficacy and vaccine production by following Good Manufacturing Practice standards

1.13.1.2 Clinical development

Clinical trials in humans are divided into four stages from initial introduction in humans (phase I) and beyond (phase IV). Clinical development ensures ethical principles of informed consent from volunteers, vaccine safety and efficacy.

Phase I clinical trials are performed on a small-scale to assess human safety from vaccines and responses of human immune system. For diseases related due to poverty conditions; this phase of trials is conducted two sub-phases. In Phase Ia, European volunteers undergo the trial while in Phase Ib volunteers from populations in Developing Countries undergo clinical trials.

Phase II clinical trials are conducted on a larger scale compared to Phase I and primarily focused on assessing the efficacy of the vaccine, against artificial infection as well as clinical disease. Additionally, safety of vaccines, their side-effects and the immune response are also evaluated.

Phase III clinical trials are conducted for vaccines which successfully complete first two stages of clinical trials. These are conducted on a large scale (number of subjects running in many hundreds) at various different sites so that efficacy of such vaccines under natural disease conditions can be evaluated. Commercially, if the vaccine is able to demonstrate safety and efficacy over predefined period then, the regulatory authorities allow the manufacturer to apply for a license to market the product for human use.

The final phase IV, focuses on detection of adverse effects which are rare and long term efficacy of the vaccine in commercial production. It is also called post-marketing surveillance.

1.13.1.3 SMART Vaccines

U.S. National Academy of Medicine (NAM) has developed a software tool — Strategic Multi-Attribute Ranking Tool (SMART)—a multicriteria decision-making process for Vaccines [108]. This is a systematic approach of selecting a relevant vaccine intervention by screening preferential attributes of candidate disease target from multiple options. This strategy renders the decision-making process transparent and gives a better perspective of available options.

The tool is already equipped with 28 core attributes for a globally acceptable vaccine (Fig.1.15).

Fig. 1.15 Core values and multicriteria decision making for vaccine development

Attributes[108] to be considered for stakeholders (developed countries, low- and middle-income countries, emerging infections and manufacturers) are shown in surrounding values.

Image replicated from the mentioned reference.[105]

Multiple vaccine targets are compared on basis of desired attributes and subsequently are given a score. After data about population, disease and characteristics of vaccine is fed to software; it produces report listing various benefit measures that are traditional by nature. They include cost effectiveness, costs or savings per year as a result of vaccine usage, premature deaths as well as incident cases prevented per year [70].

1.13.1.4 Flaviviral Vaccination

Vaccination is the most sought after strategy in controlling flaviviral global invasion. The geographical distribution of each flavivirus is partly dictated by their vector distributions. Consequently, climate change and development of transportation systems have facilitated flaviviruses to infiltrate new areas. At present, a large population is exposed to flaviviruses worldwide (WHO, global incidence of Flaviviruses). In addition, traveling through endemic countries increases the opportunity of exposure to flaviviruses. Because flaviviruses survive in different vectors and hosts without humans, eradication of flaviviruses is thought to be extremely difficult.

The challenges, currently under consideration, include (but are not limited to)

1. Flaviviruses confuse host immune system by sheer inter and intra-strain similarities. Dengue has four serotypes which have 60-70 % similarity in their surface representation to the host immune system.
2. Lack of appropriate animal model for better understanding of disease pathogenesis also impacts the progress of vaccine candidate accreditation [36]. Currently there is no animal model which resembles the human immune response to Flaviviruses, although non-human primates show similar susceptibility to Dengue infection than mouse models.
3. Gaping holes in knowledge of immune-pathogenesis in human body system.
4. Reports of antibodies helping virus to infect the cells with Fc-receptors.

5. Dynamic surface representations of the virus at various stages of infection.
6. Almost lack of accessibility of the most conserved and neutralizing epitope.
7. Flaviviral prowess of adaptation to different hosts cell receptors.

Since majority of endemic areas are located in developing countries, cost effective and uninterrupted availability of dengue vaccine in multiple doses is the major concern for policy-makers in these resource limited healthcare settings. A safe, efficacious and cost effective dengue vaccine is a high public health priority[109]. We, in our research work elucidated in following chapters, are trying to address the points 4, 5, 6 and 7 mentioned above through structural analysis of an engineered Consensus EDIII protein. We also assessed the efficiency of this protein by determining its interaction with the currency known potent cross-protective antibody vis513 as discussed before.

1.13.1.4 Dengue vaccine

Efforts to develop vaccine candidate against Dengue fever date back to 1929. But the development of such vaccine candidates faced challenges like knowledge of the disease pathogenesis being incomplete and then the presence of four serotypes makes it necessary to elicit simultaneous stable immunity against all the serotypes to counter any adverse complications. Currently numerous vaccine candidates are on the ramp with a live attenuated vaccine candidate being set to enter the markets.

1.13.2 The status of Dengue vaccines [110]

At present, there are many candidates of vaccine for Dengue are walking the ramp of clinical trials like, Dengvaxia, TetraVax-DV, DEN-Vax, TDEN PIV, V180-Merk, DNA Vaccines and Vaccine candidates from India and Vietnam. Dengvaxia stands to be the most successful so far. **Dengvaxia** is the approved trade name for a live attenuated tetravalent chimeric vaccine, which is also called as CYD-TDV. It was designed using recombinant DNA technology by swapping the PrM (pre-membrane) and E (envelope) structural genes of the yellow fever attenuated 17D strain vaccine. The swapping occurred with four of the five dengue serotypes. In

the phase III clinical trials held at Latin America and Asia, the vaccine was successful in reducing about 80% of the severe dengue cases. The vaccine was first approved in Mexico and subsequently Philippines, and Brazil in December 2015. The 3 year follow up study shows efficacy against all four serotypes.

1.13.3 Vaccine Delivery by Microneedle Patch

Commonly, intramuscular (i.m.) or subcutaneous (s.c.) are the routes used for vaccine administration. However, intradermal (i.d.) and transcutaneous (t.c.) techniques are also regaining popularity. There are several factors which strongly support the usage of the cutaneous (Fig.1.16) tissues. They include

1. Better knowledge of skin physiology and rationalization based on it for targeting the different cutaneous layers, such as the epidermis, dermis, or hypodermis
2. Improved knowledge of the plasticity of antigen-presenting cells (APCs) (i.e Langerhans cells (LC), dermal dendritic cells (DDC) and dermal macrophages) in the recruitment of immune responses.
3. The advantage of dose-sparing for some modes of cutaneous administration
4. Vaccination strategies for developing countries that avoid usage of needle will address issues like blood contamination, costly cold-chain processes, fast wide-spread dissemination and pain-free self-administration.

Microneedles is one such improved strategy to employ cutaneous immune system for vaccination. Microneedles are micron-sized needles that are coated with arrays of vaccine formulations. These are directly penetrated perpendicularly into the stratum corneum. Notably, this is the most simple and direct application into skin which does not require any prior professional training.

Fig. 1.16 Comparison of Hypodermic syringe and Micro needle[111]

Many studies documented benefits of application of microneedle for vaccine administration are as enlisted [111]

1. A live attenuated Japanese encephalitis vaccine Chimerivax in non-human primates resulted in superior immunogenicity in terms of higher neutralizing antibody titers and decreased duration of viremia compared to other routes of administration.
2. A clear example of dose-sparing was observed in case of an inactivated rabies vaccine in which higher seroconversion rates were obtained with as little as $\frac{1}{4}$ dosage of the vaccine formulation as compared to intra-muscular injection.
3. Inactivated H1N1 A/PR/8/34 virus coated microneedles resulted into increased effective antibody titers, protective immunity in form of strong Th1 bias in contrast with intramuscular option.

4. Inactivated seasonal influenza virus vaccines delivery through microneedle achieved better recall and cellular immune responses, improved induction of virus-specific memory B-cells and lung viral clearance.
5. Microneedle allows administration of dry-coated vaccines which is a remarkably helpful attribute for easy dissemination of the vaccine without costly cold storage hassle. It also eliminates the risk of contamination. This allowed to circumvent the issue of aggregation of the antigen particles by use of Trehalose in the dry-coating mixture of inactivated Influenza virus.
6. Viral Like Particle (VLP) capsid of Human Papilloma virus induced augmented neutralizing antibody titres than intramuscular immunization.
7. H1 and H5 VLP vaccines stimulated Langerhan cells, which resulted in cell morphology modulation and resulted in curtailed cell number in epidermal sheets upon administration via microneedle.
8. Microneedles are also successfully used in bacterial vaccine administrations like, tetanus, diphtheria, tuberculosis, botulism, plague and ANTHRAX.

Micro needles are reported to generate comparable immune response to intramuscular vaccination but with improved specific mucosal immunity and more balanced type1 and 2 helper T cell responses.

The i.d. route of vaccine administration presents many positive outcomes. Therefore we tried to check this option and evaluated the practical encapsulation of our Consensus EDIII protein in the layer-by-layer assembly of Microneedle. The success of this effort is described in the following chapters.

Part I AIMS And OBJECTIVES

Broad aim of this project is to analyze interaction of a therapeutic cross-protective antibody against flavivirus and its antigen at atomic level. This knowledge can be further used to engineer a better version of the antibody with perspective of passive immunization and/or developing a candidate vaccine which can elicit a cross-neutralizing immune response that can provide cross-protection over the flaviviruses or at least over four serotypes of Dengue. While pursuing these broader aims we also tried to obtain crystal structure of a consensus Dengue virus EDIII engineered to represent all four serotypes physico-chemically at tertiary fold.

Consensus ED III

Zidane et al [112] reported Consensus ED III, which is designed to have a consensus epitope of all the four serotypes with optimum similarity of the sequence and overall fold similar[113] to all the four Dengue Serotypes.

This Consensus ED III was characterized by the author [112] and concluded to be thermostable. Because of its high level similarity with all the four serotypes and its thermo-stability, it is very attractive candidate vaccine.

Most desired objective was to obtain 3D crystal structure of the complex of Consensus ED III with Antibody m366.6 as discussed ahead. Consequently, the subsidiary objectives were to express and purify the Consensus ED III in *E.coli*. and test for its ability as a vaccine.

Consensus ED III as vaccine candidate

With its potential as candidate vaccine it was planned to check its affinity towards the currently well-known cross-neutralizing antibody Ab 513 and m366.6 by SPR.

Being an ideal summarized epitope from all four serotypes, we tried to understand its molecular structure for better understanding and for better scope to improve it further as a candidate vaccine.

We also planned to check its ability to get encapsulated on Micro-needles and to release from Micro-needles in physiological conditions.

Crystal Structure of Consensus EDIII:

This Consensus EDIII is engineered to have the overall structure fold to be very close to all the EDIIIs of the four serotypes. It would be interesting to know its 3D Structure rather than speculating the similarity of the fold. Therefore we aimed to obtain its 3D crystal structure and analyze the similarity of the structure in perspective of natural immune response. We also analyzed and compared the structural similarity of the surface epitopes from various known structures of complexes of EDIII and potentially cross-protective antibodies.

m366.6 [114]

One of the interaction partner i.e the antibody was contributed by our collaborator Dr. Dimitrov, NCI in form of purified scFv named m366.6. The project revolved around this antibody, because it was human origin antibody generated from naïve Yeast Display library after B-cell panning from Dengue patient, making it most ideal for passive immunization.

This was characterized at Dr. Dimitrov's laboratory and proved to be highly cross-protective across all four genotypes and successfully neutralized all of them.

The experimental aim at our end of collaboration was to obtain 3D crystal structure of the complex of m366.6 with its antigen Envelope Domain III(ED III) of Dengue virus serotypes.

Zika virus Neutralization by m366.6

With current outbreaks of Zika and related complications word wide, we also aimed to test whether m366.6 can neutralize Zika Virus.

Part I MATERIALS AND METHODS

Materials and Methods

This chapter describes the various constructs used during the study and the experimental procedures followed to obtain pure refolded proteins. The methods to establish functional activity of the refolded proteins are also described.

2.1 Over-expression of ED III

The constructs were obtained from our laboratory [1]. The nature of the constructs is detailed in table 2.1

Table 2.1 Details of domain III expression conditions

Construct (Amino acids)	Plasmid	Tag	Expression Host/ Cleavage protease	Expression condition	Antibiotic
D1ED3 291-394	pET16b	N-terminal His ₁₀	BL-21 (DE3)/ Factor Xa	1mM IPTG at 37°C/3 hours	Ampicillin
D2Ted3 291-395	pET16b	N-terminal His ₁₀	BL-21 (DE3)/ Factor Xa	1mM IPTG at 37°C/3 hours	Ampicillin
D2Ned3 291-395	pET16b	N-terminal His ₁₀	BL-21 (DE3)/ Factor Xa	1mM IPTG at 37°C/3 hours	Ampicillin
D3Ed3 291-395	pET16b	N-terminal His ₁₀	BL-21 (DE3)/ Factor Xa	1mM IPTG at 37°C/3 hours	Ampicillin
D4Ed3 291-394	pET16b	N-terminal His ₁₀	BL-21 (DE3)/ Factor Xa	1mM IPTG at 37°C/3 hours	Ampicillin
WNEd3 299-401	pET16b	N-terminal His ₁₀	BL-21 (DE3)/ Factor Xa	1mM IPTG at 37°C/3 hours	Ampicillin

25 ml of LB broth (Novagen) with 100 µg/ml Ampicillin (USBiological) was inoculated with a single colony of BL21 (DE3) E.coli transformant cells and was incubated at 37°C overnight (~16 hours) as a pre-culture. The following day, this

pre-culture was inoculated in one Liter of LB broth with 100 μ g/ml of Ampicillin and was incubated at 37⁰C in Orbital shaker at speed 200 rpm until the OD₆₀₀ reached 0.5-0.7.

An aliquot of 1 ml was retained as an uninduced control. Then IPTG (isopropyl- β -D-1-thiogalactopyranoside) was added to final concentration of 1mM and incubation was continued at 37⁰C for further 3 hours with constant shaking at 200 rpm.

After Induction the bacterial suspension was centrifuged at 6000 rpm for 15 min at 4⁰C in Beckmann, Avanti J series. The Pellet was washed with 50 ml of Phosphate Buffered Saline twice. These washed cell pellets were stored at ~80⁰C. OD₆₀₀ matched samples taken before induction and after induction were centrifuged to pellet the cells and these cells were lysed by heating at 90⁰C with SDS-PAGE loading dye and were analyzed on 18% SDS-PAGE for putative over-expression.

2.2 Lysis of Cells

The frozen cell pellets were thawed on ice for 15 mins. One Liter cell pellet was re-suspended in 30 ml of ice-cold Lysis buffer 50 mM Tris, 1% Triton-x100, 100 mM NaCl, 1 mM DTT, 4.5 μ l protease inhibitor (Calbiochem), pH 8.2. This re-suspension of cells was lysed in French Pressure cell, Panda, at 500 bar pressure for 10 mins at 4⁰C. A small aliquot of non-lysed cell suspension was kept to visually compare with the lysate. Usually, optimum lysis is evident by a faint orange tinge and reduction in viscosity. A sample was taken for analysis on 18% SDS-PAGE from this lysate. The lysate was subjected to high-speed centrifugation at 16000 rpm for 30 mins at 4⁰C on Beckmann, Avanti J series. Supernatant was separated from the pellet having the inclusion body.

This inclusion body pellet was washed with ice-cold Wash buffer 1 (50 mM Tris, 0.5% Triton-x100, 100 mM NaCl, 1 mM DTT, pH.8.2) if the pellet looked brown as the pure inclusion body is wconditione. If the pellet looked wconditione, then it was washed with ice-cold wash buffer 2 (50 mM Tris, 100 mM NaCl, and 1 mM DTT pH.8.2). Then the pellets were washed twice to remove any residual Triton-

x100 (Fig.2.1). These washing steps were performed by homogenizing the pellet in a homogenizer in the respective wash buffer and then centrifuging them at 16000 rpm, for 15 mins at 4⁰C on Beckman, Avanti J series. After washing, the inclusion bodies were weighed and stored at -80⁰C till further use.

Fig. 2.1 Expression Profile for Inclusion body of Dengue Serotype 1 on 18% SDS-PAGE:

Lane 1: Before induction 1ml cell pellet; **2:** After induction 1 ml cell pellet; **3:** Lysate supernatant; **4:** Pellet from lysate; **5:** 1% TritonX-100 Wash; **6:** 0.5% TritonX-100 wash; **7:** without TritonX-100 wash 1, **8:** without TritonX-100 wash 2; **9:** Without TritonX-100 wash 3

2.3 Re-solubilizing the Inclusion Body

Approximately 1.5 g of Inclusion body was obtained from ~2.5 g of cell pellet from 1 Liter LB broth. The inclusion body was transferred to a sterile 50 ml beaker, with stirring. To this 10 ml of ice-cold Re-solubilizing buffer (8M Urea, 50mM Tris, pH 8.2, 1mM DTT, Filtered through 0.2µm pore-size filter) was added slowly (~1ml/min rate). The inclusion body was stirred overnight at 40C to achieve full solubilization of inclusion bodies. Next day, this inclusion body solution was centrifuged at 16000 rpm on Beckman Avanti J series for 30 mins at 4⁰C to separate the insoluble fraction from the supernatant. A sample from this supernatant was taken to check on 18% SDS-PAGE.

2.4 Refolding of EDIII

Aggregation because of exposed hydrophobic patches of denatured protein is a major hurdle in refolding. Therefore, the protein concentration was maintained at 0.2mg/ml during refolding. Refolding was carried out at 4⁰C because hydrophobic interaction is decreased at low temperatures [4]. pH was maintained at 8.2-8.5 because, it is away from PI of all the EDIIIs, in order to keep it in solution. Moreover, it is known that:

1. Near native structures are protected in inclusion bodies solubilized at alkaline pH[115],
2. Most proteins are able to refold at this pH [4], and
3. Arginine at or below neutral pH is ionic and can interfere with refolding of some proteins [116].

We adopted the strategy from [68] to maintain a proper redox buffer recipe primarily because it worked with similar proteins and secondly because it is inexpensive than the conventional redox agents.

Concentration of the re-solubilized protein in (8M Urea, 50mM Tris, 100mM NaCl, 1mM DTT pH 8.2) was measured using Nanodrop in mg/ml. Refolding of this resolubilized protein was achieved by two methods: Rapid Dilution for DENV2 TSV02 ED III and Dialysis for WNV ED III and DENV1 ED III.

Table 2.2 Summary of biochemical characteristics of recombinant domain IIIs(ED III) used in this study

Ed3	DEN1(291-395)	DEN2(291-395)	WEST NILE(299-401)	Consensus (297-408)
Mass (kDa)	14370	14669	13891	12.6
Pi	7.2	7.3	7.31	6.7

2.4.1 Rapid dilution

The denatured protein in 10 ml denaturant was introduced at 1ml/min flow-rate into chilled and stirring (500 rpm) 500 ml Refolding buffer (0.5 M Arginine,100 mM

Tris, 130 mM NaCl, 6.6 mM Beta- Mercaptoethanol, 3.7 mM Cystamine, pH 8.2) in cold room (4°C) for 48 hours. The volume of refolding buffer was adjusted such that the protein concentration on complete deposition was 0.2mg/ml.

After 48 hours the 500 ml protein solution was centrifuged in 50ml sterile falcon tubes at 4500 rpm in a cold centrifuge for 15 minutes to eliminate insoluble precipitate. A volume of 2 ml of buffer equilibrated Ni-NTA (Qiagen) slurry was added to the soluble fraction and incubated overnight at about 200 rpm in a cold room with constant stirring. Next day the soluble protein solution with the Ni-NTA slurry was poured in to a polypropylene column. Flow-through was analyzed to ensure, no ED III was lost. The resin with the EDIII was washed with 5 mM Imidazole, 0.5 M Arginine, 100 mM Tris, 150 mM NaCl, 6.6 mM Beta-Mercaptoethanol, and pH 8.2 to eliminate the weakly bound contaminants. The 10-His tagged bound protein was then eluted over ice-cold 50-500 mM gradient of Imidazole, 0.5 M Arginine, 100 mM Tris, 150 mM NaCl, and 6.6mM Beta-Mercaptoethanol, pH 8.2 in 10 ml fractions. These fractions were then analyzed on 18% SDS-PAGE. The pure EDIII protein fractions were pooled and the protein was concentrated using Vivaspin centricons with 10 kDa cut off by gentle centrifugation at 2000 rpm at 4°C to 0.5 to 1 ml volume.

2.4.2 Dialysis

The denatured protein in 10ml denaturant was diluted to 0.2 mg/ml of the protein concentration in the Re-solubilizing buffer (~300 ml). This diluted protein is then dialysed against five liters of chilled Refolding buffer (0.5M Arginine, 100mM Tris, 150mM NaCl, 6.6mM Beta-Mercaptoethanol, 3.7mM Cystamine, pH 8.2) overnight (24 hours) at 4°C. After 24 hours the dialysis bag with the protein is transferred to fresh 3 liters of chilled refolding buffer and the dialysis was continued for further 12-16 hours. Then the protein is dialysed against fresh 3 liters of chilled refolding buffer for another 12 hours. Thus the protein in 8M Urea (~300ml) was dialyzed against a total of 11 liters of refolding buffer. At this stage concentration of Urea in the protein solution was expected to be ~0.005M theoretically. This protein solution in the dialysis bag was then centrifuged at 16000 rpm for 15 mins

at 4°C to separate the soluble fraction from the precipitate. To this, 5 ml of refolding buffer equilibrated Ni-NTA resin was added and let it stir gently (~ 200 rpm) at 4°C overnight (~16 hours). Next day the protein was purified on Ni-NTA column by IMAC. A 10µl aliquot of the protein was kept for SDS-PAGE. This protein bound to the resin was washed with 5 column volumes of ice-cold 0.5 M Arginine, 100 mM Tris, 150mM NaCl, 5mM imidazole, 6.6mM Beta-Mercaptoethanol, pH 8.2 to get rid of weakly bound contaminants. The 10-His tagged bound protein was then eluted over ice-cold 50-500 mM gradient of Imidazole, 100 mM Tris, pH 8.2, 150 mM NaCl, and 6.6 mM Beta-Mercaptoethanol in 10 ml fractions. These fractions were then analyzed on 18% SDS-PAGE.

The pure ED III protein fractions were pooled and the protein is concentrated using Vivaspin centricons with 10 kDa cut off by gentle centrifugation at 2000 rpm at 4°C to 0.5ml volume.

As Arginine was found to create problems in IMAC we decided to remove it from the protocol and tried to refold West Nile Virus ED III without Arginine with the rest protocol unchanged. However these attempts failed although strategy of dialysis did show some promise. Invariably, the yield remained very low.

Arginine seemed to play very crucial role in refolding EDIII. Arginine side-chain with guanidine group interacts with aromatic groups like Tryptophan which are hydrophobic and thus suppress aggregation during refolding [116]. Success of refolding also depends on flexible reshuffling of disulfide bonds and formation of naturally correct disulfide bond formation. Here also Arginine was reported to play favorable role in refolding proteins by facilitating formation of correct disulfide bonds.[117]. Urea and Arginine in co-operation help to achieve refolded protein [118]. Therefore we re-incorporated Arginine in refolding recipe.

2.5 Gel Filtration Chromatography

The concentrated IMAC purified protein was centrifuged at a high-speed 14000rpm for 10 mins in a cold-centrifuge to remove any precipitate or aggregate which may clog the FPLC column. The concentration was measured on Nanodrop at this stage.

The protein was injected on a pre-equilibrated and calibrated Superdex-75 FPLC column (Ge Healthcare). FPLC purification was carried out at Flow rate of 0.5 ml/min, at 4°C. The buffer used was 100 mM Tris pH 8.2, 150mM NaCl, 1mM DTT. 1ml fractions were collected after the void-volume of the column. The fractions corresponding to the symmetric peak at expected volume were visualized on 18% SDS-PAGE (Fig.2.2). The pure homogeneous fractions were pooled and concentrated on Vivaspin centricon with 10 kDa cut-off at 2000 rpm at 4°C. 10% glycerol was added to this protein and subsequently aliquoted in 20 µl fractions and was flash-frozen in Liquid Nitrogen and stored at -80°C until use. (Please refer to Appendix IV for the Calibration of the column with standard molecular weight markers.)

Fig. 2.2 Ni-Affinity and Size Exclusion Profile of ED III of DEN 2 TSV 01, WNV and DENV 1

Left panel displays the Ni-Affinity purification profile on 18%SDS-PAGE reducing gels. M: Protein marker ladder, Rf: Refolded Protein before purification, FT: Flow through from column, E1-E5 elutions with 100mM Imidazole to 500mM Imidazole respectively. The corresponding Gel filtration profiles on Superdex 75 16/60 are arranged in the right panel. A. The Gel Filtration purification peaks and B. The pure fractions on 18% SDS-PAGE reducing gel.

2.6 Complex Formation and Detection

ED III and m366.6 scFv were mixed together at a molar ratio at 1.5:1. The mixture was mixed gently with a pipette and incubated at room temperature for 1 hour. Complex formation was checked on an Analytical Superdex- 75 column using 4 μ l injection. EDIII and m366.6 scFv were injected separately as controls. The analytical FPLC was run at flow-rate: 0.3 ml/min, at 4°C. The gel filtration buffer used was 100 mM Tris, pH 8.2, 150 mM NaCl, 1mM DTT.

These conditions were used for large-scale purification of the complex on preparative Superdex-75 column. On preparative Superdex-75 pre-equilibrated and calibrated column 0.5 ml of the complex was injected and 1ml fractions were collected after the void volume of the column. The fractions having pure homogeneous complex were pooled and concentrated in Vivaspin centricons with gentle (2000 rpm) centrifugation at 4°C until maximum possible concentration.

2.7 Crystallization Trials for Dengue and West Nile EDIIIs in Complex with scFv m366.6:

The crystallization trials with DEN 2 TSV01 complex with m366.6 at concentration 5mg/ml showed clear drops with PEG Ion and PEG Rx screens kits from Hampton Research. Therefore the complex of DEN1 and m366.6 at concentration 18 mg/ml was subjected to pre-crystallization screen test (PCT) kit by Hampton research. The results were promising hence we went ahead for crystallization trials. We set up total 672 screen trials for crystallization through the kits: Crystal Screen, Index, SaltRx, Peg Ion and Peg Rx from Hapton Research. In case of West Nile ED III the complex purified was very less in amount; therefore we checked only 192 conditions through the screens Peg Ion and Peg Rx from Hampton research.

In case of DENV4 ED III, the purified protein was obtained from *Visterra* and we used the approach from [68] to form the complex by mixing EDIII with scFv m366.6 in ratio 1.5: 1 respectively and incubating overnight at 4°C and then concentrating in Vivaspin concentrator with 30 kDa cut-off to retain the 42 kDa complex without the individual partner proteins. This complex was then used to set

trays Peg Ion and Peg Rx and also for the conditions used in various publications (Table: 2.3).

2.8 Consensus Envelope Domain III

2.8.1 Cloning of Consensus Domain III in pET 22b - The consensus domain III sequence was obtained from *Zidane et al* [112]

We then ordered this protein sequence with Nco1 and Xho1 at N-terminal and C-terminal end respectively from GeneScript and then sub-cloned into periplasmic expression vector pET22b (Kind gift from Dr. Shawn from IMCB).

Target sequence of the consensus domain III flanking with the desired sequence was obtained by PCR using forward Primer 5'CCGGCGATGGCCATGGCCATGGGTATGTCTTAC3' and Reverse primer 5'GGTGGTGGTGCTCGAGTTTACCGATAGAAGAACC3'. Then it was ligated to the Nco1 and Xho1 linearized pET22b vector. The ligation mixture was transformed into Top10 *E.coli*. Colony with the desired clone was confirmed by colony PCR followed by direct sequencing of the construct.

2.8.2 Expression and Purification of soluble Consensus domain III

2.8.2.1 Periplasmic Expression

pET22b with consensus sequence was transformed into *E.coli* BL21 DE3 strain. Transformed single colony was then inoculated in Super broth (SB) with 5mg/ml of Glucose and grown overnight at 37⁰C for pre-culture. Next day this pre-culture was inoculated in one liter medium of Super broth with 5mg/ml at 30⁰C. This culture was then harvested by centrifugation, and resuspended in fresh SBG5 (broth with 5 gm Glucose) medium to obtain an initial absorbance OD₆₀₀ 1.25. They were grown at 22⁰C until OD₆₀₀ 2.5 and then induced during 2 h with 0.2 mM IPTG for the expression of the recombinant consensus domain. After 2 hours of induction the cells were harvested by centrifugation and the cell pellets were frozen at -80⁰C.

2.8.2.2 Lysis of the Harvested Cell Pellet

The frozen cell pellets were thawed on ice for 15 mins. One Liter cell pellet of 5gms wet weight was re-suspended in 50 ml of ice-cold Lysis buffer 50 mM Tris-Cl pH 8.1, 50mM NaCl, 5mM β -mercaptoethanol, 4.5 μ l protease inhibitor (Calbiochem). This re-suspension of cells was lysed in French Pressure cell, Panda, at 500 bar pressure for 10 mins at 4°C.

Table 2.3 Published Crystallization Conditions of ED III- Antibody Complexes

	REF [69]	REF [68]	REF [119]	REF [88]
DENV1	24% PEG 4K, 0.1M MES pH 6.5	0.2M K-Thiocyanate 20% w/v PEG 3350		
DENV2	30% Ethanol, 12% PEG 6K, 0.1M Na Acetate			
DENV3	20% PEG 8K, 0.1M HEPES, pH 7.5	0.2M dihydrogen phosphate, 20% w/v PEG 3350		
DENV4	1.7M Ammonium Sulfate, 12.5% Glycerol, 0.1M Tris pH 8.5	0.2M K-Thiocyanate 20% w/v PEG 3350		(1)100 mM sodium acetate trihydrate pH 6.5 and 30% (w/v) polyethylene glycol 3350 (2) 100 mM sodium acetate trihydrate pH 4.5 using 30% (w/v) polyethylene glycol 3350

WNV			19% PEG 4K, 0.1M HEPES pH 8.5	
-----	--	--	--	--

2.8.3 Two-Step Purification of the Consensus Protein

Cell lysate was clarified by centrifuging at 18000 rpm for 30 minutes and subsequent syringe filtration. This clarified lysate was applied to 1ml of equilibrated Ni-resin from Quiagen. After 3 hours the Ni-resin was separated from the lysate and was washed 20 ml of with Lysis buffer and the protein was eluted on gradient of Imidazole concentration in lysis buffer. The purified fractions of the protein were further purified by Size-exclusion chromatography.

The concentrated IMAC purified protein was centrifuged at a high-speed 14000rpm for 10 mins in a cold-centrifuge to remove any precipitate or aggregate which may clog the FPLC column. The concentration was measured on Nanodrop at this stage. The protein was injected on a pre-equilibrated and calibrated Superdex-75 FPLC column (Ge Healthcare). FPLC purification was carried out at Flow rate of 0.5 ml/min, at 4°C. The buffer used was 50 mM Tris-Cl pH 8.2, 50mM NaCl, 1mM DTT. 1ml fractions were collected after the void-volume of the column. The fractions corresponding to the symmetric peak at expected volume were visualized on 18% SDS-PAGE. The pure homogeneous fractions were pooled and concentrated on Vivaspinn centricon with 10 kDa cut-off at 2000 rpm at 40°C. 10% glycerol was added to this protein and subsequently aliquoted in 200 µl fractions and was flash-frozen in Liquid Nitrogen and stored at -80°C until use.

2.8.4 Crystallization of Consensus EDIII:

Purified consensus envelope domain III was concentrated to 10 mg/ml in a volume of about 100 µl. A total of 198 crystallization conditions (Molecular Dimensions kits: JCSG *plus* and Morpheus) were screened at 20°C by using a robotic Phoenix system (Art-Robbins), using a protein-to-precipitant volume ratio of 1:1 and drops of 0.2 µl. Initial needle-shaped crystals were obtained after 24 hours in a buffer

containing 0.2 M Sodium chloride, 0.1 M Sodium citrate (pH 4.2), and 20% (wt/vol) PEG 8000.

This condition was further optimized to 0.2 M Sodium chloride, 0.2 M Sodium citrate (pH 4.2), and 25% (wt/vol) PEG 8000 with 7 mg/ml protein concentration. The crystals were still very small.

2.8.5 Micro-seeding of Consensus EDIII protein crystal

These crystals were then crushed in the buffer 0.2 M Sodium chloride, 0.2 M Sodium citrate (pH 4.2), and 25% (wt/vol) PEG 8000 to derive nuclei seeds and were seeded using Mosquito in ratio 1:1:1 of protein, seeds and the precipitant using the JCSG *plus* and Morpheus. Big plate shaped crystals were obtained in JCSG *plus* G12 condition 3.0 M Sodium chloride, 0.1 M BIS-Tris 5.5.

These crystals were further optimized to the final diffracted crystals by hanging drop optimization trials by keeping drop ratios as in seeding but overlaying 100 µl Mineral oil on 500 µl of mother liquor 3.0 M Sodium chloride, 0.1 M BIS-Tris 5.5 for 3 days.

2.8.6 Consensus EDIII Crystal Diffraction

The crystal was diffracted at MX2 Australian Synchrotron with X-ray wavelength 0.953729 Å. XDS[120] software was used for the determination of space group i.e P2. Then the collection of data was carried out using 180° C collection wedge.

Scaling and integration of the data from all collected data frames was performed on XDS software. The processed electron density was used to generate an initial model using Molecular replacement on BALBES [121] package from CCP4 online suite. Solvent content was determined using Mathews Coefficient from CCP4i package.

This model was then refined first with Non-crystallographic constraints on Refmac5 from CCP4i[122] and then performed series of refinement cycles using various constraints including low resolution refinement with jelly body constraints, TLS constraints and then was finally curated on coot[123]. The molecular co-ordinates were then visualized by Chimera software [5]. The physico-chemical parameters qualifying the structure were checked using Molprobit[124], ProSa[125], and pdbsum [126] softwares.

2.8.7 Complex Formation and Detection

The process mentioned in section 2.6 was followed for complex formation and detection.

2.8.8 Crystallization Trials

The Consensus ED III complex with m366.6 at concentration 15mg/ml was used to set about 700 (96x7) Crystallization trials with various crystal screens like Index, SaltRx, Crystal Screen, especially, screens PEG Ion and PEG Rx screens kits from Hampton Research including the published conditions mentioned in Table 2.3.

2.9 Surface Plasmon Resonance

(Collaboration with Prof. Susana Geifman Shochat. Equal contribution by Jedael from Prof. Susana's lab and author of the thesis. Guidance by Jedael is highly appreciated.)

2.9.1 Interaction Kinetics of Consensus EDIII with mAb513

The binding affinities and kinetics of interaction of mAb 513 to Consensus domain III were assessed by SPR using a Biacore 3000 (GE Healthcare) instrument.

Consensus domain III was immobilized on carboxymethylated dextran CM5 sensor chips by amine coupling as per manufacturer's instructions. The protein was captured on individual flow-cell at a flow rate of 10µl/min to 20 RUs. One of the flow-cell was used as reference cell with the same amine coupling treatment without consensus domain III. 513 mAb was diluted to 7 different concentrations 100nM to 1.625nM at dilution factor of 2 in PBS with 0.005% P20 as running buffer. Each concentration was run over the surfaces of the reference flow cell and test flow cell at 30 µl/min. A dissociation phase was analysed for 4000 seconds. Surface was regenerated by injecting 50mM HCl for 60 seconds at 30 µl/min flow rate. Kinetic parameters were determined using the kinetic function of the BIAevaluation software (Biacore) with reference subtraction. Kinetic parameters k_a (association rate constant), k_d (dissociation rate constant) were determined using Bivalent binding model.

2.9.2 Interaction Kinetics of Consensus EDIII (EDIIIC) with scFv m366.6:

An exactly similar method was applied for SPR analysis of EDIIIC interaction with scFv m366.6 as the one with Ab 513 described above. The only difference was that instead of Bivalent binding model, Languimir 1:1 model was applied to determine the k_a and k_d .

2.10 Plaque reduction neutralization (50%) test (PRNT50%) of Ab m366.6 for Zika

(in Collaboration with Dr. Shee Mei Lok , Duke-NUS, Test was performed by Jiaqi)

Day-0

BHK21 cells were seeded into 24 well plates at 2×10^5 cells per well in 500 μ l volume and the plates were incubated overnight.

Day-1

➤ Antibody Preparation

The antibody was diluted with a dilution factor of 10.

Starting from 100 μ g/ml to 0.01 μ g/ml in Phosphate Buffered Saline (PBS).

Total volume was adjusted to 10 ml.

➤ Virus Preparation

Diluted the purified Zika virus in 2% FBS-RPMI on ice to get a concentration of 300 pfu/ml.

➤ Co-incubation

Mixed 310 μ l of the different dilutions of antibody with equal volume of fixed concentrations of virus (310 μ l + 310 μ l= 620 μ l).

➤ Infection of BHK21 Cells

The mixture was incubated at 4°C for 2 hrs.

Added 200 μ l of each virus-Ab mixtures to triplicate wells of BHK21 monolayer

in 24 well plates and incubated at 37° C for 1 hr.

➤ CMC overlay

Removed the mixture from each well and added 0.5 ml of the various concentrations of CMC-overlay per well.

Incubated the plates at 37° C with 5% CO₂ for 4-6 days

Day-4

➤ Fixing the Cells

The cells were fixed at 94-96 hr after incubation, and stained the plates with 1% Crystal violet to count the plaque numbers.

2.11 Micro Needle Assembly

This work was in collaboration with Dr. Paula Hamond SMART lab, the work unless otherwise specified is done in this lab by this group. The author of this thesis has contributed purified Consensus EDIII to the amount of 100mgs in total for the standardizations and final experiments.

2.11.1 Encapsulation of EDIII Consensus protein onto the Microneedles

(In collaboration with Dr. Paula Hamond SMART, test was performed by Shareef, Author of thesis contributed the Consensus EDIII purified protein)

1. Microneedles are oxygen plasma treated for 5 minutes.
2. Spray coated with 4wt% of PNMP (UV-activated pH sensitive layer)
3. UV irradiated for 15 mins at 254nm.
4. (PS/PSS) 20 bilayers were coated using Carl Zeiss slide stainer.
 PS- Protamine Sulphate – 1mg/ml in PBS pH 5.2
 PSS – Polystyrene 4 Sulfonate – 1mg/ml in PBS pH 5.2
5. (Poly-2/PAA/EDIII/PAA) 30 tetralayers were coated using Nanostrata coating machine.
 Poly-2 – Polyamino acid esters – 1 mg/ml in 100mM NaAce buffer pH 5.2
 PAA – Polyacrylic acid – 1mg/ml in 100mM NaAce buffer pH 5.2
 EDIII-C- 40ug/ml in PBS pH 5.2
6. Dried overnight before starting release study in PBS pH 7.4
7. Microneedles were released in 500ul of PBS pH 7.4 in 24 well plate.

8. Samples were collected at different time points (3 hours, 6 hours, 22 hours, 28 and 96 hours) and were tested for presence of Consensus domain III released in the solution by ELISA.

2.11.2 Analysis of release kinetics of consensus ED III from the Microneedles:

(Release kinetics was performed by Divakara and Shareef as part of collaboration, Author of thesis contributed the purified Consensus EDIII)

The microneedles loaded with the multilayered Consensus EDIII were immersed in 200 µl of PBS pH 7.4 in a 24 well culture plate well. Then were incubated at 37 °C for one hour.

The suspension solution was then clarified by centrifuging at high speed at 4°C. The supernatant was used as test sample.

The ELISA protocol was used similar to the Direct ELISA using primary antibody protocol - Abcam (Experimental work contribution by Divakara and Sharif as part of collaboration, the author of thesis contributed Consensus EDIII)

(<http://www.abcam.com/ps/pdf/protocols/direct%20elisa%20protocol.pdf>)

The primary antibody we used is Vis 513 from Visterra at concentration of 2µg/ml. The secondary antibody was Goat Anti-Human IgG H&L (HRP) pre-adsorbed (ab7153) from Abcam.

2.11.3 Detection of Consensus EDIII in Mouse Ear:

2.11.3.1 The ear Consensus EDIII preparation:

3 Balb/c Mice were used for the experiment.

Left ear of each mouse was treated with an empty microneedle without the Consensus EDIII as Negative control and the right ear (Test) was treated with Consensus EDIII loaded microneedle. (This work in Animal models was done by Michelle Elizabeth Turvey, the rest was done by author of the thesis.)

2.11.3.2 Protocol:

The ears were dissected on ice. Snap frozen in liquid Nitrogen and were used immediately for further experiment. (They can be stored in -80 °C).

Each ear was placed in a 2ml Eppendorf tube. For each approximately 5 mg piece of tissue 300 µl of tissue extraction buffer: 0.1mM Tris,pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton-x 100 with 0.1 µM PMSF as protease inhibitor. Washed the tissue with this tissue extraction buffer 3 times to remove extraneous Protein and was crushed using a tapered syringe plunger.

This tissue was further homogenized in a homogenizer in cold room by maintaining constant agitation for 2 hours.

The lysate was then centrifuged at high speed 13000 rpm at 4°C.

The supernatant was used for detection of Consensus ED III by ELISA.

2.11.3.3 The ELISA for Detection of Consensus EDIII from Mouse Ear:

1 µg/ml of Vis 513 was prepared in Coating buffer 0.1% NaHCO₃ pH 9.6 and 100 µl was applied to the ELISA Poly-L-Lysine coated plates at 4°C for overnight.

Next day decanted the vis 513 and washed with phosphate buffered saline with 0.05% Tween 20 (PBST) thrice. Blocked the wells with 100 µl of 5% Bovine Serum albumin (BSA) in phosphate buffered saline (PBS) for half an hour at room temperature. Washed thrice with PBST. 1:2, 1:4 and 1:10 dilutions of negative control ear lysate and test ear lysates were dispensed (100 µl each) in wells in duplicates. 100 µls of the undiluted lysates of both test and negative control were also applied to plates in duplicates. Incubated at 37°C for 2 hours. Washed with PBST thrice. Then 100 µls of 1:10 diluted Anti-His antibody from Sigma Alderich was applied to the wells for 1 hour at 37°C. Washed thrice with PBST. Then 100 µls of TMB- the substrate was added to develop color, retained for 2-5 minutes to obtain optimum detectable coloration. The reaction was stopped with 1N HCl. Colorimetric detection was done at 450 nms on plate reader.

Part I RESULTS AND DISCUSSION

3.1 Consensus Domain III:

3.1.1 Periplasmic Expression of Consensus EDIII in Soluble Form:

The Soluble Expression of Consensus EDIII was an important break-through in this research work. This opened various avenues, most importantly provided a promise of very good yield, a proper homogeneous protein of good quality for interaction studies and most importantly for crystallographic success. This result is described in stepwise manner as follows:

3.1.2 Sub-cloning of Consensus EDIII and Expression in periplasm of E.coli:

Consensus EDIII gene was ordered as amino acid sequence [112] (Fig.3.1 A) from GeneScript and then was sub-cloned in to periplasmic expression vector pET 22b (Fig.3.1B).

3.1.3 Purification of Consensus Domain III

Periplasmic Expression of Consensus Domain III was obtained in soluble form from periplasmic expression. Its expression was both tested and confirmed by IMAC purification on Ni-resin. It was successfully purified using the 6x His tag on Ni-resin. (Fig. 3.2)

3.1.4 Size-Exclusion Chromatography of Consensus ED III

The IMAC pure Consensus ED III was further polished by Size Exclusion and its monomeric fraction was obtained at elution volume 81 ml on Superdex 75 16/60 FPLC column (Fig.3.2).

3.1.5 Small-scale Complex formation of Consensus EDIII with m366.6

Purified Consensus EDIII was analyzed for ability to form complex with m366.6 scFv. Purified Consensus EDIII was mixed with m366.6 scFv at 1.5:1 molar ratio respectively and incubated at 4⁰C over-night. A peak shift was observed on Analytical Size- Exclusion (Fig.3.7).

Fig. 3.1 Consensus Domain III constructs alignment and Cloning

(A) Protein Sequence of Consensus Domain III Clustal Omega alignment with other EDIIIIs.

(B) Confirmation of successful sub-cloning in pET 22b of Consensus ED III received in puc57 vector by colony PCR. PC is the PCR positive control i.e the puc57 as template, NC is no template control, Test is the sub-cloned pET22b vector.

Fig. 3.2 Purification Profile of Consensus ED III

Ni Affinity: M: Protein marker ladder, B: Before induction sample, A: After induction sample, Ft: flow through, W: wash, 10:10 mM imidazole wash, E1-E5: Elutions with 100mM Imidazole to 500mM Imidazole respectively. Top-right is Size exclusion purification of the Consensus ED III with the 18% SDS-PAGE reducing gel showing the pure fractions and the one below is Gel Filtration purification of complex with shift of peak to 11ml elution volume with the 18% SDS-PAGE reducing gel: First two lanes from the complex peak showing presence of both the scFv (28 kDa) and the consensus EDIII (12.6 kDa). The third lane shows only the Consensus EDIII (12.6 kDa).

This Consensus ED III being expressed in soluble form, it definitely was homogeneous unlike the refolded EDIIIs. Consensus ED III is also more thermostable than the other ED IIIs [112] therefore it is more advantageous in forming crystals for; stability is an important parameter which favors crystal formation. However this also did not yield crystals of the complex. This indicated that the instability of scFv m366.6 visible in form of degradation was the hurdle towards crystal formation. Consensus ED III demonstrated peak shift on Size-exclusion chromatography which indicates very strong affinity as the size-exclusion chromatography involves very high dilution of the analytes. The sustained

interaction at this dilution factor indicates stronger affinity which we have analyzed by SPR. (Results are discussed ahead)

3.2 Crystallization of Consensus EDIII protein:

The first crystal condition was obtained with buffer arrayed in row C and column 1 of the JCSG plus crystal screen. The crystals appeared in 24 hours after setting the experiment. They were very tiny needles (Fig.3.3) which were incompetent in their size to obtain any diffraction.

Consensus EDIII crystal Hit, Needles, Condition: JCSG+ C1, Conc. 10 mg/ml

Fig. 3.3 The Needle Crystal of Consensus EDIII

Therefore this crystal condition was further optimized using 24 wells grid screens by screening around this condition with 3 parameters, the concentration of protein, the concentration of precipitant and the concentration of the salt for optimum sized crystal. The best condition was with Protein 5mg/ml and 24% PEG 8K. Crystals appeared in 24 hours. (Fig 3.4.)

Consensus EDIII crystal Hit optimization, Plate Crystal, Protein Conc. 5 mg/ml, PEG 8K 24%

Fig. 3.4 The Optimized Plate Crystal of Consensus EDIII

This optimization around the first crystal condition buffer condition yielded bigger plate crystal in condition with 5 mg/ml protein concentration with the rest of the buffer conditions remaining same. This crystal diffracted to 9 Å in in-house X-ray diffraction facility. This diffraction was inadequate to provide any structure but confirmed two points. Most important one was that the crystal diffracts as a protein and has a quality lattice and second we still need to improve the size of this crystal to have better diffraction pattern. We decided to opt for Micro-seeding as our approach to get better quality and large size.

3.2.1 Micro-Seeding of Consensus EDIII crystal:

Generally, the precipitant concentration is reduced to decrease the pace of lattice formation and protein concentration is also reduced to discourage nucleation allowing a slow growth of crystal around user provided micro-seed (lattice nucleus) a crushed crystal. Along with this protocol we also tried another approach, we set a high through put trial of buffer conditions using screening robot Mosquito with commercial screen JCSG plus and Morpheus screens from Molecular Dimensions with the seed (crushed crystal) and kept the protein concentration same as 5 mg/ml. This second method of micro-seeding was more promising and provided the better crystal.

After 5 days of setting the trial screen, we obtained bigger crystals of better lattice quality in two conditions:

1. This crystal is obtained in seeding condition in row D in column 9 of the JCSG Plus screen (D9 JCSG+) from Molecular Dimensions (Fig 3.5A). This forms a beautiful hexagonal big crystal. This crystal was difficult to manipulate with the nylon loop (cryo-loop) used for fishing it out from the reservoir. It was sticking to the bottom of the crystallization screen plate. Also it was having a jelly like consistency making it very fragile for further handling. Therefore we could never scoop the full crystal in the Cryo-loop. This crystal is mildly bi-refrigent under polarizing lense.
2. This crystal grew as star shaped cluster of plates in seeding condition in row G in the 12th column of JCSG+ screen (G12 JCSG+) of molecular dimentions (Fig3.5B). We broke the star shaped cluster with pipette tip and separated the individual plates in respective buffer condition on a coverslip, before further manipulations. These plates were sturdier than the hexagonal crystal mentioned earlier. These crystals were also sticking to the bottom of the screening plate like the hexagonal crystals. These plate crystals showed some moderate bi-refringence under polarizing lens.

These crystals from both these conditions were subjected to diffraction in remote data collection from MX1 Australian beamline.

The hexagonal crystal from condition D9 JCSG + did not diffract in this data collection, but the plate obtained in condition G12 JCSG+ diffracted to 6Å resolution. This proved that the plate crystal was very promising to give us desired, result oriented diffraction in Micro-focus beamlines.

Fig. 3.5 The Micro-seeding of Consensus EDIII crystal

Therefore we further optimized this condition in 24 well grids with varied concentrations of precipitant and protein concentration around the micro-seed. We also decreased the rate of evaporation of mother-liquor by layering it with paraffin oil and consequently achieved a slower nucleation. This favors the growth of crystal with a better lattice. This ultimately developed into a bigger and better plate crystal which resulted in our current diffraction of 3.2Å in MX2 Australian beamline with complete data enough to generate a 3D Crystal structure model.

3.2.2 Crystallization and Seeding Concept in Brief:

Protein crystallization is based on the phenomenon of limit of solubility of a protein in a solution. Phase separation occurs when protein concentration is increased beyond solubility limit of the dissolving solution. As and when the conditions the solution are changed the phase separation starts. Careful control of solution conditions is at the heart of protein crystallization. Some of the important solution variables are *concentration of protein, pH, ionic strength, temperature and/or concentration of additives*.

A Schematic of protein concentration v/s precipitant concentration is shown below [127].

Fig. 3.6 Protein Crystallization Phase Diagram

Undersaturated region represents area in crystal dissolution takes place. The three zones in supersaturated region define relative levels of activation energy required for nucleation of crystals.

To assist controlled growth, seeding process is followed. Micro-seeding involves pulverizing preexisting crystal into small crystalline particles through various methods such as tissue homogenization, sonication and so on. Such seeds are introduced into the protein solution with precipitant. Important consideration is to move the system into metastable zone as in this region the nucleation is occurs at extremely slow rate.

Nucleation through formation of critical nucleus within the solution is terms as Homogenous Nucleation. Using classical nucleation theory, rate of homogeneous nucleation/volume can be expressed as

$$rate = \rho * Z * j * \exp\left(-\frac{\Delta F}{kT}\right)$$

ΔF is the free energy expended in creation of critical nucleus. The negative exponential term predicts extremely low probability that a nucleus will form at the top of the energy barrier indicated by exponential term. ρ indicates number density of molecules, j indicates rate of attachment of molecules to the nucleus and Z is the Zeldovich factor that indicates what is the probability of success of a nucleus to cross the energy barrier and grow into new phase.

Assuming nucleus to be spherical, its free energy is

$$\Delta F = \left(-\frac{4\pi}{3}\right) * R^3 * \rho_n * \Delta\mu + 4 * \pi * R^2 * \gamma$$

Which is volume energy term and surface energy term. Volume term can be interpreted as the cost of formation of spherical nucleus of radius R , whereas surface term can be interpreted as interface cost. $\Delta\mu$ is the excess of chemical potential of the phase in which nucleus develops over chemical potential of the phase from which nucleus develops. γ is the tension at the interface. Setting first derivative of the above mentioned equation to zero give free energy of the Homogenous nucleation.

Introduction of seed acts as a nucleus development site in a liquid. By reducing the free energy of nucleus formed at the surface, creates favorable conditions for nucleation. This process is known as Heterogeneous nucleation.

Assuming single nucleation barrier, the rate of heterogeneous nucleation is

$$rate = \rho_I * Z * j * \exp\left(-\frac{\Delta F^*}{kT}\right)$$

Here ΔF^* is energy hump that should be overcome for successful progress of heterogeneous nucleation and ρ_I is density of heterogeneous nucleation sites. To establish relation with homogeneous nucleation an assumption of smooth infinite plane is presumed.

$$\Delta F_{Hetero}^* = \Delta F_{Homo}^* * f(\theta)$$

θ is the angle surface and interface make at contact between nucleus and solution
[128]

3.3 Crystal Structure of Consensus ED III

3.3.1 Brief Introduction to Protein crystallization and associated terms

A protein crystal is an ordered 3D arrangement of protein molecules which periodic and repetitive. Thermodynamic requirement of reaching to lowest free-energy state is the governing principle of such arrangement. However, it is not always possible to reach such stage due to energy barriers which restrict paths to such regular arrangements.

The understanding of crystal can be better achieved by concept of *Lattice* and *Unit Cell*. A lattice is an imaginary construct wherein the points constructing lattice have fixed spatial relationship with atoms of the crystal.

The structure can be constructed by imagining three sets of planes wherein planes in each set are parallel and with equal spacing. Intersection of such planes in space creates set of parallelepiped cells. As each cell will have identical surroundings, any cell can be designated as unit cell.

Size and shape of a unit cell can be defined by 3 dimensional axes system and angles between these three axes. These six parameters are known as *lattice constants* or *lattice parameters* of the unit cell as shown below.

Various combinations of axial lengths and angles between them produces different unit cells and point lattices. However, in combination with symmetry elements

(discussed later) there exist only 7 crystal systems and **Fourteen Bravais** lattices. The lattice symbol describes type of cells namely P – primitive and I, F and C – non-primitive. The primitive nature underscores the fact that in every unit cell there exists only one lattice point while non-primitive cells contain more than one lattice point [129].

The below mentioned simple mathematical equation gives no. of lattice points per unit cell.

No. of Lattice Points per Unit cell

$$\begin{aligned}
 &= \text{No. of lattice points Interior to cell} \\
 &+ \frac{\text{No. of lattice points on the faces of a cell}}{2} \\
 &+ \frac{\text{No. of lattice points on the corners of a cell}}{8}
 \end{aligned}$$

3.3.2 Symmetry [129]

Symmetry refers to arrangement of components that arranged in a manner that by performing specific operations, the system can be in coincidence with itself.

The four macroscopic symmetry operations are

- Reflection – When an either half of a body is reflected in a plane will be in coincidence with its other half.
- Rotation – Whenever a rotation of $360^\circ/n$ brings a body in self-coincidence, it is said that the body has n-fold symmetry. E.g. a cube has two fold rotational symmetry about the plane passing through mid-points of opposite edges. A body can have only following rotation axes - 1- or 2- or 3- or 4- or 6.
- Inversion – inversion refers arrangement of points equidistantly located from center on a line through the center. In other words, inverting a body about itself will bring in self-coincidence. For example, center of a cube is its inversion center.

- Rotation-inversion – This refers to activity of rotation by $360^\circ/n$ and followed by inversion to bring about self-coincidence.

3.3.3 Lattice directions:

Aforementioned discussion on crystal structure and unit cell leads to conclude that any point in the space can be described using three-dimensional axis co-ordinates or their variants. To assess direction of a line, another line parallel to it is drawn from origin and is allowed to pass through any point in space. The co-ordinates of that point indicate the direction. These co-ordinates are written as $[xyz]$. The values of x, y and z may be integral or non-integral. Hence, they are always converted to smallest integer form. As existence of symmetry creates a family of directions which are depicted as $\langle xyz \rangle$.

Similar logic is applied while describing crystal planes. Co-ordinates of the points at which a plane intersects the axes define the indices of the plane. To overcome the difficulty of infinite co-ordinates due to parallel orientation to an axis, a scheme is devised by which the intercepts which the plane makes with crystallographic axes are inverted. Thus, if a plane makes intercepts as $1/2$, $1/3$ and 0, it runs parallel to z-axis; the miller indices are $[2\ 3\ 0]$ when converted to integers.

For hexagonal system, the miller indices are modified to include fourth axis in base plane. i.e. hexagonal system is expressed using three axes $\mathbf{a}_1, \mathbf{a}_2, \mathbf{a}_3$ in base plane each at 120° and fourth $\mathbf{c}$ vertical to the base plane. The $\mathbf{a}_3$ is related to $\mathbf{a}_1$ and $\mathbf{a}_2$ by relation

$$x + y = -i$$

Thus for hexagonal system the indices are depicted as $[x\ y\ \bar{i}\ z]$.

In the light of above discussion, consider the consensus protein whose crystallization and crystal structure determination is part of the major aim of this research. XDS software® used in MX2 Australian Synchrotron gave three axes length as $a = 56.00^\circ\text{A}$, $b = 58.85^\circ\text{A}$ and $c = 112.01^\circ\text{A}$ and angle as $\alpha = 90^\circ$, $\beta = 98^\circ 21'$ and $\gamma = 90^\circ$. Mapping this information on aforementioned crystal systems, it is

clear that the structure is Monoclinic ($a \neq b \neq c$, $\alpha=\gamma=90^\circ \neq \beta$). Given the information even volume of the unit cell can be calculated as

$$V = abc \sin \beta = 365227.06 \text{ \AA}^3$$

Above discussed symmetry elements help clarify crystal system under consideration. A point group is a group of symmetry elements that when applied on unit cell allows to develop three dimensional crystal structure. There exist 32 point groups, which consists of 5 rotation groups (1, 2, 3, 4, 6) and 5 rotation inversion groups (-1, -2(=m), -3, -4, -6) and combinations of these 10 groups. Essential elements of a point group are

- Any two operation when carried out consecutively should be represented another single operation.
- Inversion operation should be present
- Associative law of multiplication should be applicable in sequence of operations
- Existence of identity element

When such symmetry elements are combined with Bravais lattices, there exist only 230 unique combinations of three dimensional crystals. They are called as space groups. However, in proteins only l-amino acids are preferred. Application of mirror planes or inversion in proteins would create a structure with d-amino acids which does not exist in nature. Hence, not all 230 space groups are possible in proteins but only 65 space groups are possible [130].

Referring to consensus crystal, the description given by XDS software® [120, 131] used in MX2 Australian Synchrotron is P 121 or P2 in Hermann-Mauguin format can be analysed as follows:

The P refers to the primitive lattice of the Monoclinic crystal structure. It means there is only one lattice point per unit cell. The series of numerals following P – 1 2 1 – indicate applicable symmetry elements in three axes of the unit cell. As per convention in case of monoclinic cell, **b** axis is considered unique and hence, the

two fold symmetry indicated by 2 is possible about it. i.e. it is possible to bring a monoclinic crystal in self-coincidence by rotating 180° twice about **b**. For other axes, only by complete rotation of 360° it is possible to bring the crystal in self-coincidence.

3.3.4 Molecular Replacement:

Molecular replacement is an approach used to obtain phases of the electron density obtained from the diffraction. This is achieved by obtaining a similarity model of electron density with reference to a homologous protein 3D structure which is available in protein database. For our structure we used an online pipeline algorithm BALBES [121].

It is a system that uses molecular replacement technique in fully automatic mode. It is built on three major components:

- Database of protein structures reorganized for process optimization
- System control program
- Scientific programs that contain algorithms

The workflow of BALBES consists of checking and extraction of necessary information for structure determination. BALBES analyzes and sequences, parameters of unit cell as well as space group. If similarity is found, it uses PDB entry for further refinement for unit cell parameters to reduce error to less than 5% and to improve sequence identity above 90%. For undesirable R/R_{free} automated molecular-replacement runs. During these runs, similarity with existing structures in database is evaluated and group of templates is provided. Then BALBES works in decreasing order of priority and highest sequence identity. Multimer models are tried first before monomer models. Different protocols are employed [121].

3.3.5 R and R_{Free} Factor

R factor (also known as residual factor, R-Value or reliability factor) is used as a measure of accuracy of prediction of calculated crystallographic information in comparison to observed data. In protein structure determination, it is used to

measure total error (in ratio terms) between observed x-ray reflections and protein model. Mathematically,

$$R = \frac{\sum_{h,k,l} ||F_{obs}(h, k, l) - |F_{calc}(h, k, l)||}{\sum_{h,k,l} |F_{obs}(h, k, l)|}$$

F_{obs} and F_{calc} = amplitudes of measured and calculated reflections, respectively.

h,k,l are the miller indices [132] .

Under assumption of normally distributed errors, a plot of R-Factor v/s resolution (also known as Luzzati Plot) is used to analyze average error in positions of co-ordinates of protein crystal. As resolution and data quality are dependent factors, if refinement process is conducted efficiently, the R-factor for protein structures is found to be in the range of 15%-25%.

The refinement process is an optimization of a function

$$E_{cost} = \omega * \sum_{h,k,l} (|F_{obs}(h, k, l)| - |F_{calc}(h, k, l)|)^2 + \sum_{atoms} E_{geom}(x, y, z)$$

The first term is self-explanatory whereas the second terms is introduced to overcome low value of observations/parameters. The ω term is the weighing factor that determines which of the two terms dominate relatively. With high ω value leads to low R-value but geometry is poor whereas low ω value leads to good geometry but high R-value. Hence, optimization is required to arrive at global minimum [133].

However, it is possible to overfit using data without much improvement or even reduction in quality of analysis. Hence, A.T. Bruenger introduced, concept of R_{Free} . Here, diffraction data is partitioned into two sets. Approximately 5% -10% of observations are separated into one set termed as *Test T* and remaining in another set termed as *Working A*. T is omitted in modeling process and only A is considered. R is calculated as R_{Free} for T set.

$$R_T^{Free} = \frac{\sum_{h,k,l \in test\ set} ||F_{obs}(h, k, l) - |F_{calc}(h, k, l)||}{\sum_{h,k,l \in test\ set} |F_{obs}(h, k, l)|}$$

R_{Free} indicates information contained by atomic model. For perfect data and atomic model, $R = 0$ and $R_{\text{Free}} = 0$ also. However, in case of imperfections, with the assumption that distribution of noise in data is independent, R_{Free} is will not be biased as R may be due to overfitting [132] .

3.3.6 $CC_{1/2}$

As strength of diffraction is directly proportional to resolution, a high resolution cut-off is determined so that weak data can be discarded and better model can be developed. Typical limit for the cut-off is when R_{merge} exceeds $\sim 0.6 - 0.8$ and signal to noise ratio derived empirically drops to less than ~ 2.0 . The values selected by using these criteria are proven inadequate because the area that is discarded also contains information for development of better model.

Hence, Karplus and Diederich (2012) [134] developed Correlation Co-efficient (CC) concept as a measure of data quality and assessing similarity between model and data. The unmerged experimental data is divided into two parts. While separating data, the observations are randomly chosen. Average of each half data set is calculated. As random choice is employed, variances of two sets are same. CC (denoted as $CC_{1/2}$) between average intensities of each set is calculated and statistically tested for significance. Hence, it indicates statistically validated value to define high resolution cut-off. As the aim is to find CC_{true} , an analytical relationship is developed between $CC_{1/2}$ and CC_{true} (CC^*).

$$CC^* = \sqrt{\frac{2CC_{1/2}}{1 + CC_{1/2}}}$$

Underlying assumption of mutual independence of errors, if invalid, indicate presence of systematic errors. Such errors may or may not affect model. If all intensities are offset positively, which is a systematic error, independence of errors doesn't get impacted and has no influence on CC^* and $CC_{1/2}$. However, this error reduces R_{merge} but the ratio $R_{\text{work}}/R_{\text{free}}$. This dichotomy of data and model R values is an indicator of inferiority of approach for evaluation of model quality using R [135].

Results And Discussion Continues:

3.3.7 X-ray Data Collection:

The diffraction pattern obtained is as in fig.3.7

X-ray Diffraction of Consensus EDIII crystal at MX2 Australian Synchrotron at Resolution 3.2 Å
Courtesy: Abbas El Sahili who helped to diffract the Crystal at MX2 Australian Synchrotron

Fig. 3.7 The X-ray Diffraction Pattern of Consensus EDIII crystal

The crystal efficiently diffracted the X-rays and the best diffraction that we obtained was at 3.25 Å resolution. The unit cell parameters are 56.00/58.85/112.01/90/98.21/90 which means the unit cell is a rectangle with length of sides 56, 58.85 and 112.01 in Å with angles 90, 98.21 and 90 in degrees. These dimensions of unit cell (Fig.3.8) classifies it to space group P2 and P 121 in specifics. Therefore complete data set was obtained using 180⁰ collection wedge with total reflections 34630 (Table 3.1). The overall CC_{1/2} value of 97.8 and 59.7 at the outer most shell indicates that the quality of the electron density data is optimal as discussed before in brief introduction.

Table 3.1 X-ray Diffraction Data Processing and Refinement Parameters

Dataset	Description
	AUS-MX2 (25072017)
Wavelength (Å)	0.953729
Resolution (Å)	50-3.25 (3.44-2.24)
Space group	P2
Unit cell	56.00/58.85/112.01/ 90/98.21/90
Collection wedge	180°
Total reflections	34630 (4598)
Unique reflections	20365 (2943)
Multiplicity	1.7
Completeness (%)	90.3 (81.1)
Mean I/sigma I (<i>I</i>)	4.88 (1.24)
Rmerge	12.1 (52.8)
CC (1/2)	97.8 (59.7)
Correlation Coefficient	0.907
R-work	0.2240
R-free	0.2668
Number of non-hydrogen atoms	3884
Water Molecules	64
RMSDs from Ideality	
Bond Angles	2.048
Bond Lengths (Å)	0.015
Ramachandran favored (%)	95.84
Ramachandran outliers (%)	4.16
Average B-factor	61.407

Values in parenthesis are those for the last shell. CC1/2= percentage of correlation between intensities from random half-dataset (P. A. Karplus, K. Diederichs, Science 2012, 336, 1030–1033).

Fig. 3.8 Unit Cell of EDIIC

Graphic Generated on Chimera[5]

The processed electron density map after integration of all collected frames and scaling was subjected to molecular replacement by generating the phases through Balbes algorithm pipeline on CCP4 online suite. The initial model of the structure obtained, had only 4 molecules in the asymmetric cell. This did not agree with the Mathew's co-efficient (CCP4i package) results which calculates molecules in asymmetric unit in accordance to the solvent content. Protein crystals contain ~50% solvent content. Mathew's co-efficient calculation suggested 6 molecules in the unit cell for ~50% solvent content. Therefore we used our model from Balbes for molecular replacement of our electron density data on Phaser in ccp4 to obtain more accurate asymmetric unit. The model from this molecular replacement finally showed 5 molecules and a little part of 6th molecule in the asymmetric unit (Fig.3.8). This version of model we considered for further refinement of the structure as it was in agreement with the Mathew's coefficient result showing 6 molecules in unit cell.

3.4 Refinement of the structure model:

The unit cell has 5 intact copies of the EDIIC molecules and is of monoclinic lattice (Space group P2). This offered a cue towards existence of Non-crystallographic symmetry (NCS) in the protein moiety. Therefore we used NCS restraint in the Refmac5 algorithm in the CCP4i package. The Rfactor reduced to 0.30 from 0.32 and the Rfree reduced to 0.35 from 0.36. This indicated that we were trading the right path. Therefore we ran multiple cycles of the NCS restraint in Refmac5 until we reached the best values of Rfactor 0.29 and Rfree 0.30.

Then we ran the low resolution refinement algorithm from CCP4i suite because our crystal diffracted at a low resolution of 3.2Å. This showed improvement of the Rfactor to 0.24 and the Rfree to 0.286.

After this software Buster was used to refine the model further using TLS restraint to achieve better values of Rfactor to 0.24 and Rfree to 0.27. TLS restraint curates the structure for correct di-hedral and rotational angles. Then we used coot to correct the fitting of model to electron density with correct rotamer arrangements manually.

Structure refinement is still underway with present Rfactor of 22.4 and Rfree to 26.6.

3.5 The Quality Check of the Structure:

The refinement process was verified by intermittent Quality checks by softwares Molprobity[124] , PDSum [126]and ProSa[125]. ProSa reviewed the structure and validated it as a good quality structure with Z-score -5.35 which means the model fits within the ensemble of values typically found for this size of protein. The plot of Residue score never exceeds zero and the energy value is negative over the stretch of 40 residues. This is also indicating a good quality structure.

MolProbity Score was 2.69. This means the quality of the structure matches with the parameters of structure at resolution 2.69Å. For the structure obtained at resolution 3.2Å this is favorable outcome. However this program revealed that the di-hedral angles and B-factors still need improvisation. The PDBsum software uses

ProCheck to analyze the structure quality. It also highlighted the difficulty in Ramachandran Plot agreement with the structure. The structure still needs to be refined to perfection of rotation angles.

Fig. 3.9: Consensus EDIII motif comparison with All Four Serotypes

Figures created on Chimera[5]

3.5.1 Functional Efficiency of Consensus EDIII as Potential Vaccine Candidate:

Currently available published crystal structures of Cross-neutralizing antibodies in complex with their antigen as Dengue EDIII provide reference of atomic details of this interaction. We chose Vis 513, 4e11, 3e31 and 2D73 to assess functionality of Consensus EDIII. The process of improvising involved functionality as a parameter with the tertiary structure fold structure alignment conservation as a basic criteria for selection. This ensured that the functional core of the protein was retained across the four Dengue serotypes along with the basic skeleton of solvent exposed motif. We tested this by aligning the Consensus EDIII structure with all four serotype EDIIIs on Chimera graphical software[5] (Fig.3.9). Residue to residue distances (Å) of alignment was obtained using PDBeFold online software [136].

3.5.1.1 Vis 513 [88] epitope Alignment:

This antibody is evolved from its predecessor 4E11 by computational engineering of the scaffold. The improved paratope advances to the DE loop, covering the region of residues numbered 362-365 with a prominent impact at Valine 364 on Dengue serotype 4. The Distance in (Å) of the 3D structure alignment of the Consensus EDIII in this region with all four Dengue serotypes ranges from 0.2-0.8. The A-strand epitopes region of 513 on Consensus EDIII aligns with the four Dengue serotypes at RMSD of 0.8. The epitopes of this highly efficient cross-protective mAb for Dengue serotypes 2, 3 and 4 lie on the region on B strand also strike a very significant similarity of Distance 0.9 Å with Consensus EDIII in this region. This proves that the whole foot-print of Vis 513 is stamped on Consensus EDIII with intact tertiary structural similarity.

3.5.1.2 4E11 [69] epitope Alignment:

The A strand and B strand epitopes of 4E11 are similar to vis 513. Therefore the foot-print matches exactly with the vis 513 as already described. This antibody additionally has its interaction in the FG loop extending to G strand at residues leucine 387 and Tryptophan at 391. Both these epitopes are highly conserved in flaviviruses and the structural alignment of this region with Consensus EDIII shows high similarity with distance values 0.3Å for the Tryptophan and 1.1 for the Leucine.

3.5.1.3 Ab 2D73 epitope Alignment:

This antibody also has its foot-print similar to the vis 513 with addition of AB loop residues. The AB loop of Consensus EDIII structurally aligns better with Dengue serotype 4 EDIII with distances ranging from 0.3 to 0.9Å than other serotype EDIIIs with the distance more than 1 reaching 1.83.

3.5.1.4 Ab 3E31 epitope Alignment:

This Ab epitopes are overlapping with 2D73 on AB loop, so the structural alignment of Consensus EDIII in this region matches with the 2D73 alignment.

This antibody extends its footprint to E-loop in the region of residue 368 and 370. The RMSD alignment in E loop of Consensus EDIII is close to 0.19 in case of

Dengue serotype 4 EDIII with other serotype EDIIIs aligning to Distance ranging between 0.3 to 0.7Å structurally.

3.6 Therapeutic Anti-EDIII Monoclonal antibodies Epitopes and Significance of Consensus EDIII:

The epitope sites of the potent cross-protective antibodies as discussed above align well with Consensus EDIII for all four serotypes of Dengue. As a vaccine perspective the epitope sites are discussed for their significance as follows:

3.6.1 Neutralization Mechanism Similarity Features of The successful monoclonal Antibodies:

Target site at Three-fold axis on Viral Icosahedron:

The remarkable similarity in these antibodies is in their target site at the 3-fold rotational axis. This site is the most dynamic site for the thermal expansion that affects this site and induces an arrangement like pre-fusion intermediate and at the very event of fusion the pre-fusion intermediate dislodges the herring bone E-protein dimer [33, 137, 138].

3.6.2 Stable B-molecule of E-protein at three-fold axis:

This site has three characteristic positions of the A, B and C molecules of the E-protein as discussed in Introduction (Fig.1.8). Interestingly the B protein is comparatively more stable and least displaced of the three during fusogenic movement [138]. Ab E16 is known to have its target site on this molecule B along with C. The 1A1D2 Ab has its target site on molecule A and C. We can deduce some facts from this observation. E16 did not require thermal expansion of virus for neutralization whereas 1A1D2 suffers this limitation. This means the target site at molecule B is accessible without ‘Breathing’ of the viral particle. The DE loop epitope of E16 not shared by 1A1D2 ab might be availing access of molecule B to the E16 [138]. These DE loop epitope stretch matches with Consensus EDIII as discussed before.

The efficient cross-protective ab 513 [88] also is observed to arrest virus at pre-fusion intermediate post-attachment like E16 and also has DE loop epitope. This common feature with E16 indicates possibility of this antibody having its foot print on the stable molecule B at the three-fold viral axis.

In case of 3E31 [89] the epitope has very little presence on DE Loop. This may be attributed to its dependency on thermal expansion of virus for effective neutralization, even though it has foot print on the B molecule. This means that the antibody should have a sizable footprint on the DE loop. The epitope of 3E31 is found to be immunodominant epitope of natural human humoral immune response.

The epitope of Ab 2D73 [139] is on the 3 fold axis on viral envelop but is not immunodominant in human humoral immune response. The epitope extends to c-terminal residues of EDIII 390-395. This antibody does not require thermal expansion and more strikingly forces pre-mature fusion at neutral pH. This ab also should have its target site on B-molecule, more so because it can interact with the dimer on mature smooth virus without any pre-fusion intermediate conformation. Consensus EDIII has these sites matched with the other four serotypes.

3.6.3 Three-fold axis – stoichiometry of ab binding [138, 140]:

The epitopes are mostly on the lateral ridge of the EDIII. These epitopes are also exposed at the five-fold axis at the vertex of the viral icosahedron. There is little space for one paratope to engage the target. Also because of this even if the paratope interacts, it will interact with all five EDIIIs together. This indicates that only 12 antibody molecules can occupy the 12 vertexes in total and 12x5 i.e 60 E-proteins would be engaged. In three-fold axis each paratope engages 1 molecule and each molecule of the antibody engages 2 E- protein molecule which increases the occupancy of the antibody two-folds to 120 at the three fold axis. This also means that the two antibody molecules are placed closer to each other upon interaction helping trigger the Cq1 complement molecule for aided neutralization given the fact that mostly all these antibodies are of class IgG1 [34]. Therefore a potent vaccine should present these epitopes on the threefold axis for better efficiency. Consensus

EDIII fold at this site is similar to all the four serotypes and contains the important epitopes raising it as a potent vaccine candidate.

3.6.4 Importance of Lysine 310 epitope [69]:

All the cross-protective potent antibodies are found to interact with Lysin 310. This residue is positioned on the Tryptophan at 101 on EDII where lies the fusion tip of fusion loop- the most dominant epitope of human humoral immune system and the most conserved region. These potent antibodies might be destabilizing the tight E-protein dimer arrangement irreversibly to expose the fusion tip which might get attacked by the targeting sera with high concentration of Anti-Fusion loop antibodies. This epitope site being conserved is harbored on Consensus EDIII.

3.6.5 Importance of target site between residues 323 to 327:

This site harbors the Tyrosine at 326 [112] which interacts with 303 and 304 and is very important for viral folding. Therefore these antibodies influence viral folding in various ways depending on the biochemistry of their other epitope electrostatic tinkering. They seem to influence at the step of extension of stem-region that occurs before the trimerization arrangement of fusion. This is also a well conserved epitope so is there on the Consensus EDIII.

3.6.6 Target site ensures lysosomal clearance:

All these antibodies let the internalization take place. This may be the reason, these antibodies elicit practically no cytokine storm. The epitopes are mostly affecting viral folding and fusion which are the most vital steps for viral replication. Therefore these sites don't depend on complement mediated clearance which intern depends on the Fc portion of the antibody.

3.6.7 Scope for scFv mediated Immunotherapy:

This mode of neutralization opens the scope for scFv fragments as immunotherapy. scFv is easy to administer than the whole Ab and also can be generated economically in ample amounts by recombinant technology in prokaryotic system.

Host genetic factors like polymorphism contributing as vitally important players in immune response may affect the final outcome of infection of Dengue virus. This may complicate vaccination success in some individuals. Immunotherapy can be a favorable option in such cases.

3.6.8 Brief Introduction of Ab 513 Neutralizing Efficiency for Dengue serotype four:

The Ab513 as mentioned previously in the introduction is a strategically engineered [88] antibody over its parent antibody 4E11 [69]. The Ab513 turned out be more efficient especially in case of DENV4 in comparison to prior version. The parent antibody 4E11 and its modified version 4E5A had very low affinity towards DENV 4 as compared to Ab513. The deliberate re-design of the Ab 513 was based on Structure guided analysis and Epitope-Paratope connectivity analysis for improved affinity for DENV 4. EPC analysis found on 7 unique residues on DENV-4 (Signature residues of DENV4) with four of these clustered in the DE-loop β -sheets (residue 358-365). The Ab region CDRH1 interacting with these signature residues at DE-loop was having an Elbow region restricting reach of the Ab. This region was improved by deletion of residues in the region 25-25 of CDR-H1. This favored more efficient electrostatic interaction with negatively charged residue 360-363 of EDIII of DENV-1, DENV-2 and DENV-3 as well. Ab513 interacts with the A-strand of EDIII with its light and heavy chains engaging the G and A β strands respectively [141]. The Consensus EDIII has similar structure alignment at DE loop and thus will prove to be a cross-protective antigen generating Ab 513 like anti-sera which would encompass the Dengue serotype 4.

Please refer to Table: AP II for comparison of epitopes of Ab 513 with its parent 4E11 and an antibody which failed to neutralize DENV 4 totally, 1A1D2 [142]. This comparison reveals that Ab 513 engages DE loop of the EDIII in addition to other epitopes shared by other two antibodies. This loop is less flexible than other loops and more exposed to solvent at 5-fold vertex of icosahedral virus (Table API). The Ab513 very efficiently engages G-strand of ED III and B-strand at region where they interact with ED II of the partner sE monomer. As the antibody neutralizes the

virus after internalization, it might be locking the virus in pre-fusion or post-fusion state. It is observed that DENV most probably exists in form of pre-fusion intermediate in human body at 37°C [143]. It can be hypothesized from this fact that the Ab 513 binds this pre-fusion intermediate of virus and lock it in the state after internalization.

With its very strong affinity the Ab 513 might be able to successfully compete with the more dominant non-neutralizing antibodies like Anti-prM which pose hindrance to neutralizing antibodies.

3.7 Surface Plasmon Resonance (SPR) of Consensus ED III with mAb 513

The consensus ED III has similarity ranging from 71% with DENV1 ED III to 61 % with DENV4 ED III. This makes it an attractive candidate as Dengue vaccine which needs to be tested on that direction. With this perspective we tried to test its affinity with the potently neutralizing antibody Ab 513 and our m366.6 by using Surface Plasmon resonance in kind collaboration with Prof. Susana Geifman Shochat's group. The antibody Ab 513 was kindly provided by our collaborators (Dr. Paula Hamond group, SMART).

Consensus ED III was non-specifically and very avidly sticking to the SPR-Chip Surface. Therefore we decided to immobilize Consensus ED III on the chip instead of the Antibody.

As the mAb 513 IgG is having two paratopes for binding the ligand Dengue Consensus domain, perfect fit was obtained through Bivalent Fit (Fig 3.10).

We could not obtain accurate values of the second binding which may be attributed to crowding of the antibody molecules on the chip surface.

The binding was very strong involving both affinity and strong avidity.

We tried several approaches to achieve better dissociation viz: Reducing the amount of immobilized ligand, 1 hour dissociation as shown in the figure and 5 hour

dissociation (results not shown). But even after 5 hour of dissociation experiment we could obtain only 5% of dissociation at 100nM concentration of mAb 513.

The experiment proved that the interaction between the Dengue Consensus domain III with mAb513 is very strong and the final K_D calculated by Bivalent fit is 14.7 picomolar.

Fig. 3.10 SPR results for Analysis of Affinity between Consensus ED III Ab513

A. Sensorgram was run for 1 hour in anticipation of dissociation over a chip with 20RU Dengue Consensus domain III. mAb 513 was flown over this chip at different concentrations 100nM to 1.625nM at 2 fold dilution as shown in the legend on the right side of the sensorgram. B. The first 300 seconds run from A is enlarged. C. Response Units (RUs) obtained of each dilution of mAb513 before and after 1hour and corresponding dissociation after 1 hour are tabulated. D. Values of $ka1$ and $kd1$ obtained after bivalent fit are shown in the table. These values for the second binding could not be obtained. Hence the final value was calculated based on the first binding which turns out to be 14.7 picomolar.

Ab 513 very strongly binds to the Consensus ED III as evident from the SPR experiment. The K_D value is in picomolar range which is a much better interaction as compared to nanomolar K_D of other DENV serotypes [88]. Remarkably, the DE loop epitopes of Ab 513 (Table: APII) on the Consensus ED III are more similar to DENV4 as compared to other serotypes. All other strand A epitopes and most on

strand B are conserved in all serotypes. Consensus Domain III is thermostable [112] making it ideal candidate for vaccine.

3.7.1 Small Scale Analysis of Complex formation on Analytical Superdex 75 5/150

Complex formation of m366.6 with EDIIIs of DENVT2, DENV1 and WNV was tested on a smaller scale so as to observe the peak shift. This also helped to optimize the ratios of the component proteins (EDIII and m366.6 scFv) for large-scale purifications of these complexes. The peak-shift is as depicted in the (Fig: 3.12) of DENV1 ED III as a representative profile.

The purified and seemingly homogenous ED III proteins successfully formed complex with m366.6 scFv as evident from the shift of peak observed on analytical size-exclusion. Small-scale Complex formation of WNVEDIII with m366.6.

3.7.2 Complex of m366.6 with DEN2TEDIII

The small-scale experiment showed that DEN2T ED III was too much in excess in the complex although it is advised to have the ligand in excess in moderation if the aim is to get a crystal. Therefore 1.1:1(DEN2T ED III: m366.6) molar ratio was used for the large-scale purification of the complex on preparative Superdex 75 16/60 column which had DEN2TEDIII in excess in moderation as desired (Fig3.13).

The fractions corresponding to the peak of complex were pooled and concentrated to concentration of 5mg/ml in 200 µl. This protein was screened for crystallization in PEG Ion and PEG Rx conditions from Hampton Research. As evident from the SDS-PAGE, the m366.6 scFv is degrading by a small fraction. To quench this difficulty we actually let the scFv degrade by keeping it at room temperature. After two weeks the scFv was completely degraded to a single band on SDS-PAGE. We adopted this strategy in our further course of study.

3.7.3 Complex of m366.6 with WNVEDIII on Superdex 75 10/300

Very low amount of complex was formed due to the aggregation observed in small-scale experiment. The complex was purified on Superdex 75 10/300 to obtain it on a larger scale 1mg/ml in total 0.5 ml. The fractions were analyzed on 18% SDS-PAGE gel to validate complex formation (Fig.3.13).

3.7.4 Complex of m366.6 with DENV1EDIII on Superdex 75 16/60

The large-scale complex of DENV1EDIII (Fig.3.13) was very robust and showed no precipitation during concentration. We could concentrate this complex to 18 mg/ml of concentration in 45 μ l which (the volume) is very less for large number of trials hence we performed few known trials by vapor-diffusion in 24 well plate.

Fig. 3.13 Large Scale purification of ED IIIs in Complex with m366.6

M: Protein Marker Ladder, Fv: scFv control, c: concentrated complex, Ed3: ED III alone.

The aim of the Refolding and purification of the ED IIIs and their complex with the scFv m366.6 was to obtain good crystals to get crystal structure of the complex to have a better understanding of the interaction at atomic level.

However, we did not get any crystal condition so far. We tried repeating the crystal trials with fresh proteins but we had limitation of the scFv m366.6 amount even though the collaborators were kind enough to try to send whenever we asked.

There are many angles to look at this failure but the prominent were two. One was that the very method of refolding suffers limitation of generating heterogeneous species of protein. Secondly, the degradation of scFv was obviously imparting its own heterogeneity. We decided to reduce the heterogeneity at least on the part of ED III by trying Periplasmic Expression of soluble Consensus ED III.

3.8 Zika Virus is not neutralized by m366.6

As the antibody m366.6, we had was a cross-protective antibody across flaviviruses like Dengue all four serotypes including West Nile Virus, we hoped it may also encompass Zika virus. Therefore we tried to check its neutralization ability of Zika virus by Plaque reduction Neutralization (PRNT) test. However the Antibody failed to neutralize Zika virus (Fig 3.15). As Prof. Dr. Shee Mei Lok, Duke-NUS had the required resources especially the Zika virus and required working facility, we collaborated with her for our experiments. Zika structure is thermostable. This explains its reach of CNS and Sperm. It invades fetal brain by binding TIM1 cofactor expressed on the surface of placenta [144]. It is observed to infect Hofbauer cells (Placental macrophages) before its entry into placenta leading to birth defects [145]. In case of Neural system, Zika is found to infect Neural Progenitor cells of both Fetus and adults [146] [147] [142].

ED III being the site of receptor binding and fusion, it is worth exploring this region for targeting the virus.

Zika Structure reveals that it is a very compact structure as compared to Dengue virus [31]. It did not exhibit the expansion at 37°C as Dengue virus [143]. At fivefold axis also the Zika ED III is pressed close to virion surface of Zika. This

means Zika Envelope protein does not breathe like Dengue thereby hiding many epitopes on EDIII from antibodies. The neutralizing antibody EDE1 [91] interacts with E strand of Zika ED III as compared to its interaction with A and B strands in case of Dengue virus. The interaction of this antibody induces changes in DENV 2 structure but the Zika structure remains intact upon binding.

Consensus Domain III has 50 % sequence identity with Zika ED III (Table 3.1). It can be further engineered to encompass Zika ED III in the region of quaternary epitope like E strand region which is accessible on Zika surface which is conserved in flaviviruses [91]. This will improve the Consensus ED III to be a better vaccine candidate.

Fig. 3.15 PRNT assay for testing m366.6 neutralization of Zika Virus

A: PRNT test for mAb m366.6 in duplicates against Dengue serotype 2 as positive control and against Zika virus as test candidate. Plaques of virus are visible in all the wells for Zika virus indicating no neutralization. **B:** Comparative plot of number of plaques for each 10 fold dilution of mAb m366.6 against Dengue serotype 2 and Zika virus.

Collaboration with Dr. Shee Mei Lok , Duke-NUS, Test was performed by Jiaqi

The m366.6 does not neutralize Zika virus. This may be a cue that the epitopes of this antibody may require thermal expansion of the virus particle at physiological temperature. The epitopes thus may be on A strand, AB loop mimicking the foot prints of ab 1A1D-2, 4E11 and would be prominently displayed on A and C molecules of E protein on viral surface at the three fold axis as described [148].

Table 3.2 Zika ED III Sequence Identity Matrix with other ED IIIs

	Den1EDIII	Den4 EDIII	Den3 EDIII	Den2 EDIII	Zika ED III	Consensus EdIII	West Nile ED III
Den1 EDIII		0.54	0.7	0.63	0.485	0.8	0.436
Den4 EDIII	0.54	ID	0.49	0.58	0.475	0.66	0.417
Den3 EDIII	0.7	0.49	ID	0.57	0.446	0.72	0.359
Den2 EDIII	0.63	0.58	0.57	ID	0.436	0.73	0.417
Zika EDIII	0.485	0.475	0.446	0.436	ID	0.495	0.563
Consensus EDIII	0.8	0.66	0.72	0.73	0.495	ID	0.436
West Nile ED III	0.436	0.417	0.359	0.417	0.563	0.436	ID

3.9 Surface Plasmon Resonance (SPR) of Consensus EDIII with scFv m366.6

Fig. 3.16: SPR Results for Consensus EDIII interaction with scFv m366.6

- 1) Sensorgram was run for 350 over a chip with 17RU Dengue Consensus domain III. mAb 513 was flown over this chip at different concentrations 100nM to 1.625nM at 2 fold dilution as shown in the legend on the right side of the sensorgram. B. color code of the different dilutions concentration in duplicates, duplicate denoted with '_2' C. Values of k_a and k_d obtained after 1:1 Languimeer fit are shown in the table. Index of Ta

Consensus EDIII showed a tight interaction with scFv m366.6 (Fig.3.16). Single chain fragment variable-scFv is a monovalent probe as compared to monoclonal antibody as in the previously mentioned case of mAb 513. Therefore the mode of fitting we used in case of scFv m366.6 is 1:1 Langmuir. The fitting to this model was not very perfect. Therefore we also tried the bivalent fit model (Data not shown here) owing to the degradation observed in m366.6 which would impart two moieties of interaction as the degraded fraction retains its binding capacity to EDIII. This model also did not give perfect fit of the profile. When the two fits were compared the 1:1 Langmuir demonstrates strong interaction of the two molecules corroborating with the peak shift on size-exclusion column as complex. There is no dissociation at the end of 350 seconds. We anticipated this outcome from SPR results of mAb 513 and therefore we did not continue the dissociation beyond 350 seconds in this case of scFv m366.6. Instead we invested more time in improving the fit by reducing the amount of Consensus EDIII immobilization on the chip so as to avoid any overcrowding of interacting scFv molecules on the chip. The best fit still remains to be the one presented here. However the result is definitely indicating an interaction strong enough to have a K_D value of 6.5 picomoles.

The Consensus Domain III displayed a very strong affinity interaction with Ab 513 and scFv m366.6. The K_D value which reached picomolar range is comparatively better than all other ED IIIs tested against Ab 513 [72, 88]. This result prompted that this Consensus domain III with its strong binding to cross-protective antibody, which can be easily produced on a large scale in soluble form, can be a good candidate for vaccine. Therefore we collaborated with Dr. Paula Hamond (vaccine development group, SMART) to further test this ED III in this regard.

3.10 Crystal of complex of Consensus envelope domain III and scFv m366.6:

Previous trials for crystallization of this complex were unable to show any crystal conditions and we were left with very small quantity of purified complex of scFv

m366.6 with consensus ED III left with us. Therefore we tried only one crystal screen JCSG+ for this one possible screening which we had not tried before. This screening showed very miniscule micro-needles under a very high magnification (1000x) microscope lens in JCSG+ screen in row H column 9 (H9), 10 (Fig 3.17) and 11 after 3 days of setting the screen.

Fig. 3.17 Micro-needle crystal of complex of Consensus EDIII with scFv m366.6

This successful nucleation after repeated previous attempts had only one difference i.e this time we used JCSG+ screen from Molecular Dimensions. This condition was reproducible to generate the microneedles. Micro-needles being too small, micro-seeding was the only possible step ahead. The micro-needles in condition H9 were better formed so we used the micro-needles from this drop for our micro-seeding experiments further.

3.10.1 Micro-seeding of Complex micro-needles:

We obtained a couple of crystal conditions in high-through put micro-seeding in JCSG+ screen in buffer condition grid screens A1 and D11.

Microseeding of crystal of Complex of m366.6 scFv with Consensus EDIII:
Condition JCSG+ A1

Fig. 3.18 Micro-seeding Condition of EDIII in complex with scFv m366.6

First picture is the Micro-seed condition in JCSG+ A1 , Second shows bi-refrference under polarizing lens, third shows the condition under Ultra violate wavelength with the illumination confirming it as protein nucleation

These crystal conditions were confirmed to be of protein by UV-illumination in the Crystallization Robot. Protein crystals absorb UV light at 280 nm wavelength and hence light up under UV light distinguishing them from the salt crystals (Fig.3.18 and 3.19)).

Microseeding of crystal of Complex of m366.6 scFv with Consensus EDIII:
Condition JCSG+ D11

Fig. 3.19 Micro-seeding Condition 2 of EDIII in complex with scFv m366.6

First picture is the Micro-seed condition in JCSG+ D11 with bi-refrference under polarizing lens, second shows the condition under Ultra violate wavelength with the illumination confirming it as protein nucleation. (The needle cluster broke during handling.)

These crystals did not diffract in MX2 Australian Light Source. The size is still very small for diffraction. This means that we have the nucleation condition for the complex but we still need to optimize the seeding condition.

3.11 Consensus EDIII A Potential Vaccine Candidate:

The Consensus EDIII protein was derived from the multiple alignments of strains isolated from complicated cases of DHF and fatal outcome representing 50% of the cases. This establishes the Consensus EDIII as a clinical isolate subunit. This multiple alignment applied was not restricted to only the primary structure of the candidate proteins but also existed in the tertiary structure ensuring a more functionally relevant similarity. This extensive mining sustaining the chemical and physical parameters additionally resulted in revealing the functionally relevant variation boundaries. Consensus constructs of any species, usually are observed to be more stable and are more significant for viable biological functions required for the organism's survival. The stability of the Consensus EDIII was found to be greater or equal to the individual counterparts from the four Dengue serotypes [112].

3.11.1 Thermostability of Consensus EDIII:

The Consensus EDIII attracts a particular interest for its striking thermostability with T_m (Melting Temperature) of 86°C [112]. The residues found to contribute this remarkable feature were Valine at position 320 and 322 with Isoleucine at position 339. These hydrophobic residues belong to the buried core of the protein. Threonine 303 and Serine 385 from solvent exposed surface also were found to positively influence the thermostability of this protein.

Fig. 3.20: Electrostatic Solvent Accessible Surface of Consensus EDIII comparison with All Four Serotypes

Ribbon representation of EDIII of cyan: Den1 (pdb id: 3irc); Yellow: Den2 (pdb id: 2jsf); bottle green: Den3 (pdb id: 3vtt); Pink: Den4 (pdb id: 3we1); Grey: Consensus EDIII. Solvent accessible area DE loop, N-terminal A strand, BC loop, FG loop are colored in orange. The surface in Circle matches with Dengue Serotype4 and the surface in the Square matches with the others. Electrostatic Surface Figure generated on Chimera [5] Red: Negative charge, Blue: positive charge, Wconditione : Neutral

3.11.2 Electrostatic Solvent Accessible Surface Comparison of Consensus EDIII:

The N-terminal A-strand, BC loop, DE loop and FG loop are solvent accessible areas of EDIII on mature and thermally expanded virus[148]. This region includes the lateral ridge of the EDIII. This region is mostly targeted by the cross-protective neutralizing antibodies with little nuances in their epitopes. The electrostatic property of this surface affects the efficiency of the cross-protection. Therefore we compared the electrostatic surface of Consensus EDIII with the four serotype Dengue virus surface (Fig3.20). The Serotype4 virus has the most distinctly neutral electrostatic surface and different distribution of positive and negative charges. The

Consensus EDIII is found to strike a balance by being inclusive of Serotype 4, in the N-terminal region of the solvent exposed area, especially the immunologically favored area of serotype 4 specific neutralizing antibodies in the A-strand [149].

The Consensus EDIII protein was engineered from repertoire of clinical isolates of each Dengue serotype with DHF and fatal outcome. We obtained a 3D structure of this Consensus EDIII to explore it as a potential vaccine candidate against Dengue. The structural alignment analysis of 3D structures of this Consensus EDIII with the Dengue serotype EDIII reveals the inherent similarity in these proteins. We further analyzed this alignment to check foot-prints of cross-protective antibodies on the Consensus EDIII. The Consensus protein scores very high as potential vaccine candidate as the alignments show very relevant and significant conservation of the foot-print of these antibodies.

Surface Plasmon Resonance (SPR) experiments of the antibody 513 with Consensus EDIII shows picomolar interaction. This binding is stronger than the four serotypes EDIIIs of flaviviruses that have nanomolar interaction. The two molecules did not dissociate indicating high avidity. This very strong interaction promises a high probability that Consensus EDIII will induce vis 513 like paratope in host upon administration as a vaccine. This anticipation is reinforced in context of human immune system when SPR experiments with m366.6 an antibody generated in human host demonstrated picomolar interaction with Consensus EDIII.

The anti-serum from dengue serotype 4 infected mice showed comparatively weak interaction with this Consensus EDIII [150]. This experiment result infers that the Consensus EDIII does not have an immune-dominant epitope in case of Dengue serotype 4 as compared to other serotypes. However our alignment results proved that this Consensus EDIII has foot-print of efficient cross-protectors like Vis 513, 3E31, 2D73 with strong neutralizing ability against Dengue serotype 4. Therefore this Consensus EDIII is a strong contender as a vaccine in spite of the report. Secondly, it is established that the mouse humoral immune response is different from human immune response. In addition, the anti-serum in this study is not in response to the Consensus EDIII administration but is triggered by the Dengue viral

particle. This, supports the Consensus EDIII as a vaccine candidate because the natural immune-response can be tweaked towards this desirable non-immunodominant epitope of potentially cross-neutralizing antibodies.

The Consensus EDIII is an attractive vaccine candidate also because of its remarkable thermostability. This will facilitate an easy logistics of this molecule to the tropical global sites of high flaviviral incidence.

We explored administration of the Consensus EDIII via Skin through self-dissolving Micro-needle assembly. We show a successful assembly of Consensus EDIII in this multilayered assembly and delivery inside mouse skin through this skin-patch.

3.12 Assembly of Consensus EDIII in Micro Needle

3.12.1 Successful Encapsulation and Release of Consensus Domain III from Dissolving Type Microneedle

Two members of the family of poly (β -amino esters) designated as poly-1 and poly-2 were used for arranging polyanion and polycation layers called polyelectrolyte Multilayer (PEM) assembly. These electrolyte are known for their hydrolytic degradation in physiological conditions. This property is utilized to manufacture a PEM film that is useful to inject macromolecular solutions in the skin. Success of poly-1 was previously established by developing layer by layer (LbL) films (Fig3.21)[151]. Multilayer assembly consisting Consensus envelop domain III (Consensus ED III) is examined to test its utility as protein cargo sample which is a potential vaccine candidate (Fig. 3.22).

Aqueous buffers containing poly-1 or poly-2 and Consensus ED III were prepared. Using these buffers, PEM films were developed on glass slides using phenomenon of alternate adsorption. Electrostatic interaction that exists between polymer and protein was employed for assembly mediation and fluorophore-conjugated ova that gets absorbed was used to monitor protein incorporation. The film assembly was tested between pH range of 5-7. The underlying consensus EDIII had pI of approx. 5.2 whereas poly-2 indicated pK_a in the range of 4.4 to 8.1. At pH level 4, it was

observed that Consensus EDIII protein amount adsorbed was maximum. 40 bilayer films were contained Consensus EDIII protein that was approx. $5\mu\text{g}/\text{cm}$ (Fig 3.22).

Fig. 3.21 Layer-By-Layer Micro-Needle Assembly

(A) Layering is achieved by arranging positive and negative charged solutions of desired proteins and polymers or other reagents like adjuvants with complimentary electrochemistry. (B) The Micro-needle patch (C) The polycations and polyanions are alternatively sprayed on the microneedles. Consensus EDIII is positively charged layer at pH 4.0. (D) These multilayers can be customized as per user's need with components like Adjuvants. (E) Encapsulated Micro-needle representation. Figure contribution: Michelle E Turvey, Abdul RM Sharif and Paula T Hammond, Infectious Diseases-Interdisciplinary Research Group (ID-IRG), SMART

This amount of dose per area was found to be sufficient for the purpose of vaccination based on historical studies. Rate as low as $3\mu\text{g}$ were found in historical studies as the rate of antibody response for doses of antigen for transcutaneous application.

Rehydrated assembled PEM films that were dried, exhibited release of protein at a rapid rate. Rehydration was carried out in phosphate buffered saline that had pH of 7.4. PNMP (poly(o-nitrobenzyl-methacrylate-co-methyl-methacrylate-co-poly(ethylene-glycol)-methacrylate) substrate was first spray coated on the O-plasma treated microneedle base before applying the LBL assembly. o-nitrobenzyl groups on PNMP break away upon uv exposure. This cleavage renders the PNMP soluble in aqueous solution at physiological pH. Before cleavage of the o-

nitrobenzyl groups, PNMP is organic soluble. This property is used for release of multi layers at physiological conditions of biological systems.

Fig. 3.22 Consensus EDIII Encapsulation Assembly on Microneedle

Figure contribution: Michelle E Turvey, Abdul RM Sharif and Paula T Hammond, Infectious Diseases-Interdisciplinary Research Group (ID-IRG), SMART

The amount of encapsulated EDIIC was estimated by release of microneedles in PBS pH7.4 at 37°C over the period of 7 days. At the end of 7 days, the released EDIIC was quantified by ELISA.

Fig. 3.23 Determination of Encapsulation Amount on Microneedle

Quantification of EDIIC protein assembled in LbL films of (Poly1/EDIIC)_n and (Poly1/PAA/EDIIC)₁₀ on microneedles. Data represent mean±sem (N=3). Poly1 is a bio-degradable poly(beta-aminoester) polymer. It is clear that addition of PAA helped improve the encapsulation by 6-fold compared to the double layered encapsulation. Figure contribution: Divakara SSM Uppu, Abdul RM Sharif and Paula T Hammond, Infectious Diseases-Interdisciplinary Research Group (ID-IRG), SMART

The experiment results indicate that the amount of total EDIIC that got encapsulated was increased by approximately five folds with addition of layer of PAA (Fig.3.23) to 60µgs per micro needle after 10 trilayers as compared to 15 bilayers which could encapsulate only 10 µgs. Therefore trilayers were adopted as the optimized strategy further.

The release kinetics was as plotted from these micro-needles in-vitro as depicted in fig.3.24. This was achieved by assaying the amount of protein released over a period of 10 days. Sampling was done each day. Release sample from each day was checked by ELISA for the amount of EDIIC released for four micro-needles.

The results exhibited a plateau approximately at 7th day where the release reached its maxima which is about 10µgs as seen in fig.3.24.

Fig. 3.24 Release Kinetics of Consensus EDIII from Microneedle assembly

Figure contribution: Michelle E Turvey, Abdul RM Sharif and Paula T Hammond, Infectious Diseases-Interdisciplinary Research Group (ID-IRG), SMART

Further testing was done to test integrity of structure of ova during integration into the film and release from the film later during process of dissolution. Both non-reducing SDS PAGE and native PAGE were used. Supernatants were taken from assembled films that were developed at different pH values. These films were dried and rehydrated for 30 min in PBS. Tests were a success as, the protein released from the films showed that it existed in monomeric form and was at the same position as found in native PAGE gel. Moreover, the released protein didn't aggregate with itself or with poly-2. This lack of aggregation confirms that the protein is well suited for therapeutics or for understanding responses of antibody to antigens of vaccines, as irreversible complexes doesn't occur.

3.12.2 Detection of Fluorescent labeled EDIIIC in Skin:

The fluorescently labelled protein was visualized under Fluorescent microscope in skin. The blue fluorescence is clearly visible in the test skin samples (Fig.3.25). This demonstrates success of PEM to deliver the cargo of EDIIIC a potential vaccine candidate in the dermis.

Fig 3.25 Fluorescent Visualization of EDIHC protein in layer-by-layer (LbL) films on microneedles and Delivery in Mouse Ear Skin.

A. Representative confocal images of PLLA (PolyL-lactic acid) microneedles coated with a base polymeric release layer and then EDIHC protein conjugated with AlexaFluor 430 (right) was assembled in LbL films of (Poly1/EDIHC)15. Scale bar, 100 μm. Poly1 is a bio-degradable poly(beta-aminoester) polymer. B. Confocal images of the Alexafluor430-EDIHC at the puncture sites of mice ear skin post-microneedle application. BF-bright field. Red arrows indicate the puncture marks. Scale bar, 200 mm. Confocal images were taken using a Zeiss Axio Observer Z1 LSM 700 (488 nm laser) at 10X objective.

Figure contribution: Divakara SSM Uppu, Abdul RM Sharif and Paula T Hammond, Infectious Diseases-Interdisciplinary Research Group (ID-IRG), SMART

3.12.3 Functional Viability of Delivered EDIII in Skin tissue:

3.26: Efficiency of Microneedle to Lodge EDIII in Mouse Ear through Skin by ELISA

Murine ear skin was used to perform this experiment. The stratum corneum, the barrier that must be bypassed so that vaccine reaches immune cells, was disrupted by repeated tape stripping. This process is painless and expedites transfer rate of vaccine molecules. It is also observed to elicit some built-in adjuvant response by activation of Langerhan Cells (LC) and keratinocytes [151]. The Consensus EDIII in ear lysates was checked by ELISA (Fig.3.26). The qualitative ELISA experiment clearly demonstrates success of Microneedle Delivery of Consensus EDIII in the mouse ear through skin.

Note on the Collaboration on this Microneedle work: As we were able to generate soluble purified Consensus EDIII, we were invited for this collaboration by Dr. Paula T Hammond from Infectious Diseases-Interdisciplinary Research Group (ID-IRG), SMART. All the required resources for Micro Needle assembly are from her laboratory. The author of this thesis majorly contributed the pure Consensus protein to the amount of approximately 100 mgs.

3.12.4 Dengue Vaccine Perspectives:

Dengue immune response engages all the faculties of the immune system including innate immunity and both arms of acquired immune system: the T-cell immunity and B-cell-immunity. The successful clearance of virus without severe disease exhibits a controlled orchestra of all players including the contribution of regulatory pathways. This is the outcome in most cases. But in some cases the critical balance is lost due to several factors including viral evasion mechanisms.

This calls for contemplation on vaccine perspectives.

Humans have sizable but lesser fraction of anti-EDIII population in their sera [140]. Secondly, it is observed that the previous memory B-cell or T-cell clones are expanded with practically little contribution from the D (Diversity) gene in somatic hyper mutation in B-cell maturation in secondary infection [87]. Therefore the sera generally is elicited at immunodominant epitopes with peptide residues 309-320 [34] on N terminal region of the EDIII which is not very much surface exposed on virus. Vaccination of a non-immunodominant [88] but functionally relevant epitope could aid the immune system to generate efficient broad-neutralizing population of antibodies.

Another observation that the anti-sera is more potent [140] at the secondary infection than the primary infection highlights the significance of vaccination of naïve population of individuals in endemic area or the travelers for better results.

Time lapse between two successive heterologous infections is observed to dictate the clinical outcome of the disease [152]. It requires 2.6 years between the clinical non-severe dengue fever to lose the protection of the cross-protective sera for the successive heterologous infection to manifest as severe hemorrhagic fever. This means a proper cross-protective vaccine administration in due time will restore the protection from severe outcome. Most desirably the vaccine will stop progression of asymptomatic case to a sub-clinical case which shows a time-period of 1.6 years. Another factor which underscores importance of a cross-protective vaccine is evolution of virulent pathogenic genotypes [152] from within the serotypes that escape the normal immune response. A vaccine that encompasses conserved regions

that are vitally important for viral survival would efficiently tackle this problem of viral evolution.

3.12.5 Vaccination through Skin:

Subcutaneous or intra-dermal administration of vaccine triggers highly active migration of Dendritic Cells (Dc) to regional lymphnodes within 24 hours [153].

Mast cell degranulation is observed to be an early stage of dengue infection. High amount of chymase a, chemokine released by activated mast cells, in circulation observed in less severe dengue disease outcome. This stimulation of mast cells possibly recruit Natural Killer cells to the infected site. Low levels of chymase were detected in severe Disease cases. In addition, Plasma-cytoide Dendritic cells in skin offer protection resulting in better disease clearance by secreting type 1 interferon at the early stages of the infection [154].

The langerhan cells (Lc) in skin layers ensure proper balance of activation and regulation of memory T-cells that are abundantly present in dermal tissue[155].

High population of Lcs is found at dermal infection site in 24 hours [156], Abs are produced more rapidly if the vaccine is administered through skin than muscle. Secondary response is more rapid in 12 hours [153] with this route. CD1a positive Dcs are targeted first by denv upon dermal entry [157]. These Dcs express FcγII receptor and show no enhancement of disease even if the anti-sera is diluted. IL-12p70 is required by dermal Dcs to mature and induce TC1 polarized immunity [158].

Tcel-Dendric cell synapse very much important for strong neutralizing immune response that is established by IL-12 [159]. LPS as adjuvant is shown to establish and strengthen[160] this tight synapse for better antigen-presentation.

EDIII is not observed to trigger disease enhancement when administered as vaccine [161]. Instead a similar Consensus EDIII was found to induce neutaralizing immune respose to DENV2 in non-human primates who are susceptible to Dengue Fever [36]

Conclusions

We successfully achieved periplasmic expression of Consensus EDIII in soluble form. This Consensus EDIII showed a very strong interaction of picomolar KD value with efficient cross-protective antibodies like Vis513 and m366.6 by Surface Plasmon Resonance. We generated 3D crystal structure of this engineered Consensus EDIII of flavivirus which is thermostable.

The structural alignments of Consensus EDIII with all the Envelope domain IIIs of the Dengue viral serotypes proved that the epitopes of the potent cross-neutralizing antibodies are present on the surface of the Consensus EDIII with its conformational fold intact. This establishes it as a potential vaccine candidate. We have further demonstrated a route of administration of this vaccine candidate through skin by application through micro-needle patch. The Consensus EDIII was assembled in the polyelectrolyte assembly and was successfully administered in the ear skin of mouse.

We have obtained the buffer conditions for micro-crystal of the complex of Consensus EDIII and scFv m366.6.

Our refolded West Nile EDIII interacts with m366.6 is an additional valuable information from this result.

Future Work

At present the structure of Consensus EDIII is underway for improved refinement. The resolution of 3.2 Å is comparatively a lower resolution and hence the refinement process faces difficulty. Therefore a software suit Buster would be used through its High resolution constraint algorithm with a hope of having a perfectly refined structure.

As we have mined the conditions of the micro-crystal for the complex of scFv m366.6 and Consensus EDIII, the most prioritized future work is to optimize it further to get better crystals and subsequent crystal structure of this complex. Consensus EDIII complex with mAb 513 also a very promising future plan. These crystal structures would give the atomic basis of this interaction. With this information better vaccine and improved immunotherapy can be designed.

In case of the micro-needle administration, the spleen and regional lymphnode can be assessed after Consensus EDIII delivery to check the response of acquired immunity. Subsequently the protection offered by this vaccine candidate can be assessed in suitable animal model.

This Consensus EDIII can be further explored as a high-throughput diagnostic probe for faster detection of Dengue infection in endemic areas.

**Part II: TRANSIENT INTERACTION BETWEEN
GTP-EXCHANGER VAV1 AND HISTONE
METHYLTRANSFERASE EZH2**

Part II ABSTRACT

Vav1 proteins are GDP/GTP exchange factors for Rho family signal transducers belonging to Vav family which also includes Vav2 and Vav3 proteins. They are implicated in various carcinomas; therefore they are also classified as proto-oncogenes. Ezh2 is a polycomb group Histone Methyltransferase which is reported to be recruited by Vav1 for interaction with Talin. This is Integrin pathway of Leukocyte trans-migration. The recruitment of Ezh2 by Vav1 to interact with Talin helps the cells detach from extra-cellular matrix and facilitates their migration. This mechanism possibly explains these two protein's role in poor prognosis of Carcinomas as reported. Therefore, this project aims at exploring the binding interface of these proteins at atomic level by NMR and/or Crystallography.

We have purified the interacting domains (Vav1) and Fragments (Ezh2) expressed in *E.coli*. Attempts are ongoing to get the interaction residues by NMR or Crystallography. Currently we have obtained 3D NMR structure of Ezh2 fragment (201-252). We have also tried to assess this interaction through computational simulation by molecular dynamics with two mutations in Ezh2 residues, hypothesized to improve this interaction. The double-mutant is generated and its expression and purification is achieved.

Part II INTRODUCTION

4.1 Vav Proteins

Vav proteins are members of a group of molecules participating in signal transduction. They are omnipresent in life with the exception of unicellular organisms. Genomic studies [162] suggest that the function of Vav proteins seems to be co-evolved with tyrosine kinase pathways, taking part in signal transduction of biological responses linked to the cytoskeleton and gene transcription. Vav proteins are best known for their association with tyrosine phosphorylation controlled functions as GDP/GTP exchange factors for Rho/Rac molecules. This function crucially depends on accurately orchestrated interactions and related up-stream and down-stream effectors phosphorylating Vav proteins. Vav proteins are also reported to contribute other cellular functions assuming role of adaptor molecules. The Vav family proteins have a distinct modular organization composed of CH domain, the DH-PH cassette, ZF region, and SH2, SH3 region [163]. Vav proteins as guanosine nucleotide exchange factors for Rho/Rac proteins [164-167] catalyze the release of GDP from the inactive GTPases, leading to subsequent incorporation of GTP and induces conformational morphogenesis of GTPases required for further downstream interactions with effectors. This conformational change is reported to facilitate releasing the inhibitory Rho GDI molecules linked to GDP-bound Rho/Rac proteins, resulting in recruitment of the GTPases to the plasma membrane

These proteins are reported [168] to have significant role in actin regulatory pathways, immune cell development and stimulation, neuronal development, and angiogenesis.

Fig. 4.1 The cardinal regulatory pathways in which Vav is involved as an organizer of the cytoskeleton. *Image Replicated From [168]*

Vav family proteins are significant players in cell signaling cascades and related biological processes like Integrin pathway (Fig.4.1). Integrins are heterodimeric transmembrane receptors contribute in cell adhesion to the matrix and inter-cellular attachments thereby providing link between extracellular matrix to the cytoskeleton[168]. Subsequent interactions with the actin cytoskeleton activate several important cellular cascades involved in cell-substrate or cell-cell adhesions

and cell spreading. Common feature of cellular transformation are the disruption of actin filaments of cytoskeleton and reduced focal adhesions. These transformations are significantly linked to anchorage-independent growth and cellular malignancy, marking Actin filaments role in oncogenic transformation [169, 170]. Regulators and effectors of Rho family GTPases play very important role in maintaining normal cellular behaviors and hence are generally the major subversion targets in the events of oncogenic transformation. Accordingly, Vav1 was originally identified as oncogene in vitro [171].

4.1.1 Vav proteins have a modular multi-domain organization

(Refer to figure 4.2)

1. **calponin homology (CH)** : This domain is involved in Ca^{+2} mobilization
2. **Acidic (Ac)**: Bears 3 regulatory tyrosines in mammalian Vav
3. **Dbl homology (DH)**: A conserved region catalyzing GDP for GTP exchange on Rac/Rho GTPases, contributing to cytoskeleton reorganization.
4. **pleckstrin homology (PH)**: Engages phosphatidylinositol 3-kinase (PI3K)–generated lipid products recruiting it to the inner face of the plasma membrane
5. **zinc finger (ZF)**
6. **Src homology 3 (SH3)**: Interacts proline-rich proteins
7. **Src homology 2 (SH2)**: interacts with proteins that contain phosphorylated tyrosines
8. **Second SH3**

The DH domain catalyzes guanine nucleotide exchange in Rho family GTPases [172]. The five N-terminal domains (abbreviated CADPZ; other fragments are designated analogously) together, regulate the biochemical activity of the DH domain; the C-terminal Src homology domains presumably direct cellular localization [163].

Fig. 4.2 C-terminal Src homology domains presumably direct cellular localization [Image Modified from [173]]

4.1.2 Cooperative dynamics of Vav1 physiology

Phosphorylation at Tyr174 relieves a core auto inhibitory conformation of Vav1 locked by C-terminal helix of Ac domain blocking the active site at DH domain [172, 174-176]. An Ac-DH fragment of Vav1 is phosphorylated at two tyrosine Tyr 142 and Tyr 160 [175, 177] in addition to previously known Tyr 174. Phosphorylation at Tyr 142 and Tyr160 were found to relax the modular controlled conformation of Vav1 making Tyr 174 more accessible to the downstream kinases. The core process at Tyr 174 provides approximately 10-fold suppression and the Tyr 142 and Tyr 160 switches further tighten the inhibitory (helix-bound) state to additional 10-fold. This layered energetic architecture of modular Vav1 enables the critical kinetics of rapid activation upon effector stimulation and robust inhibition at ground state. The Tyr 142 and Tyr 160 residues are situated in CH domain of Vav1 (Appendix IX, Fig. AP IV). This indicates a conformational change in CH domain upon phosphorylation would influence the auto-inhibition of Vav1 protein.

4.2 Ezh2 (Enhancer of Zeste Homologue) Protein

Differentiation of somatic cell, committed to a particular lineage of tissue is due to selective subset of active genes, muting the rest of the genome. This lineage specific genome expression is achieved by modifications in the chromatin structure accordingly. This epigenetic modification of chromatin is maintained through generations by balancing actions of transcriptional activators of the trithorax group (TrxG) proteins and repressors of the polycomb group (PcG) proteins [178].

Ezh2 is reported in literature [178] to have role in chromatin remodeling, lymphocyte development and activation. Ezh2 from the PcG group has a distinct, highly conserved SET domain which catalyzes the H3 methyltransferase (HMTase) activity. It methylates lysine 9 (H3-K9) and lysine 27 (H3-K27) of the histone3 in chromatin [178]. H3-K9 or H3-K4 by separate HMTase ensures stable gene expression or transcriptional activation respectively [178]. Ezh2 role is sighted in various reports in B-cell division with up-regulation in proliferating germinal centers of B-cell and mitogen-stimulated lymphocytes. Ezh2 is found in increased concentrations embryonic lymphopoiesis at fetal liver and thymus. Association of Ezh2 with Vav underscores its role in lymphocyte activation.

Ezh2 is predominantly localized in cellular nucleus as expected in agreement with its role in epigenetic modification. But surprisingly Su et al [178] reported its cytosolic presence in fibroblasts and peripheral T lymphocytes. They further demonstrated posttranslational modification management of proximal receptor-mediated signal transduction in mammalian cells by cytosolic Ezh2. They found that cytosolic Ezh2 form a methyltransferase complex similar to its nuclear version but the cytosolic complex includes Vav1 unlike the nuclear complex. The study confirmed direct role of Ezh2 in Vav1 driven TCR-mediated actin polymerization resulting in T cell activation. Interaction with Vav suggested that Ezh2 is operational upstream of small GTPases and controls signals between the receptor on the surface and small GTPases. The PRC2 complex is explained in detail in the reference [179] (Fig.4.4).

4.2.1 Ezh2 in Cellular Transformation

Recently, Ezh2 role in regulation of integrin signaling and adhesion dynamics of neutrophils and dendritic cell was explored in cytosol. Ezh2 was responsible for clearing of focal adhesions of leukocytes by interfering the binding of Talin with F-actine. Direct methylation of Talin by Ezh2 induced this clearing of focal adhesions. Gunawan et al [180] demonstrated that interaction of Vav1 and Ezh2 was required for such methylation of Talin and resultant disassembly of adhesion structures (Fig.4.3). It was further established that the Ezh2-interaction domain of vav1 is highly conserved in the members of vav family and thereby Ezh2-Vav-Talin complexes were observed in other non-hematopoietic cells.

Reports of Ezh2 over-expression leading to down-regulation of adhesion molecules [119], cell cycle regulators and transcription factors indicate role of Ezh2 in cellular transformation. Overexpression of Ezh2 is also linked with impairment of DNA repair machinery. Ezh2 is found to be responsible for poor prognosis in cellular transformation and to promote metastasis [181]. The mechanism of regulation of adhesion and migration via direct methylation of Talin seems to be conserved across different cell types and may hint towards role of Ezh2 in affecting cell-adhesive properties in the events of cellular transformations. Targeted disruption of interactions between Vav1 and Ezh2 abolished this regulatory effect of Ezh2 on Talin [180]. This may prove to be a promising treatment option in cases of Ezh2 and Vav1 linked carcinomas.

Fig. 4.3 Rapid cell migration requires coordinated action of VAV1 and Ezh2 along with other cellular partners (Image replicated from[182])

4.2.2 Treatment is to target Ezh2 interaction and not Ezh2

Even though Ezh2 is established as a tumor-marker, Ezh2 specific methyltransferase inhibitors are not the desired treatment options in Ezh2-linked carcinoma because Ezh2 is found to be crucial for maintenance of various somatic cells like, muscle, skin, hematopoietic and neuronal stem cells [183] [184]. Ezh2 is also reported to exhibit tumor-suppressor role [185]. Therefore any route to disrupt the interaction of VAV with Ezh2 is most desired outcome.

4.3 Vav1CH domain and Ezh2 interactions

Ezh2 is reported to interact with N-terminal region of vav1 in the CH domain [186]. This region of CH domain is highly conserved among the Vav family and has charged interactive surface. One part of this surface is positively charged and the other end is negatively charged.

Therefore it can be assumed that the Vav-Ezh2 interaction would be a predominantly electrostatic in nature. This is one of the conserved features of the CH domain interactions. Another feature is its favoritism to alpha helical partner motif for its interaction. The fragment of Ezh2 presumed to be interacting with this region is also alpha helical strengthening (Fig.4.4) the assumption.

Fig. 4.4 PRC2 complex structure with EZH2

(Inset fragment is the interest of this study), Ezh2 (Orange), EED (green) and SUZ12-VEFS (pink) in ribbon representation (Image modified from [187])

Part II AIMS AND OBJECTIVES

The aim of this project is to obtain the binding interface of two proteins Vav1 a GTP exchanger and Ezh2 which is a Histone Methyltransferase belonging to Polycomb Group protein Complex 2 (PRC2).

They are already proven to interact in the region of CH domain in case of Vav1 and in the region of residues 230 to 250 in case of Ezh2.

Therefore the aim was broken down into following objectives:

1. To express full length Vav1 in *E.coli*
2. To express Ezh2 fragment containing region of residues 230 to 250
3. Co-crystallization of this complex.
4. Another subsidiary option was to express CH domain of Vav1 and its co-crystallization with Ezh2 fragment.

During the course of the interesting journey of this project, we realized that the transient interaction between these two proteins would be better captured by NMR spectroscopy which is capable of structure determination in dynamic state like transient protein interaction than Crystallization which depends on stability of the molecules that can form nucleus of the crystal.

Therefore our current objectives also include the NMR structure of the interface along with co-crystallization.

Part II MATERIALS AND METHODS

5.1 Vav1

5.1.1 Vav1-CADPZ

5.1.1.1 Construct Vav1-CADPZ

Human Vav1 (Accession No NM-005428) residues 1-584 cloned into mbp-vector-p15Cool2-MBP-tev in E.coli BL21 DE3 was obtained from Prof. Su Ishin's laboratory

There is a Tev protease site in between the MBP+ 6 HIS tag and Vav1-CADPZ

5.1.1.2 Expression of Vav-1 CADPZ

BL21 DE3 was inoculated in LB media and was grown over-night at 37°C as preculture. Next day this culture was further inoculated into 1liter LB in 2 Liter Erline-Mayor Flask and incubated at 37°C till the OD₆₀₀ was reached to 0.7. At this OD₆₀₀ it was transferred to 4°C before induction with 0.2 mM IPTG. After induction it was incubated at 16°C for over-night.

5.1.1.3 Lysis of Cells

The frozen cell pellets were thawed on ice for 15 mins. One Liter cell pellet of 2gms wet weight was re-suspended in 30 ml of ice-cold Lysis buffer 50 mM Tris-Cl pH 7.4, 50mM NaCl, 2mMDTT, 5% Glycerol, 4.5µl protease inhibitor (Calbiochem). This re-suspension of cells was lysed in French Pressure cell, Panda, at 500 bar pressure for 10 mins at 4°C.

5.1.1.4 Two-Step Purifiaction of the Vav-1 CADPZ

Cell lysate was clarified by centrifuging at 18000 rpm for 30 minutes and subsequent syringe filtration. This clarified lysate was applied to 1ml of equilibrated Ni-resin from Quiagen. After 3 hours the Ni-resin was separated from the lysate and was washed 20 ml of with Lysis buffer and the protein was eluted on gradient of Imidazole concentration in lysis buffer.

5.1.1.5 Tev protease digestion

The IMAC purified protein was buffer exchanged to lysis buffer composition and was treated with Tev protease at 1:10 ratio (Tev:Protein) and was incubated overnight at 4°C.

The digested protein was further purified by Size-exclusion chromatography.

5.1.1.6 Size-exclusion Chromatography

The concentrated IMAC purified protein was centrifuged at a high-speed 14000rpm for 10 mins in a cold-centrifuge to remove any precipitate or aggregate which may clog the FPLC column. The concentration was measured on Nanodrop at this stage. The protein was injected on a pre-equilibrated and calibrated Superdex-200 FPLC column (Ge Healthcare). FPLC purification was conducted at Flow rate of 0.5 ml/min, at 4°C. The buffer used was 50 mM Tris-Cl pH 7.4, 50mM NaCl, 5% glycerol, 2mM DTT. 1ml fractions were collected after the void-volume of the column. The fractions corresponding to the symmetric peak at expected volume were visualized on 18% SDS-PAGE. The pure homogeneous fractions were pooled and concentrated on Vivaspın centricon with 30 kDa cut-off at 2000 rpm at 4°C.

5.1.2 Vav1 1-172 mouse Construct

Mouse Vav-1 construct (Acc. No. AAH20487.1) with residues from 1-172 (red inset) in cassette of pGEX6T vector was obtained from Prof. Su Ishin.

The expression is similar to Vav1 (CADPZ) construct, 1st step of purification was performed by applying the clarified lysate to Glutathione-S- transferase resin. The resin was washed with the lysis buffer and the protein was eluted in lysis buffer

Expression process is similar to Vav1 (1-172) and Vav1 CADPZ including lysis of cells with the lysis buffer similar to Vav1 CADPZ construct. A two step-purification was performed comprising of Ni-affinity and following Gel filtration on Superdex 75 16/60.

5.2.3 Ezh2 Double Mutant:

Wild type mouse Ezh2 construct containing amino acid residues 201-255 with His-tag in the pET28 vector were obtained from Prof. Su I H-sin (SBS, NTU). The wild type Mouse Ezh2 construct (Residue 201-255) in pET28 vector was used as template to generate a double mutant with site directed mutation from Lysine to Arginine at position 234 and 245.

5.3 Site directed mutagenesis was performed using Phusion Flash High-Fidelity PCR master mix with following Primers:

Ezh2 K234R forward:

tca atg ttt cca gat aga ggc acc gca gaa

Ezh2 K234R reverse:

tct tct gcg gtg cct cta tct gga aac att

Ezh2 K245R forward:

gaa ctg aaa gaa aaa tat cgt gaa ctc acg gag cag cag

Ezh2 K245R reverse:

ctg ctg ctc cgt gag ttc acg ata ttt ttc ttt cag ttc

The double mutation was confirmed by sequencing.

5.4 Expression and Purification of Ezh2 (Residues 201-255 Mouse)

Double mutant (Ezh2M)

NCBI Accession Number: AAH79538.1 of the wild type construct which is used as template for site-directed mutagenesis.

Expression and purification was performed employing similar method as the wild type Ezh2 construct as mentioned earlier.

The wild type and double mutant Ezh2 were labelled with ^{15}N and ^{13}C by growing the recombinant E.coli expression strain in M9 medium with 1gm of ^{15}N - NH_4Cl

and 2gms of ^{13}C - Glucose as sole source of Carbo and Nitrogen source. The rest of the methods were similar as mentioned before.

5.5 Circular Dichroism of Ezh2 (res 201-252)

The CD spectra were recorded on a Jasco J810 spectro-polarimeter using three accumulations of data at 0.1 nm intervals and were smoothed using the noise reduction routines provided with the instrument, including solvent background subtraction. The buffer contained 50mM Tris/HCl, 50mM Sodium Chloride pH 7.4 and the protein concentration was 0.1 mg /ml.

5.5.1 Co-Crystallization of Vav1 (mouse 1-172) and wild type Ezh2 (mouse 201-255):

The homogenously purified Vav1 (mouse1-172) and Ezh2 (mouse 201-255) were mixed in an eppendorf tube on ice in 1:1.5 molar ratio with 30mg/ml concentration together. This combination was set for crystallization trials using PegIon screen from Hampton Research. The ratio of precipitant and test sample was 1:1 in the screening drops. Both proteins individually were used as controls in this screening by setting the individual protein alongside the Cocrystallization test drop.

5.6 Nuclear Magnetic Resonance

(NMR experiments were performed by Li Yan from CongBao lab, ETC, Biopolis)

All the NMR experiments were collected at 25°C on a Bruker 700 or 600 MHz magnet equipped with a cryoprobe. The NMR data were processed using TopSpin 2.1 (www.bruker-biospin.com) and analyzed using CARA (www.nmr.ch). Sequence-specific assignment of backbone $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}'$ and $^{13}\text{C}^{\alpha}$ was achieved by using 2D ^1H - ^{15}N -HSQC, 3D HNCACB, HN(CO)CACB, HNCA, HN(CO)CA, HNCO and HN(CA)CO experiments on a $^{15}\text{N}/^{13}\text{C}$ -labeled EZH2 protein. Side-chain resonances were assigned using 3D HBHA (CO) NH, (H) CCH-TOCSY and HCCH-TOCSY. The secondary structure was analyzed using TALOS+ based on the obtained backbone chemical shifts [188]. NOE distance restraints were obtained from ^{15}N -NOESY-HSQC and ^{13}C -NOESY-HSQC spectra (both mixing time 120ms). Short-range and medium range NOE connectivities were used to establish

sequence-specific ^1H NMR assignments and to identify elements of the regular secondary structure. Structure calculations were performed using CYANA 3.0 [189, 190] and visualized using PyMOL (Delano Scientific) [159] [160] [161]. Structure Alignment of NMR Ezh2 structure obtained with the published Structure of Ezh2 in PRC2 complex

Most recently the crystal structure of the whole human PRC2 complex is published [2]. We also have our NMR structure of the Ezh2 construct (residues 201-252). Atomic Structure co-ordinates of the two structures were aligned with each other using computational tool PyMOL. The backbone and side-chains were matched to check orientation difference.

5.7 Vav1 and Ezh2 interaction Computational Model

Vav1 CH domain (PDB ID-3ky9, residues 1-126) and C-terminal helix (Residues 233-255) of Ezh2 NMR structure obtained were used for the Simulation (Fig 5.1).

Fig. 5.1 Structure Motifs selected for Hex Dock Prediction of interaction
Cyan fragment on Vav1 motif at extreme left is hypothesized to interact with Wild type and Double mutant Ezh2 fragments in grey

Both the proteins were first docked using Hex software [191] with reference to the mutation data reported by our collaborators as represented in Fig.6.1. This comparative model is just a representation of putative interaction residues predicted

from the series of mutation studies in Prof. Su I-hsin's laboratory. The molecules did not show any interaction in Docking experiments. Moreover the Ezh2 molecule was standing apart at a different portion (residues 112-116) of the Vav1 than the desired position at residues 89 to 104 at the lowest energy. But we preferred this position than the next where it stood at the opposite side altogether. The lowest energy interaction model was further used to generate the Molecular Dynamics Simulation through QuickMD application in VMD software [192]. Before proceeding to Simulations the molecules were put in a water-box of sufficient volume that extended to 15Å from the couple of the molecules from all the three dimensions and were equilibrated to 37°C and 150mM NaCl.

Fig.5.2 Conjugate Gradient Minimization for Sample Preparation in MD

The systems described were subjected to conjugate gradient minimization (Fig. 5.2) for 1000 steps and subsequently heated to 310 K in <4 ps. The procedure was followed by a 0.5 ns equilibration with both proteins fixed under an NPT ensemble. Finally, 30ns production simulations under an NPT ensemble were performed. To ensure that the starting structures retain the key interactions, the hydrogen bond and electrostatic network was maintained by imposing virtual springs to constrain their distances. The constants of these virtual springs were set to 50 kcal mol⁻¹ Å⁻² during minimization and 20 kcal mol⁻¹ Å⁻² during NPT ensemble equilibration. The constraints were removed in the final 30 ns simulation. Subsequently the simulations were performed using NAMD version 2.9 [193] assuming the CHARMM27 force field with CMAP corrections for the protein and assuming the TIP3P model for water molecules. 1500 frames of interaction were generated over

the time of 30ns on quickMD [194]. Periodic boundary conditions were assumed, and the particle mesh Ewald summation method was employed for the evaluation of Coulomb forces. The van der Waals energy was calculated using a cutoff of 12 Å. The temperature and pressure were maintained at 310 K and 1 atm, respectively, using a Langevin thermostat with a damping constant of 1 ps⁻¹ and Nose-Hoover Langevin piston methods. The integration time step was 1 fs. Residue-based interaction energies between Vav1 and Ezh2 were calculated using a modified generalized Born (GB) model. This model considers van der Waals interaction, electrostatics, and a solvation energy component that takes solvent polarization into account. Trajectory was run only once as our lab had limited resource like server access.

The same protocol was used to build Molecular Dynamics Simulation of double mutant Ezh2 K234, 245. The mutations were generated on VMD.

Part II RESULTS AND DISCUSSION

6.1 Brief Introduction:

Vav1 N-terminal CH domain, especially the residues after first 60 interact with Ezh2. Our collaborator Prof. Su I-hsin and group performed extensive mutation studies and pull down assays and proposed a model of interacting residues, which is represented as in (Fig.6.1).

Fig. 6.1 Comparative Model of Vav1-Ezh2 Interaction

Positively charged Lysine residues on Ezh2 are predicted to interact with negatively charged acidic residues glutamate and aspartate on Vav1 CH domain. With this initial hypothesis from our collaborator we designed our experiments to get structural analytical evidence of this proposed interaction. Our first approach was to get the structure of this interaction by crystallography. But later we also tried NMR to achieve the goal as we realized that the interaction is transient in nature. During the course of our crystallography and NMR experiments, we also tried to analyze this interaction by Molecular dynamics. Our results are discussed in following chapters with respect to different constructs that we used for our structure determination experiments.

6.2 Vav1 Expression and Purification

6.2.1 Vav1

6.2.1.1 Vav1 (MBP-His-CADPZ- human)

Vav1 (MBP-His-CADPZ) lysate was subjected to Ni-affinity purification by applying to 2ml Ni-resin. This construct was better purified by Ni affinity (Fig.6.1A) than on Amylose resin (Data not shown). Upon Tev digestion both the fractions bound to Ni-beads (Fig 6.1 B) therefore the Vav1 (CADPZ) was further purified on size exclusion S200 10/300 FPLC column (Fig 6.1 C and D)

Fig. 6.2 Vav1 (CADPZ) purification profile

(A) Ni-affinity separation. M: Protein marker ladder, L: Lysate, FT: Flow through, E1-E5: elution fractions from 100 mM – 500 mM Imidazole respectively (B) After TEV protease digestion Ni-Affinity purification. (C) Digest was put on Size Exclusion S 200 10/300 (D) Vav1 Fractions from (C) re-purified on S 200 10/300.

The yield was too low for crystallization (10µg/Liter). Therefore attempts were made to scale up the expression.

We were able to purify this Vav1 protein construct with first five domains intact. But, the yield invariably remained very low for Crystallization purpose. This was not economical and very time consuming. Therefore we decided to use the smaller Vav1 construct with first 172 amino acid residues derived from mouse. This construct also was from Prof. Su I H-sin's group.

6.2.2 GST-Vav1 (1-172 mouse) Purification

Vav1 (1-172) construct was first purified on Glutathione-S-Transferase Resin and then further polished using Size Exclusion on S200 16/60 FPLC column (Fig 6.2).

Fig. 6.3 Purification profile of GST-Vav1-1-172

This construct had very good yield after purification of about 6.6 mgs/Liter of culture. We obtained one crystal condition from co-crystallization of this protein with Ezh2 construct with 201-255 residues. The data is not shown here because of lack of diffraction data of the small crystal which we diffracted in IMCB in-house X-ray source. It was too small for adequate diffraction to obtain crystal structure.

Further, we could not reproduce this crystal condition which can be ascribed to the fact that the interaction is transient in nature imparting its own non-reproducibility.

Further we considered this construct for NMR experiments which needed its separation from the big GST tag to make it smaller in size suitable for NMR calculations.

We are in process of getting a pure cleaved Vav1 (1-172) without GST tag.

6.3 Ezh2 (res. 201-252 mouse)

The two step purification is as depicted in (Fig6.4 A and B). This construct results into a very stable protein.

Fig. 6.4 Ezh2 wild type construct two-step purification and CD spectrum of the Construct

A: Ni-affinity purification of wild type Ezh2 mouse (201-255) construct: L- cell lysate, M- molecular marker, FT-Flow through, W-10mM Imidazole wash, E1-E4-Elution Fraction over gradient of Imidazole concentration from 100mM to 400mM, B: Gel filtration on column S75 16/60 and corresponding 18% SDS-PAGE reducing gel of the purified fractions. C; Circular Dichroism chart showing alpha-helix of Ezh2 mouse (201-255) purified protein.

6.3.1 Ezh2 (201-255 mouse) Secondary Structure by Circular Dichroism (CD)

The CD spectrum obtained (Fig 6.4C) clearly demonstrates an alpha helical structure with dips at 208 nms and 222 nms wavelength [195].

6.3.2 NMR Results

Fig. 6.5 ^1H - ^{15}N HSQC Backbone Assignments of Wild Type Ezh2 Mouse (res.201-255)

NMR experiments were performed by Li Yan from CongBao lab, ETC, Biopolis

^1H - ^{15}N -HSQC spectra of EZH2 protein, with cross peaks labeled by one-letter code and residue number.

Backbone assignments were obtained by ^1H - ^{15}N -HSQC spectra of EZH2 protein as shown in Fig.6.5. Each peak represents individual residue of the protein.

HSQC using ^{15}N is a preferred experiment wherein structure of protein is determined using NMR method. Its simplicity and speed are the key determinants. Hence, it is employed to screen proteins that may be suitable for determination of structure by NMR. The media is labelled with ^{15}N is used to grow cells in which protein is expressed.

With only proline residue as an exception, the other residues of protein has an amide proton connected to each of it. Each such proton causes a peak in HSQC spectrum. Thus HSQC is instrumental in explaining relationship between ^{15}N and the amide

proton. The spectrum obtained is similar to a topographic map and demonstrates chemical shifts for ^1H and ^{15}N . If a protein is properly expressed, then the spectrum shows one peak for proton on backbone chain, and one peak for indole NH that is found on tryptophan. Amide group of residues found on sidechain – Asn and Gln – also show peaks. Properly folded and unfolded proteins give spectrum with sharp lines whereas less quality spectrum indicates existence of partially folded proteins.

As wide variety of chemical environment is found in the β -Sheet proteins, its spectra are better dispersed than proteins that contain α -helix structure. If there are random signal regions, then the number of signals in those regions is an indication of disorderliness that can be found in protein chain. Such information can be exploited by a X-ray Crystallographer when developing crystals.

To determine solution structure of protein using NMR requires assignment of resonance. It consists of determining chemical shift of every atom in protein. Triple resonance NMR experiment series output is generated. The experiment establishes connection between ^1H and ^{15}N and ^{13}C and then use the pattern mapping for preliminary protein sequence determination. Further processing involves calculating distance between protons, dihedral angles and hydrogen bonds.

The proton dipolar interaction utilizes the property that protons within radius of $6 \text{ \AA}$ in space sense presence of each other. The interaction is the nuclear Overhauser effect (NOE) of ^1H and ^1H and is an indirect indication of distance between hydrogen nuclei.

Covalent structure of molecule contains number of nuclei. Those sets of two nuclei that are less than three covalent bonds away demonstrate interactions termed as J coupling. This property is crucial for the aforementioned successful implementation of triple resonance spectrum wherein unique nucleus is assigned to each NMR signal.

Combining information on assignments, restraints such as bond lengths, J coupling and well known atomic properties results in three dimensional structure [196].

Fig. 6.6 Secondary structural analysis of EZH2.

NMR experiments were performed by Li Yan from CongBao lab, ETC, Biopolis

*** The residue no.1 corresponds to residue no. 202 of Wild type mouse Ezh2 construct.** (A) TALOS+ analysis of EZH2. The probability (0-1) to be a helix or not is plotted against residue number. (B) The difference of $C\alpha$ chemical shift from the value from a random-coil structure is plotted against residue number. (C) Dynamic analysis of Ezh2. The S^2 values derived from TALOS+ are plotted based on the backbone resonance chemical shifts.

Talos+ is a secondary structure prediction algorithm based on phi and psi torsion angle constraints exerted by a particular secondary structure. The accuracy of the prediction depends on the quality of the 3D structure under examination. In our case the prediction matches residue to residue with the Sequential NOE Plot described ahead. Thereby the Talos+ prediction and the sequential NOE plot both validate each other.

Fig. 6.7 Sequential NOE Plot

Sequential NOE connectivity of Wild Type Ezh2 (mouse 201-255) between each 4 residues is termed as $d_{\alpha N}(i, i+4)$ is a unique feature of α -Helix, The alphabets arranged on top are the amino acid sequence of Wild Type Ezh2 (mouse 201-255) represented as one letter code. NMR experiments were performed by Li Yan from CongBao lab, ETC, Biopolis

6.3.3 NOE Plots

The distance between hydrogen atoms (protons) located in residues is important for better understanding of secondary structure of polypeptides. The amino acid residues (consisting sequence of N, α and C on main branch) are denoted as i , $i+1$, $i+2$, sequentially. So $d_{\alpha N}$ refers to ^1H - ^1H distance of interest located on residues located sequentially whereas $d_{\alpha N(i, i+4)}$ refers to ^1H - ^1H bond from αC of residue at location i to N of residue located at $i+4$. Any connectivity beyond $i+1$ is considered as medium range.

Of the two types of regular secondary structures, α -helix is predominant. Some of the important general physical properties of α -helix are (<http://www.lorieau.com/nmr/2013/09/12/NOE-patterns.html>):

- No. of Residues for every turn = 3.6
- Translation distance = $1.5 \text{ \AA}$
- Pitch of $5.4 \text{ \AA}$ ($1.5 * 3.6$)
- Hydrogen bonding between 1st residue to 4th residue

Of the above the last one is the unique feature of α -helix. Hydrogen bond between residue at position i to residue at position $i+4$ i.e $d_{\alpha N(i,i+4)}$ (Fig.6.8) is only found in α -helix

(https://en.wikibooks.org/wiki/Structural_Biochemistry/Proteins/Structures#Determination_of_Primary_Structure:_Amino_Acid_Sequencing).

Fig. 6.8 NOE i+4 of α Helix

Diagrammatically, the accompanying sequential NOE plot shows the presence of $d_{\alpha N(i,i+4)}$ confirming α -helix. Each horizontal line indicates the links between residues which have hydrogen atom connections [197, 198].

Referring to (Fig.6.7) residue K just before location 250, the $d_{\alpha N(i,i+4)}$ – the last row – the connector link – the last line – is stretched to residue E just below location 250. So designating residue K as i , E as $i+1$, L as $i+2$, T as $i+3$, E becomes $i+4$. Thus confirming $d_{\alpha N(i,i+4)}$

Fig. 6.9 NOE Strip showing Helix Turn

NMR experiments were performed by Li Yan from CongBao lab, ETC, Biopolis

The ^1H - ^1H NOE strips (Fig. 6.9) indicate that the Alanine at position 237 in the C-terminal helix is neighboring residue to the Phenyl Alanine at position 224 in N-terminal helix. This establishes the Helix-turn-Helix motif of the final 3D structure of Wild type Ezh2 (mouse Res. 201-255) construct (Fig 6.10).

Note about this Collaboration: Our lab being a crystallography lab we collaborated with Dr.CongBao from ETC, Biopolis for this work for his expertise in NMR.

Fig. 6.10 3D Structure of Ezh2

On the Left: ensemble of 20 different dynamic positions captured of Ezh2. On the right: the one best position selected from the ensemble with both its N and C terminus.

The Ezh2 structures [2] and [187] were published at the same time as we were at our final stages of obtaining our Ezh2 NMR structure. When we started our NMR experiments these structures were not available.

None the less, the structure we obtained is of the fragment that is hypothesized to interact with Vav1 and in our study we use it as purified fragment. Therefore the isolated structure is relevant to this study. With these available structures of the complete Ezh2 in the PRC2 complex, we were able to compare our fragment structure with these biologically viable structures.

Fig. 6.11 Aligned 3D structures of Ezh2 NMR structure obtained with Published Ezh2 in PRC2 complex[2].

Computational pairwise alignment tracing α -Carbon atom on Chimera was performed. Over all Root Mean Square Deviations (RMSD) of the atom positions was 1.41 Å. This was showing that the structures were not very similar considering 100% sequence similarity in the chosen region of residues 222-248. This may be because the published structure is with an inhibitor. However the most relevant fact for our experimental interest was that the secondary structures tertiary folds of both molecules were 100% similar. The RMSD calculated only for the region of res.234-248 reduced to 0.9 Å. This is the stretch of the fragment that has the residues that are hypothesized to interact with Vav1. The overall fold of the protein also matches as seen in fig 6.11. This is encouraging because this proves that our Ezh2 fragment behaves as in native conditions in isolation.

The Ezh2 fragment 201-255 3D-NMR structure (Fig.6.10) is as expected an α -helix structure. More remarkably, α -helix turn α -helix motif observed is similar to Sant domain 1 arconditionecture [2]. C-terminal helix is the one which bears the residues which are hypothesized to interact with Vav1 CH domain.

The Sant 1 domain isoelectric pH matches with Ezh2 construct to be very acidic as 4.6. This explains the transient nature of the interaction. Both the faces, which

interact, demonstrate predominantly negative charges. It is most intriguing how this interaction establishes which will be revealed upon acquisition of the atomic structure of the interaction. From Fig.6.11, it is evident that the Ezh2 construct assumes the same orientation as is present in native cellular conditions as previously discussed. The ensemble of 20 different dynamic positions of Ezh2 indicates that the wild type Ezh2 is very dynamic in solution. This may be contributing to the transient nature of the interaction.

In our ongoing work we are pursuing this atomic structure of the interaction by two approaches. One approach is by NMR we will try to get the interacting residues at the interface. Second approach is co-crystal X-ray structure of the complex.

6.3.4 GST-Vav1 (mouse 1-172) and Wild Type Ezh2 (mouse 201-255) Co-crystallization:

The GST-Vav1 (mouse 1-172) and Wild Type Ezh2 (mouse 201-255) interaction is comparatively a transient association of 0.1 μ M KD (SPR results from Prof. Su I-hsin group) than the previously described antigen-antibody interactions with nanomolar KD. Therefore the complex of Vav1 (mouse 1-172) and Wild Type Ezh2 (mouse 201-255) did not survive the severe dilution on Gel filtration by size-exclusion chromatography. Hence, we opted for co-crystallization approach by mixing these pure proteins Vav1 (mouse 1-172) and Wild Type Ezh2 (mouse 201-255) in 1:1.5 molar ratio respectively before setting up the crystallization trials. We got a condition in 5 days in Peg-Ion Screen H4 (0.07 mM Bis-tris Propane pH 7.6, 0.003% Citric Acid, 20% PEG 3350). Fig 6.12

*Vav-1 + Ezh2 (1:1.5) co-crystallization
0.07 mM Bis-tris Propane pH 7.6, 0.003% Citric
Acid, 20% PEG 3350*

Fig. 6.12 GST-Vav1 (mouse 1-172) and Wild Type Ezh2 (mouse 201-255)

Co-crystallization Condition.

This crystal was very small for optimal diffraction. However it was confirmed as a protein crystal in an in-house X-ray diffraction. We did not have any proof of interaction on Size-exclusion column as in case of our previous study of antigen-antibody other than the SPR result from our collaborator. However, we had set individual protein crystallization drops along with this co-crystallization trials in the same screening plates for control. This crystal was formed only in the co-crystallization drop. Therefore there is higher probability that the crystal is of the complex over solitary partners.

Unfortunately, the condition of the crystal did not reproduce the crystal in repeat experiments. It was highly intriguing to pinpoint any reason for this problem. The most proactive approach was to check around this condition with different concentrations of the proteins and the associated reagents in the buffer condition to address the problem of weak interaction. We tried this and we also are currently trying the buffer screening from the scratch.

6.3.5 Computational model of Vav1 and Ezh2 Interaction

Hex software generated ensemble of 2000 docked models for each of the interaction involving wild type Ezh2 and the Mutant. We chose the model with thermodynamically lowest energy and which was agreeing with our prediction of interaction (Fig.6.1).

MD Simulation data shows (Fig.6.13) interaction of K234 of Ezh2 with D104 of Vav1. Out of 1500 frames of the simulation, this interaction stayed till the first 1081 frames which translate to 21 nanoseconds (Fig 6.14).

Mutations K234R and K245R are shown to strengthen the interaction by our collaborators (Unpublished data). MD simulation data (Fig.6.13 and 6.14 Mutant) agrees with the hypothesis and shows that the interaction persists the full 30ns Simulation. This is stronger interaction as compared to the Wild type interaction. From the model it looks like the R (Arginine) at position 245 does not interact with Vav1 but in turn balances the intra molecular negative charges and thereby enhances the contact. The simulation reveals that the K245 R mutation gradually engages both the E 246 and 249 residues in the same molecule. Also it is observed that at 15 nanoseconds K234R interaction with CH domain residues D 98 and 104 is improved (The bond lengths in Fig. 6.14).

Fig. 6.13 MD Simulation of Vav1 and Ezh2

Ezh2 fragment (Res 234-252) in Grey and Vav1 CH domain (PDB 3ky9 residues 2-126) in Cyan. Interacting residues are represented as sticks. The mutated residues on Ezh2 fragment (K234R and K245R are in black circles) MD simulation was performed over the total time of 30 nanoseconds (ns)

Fig. 6.14 Molecular Dynamic Simulation Analysis of affinity interaction of Wild type and Double mutant (L234R and L245R) Ezh2 with CH domain of Vav1 over time scale

Ezh2 fragment (Res 234-252) in Grey and Vav1 CH domain (PDB 3ky9 residues 2-126) in Cyan. Interacting residues are represented as sticks. The mutated residues on Ezh2 fragment (K234R and K245R) are in black circles. At 21 ns Wild type Ezh2 is totally dissociated from CH domain of Vav1 in contrast to the double mutant Ezh2. Double mutant Ezh2 is observed to have improved contact with CH domain gradually with time.

6.3.6 Ezh2 Double Mutant (K234, 245R) Expression and Purification:

Prof. Su I-hsin's group reported that double mutation of Lysine to Arginine in Ezh2 at positions 234 and 245 strengthen the interaction between Ezh2 and Vav1 as compared to Wild type by Pull down assay.

Our MD simulation also corroborates with this hypothesis.

Therefore we created a double mutant of the Ezh2 construct (Residues 201-255). The expression and purification was successfully achieved and the results are shown in the figure.6.15

Fig. 6.15: Ezh2 Double Mutant Purification Profile

A: Nickle-Affinity Purification of Ezh2 Double mutant.

SDS PAGE shows purified elutions over gradient of imidazole in last 5 wells.

M: Standard marker, L: lysate; FT: Flow through; W: Wash with 15% Imidazole

B: Purification of Ni-affinity purified Ezh2 double mutant on Superdex 75 16/60 column by size exclusion chromatography. Purity checked on SDS-PAGE gel

We are optimizing an Iso-Thermal Calorimetry experiment of this double mutant as a ligand with Vav1 (Residues 1-172). We have set co-crystallization trials of these proteins and we have generated Carbon and Nitrogen labelled proteins with an aim to try NMR experimentation of the interaction.

It is clear from the NMR structure of the wild type Ezh2 construct that it forms a tight dimer which is also apparent in the Size-exclusion chromatography as its elution volume. The double mutant Ezh2 construct elutes at exactly same elution volume indicating that it also is a dimer. This means this Ezh2 construct will recruit two molecules of vav1. This will complicate NMR experiment outcome.

Part II CONCLUSIONS

During our effort to capture 3D atomic structure of the already reported interaction of Vav1 and Ezh2 we could churn out some facts as important milestones towards the goal.

Computationally the interaction was analyzed with limited resources considering our lab-setting as crystallography lab. The interaction involving Wild type Ezh2 was found to be transient in nature with prominent presence of acidic residues. Still the Lysine at position 234 was found to contact Vav1 interacting surface with predominantly acidic residues. This might be playing important role in establishing this weak interaction. The double mutant with Arginine at position 234 and 245 on Ezh2 was found to strengthen this interaction further. Arginine at 234 anchors on the residue 104 on the Vav1 and Arginine at 245 found to interact with intramolecular acidic residues on Ezh2 strand.

The co-crystallization success offers a hope of obtaining a crystal structure of this interaction.

The NMR structure of Wild Type Ezh2 was planned because no structure was available at the time. But, full Ezh2 in PRC2 complex was published during our course of study. Our NMR structure still gives information that the mouse Ezh2 has the same tertiary structure and the construct behaves and folds exactly in similar fashion as in physiological condition. Another additional information provided by our NMR structure is that the structure is dynamic in solution than the published one in crystallographically fixed condition. The NMR ensemble of Ezh2 construct shows that it is very dynamic.

Part II ONGOING WORK

Co-Crystallization will be tried with the mutant version of Ezh2 fragment with K234R and K245R mutations which are recently observed by our collaborators to make the interaction 10 times stronger. This will hopefully impart stability to the interaction making it feasible for Crystallization.

The Cystein in position 213 in Ezh2 may be forming tight di-sulphide bond with neighboring molecule imparting the dimer of the Ezh2. We have ordered a trimmed peptide starting from residue 218-250 of the double mutant version. This should facilitate NMR and crystallization both.

Meanwhile, the K_D value of this improved interaction of this mutant will be measured either by SPR or ITC (Isothermal Calorimetry).

EPILOGUE

The two projects that this thesis addresses were linked at one similarity that both of them involved protein-protein interaction. At the end of the journey, after relevant literature review, a striking relation is revealing itself.

Both these projects converge at the juncture of immunological response. Dengue virus exhibits a very characteristic ability to evoke a strong immune response from all the faculties of immune system including the innate response, Antigen presenting cells, and all the arms of acquired immune system. The healthy immune response involves both the pro-inflammatory pathways and the regulatory pathways in proper balance. The immune response is ideal so Dengue virus is being considered as a treatment to generate vaccine for cancers[160]. The T-cell mediated response was of significance here because it triggered a strong inflammatory response that aided the vaccination towards the cancer.

The severe disease in Dengue infected cases demonstrate flawed balance of intricate cytokine signaling and movement of cells is increased by decreased adhesion through actin-mediated pathway. In T-cell mediated signaling in this cell migrations the Vav1-Ezh2 also are found to influence there effect. This convergence opens up avenues for research further to analyze if Vav1-Ezh2 interaction can play as a switch to control the non-regulated cytokine storm in severe Dengue cases?

APPENDIX

Appendix I: Reported Envelope domain III residues and their significance

Table AP I: Summary of reported significances of Envelop domain III

ED3 Residues	Significance
Lys 291 and 295	Interaction with Glycosaminoglycans (Heparan Sulfate receptors) [199]
	Possible role in rearrangement between pre and post fusion for DI-DIII linker at 291-301 [199]
K305, K307	Solvent accessible residues on A strand at Five-fold pore of icosahedral virus [199]
K310	Solvent accessible , covers Tryptophan 101 at tip of fusion loop of partner monomer [69, 199]
A strand Res 306-314	Solvent exposed residues with Central bulge at 309-312 [200]
Residue 305,310,311	Target of cross reactive antibody [201]
Residue 305, 388 CD loop region	Target of type specific antibody [201]
B Strand Res T320- Y326	Exposed on viral particle, interaction with ED II of partner E protein [200]
E strand R350- I351	Solvent exposed, interaction with ED II of partner E protein [200]
G strand V 365-E 370	Solvent exposed, interaction with ED II of partner E protein [200]
C strand and F strand	Interaction with EDI of partner E protein [200]
I 368	Salt-bridge with R9 of DI at neutral pH and interaction with H 317 at endosomal low pH [202]
BC(325-334) and DE Loop(358-365)	Face Five-fold axis , the most exposed on icosahedral virus, cross-protective antibody target [200]
Residues at N and C termini	Highly dynamic [202]
BC, CD and EF loops	More flexible than β -strands [202]
DE loop	comparatively less flexible and solvent accessible [202]
FG loop (380 -389)	Mosquito cell interactions [199]
Tyrosine 326	Critical for folding of virus, forms Hydrogen bonds with 303 and 304.[112]

**Appendix II: Comparison of Envelope domain III Epitopes of Ab 513, 4E11
1A1D2 3E31 and 2D73**

Table AP II: Epitopes of Ab 513, 4E11, 1A1D2, 3E31 and 2D73 at a glance.

Antibody	Total Contacts	Buried Area Å ²		Epitopes	Location	Serotypes	Interaction
Ab513[88]	20 H-bonds, 13 salt-bridges		898	Glu 362	DE loop	denv1 and3	Electrostatic
				Asp 362	DE loop	denv2	Electrostatic
				Glu 363	DE loop	denv3	Electrostatic
				Glu 311	A strand	All 4	Electrostatic
				Lys 310	A strand	All 4	Electrostatic
				Lys323	B strand	denv 4 and 3	Electrostatic
				Val 364	G strand	denv 4	Hydrophobic
				Lys 305	A strand	denv 4 and2	
				Ala 329	B strand	denv 4 and2	
				Glu 327	B strand	denv 4	H-bond
Ab4E11[69]	16 H-bonds, 8 salt bridges			308-312	A strand	Core Epitope	
				387, 389,391	G Strand	in AG patch	
				Lys 310	A strand	All 4	Salt-bridge
				Glu 311	A strand	All 4	Salt-bridge
				Asp 309	A strand	Denv 4	
				Glu 309	A strand	denv 1	H-bond
				Lys 307	A strand	denv1	H-bond
				Glu 325	B strand	denv 3	H-bond
				Glu 327	B strand	denv3	H-bond
1A1D2[142]	8 H-bonds		940	Phe 306	A strand	All 4	H-bond
				Asn 390	G strand	denv 2 and 3	H- Bond
				Lys 388	G strand	denv 1,2 and 3	H-bond
				Lys 305	A strand	denv 2 and 4	H-bond
				Lys 310	A strand	All 4	H-bond
				Pro 364	E strand	denv1,2 and 3	H-bond

Table AP II: Epitopes of Ab 513, 4E11, 1A1D2, 3E31 and 2D73 at a glance.

Antibody	Total Contacts	Buried Area Å ²	Epitopes	Location	Serotypes	Interaction
Ab3E31[89]	8 H-bonds,2 salt-bridges	1210	Gln 316	AB loop	All 4	Electrostatic
			His 317	AB loop	All 4	Electrostatic
			Glu 368	E strand	All4	Electrostatic
			Glu 370	E strand	All 4	Electrostatic
			Ala 313-315	A strand	313 Denv 2,3,4, rest all 4	Vander Waal
			Lys323	B strand	denv 4 and 3	Vander Waal
			Val 321	B strand	Zika, Denv1	Vander Waal
			Ile352	D loop	All denv and zika	Vander Wall
Ab2D73[92]	16 H-bonds, 6 salt bridges	1640	Ile308,Asp309	A strand	Denv4	H-bond
			Glu 311,314	Astrand	All 4	H-bond
			Gln 316	A strand	All 4	H-bond
			Lys 323	B strand	Denv 2,3, 4	H-bond
			Tyr 377	F strand	Denv 1,2,4	H-bond
			His 390	C-terminus	Denv4	H-bond
			Phe 392	C-terminus	denv1,2,4	H-bond
			Lys 394	C-terminus	All 4	H-bond
			Val 364	DE loop	Denv4	Vander Waal
				G strand	denv 2 and 3	H- Bond
				G strand	denv 1,2 and 3	H-bond
				A strand	denv 2 and 4	H-bond
				A strand	All 4	H-bond
				E strand	denv1,2 and 3	H-bond

Appendix III: Flaviviridae Phylogenetic Tree

Fig.AP V: Phylogenetic tree based on the analysis (neighbour joining) of aligned conserved motifs of the RNA dependent RNA polymerase (RdRp). Shown are selected members of the family. A distance scale corresponding to amino acid substitutions per position is shown [3].

Appendix IV: Modular Structure of Vav1

Fig. AP VI: Vav1 Auto inhibition

Domains of Vav1 are colored: CH (gold), Ac (red), DH (blue), PH (green), ZF (magenta), SH3 (wconditione), SH2 (wconditione).

(A) Domain architecture of Vav1 and thermodynamic model for cooperative inhibition. Solid lines represent direct physical contacts; dashed line represents thermodynamic coupling between core and modulatory equilibria. (B) Schematic representation of autoinhibited CADPZ. Dotted lines outline C-terminal helix of DH domain lying behind PH and CH domains. (C) Ribbon diagram of Vav1 CADPZ. Dashed lines indicate regions not observed in the electron density map. Side chains of tyrosines 142, 160, and 174 are shown as sticks. DH active site is circled. The boxed area depicts the regions of inter-domain contacts.

REFERENCES

1. Rajamanonmani, R., et al., *On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes*. J Gen Virol, 2009. **90**(Pt 4): p. 799-809.
2. Justin, N., et al., *Structural basis of oncogenic histone H3K27M inhibition of human polycomb repressive complex 2*. Nature communications, 2016. **7**.
3. Romero-Brey, I. and R. Bartenschlager, *Membranous replication factories induced by plus-strand RNA viruses*. Viruses, 2014. **6**(7): p. 2826-2857.
4. Lok, S.-M., *The interplay of dengue virus morphological diversity and human antibodies*. Trends in microbiology, 2016. **24**(4): p. 284-293.
5. Pettersen, E.F., et al., *UCSF Chimera—a visualization system for exploratory research and analysis*. Journal of computational chemistry, 2004. **25**(13): p. 1605-1612.
6. Li, L., et al., *The flavivirus precursor membrane-envelope protein complex: structure and maturation*. Science, 2008. **319**(5871): p. 1830-1834.
7. Ferlenghi, I., et al., *Molecular organization of a recombinant subviral particle from tick-borne encephalitis virus*. Mol Cell, 2001. **7**(3): p. 593-602.
8. Allison, S.L., et al., *Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E*. Journal of virology, 1999. **73**(7): p. 5605-5612.
9. Kuhn, R.J., et al., *Structure of dengue virus: implications for flavivirus organization, maturation, and fusion*. Cell, 2002. **108**(5): p. 717-25.
10. Gea-Banacloche, J., et al., *West Nile virus: pathogenesis and therapeutic options*. Ann Intern Med, 2004. **140**(7): p. 545-53.
11. Heinz, F.X. and S.L. Allison, *Flavivirus structure and membrane fusion*. Advances in virus research, 2003. **59**: p. 63-97.
12. Simons, J.N., et al., *Isolation of novel virus-like sequences associated with human hepatitis*. Nat Med, 1995. **1**(6): p. 564-9.
13. Gould, E.A., et al., *Origins, evolution, and vector/host coadaptations within the genus Flavivirus*. Adv Virus Res, 2003. **59**: p. 277-314.
14. Mostashari, F., et al., *Epidemic West Nile encephalitis, New York, 1999: results of a household-based seroepidemiological survey*. Lancet, 2001. **358**(9278): p. 261-4.
15. Kramer, L.D., L.M. Styer, and G.D. Ebel, *A global perspective on the epidemiology of West Nile virus*. Annu Rev Entomol, 2008. **53**: p. 61-81.
16. Wilder-Smith, A. and P. Byass, *The elusive global burden of dengue*. The Lancet Infectious Diseases. **16**(6): p. 629-631.
17. Shepard, D.S., et al., *The global economic burden of dengue: a systematic analysis*. The Lancet Infectious Diseases, 2016.
18. Messina, J.P., et al., *Global spread of dengue virus types: mapping the 70 year history*. Trends Microbiol, 2014. **22**(3): p. 138-46.

19. De Filette, M., et al., *Recent progress in West Nile virus diagnosis and vaccination*. Vet Res, 2012. **43**: p. 16.
20. Kramer, L.D., J. Li, and P.Y. Shi, *West Nile virus*. Lancet Neurol, 2007. **6**(2): p. 171-81.
21. Hayes, E.B., et al., *Epidemiology and transmission dynamics of West Nile virus disease*. Emerg Infect Dis, 2005. **11**(8): p. 1167-73.
22. Chan, M., *Zika Strategic Response Plan*. 2016. p. World Health Organization.
23. Chan, M., *Zika Situation Report*. 2016f, World Health Organization.
24. Lindenbach, B.D. and C.M. Rice, *Molecular biology of flaviviruses*. Adv Virus Res, 2003. **59**: p. 23-61.
25. Thullier, P., et al., *Mapping of a dengue virus neutralizing epitope critical for the infectivity of all serotypes: insight into the neutralization mechanism*. J Gen Virol, 2001. **82**(Pt 8): p. 1885-92.
26. Chu, J.J. and M.L. Ng, *Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway*. J Virol, 2004. **78**(19): p. 10543-55.
27. Deubel, V., et al., *Variations in biological features of West Nile viruses*. Ann N Y Acad Sci, 2001. **951**: p. 195-206.
28. Zhang, W., et al., *Visualization of membrane protein domains by cryo-electron microscopy of dengue virus*. Nat Struct Biol, 2003. **10**(11): p. 907-12.
29. Zhang, Y., et al., *Structures of immature flavivirus particles*. Embo j, 2003. **22**(11): p. 2604-13.
30. Fibriansah, G., et al., *Structural changes in dengue virus when exposed to a temperature of 37 degrees C* J Virol. **87**: 7585–7592. 2013.
31. Kostyuchenko, V.A., et al., *Structure of the thermally stable Zika virus*. Nature, 2016. **533**(7603): p. 425-8.
32. Mukhopadhyay, S., R.J. Kuhn, and M.G. Rossmann, *A structural perspective of the flavivirus life cycle*. Nat Rev Microbiol, 2005. **3**(1): p. 13-22.
33. Modis, Y., et al., *Structure of the dengue virus envelope protein after membrane fusion*. Nature, 2004. **427**(6972): p. 313-9.
34. Li, X.-Q., et al., *Dengue virus envelope domain III immunization elicits predominantly cross-reactive, poorly neutralizing antibodies localized to the AB loop: implications for dengue vaccine design*. Journal of General Virology, 2013. **94**(10): p. 2191-2201.
35. de Oliveira Trugilho, M.R., et al., *Platelet proteome reveals novel pathways of platelet activation and platelet-mediated immunoregulation in dengue*. PLoS Pathogens, 2017. **13**(5): p. e1006385.
36. Chen, H.-W., et al., *A consensus envelope protein domain III can induce neutralizing antibody responses against serotype 2 of dengue virus in non-human primates*. Archives of virology, 2013. **158**(7): p. 1523-1531.

37. Dejnirattisai, W., et al., *Cross-reacting antibodies enhance dengue virus infection in humans*. Science, 2010. **328**(5979): p. 745-8.
38. Modis, Y., et al., *Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein*. J Virol, 2005. **79**(2): p. 1223-31.
39. Roehrig, J.T., R.A. Bolin, and R.G. Kelly, *Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica*. Virology, 1998. **246**(2): p. 317-28.
40. Modis, Y., et al., *A ligand-binding pocket in the dengue virus envelope glycoprotein*. Proc Natl Acad Sci U S A, 2003. **100**(12): p. 6986-91.
41. Crill, W.D. and G.J. Chang, *Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes*. J Virol, 2004. **78**(24): p. 13975-86.
42. Stiasny, K., et al., *Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites*. J Virol, 2006. **80**(19): p. 9557-68.
43. Wu, K.P., et al., *Structural basis of a flavivirus recognized by its neutralizing antibody: solution structure of the domain III of the Japanese encephalitis virus envelope protein*. J Biol Chem, 2003. **278**(46): p. 46007-13.
44. Volk, D.E., et al., *Solution structure and antibody binding studies of the envelope protein domain III from the New York strain of West Nile virus*. J Biol Chem, 2004. **279**(37): p. 38755-61.
45. Volk, D.E., et al., *Solution Structure of the Envelope Protein Domain III of dengue-4 virus*. Virology, 2007. **364**(1): p. 147-54.
46. Huang, K.C., et al., *Solution structure and neutralizing antibody binding studies of domain III of the dengue-2 virus envelope protein*. Proteins, 2008. **70**(3): p. 1116-9.
47. Wang, E., et al., *Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses*. J Virol, 2000. **74**(7): p. 3227-34.
48. Huerta, V., et al., *Characterization of the interaction of domain III of the envelope protein of dengue virus with putative receptors from CHO cells*. Virus Res, 2008. **137**(2): p. 225-34.
49. Hung, J.J., et al., *An external loop region of domain III of dengue virus type 2 envelope protein is involved in serotype-specific binding to mosquito but not mammalian cells*. J Virol, 2004. **78**(1): p. 378-88.
50. Hahn, C.S., et al., *Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it*. Proc Natl Acad Sci U S A, 1987. **84**(7): p. 2019-23.
51. Jennings, A.D., et al., *Analysis of a yellow fever virus isolated from a fatal case of vaccine-associated human encephalitis*. J Infect Dis, 1994. **169**(3): p. 512-8.
52. Wang, T., et al., *Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis*. Nat Med, 2004. **10**(12): p. 1366-73.

53. Chen, Y., et al., *Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate*. Nat Med, 1997. **3**(8): p. 866-71.
54. Pokidysheva, E., et al., *Cryo-EM reconstruction of dengue virus in complex with the carbohydrate recognition domain of DC-SIGN*. Cell, 2006. **124**(3): p. 485-93.
55. Navarro-Sanchez, E., et al., *Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses*. EMBO Rep, 2003. **4**(7): p. 723-8.
56. Miller, J.L., *The Mannose Receptor Mediates Dengue Virus Infection of Macrophages*. 2008. **4**(2).
57. Thepparit, C. and D.R. Smith, *Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor*. J Virol, 2004. **78**(22): p. 12647-56.
58. Tio, P.H., W.W. Jong, and M.J. Cardoso, *Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3*. Virol J, 2005. **2**: p. 25.
59. Munoz, M.L., et al., *Putative dengue virus receptors from mosquito cells*. FEMS Microbiol Lett, 1998. **168**(2): p. 251-8.
60. Peiris, J.S. and J.S. Porterfield, *Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines*. Nature, 1979. **282**(5738): p. 509-11.
61. Beasley, D.W. and A.D. Barrett, *Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein*. J Virol, 2002. **76**(24): p. 13097-100.
62. Lin, C.W. and S.C. Wu, *A functional epitope determinant on domain III of the Japanese encephalitis virus envelope protein interacted with neutralizing-antibody combining sites*. J Virol, 2003. **77**(4): p. 2600-6.
63. Nawa, M., *Effects of bafilomycin A1 on Japanese encephalitis virus in C6/36 mosquito cells*. Arch Virol, 1998. **143**(8): p. 1555-68.
64. Bressanelli, S., et al., *Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation*. Embo j, 2004. **23**(4): p. 728-38.
65. Harrison, S.C., *Mechanism of membrane fusion by viral envelope proteins*. Adv Virus Res, 2005. **64**: p. 231-61.
66. Harrison, S.C., *Viral membrane fusion*. Nat Struct Mol Biol, 2008. **15**(7): p. 690-8.
67. Weissenhorn, W., A. Hinz, and Y. Gaudin.
68. Midgley, C.M., et al., *Structural analysis of a dengue cross-reactive antibody complexed with envelope domain III reveals the molecular basis of cross-reactivity*. The Journal of Immunology, 2012. **188**(10): p. 4971-4979.

69. Cockburn, J.J., et al., *Mechanism of dengue virus broad cross-neutralization by a monoclonal antibody*. Structure, 2012. **20**(2): p. 303-14.
70. Gubler, D.J., *Dengue and Dengue Hemorrhagic Fever*. Clin Microbiol Rev, 1998. **11**(3): p. 480-96.
71. Gibbons, R.V. and D.W. Vaughn, *Dengue: an escalating problem*. Bmj, 2002. **324**(7353): p. 1563-6.
72. Brandt, W.E., et al., *Infection enhancement of dengue type 2 virus in the U-937 human monocyte cell line by antibodies to flavivirus cross-reactive determinants*. Infect Immun, 1982. **36**(3): p. 1036-41.
73. Samuel, M.A. and M.S. Diamond, *Pathogenesis of West Nile Virus Infection: a Balance between Virulence, Innate and Adaptive Immunity, and Viral Evasion*. J Virol, 2006. **80**(19): p. 9349-60.
74. Hamel, R., et al., *Biology of Zika Virus Infection in Human Skin Cells*. J Virol, 2015. **89**(17): p. 8880-96.
75. Lai, C.Y., et al., *Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II*. J Virol, 2008. **82**(13): p. 6631-43.
76. Halstead, S.B., *Dengue Antibody-Dependent Enhancement: Knowns and Unknowns*. Microbiol Spectr, 2014. **2**(6).
77. Fagbami, A.H., et al., *Cross-infection enhancement among African flaviviruses by immune mouse ascitic fluids*. Cytobios, 1987. **49**(196): p. 49-55.
78. Ludwig, G.V., et al., *Viremic enhancement due to transovarially acquired antibodies to St. Louis encephalitis virus in birds*. J Wildl Dis, 1986. **22**(3): p. 326-34.
79. Moi, M.L., T. Takasaki, and I. Kurane, *Human antibody response to dengue virus: implications for dengue vaccine design*. Tropical Medicine and Health, 2016. **44**(1): p. 1.
80. Guzman, M.G. and S. Vazquez, *The Complexity of Antibody-Dependent Enhancement of Dengue Virus Infection*. Viruses, 2010. **2**(12): p. 2649-62.
81. Rodenhuis-Zybert, I.A., et al., *Immature dengue virus: a veiled pathogen?* (1553-7374 (Electronic)).
82. Schmidt, A.C., *Response to dengue fever--the good, the bad, and the ugly?* N Engl J Med, 2010. **363**(5): p. 484-7.
83. Beltramello, M., et al., *The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity*. Cell Host Microbe, 2010. **8**(3): p. 271-83.
84. Wahala, W.M., et al., *Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody*. Virology, 2009. **392**(1): p. 103-13.

85. Wahala, W.M., et al., *Recombinant dengue type 2 viruses with altered e protein domain III epitopes are efficiently neutralized by human immune sera*. J Virol, 2012. **86**(7): p. 4019-23.
86. Williams, K.L., et al., *Antibodies targeting dengue virus envelope domain III are not required for serotype-specific protection or prevention of enhancement in vivo*. Virology, 2012. **429**(1): p. 12-20.
87. Parameswaran, P., et al., *Convergent antibody signatures in human dengue*. Cell host & microbe, 2013. **13**(6): p. 691-700.
88. Robinson, L.N., et al., *Structure-Guided Design of an Anti-dengue Antibody Directed to a Non-immunodominant Epitope*. Cell, 2015. **162**(3): p. 493-504.
89. Li, J., et al., *Structural and Functional Characterization of a Cross-Reactive Dengue Virus Neutralizing Antibody that Recognizes a Cryptic Epitope*. Structure, 2017.
90. Tharakaraman, K., et al., *Redesign of a cross-reactive antibody to dengue virus with broad-spectrum activity and increased in vivo potency*. Proceedings of the National Academy of Sciences, 2013. **110**(17): p. E1555-E1564.
91. Barba-Spaeth, G., et al., *Structural basis of potent Zika-dengue virus antibody cross-neutralization*. Nature, 2016. **536**(7614): p. 48-53.
92. 黄艳芬, 登革病毒包膜蛋白 III 区单抗中和作用与作用机制研究以及登革病毒初次感染患者血清中和抗体反应分析. 2013, 万方数据资源系统.
93. Pierson, T.C., et al., *Structural insights into the mechanisms of antibody-mediated neutralization of flavivirus infection: implications for vaccine development*. Cell Host Microbe, 2008. **4**(3): p. 229-38.
94. Acosta, E.G. and R. Bartenschlager, *Paradoxical role of antibodies in dengue virus infections: considerations for prophylactic vaccine development*. Expert review of vaccines, 2016. **15**(4): p. 467-482.
95. Kohler, G. and C. Milstein, *Continuous cultures of fused cells secreting antibody of predefined specificity*. Nature, 1975. **256**(5517): p. 495-7.
96. Brien, J.D., et al., *Genotype-specific neutralization and protection by antibodies against dengue virus type 3*. J Virol, 2010. **84**(20): p. 10630-43.
97. de Alwis, R., et al., *In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection*. PLoS Negl Trop Dis, 2011. **5**(6): p. e1188.
98. Lai, C.Y., et al., *Analysis of cross-reactive antibodies recognizing the fusion loop of envelope protein and correlation with neutralizing antibody titers in Nicaraguan dengue cases*. PLoS Negl Trop Dis, 2013. **7**(9): p. e2451.
99. Smith, S.A., et al., *The Potent and Broadly Neutralizing Human Dengue Virus-Specific Monoclonal Antibody 1C19 Reveals a Unique*

- Cross-Reactive Epitope on the bc Loop of Domain II of the Envelope Protein*. mBio, 2013. **4**(6).
100. Weisser, N.E. and J.C. Hall, *Applications of single-chain variable fragment antibodies in therapeutics and diagnostics*. Biotechnol Adv, 2009. **27**(4): p. 502-20.
 101. Crivianu-Gaita, V. and M. Thompson, *Aptamers, antibody scFv, and antibody Fab' fragments: An overview and comparison of three of the most versatile biosensor biorecognition elements*. Biosens Bioelectron, 2016. **85**: p. 32-45.
 102. Pasman, Y. and A.K. Kaushik, *VH and VL Domains of Polyspecific IgM and Monospecific IgG Antibodies Contribute Differentially to Antigen Recognition and Virus Neutralization Functions*. Scand J Immunol, 2016. **84**(1): p. 28-38.
 103. Brekke, O.H. and I. Sandlie, *Therapeutic antibodies for human diseases at the dawn of the twenty-first century*. Nat Rev Drug Discov, 2003. **2**(1): p. 52-62.
 104. Beck, A., et al., *Strategies and challenges for the next generation of therapeutic antibodies*. Nat Rev Immunol, 2010. **10**(5): p. 345-52.
 105. Reichert, J.M., *Marketed therapeutic antibodies compendium*. MAbs, 2012. **4**(3): p. 413-5.
 106. Philadelphia, T.C.o.P.o. 2016 [cited 2016 August 05]; Top 20 Questions about Vaccination]. Available from: <http://www.historyofvaccines.org/content/articles/top-20-questions-about-vaccination#1>.
 107. Initiative, E.V. *Stages of vaccine development*. 2016 [cited 2016 August 07]; Available from: <http://www.euvaccine.eu/vaccines-diseases/vaccines/stages-development>.
 108. Barocchi, M.A., S. Black, and R. Rappuoli, *Multicriteria decision analysis and core values for enhancing vaccine-related decision-making*. Science translational medicine, 2016. **8**(345): p. 345ps14-345ps14.
 109. Ghosh, A. and L. Dar, *Dengue vaccines: challenges, development, current status and prospects*. Indian J Med Microbiol, 2015. **33**(1): p. 3-15.
 110. Wikipedia. *Dengue Vaccine*. 2016 [cited 2016 August 07]; Available from: https://en.wikipedia.org/wiki/Dengue_vaccine.
 111. Suh, H., J. Shin, and Y.C. Kim, *Microneedle patches for vaccine delivery*. Clin Exp Vaccine Res, 2014. **3**(1): p. 42-9.
 112. Zidane, N., et al., *Thermodynamic stability of domain III from the envelope protein of flaviviruses and its improvement by molecular design*. Protein Eng Des Sel, 2013. **26**(6): p. 389-99.
 113. Danecek, P., W. Lu, and C.H. Schein, *PCP consensus sequences of flaviviruses: correlating variance with vector competence and disease phenotype*. Journal of molecular biology, 2010. **396**(3): p. 550-563.

114. Dimitrov, D.S. and Z. Zhu, *Cross-reactive antibodies against dengue virus and uses thereof*. 2017, Google Patents.
115. Patra, A.K., et al., *Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from Escherichia coli*. Protein Expr Purif, 2000. **18**(2): p. 182-92.
116. Tsumoto, K., et al., *Role of arginine in protein refolding, solubilization, and purification*. Biotechnol Prog, 2004. **20**(5): p. 1301-8.
117. Fan, X., et al., *Improving the refolding of NTA protein by urea gradient and arginine gradient size-exclusion chromatography*. J Biochem Biophys Methods, 2008. **70**(6): p. 1130-8.
118. Chen, J., et al., *Cooperative effects of urea and L-arginine on protein refolding*. Protein Expr Purif, 2009. **66**(1): p. 82-90.
119. Barsotti, A.M., et al., *Epigenetic reprogramming by tumor-derived EZH2 gain-of-function mutations promotes aggressive 3D cell morphologies and enhances melanoma tumor growth*. Oncotarget, 2015. **6**(5): p. 2928.
120. Krug, M., et al., *XDSAPP: a graphical user interface for the convenient processing of diffraction data using XDS*. Journal of Applied Crystallography, 2012. **45**(3): p. 568-572.
121. Long, F., et al., *BALBES: a molecular-replacement pipeline*. Acta Crystallographica Section D: Biological Crystallography, 2008. **64**(1): p. 125-132.
122. Winn, M., *An overview of the CCP4 project in protein crystallography: an example of a collaborative project*. Journal of synchrotron radiation, 2003. **10**(1): p. 23-25.
123. Emsley, P. and K. Cowtan, *Coot: model-building tools for molecular graphics*. Acta Crystallographica Section D: Biological Crystallography, 2004. **60**(12): p. 2126-2132.
124. Chen, V.B., et al., *MolProbity: all-atom structure validation for macromolecular crystallography*. Acta Crystallographica Section D: Biological Crystallography, 2010. **66**(1): p. 12-21.
125. Wiederstein, M. and M.J. Sippl, *ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins*. Nucleic acids research, 2007. **35**(suppl_2): p. W407-W410.
126. Laskowski, R.A., *PDBsum: summaries and analyses of PDB structures*. Nucleic acids research, 2001. **29**(1): p. 221-222.
127. Asherie, N., *Protein crystallization and phase diagrams*. Methods, 2004. **34**(3): p. 266-272.
128. Sear, R.P., *Nucleation: theory and applications to protein solutions and colloidal suspensions*. Journal of Physics: Condensed Matter, 2007. **19**(3): p. 033101.
129. Cullity, B.D., S.R. Cullity, and S. Stock, *Elements of X-ray Diffraction*. 2001.
130. Drenth, J., *Principles of protein X-ray crystallography*. 2007: Springer Science & Business Media.

131. Kabsch, W., Xds. *Acta Crystallographica Section D: Biological Crystallography*, 2010. **66**(2): p. 125-132.
132. Brünger, A.T., *Free R value: a novel statistical quantity for assessing the accuracy of crystal structures*. *Nature*, 1992. **355**(6359): p. 472-475.
133. Vitkup, D., et al., *Why protein R-factors are so large: A self-consistent analysis*. *Proteins: Structure, Function, and Bioinformatics*, 2002. **46**(4): p. 345-354.
134. Karplus, P.A. and K. Diederichs, *Linking crystallographic model and data quality*. *Science*, 2012. **336**(6084): p. 1030-1033.
135. Diederichs, K. and P.A. Karplus, *Better models by discarding data?* *Acta Crystallographica Section D: Biological Crystallography*, 2013. **69**(7): p. 1215-1222.
136. Finn, J.A., J. Koehler Leman, and J.R. Willis, *Improving Loop Modeling of the Antibody Complementarity-Determining Region 3 Using Knowledge-Based Restraints*. 2016. **11**(5): p. e0154811.
137. Fibriansah, G., et al., *A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface*. *EMBO Mol Med*, 2014. **6**(3): p. 358-71.
138. Lok, S.M., et al., *Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins*. *Nat Struct Mol Biol*, 2008. **15**(3): p. 312-7.
139. 李洁, 登革病毒包膜 E 蛋白 III 区中和抗体的功能及结构分析. 2013, 南方医科大学.
140. Dowd, K.A. and T.C. Pierson, *Antibody-mediated neutralization of flaviviruses: a reductionist view*. *Virology*, 2011. **411**(2): p. 306-315.
141. Chothia, C., et al., *Conformations of immunoglobulin hypervariable regions*. *Nature*, 1989. **342**(6252): p. 877-83.
142. Dang, J., et al., *Zika Virus Depletes Neural Progenitors in Human Cerebral Organoids through Activation of the Innate Immune Receptor TLR3*. *Cell stem cell*, 2016.
143. Zhang, X., et al., *Dengue structure differs at the temperatures of its human and mosquito hosts*. *Proceedings of the National Academy of Sciences*, 2013. **110**(17): p. 6795-6799.
144. Tabata, T., et al., *Zika virus targets different primary human placental cells, suggesting two routes for vertical transmission*. *Cell Host & Microbe*, 2016. **20**(2): p. 155-166.
145. Quicke, K.M., et al., *Zika Virus Infects Human Placental Macrophages*. *Cell host & microbe*, 2016.
146. Miner, J.J. and M.S. Diamond, *Understanding How Zika Virus Enters and Infects Neural Target Cells*. *Cell stem cell*, 2016. **18**(5): p. 559-560.
147. Li, H., et al., *Zika virus infects neural progenitors in the adult mouse brain and alters proliferation*. *Cell Stem Cell*, 2016.

148. Lok, S.-M., et al., *Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins*. Nature structural & molecular biology, 2008. **15**(3): p. 312-317.
149. Kulkarnia, M.R., et al., *Analysis of Sero-Specific Immune Responses using Epitope Grafted Dengue ED3 Mutants*.
150. Bowen, D.M., et al., *Simplifying complex sequence information: a PCP-consensus protein binds antibodies against all four dengue serotypes*. Vaccine, 2012. **30**(42): p. 6081-6087.
151. Su, X., et al., *Layer-by-layer-assembled multilayer films for transcutaneous drug and vaccine delivery*. Acs Nano, 2009. **3**(11): p. 3719-3729.
152. Endy, T.P., *Human immune responses to dengue virus infection: lessons learned from prospective cohort studies*. Frontiers in immunology, 2014. **5**.
153. TAWEECHASUPAPONG, S., et al., *Langerhans cell density and serological changes following intradermal immunisation of mice with dengue 2 virus*. Journal of medical microbiology, 1996. **45**(2): p. 138-145.
154. Srikiatkachorn, A., A. Mathew, and A.L. Rothman. *Immune-mediated cytokine storm and its role in severe dengue*. in *Seminars in Immunopathology*. 2017. Springer.
155. Seneschal, J., et al., *Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells*. Immunity, 2012. **36**(5): p. 873-884.
156. Garcia, M., et al., *Skin innate immune response to flaviviral infection*. European Cytokine Network, 2017. **28**(2): p. 41-51.
157. Wu, S.-J.L., et al., *Human skin Langerhans cells are targets of dengue virus infection*. Nature medicine, 2000. **6**(7): p. 816-820.
158. Carreno, B.M., et al., *IL-12p70-producing patient DC vaccine elicits Tc1-polarized immunity*. The Journal of clinical investigation, 2013. **123**(8): p. 3383.
159. Nielsen, D.G., *The relationship of interacting immunological components in dengue pathogenesis*. Virology journal, 2009. **6**(1): p. 211.
160. Lyday, B.W., *Compositions and methods for combination therapy with dengue virus and dendritic cells*. 2016, Google Patents.
161. Campos, J.L.S., et al., *DNA-immunisation with dengue virus E protein domains I/II, but not domain III, enhances Zika, West Nile and Yellow Fever virus infection*. PloS one, 2017. **12**(7): p. e0181734.
162. Bustelo, X.R., *Vav proteins, adaptors and cell signaling*. Oncogene, 2001. **20**(44).
163. Bustelo, X.R., *Vav proteins, adaptors and cell signaling*. Oncogene, 2001. **20**(44): p. 6372-81.
164. Crespo, P., et al., *Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product*. Nature, 1997. **385**(6612): p. 169-72.

165. Movilla, N. and X.R. Bustelo, *Biological and regulatory properties of Vav-3, a new member of the Vav family of oncoproteins*. Molecular and cellular biology, 1999. **19**(11): p. 7870-7885.
166. Han, J., et al., *Lck regulates Vav activation of members of the Rho family of GTPases*. Mol Cell Biol, 1997. **17**(3): p. 1346-53.
167. Schuebel, K.E., et al., *Phosphorylation-dependent and constitutive activation of Rho proteins by wild-type and oncogenic Vav-2*. Embo j, 1998. **17**(22): p. 6608-21.
168. Hornstein, I., A. Alcover, and S. Katzav, *Vav proteins, masters of the world of cytoskeleton organization*. Cell Signal, 2004. **16**(1): p. 1-11.
169. Frame, M.C. and V.G. Brunton, *Advances in Rho-dependent actin regulation and oncogenic transformation*. Curr Opin Genet Dev, 2002. **12**(1): p. 36-43.
170. Pawlak, G. and D.M. Helfman, *Cytoskeletal changes in cell transformation and tumorigenesis*. Curr Opin Genet Dev, 2001. **11**(1): p. 41-7.
171. Katzav, S., D. Martin-Zanca, and M. Barbacid, *vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells*. Embo j, 1989. **8**(8): p. 2283-90.
172. Abe, K., et al., *Involvement of NH(2)-terminal sequences in the negative regulation of Vav signaling and transforming activity*. J Biol Chem, 1999. **274**(43): p. 30410-8.
173. Katzav, S., *Vav1: an oncogene that regulates specific transcriptional activation of T cells*. Blood, 2004. **103**(7): p. 2443-51.
174. Aghazadeh, B., et al., *Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation*. Cell, 2000. **102**(5): p. 625-33.
175. Lopez-Lago, M., et al., *Tyrosine phosphorylation mediates both activation and downmodulation of the biological activity of Vav*. Mol Cell Biol, 2000. **20**(5): p. 1678-91.
176. Han, J., et al., *Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav*. Science, 1998. **279**(5350): p. 558-60.
177. Miletic, A.V., et al., *Vav1 acidic region tyrosine 174 is required for the formation of T cell receptor-induced microclusters and is essential in T cell development and activation*. J Biol Chem, 2006. **281**(50): p. 38257-65.
178. Su, I.-h., et al., *Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement*. Nature immunology, 2003. **4**(2): p. 124-131.
179. Eckert, R.L., et al., *Polycomb group proteins are key regulators of keratinocyte function*. J Invest Dermatol, 2011. **131**(2): p. 295-301.
180. Gunawan, M., et al., *The methyltransferase Ezh2 controls cell adhesion and migration through direct methylation of the extranuclear regulatory protein talin*. Nature immunology, 2015. **16**(5): p. 505-516.

181. Campbell, S., et al., *Polycomb repressive complex 2 contributes to DNA double-strand break repair*. Cell cycle, 2013. **12**(16): p. 2675-2683.
182. Wehrle-Haller, B., *Cytoplasmic methylation fuels leukocyte invasion*. Nat Immunol, 2015. **16**(5): p. 441-3.
183. Chen, Y.-H., M.-C. Hung, and L.-Y. Li, *EZH2: a pivotal regulator in controlling cell differentiation*. American journal of translational research, 2012. **4**(4): p. 364.
184. Ezhkova, E., et al., *Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells*. Cell, 2009. **136**(6): p. 1122-1135.
185. de Vries, N.A., et al., *Prolonged Ezh2 depletion in glioblastoma causes a robust switch in cell fate resulting in tumor progression*. Cell reports, 2015. **10**(3): p. 383-397.
186. Hobert, O., B. Jallal, and A. Ullrich, *Interaction of Vav with ENX-1, a putative transcriptional regulator of homeobox gene expression*. Mol Cell Biol, 1996. **16**(6): p. 3066-73.
187. Brooun, A., et al., *Polycomb repressive complex 2 structure with inhibitor reveals a mechanism of activation and drug resistance*. Nature communications, 2016. **7**.
188. Shen, Y., et al., *TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts*. J Biomol NMR, 2009. **44**(4): p. 213-23.
189. Guntert, P., C. Mumenthaler, and K. Wuthrich, *Torsion angle dynamics for NMR structure calculation with the new program DYANA*. Journal of Molecular Biology, 1997. **273**(1): p. 283-98.
190. Herrmann, T., P. Guntert, and K. Wuthrich, *Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA*. Journal of Molecular Biology, 2002. **319**(1): p. 209-227.
191. Ritchie, D.W. and G.J. Kemp, *Protein docking using spherical polar Fourier correlations*. Proteins: Structure, Function, and Bioinformatics, 2000. **39**(2): p. 178-194.
192. Humphrey, W., A. Dalke, and K. Schulten, *VMD: visual molecular dynamics*. Journal of molecular graphics, 1996. **14**(1): p. 33-38.
193. Phillips, J.C., et al., *Scalable molecular dynamics with NAMD*. Journal of computational chemistry, 2005. **26**(16): p. 1781-1802.
194. Ribeiro, J.V., et al., *QwikMD—Integrative Molecular Dynamics Toolkit for Novices and Experts*. Scientific reports, 2016. **6**.
195. Greenfield, N.J., *Using circular dichroism spectra to estimate protein secondary structure*. Nature protocols, 2006. **1**(6): p. 2876-2890.
196. Kwan, A.H., et al., *Macromolecular NMR spectroscopy for the non-spectroscopist*. The FEBS journal, 2011. **278**(5): p. 687-703.
197. Wüthrich, K., M. Billeter, and W. Braun, *Polypeptide secondary structure determination by nuclear magnetic resonance observation of short proton-proton distances*, in *Nmr In Structural Biology: A*

- Collection of Papers by Kurt Wüthrich*. 1995, World Scientific. p. 218-243.
198. Wagner, G., et al., *Nuclear magnetic resonance identification of "half-turn" and 310-helix secondary structure in rabbit liver metallothionein-2*. Journal of molecular biology, 1986. **187**(1): p. 131-135.
 199. Watterson, D., B. Kobe, and P.R. Young, *Residues in domain III of the dengue virus envelope glycoprotein involved in cell-surface glycosaminoglycan binding*. J Gen Virol, 2012. **93**(Pt 1): p. 72-82.
 200. Soares, R.O. and A. Caliri, *Stereochemical features of the envelope protein Domain III of dengue virus reveals putative antigenic site in the five-fold symmetry axis*. Biochim Biophys Acta, 2013. **1834**(1): p. 221-30.
 201. Crill, W.D., et al., *Humoral immune responses of dengue fever patients using epitope-specific serotype-2 virus-like particle antigens*. PLoS One, 2009. **4**(4): p. e4991.
 202. Moraes, A.H., et al., *Antibody Binding Modulates Conformational Exchange in Domain III of Dengue Virus E Protein*. 2016. **90**(4): p. 1802-11.