

DENGUE DIAGNOSIS

A multi-pronged attack



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Issues that are faced in Dengue:

A Disease that requires a multi-prong attack

Dengue

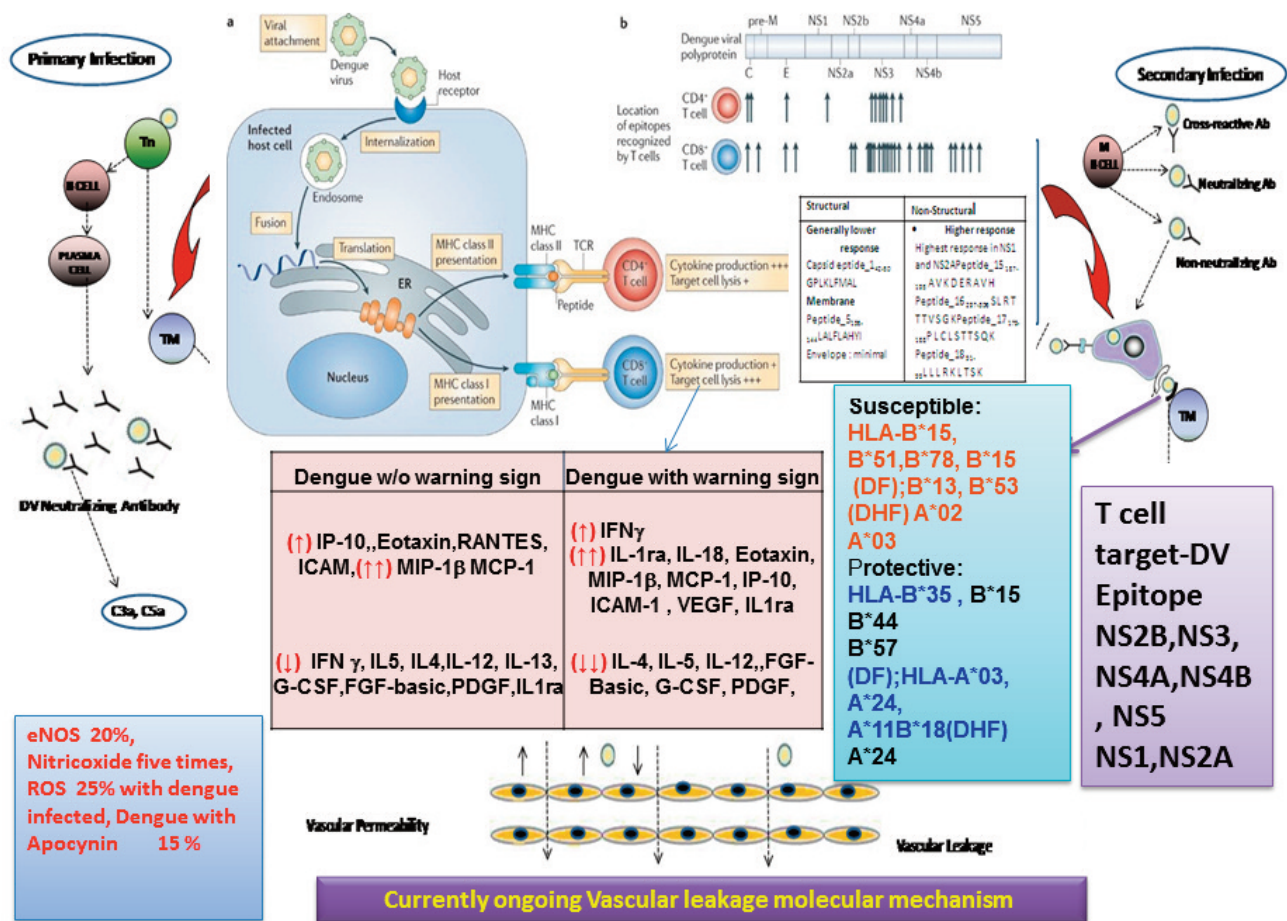
Dengue, in recent decades has become one of the most uncontrolled neglected infectious diseases especially in the tropical and sub-tropical regions of the world. The earliest records on diseases that were clinically compatible to dengue were from the Chin Dynasty (265 to 420 A.D.) and from Northern Sung Dynasty (992 A.D.). Since 1960s, dengue incidences around the world have increased by more than 30-fold, with more than 100 countries in all continents being affected. Recently, it was estimated that approximately 390 million dengue infections occurs yearly. It is believed that the societal and ecological changes/movement during the world wars increased vector-borne diseases, and dengue hyperendemicity began in the Southeast Asian regions, therein triggering epidemics of DHF. Dengue viruses can be asymptomatic or inapparent, or may cause a wide spectrum of symptoms that may range from classical fever to plasma leakage, hemorrhage, shock and even death. Dengue, despite being around for centuries, has neither commercialised vaccines nor anti-dengue drugs. This relatively slow progress in dengue vaccination and drug discovery is mainly because (i) the vaccine needs to be able to protect against all 4 DENV serotypes; (ii) the lack of protective immune correlates; (iii) the absence of reliable animal models to represent dengue and (iv) the controversial and limited understanding of dengue pathogenesis.

Dengue – Laboratory diagnosis

Dengue diagnosis is not only important for clinical management of patients, but also for epidemiological surveillance, outbreak intervention and vaccine development and monitoring. Due to the absence of pathognomonic clinical features that can distinguish dengue from other febrile illnesses, laboratory confirmation is an essential part of diagnosing dengue. Ideally, a dengue diagnostic test should be rapid, simple, with high sensitivity and specificity, able to serotype and differentiate primary and secondary infections. Despite the many efforts to create a single assay that could confirm dengue, that goal has not been reached. Nevertheless, dengue diagnostics have come a long way, with many researchers around the world attempting for a more efficient and reliable diagnostic method.

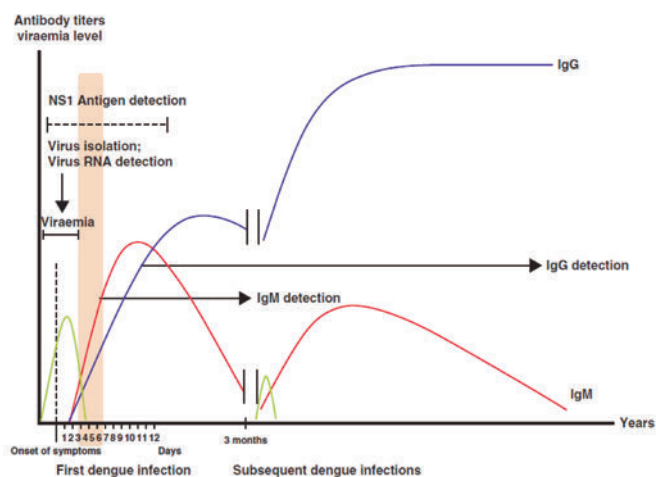
The main hurdle in developing an ideal diagnostic assay for dengue would lie in the complicated pathogenesis of dengue and the fact that multiple sequential infections occurs in dengue endemic areas. Understanding the clinical conditions of dengue patients is essential for appropriate usage of current dengue diagnostics which are mostly serological-based, nucleic acid-based and antigen based. Dengue diagnosis is divided into two main phases, the early phase and the late phase. In the early phase, the approach of diagnosis is via viral detection, viral RT-PCRs, and antigen detection. Meanwhile, the later phase of diagnosis is mainly through serological testing.

We have been actively involved in the development of diagnostic kits. An in-house method for the detection of dengue IgM via



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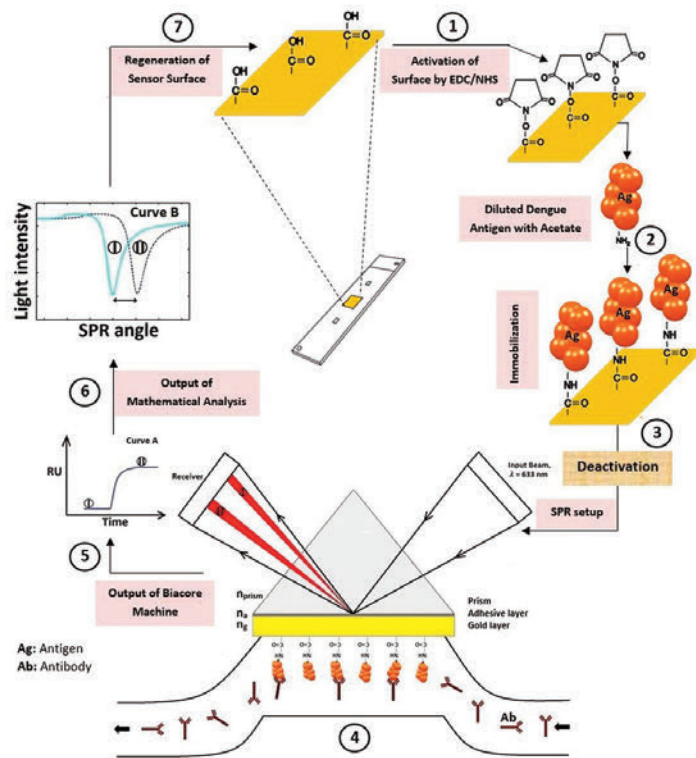
IgM capture ELISA, which is simple and can provide an early diagnosis for dengue infection and a multiplex dengue viral RNA detection assays were developed and evaluated. In the evaluations that were carried out, the sensitivity of the assay was found to be advantageous, showing good sensitivity and specificity and the results obtained were less subjective than the HI method.



Rathakrishnan A, and Sekaran S D (2013)

We have been actively involved in evaluation projects of dengue diagnostic kits conducted by the WHO and collaborating partners around the world as well as with companies that are commercialising these kits. These evaluations revealed that the NS1 detection rate is inversely proportional while the IgM detection rate is directly proportional to the presence of IgG antibodies. Hence, with such findings, this information helps in balancing the use of NS1 kits which are not entirely solely dependable as a single assay for the detection of dengue infection. Combining this assay with an IgM and/or IgG assay will increase the sensitivity of detection, especially in areas with a higher prevalence of secondary dengue virus infections.

Newer technologies are being applied to fine-tune available diagnostic arrays and also to design new assays that fit the ideal test concept. In collaboration with the Faculty of Engineering, UM are involved in the application of biosensors in detecting dengue. Two papers



Jahanshahi P, Zalnezhad E, Sekaran S D, and Adikan F R M (2014)

have since been published revolving around the use of surface plasmon resonance (SPR) as a medical diagnosis technique with high sensitivity and specificity in detection of dengue. In this research, a new method based on SPR is proposed for rapid, 10-minute detection of the anti-dengue virus in human serum samples. This novel technique, known as rapid immunoglobulin M (IgM)-based dengue diagnostic test, can be utilised quickly and easily at the point of care. According to the results, a serum volume of only 1 μ l from a dengue patient (as a minimised volume) is required to indicate SPR angle variation to determine the ratio of each dengue serotype in samples with 83-93% sensitivity and 100% specificity. This breakthrough is a stepping stone towards faster and better diagnostic results.

With many tests and assays that have been carried out, we are still in the search to improve better diagnostics of dengue infection. Our laboratory thrives to develop and test for new avenues in making dengue diagnosis more robust and less cumbersome.

Dengue – The Pathogenesis

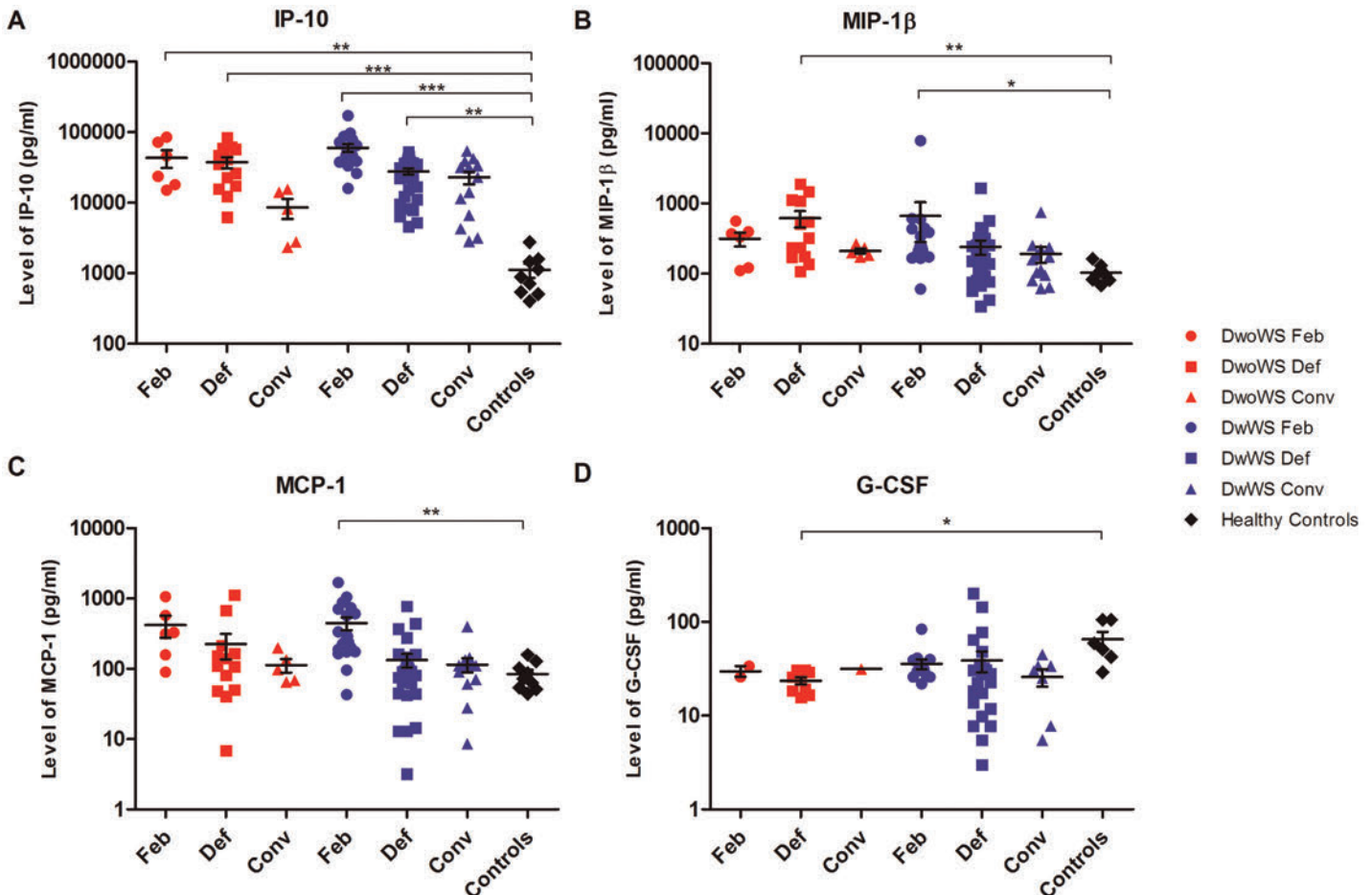
These past few decades have seen a surge in dengue research – in trying to understand the disease, the cause and the trigger. However, the findings have been frustrating where many studies have seen controversial findings and mind-boggling correlations. Our lab has been part of the worldwide researchers who have embarked on finding the cause and correlates of severe dengue and the occurrence of asymptomatic dengue cases.

Dengue viruses can evade our immune system in various manners which may include direct immunosuppression, antigenic variability, passive evasion of the innate immunity and active inhibition of the system. Another postulated dengue pathogenesis is that virulence of different DENV virus strains cause discrepancy in the levels of dengue severity. Furthermore, the sequence of infection by the 4 DENV may also be a risk for manifesting severe symptoms of dengue. Mostly though, dengue has been believed to be an immune-mediated disease whereby most severe cases seemed to have occurred in secondary infections. A dengue-infected person develops lifelong immunity towards the first infecting DENV serotype; however, this immunity is only partially conferred for heterologous dengue serotypes in subsequent infections. Severe dengue cases have been linked to secondary infections as evidenced by retrospective clinical studies and also manifestation of severe dengue in babies with maternal dengue antibodies. In this perspective, the common explanations for dengue pathogenesis include (i) dengue antibody-dependent enhancement (ADE); and (ii) cross-reactive cytotoxic T cells whereby in secondary infections T cell activation would be greater and more rapid or via original antigenic sin to be with lower affinity and avidity towards subsequent heterologous dengue serotypes. Consequently, the atypical activation of T cells and reaction of enhancing antibodies can trigger an impromptu/inappropriate cascade of signaling events,

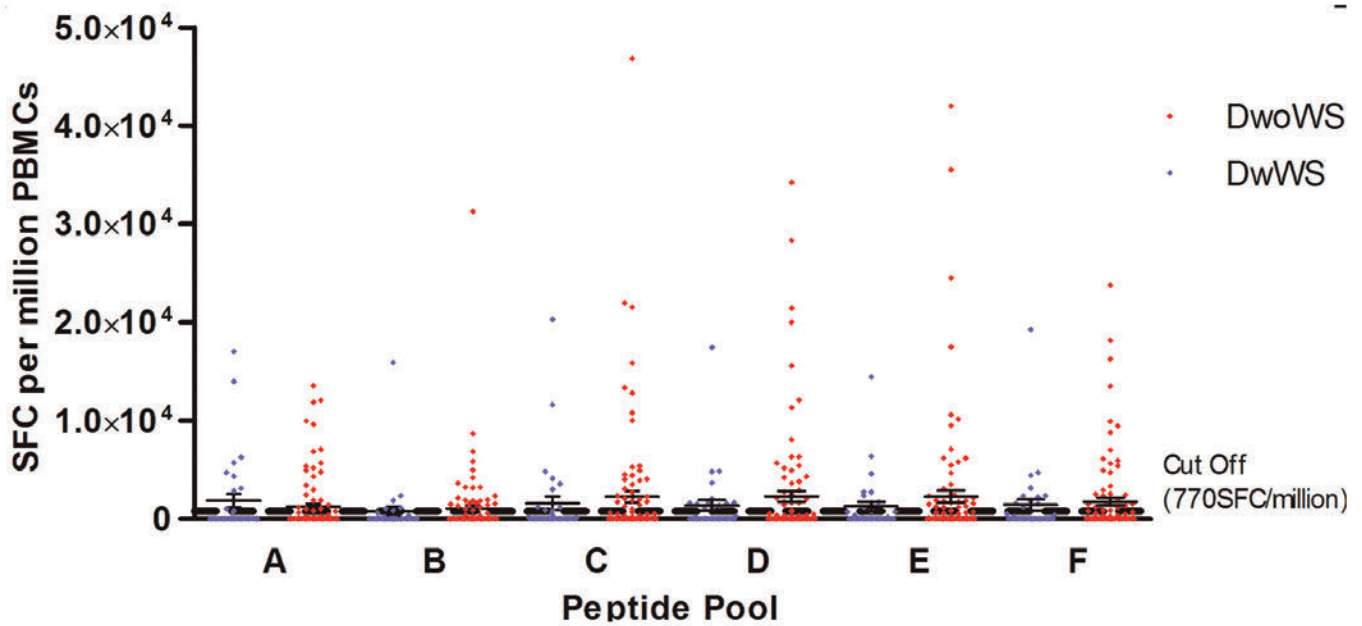
Dengue – Asymptomatic and its relatedness to possible protection

creating cytokine storms that have been correlated with severity in dengue infections. Furthermore, various host genetic factors have been implied in increased dengue severity such as vitamin D receptor, TNF, Fc γ R2, CTLA4, TGF β 1 and the HLA family of genes while TAP1 and TAP2 have been shown to confer susceptibility to/ protection against dengue severity. Having a strong virology and immunology background, we investigated immune correlates in dengue severity which included HLA association with dengue susceptibility/protection, T cell responses of dengue infected patients, cytokine profiling of dengue patients at different phases of illness. This has resulted in various publications in peer-reviewed journals including Clinical and Vaccine Immunology and PLoS One.

Recently, it was estimated that among the 390 million cases of dengue annually, about 75% were mild or asymptomatic cases – an implication that dengue reservoirs might be larger than expected. With the understanding that the asymptomatic dengue cases should not be taken lightly, our lab has investigated the asymptomatic dengue cohort to search for predisposing markers that constitute protection or lack of clinical manifestations in these individuals. Applying microarray and high throughput quantitative PCR to assess the molecular basis of the human genomics, we focused on the gene expressions of immunological responses in the asymptomatic individuals during dengue infections. Based on comprehensive bioinformatics software Spotfire[®] DecisionSite used in microarray analysis, our study was able to show



Rathakrishnan A, Wang S M, Hu Y, Khan A M, Ponnampalavanar S, Lum L C S, and Sekaran S D (2012)



Rathakrishnan A, Klekamp B, Wang S M, Komarasamy T V, Natkunam S K, Sathar J, and Sekaran S D (2014)

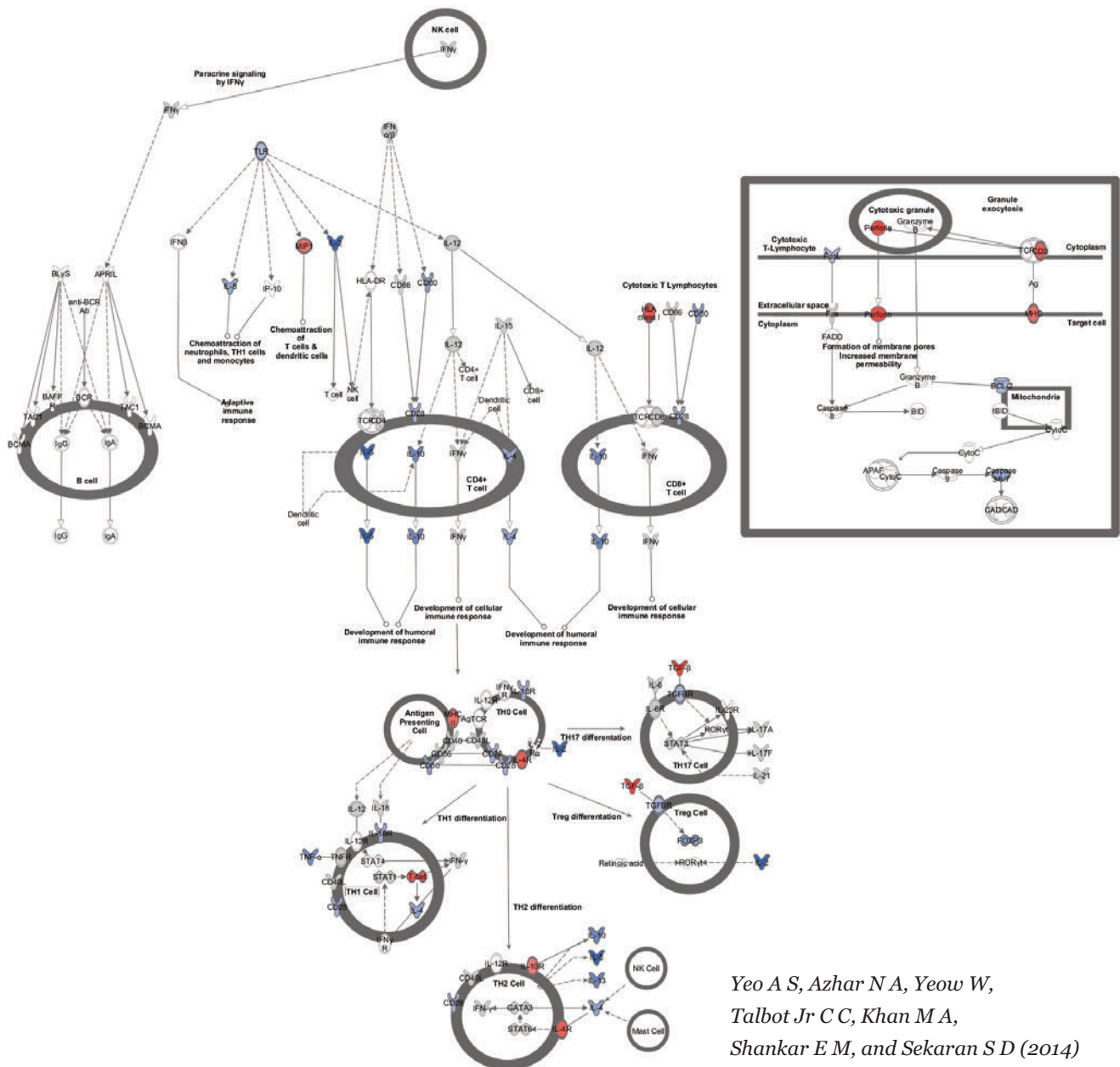
each gene's asymptomatic to symptomatic fold changes and the comparisons' statistical significance which was depicted in a volcano plot. The near symmetrical distribution of genes in the plot provided confidence in reliability of the expression values of which was essential in elucidating a significant difference between the dengue patients and the asymptomatic individuals in terms of their immunological genes' responses. Our study then proceeded to supervised hierarchical clustering which revealed a heat map depicting clarity that the gene expression patterns of the asymptomatic household members' were different from the patients'. We revealed involvement of broad down-regulation of host defense responses, generally the innate and adaptive immune responses and the matrix metalloprotease genes in asymptomatic individuals against symptomatic patients. Although a wide spectrum of genes were down regulated, our study highlighted genes of TNF α (TNF), IL8, C1S, factor B (CFB), IL2, IL3, IL4, IL5, IL8, IL9, IL10 and IL13, CD80, CD28, and IL18, MMP8, MMP10, MMP12, MMP15, MMP16, and MMP24. On the contrary, we also observed selective up-regulation of distinct genes that have been associated with protection. Those up regulated genes were RANTES (CCL5), MIP-1 α (CCL3L1/CCL3L3), MIP-1 β (CCL4L1), TGF β (TGFB), and TIMP1. This study giving the preliminary information on biomarkers that showed differences of the gene expressions of

DENV asymptomatic individuals in comparison with the DENV infected patients has since been accepted for publication in PLoS One. Our findings highlight the potential association of certain host genes conferring protection against clinical dengue. DENV infection among asymptomatic individuals, as compared to clinical dengue patients provides myriad information with regards to gene expression of immune correlates. These data are valuable to better explore the mysteries behind the poorly understood immunopathogenesis of subclinical dengue infection.

Dengue – Endothelium dysfunction

More recently, we have endeavored the field of endothelium dysfunction of infectious diseases. This is mainly spurred by her continuous investigations of immunopathogenesis of dengue infection. The hallmark of severe dengue is plasma leakage and hemorrhage and the "organ" crucial for regulating electrolyte content of intra- and extravascular spaces is the endothelium layer. The endothelium is also an important area of immunity because blood coagulation, platelet adhesion, and immune cells interactions occur here.

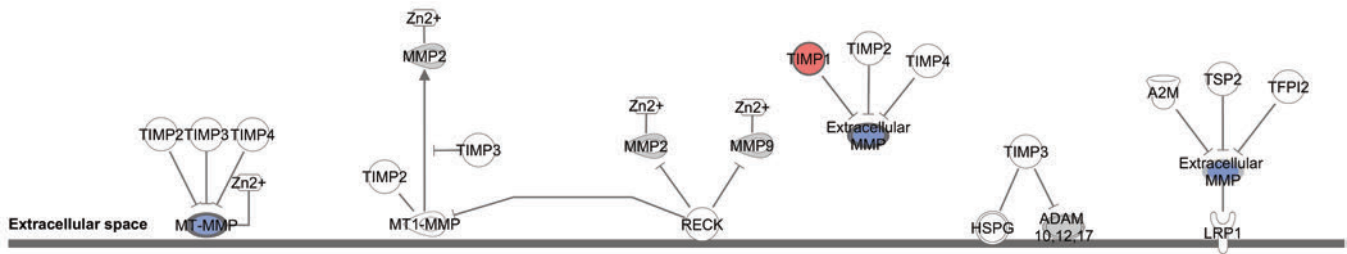
In vitro, endothelial cells (ECs) have been shown to be permissive to DENV which was once again demonstrated by our team in various endothelial cells of different origins (including



Yeou A S, Azhar N A, Yeow W, Talbot Jr C C, Khan M A, Shankar E M, and Sekaran S D (2014)

dermal, hepatic, brain and lungs). Previously it has been demonstrated that ECs that are exposed to DENV or dengue antibodies are known to release various cytokines. We have demonstrated that exposing ECs to certain cytokines such TNF- α and MCPs-1 seemed to induce vascular permeability, observed via damage induced on the ECs junctional proteins. In collaboration with investigators at the Johns Hopkins University and PUGSOM, we are currently investigating the endothelial dysfunction caused by dengue virus infection. With the purchase of the Electric Cell-substrate Impedance Sensing (ECIS) system, the effect of dengue infections on endothelial cells is being probed real time in a non-label method by monitoring morphological changes,

cell locomotion, and other behaviors directed by the cell's cytoskeleton. Preliminary results indicate that the microvascular ECs examined were activated immediately upon dengue infection at high virus titers. This modulation of the endothelial cells appears to be caused by modulations of the junctional proteins via the paracellular pathway. Currently, the gene expression of DENV-infected microvascular ECs as early as the moment of infection is being scrutinised. In other studies which analysed gene expression of DENV-infected ECs at 24-48 hours post infection revealed that the cellular pathway, immune response and inflammation genes were upregulated, whereas the cytoskeletal and membrane structure genes were downregulated



Yeo A S, Azhar N A, Yeow W, Talbot Jr C C, Khan M A, Shankar E M, and Sekaran S D (2014)

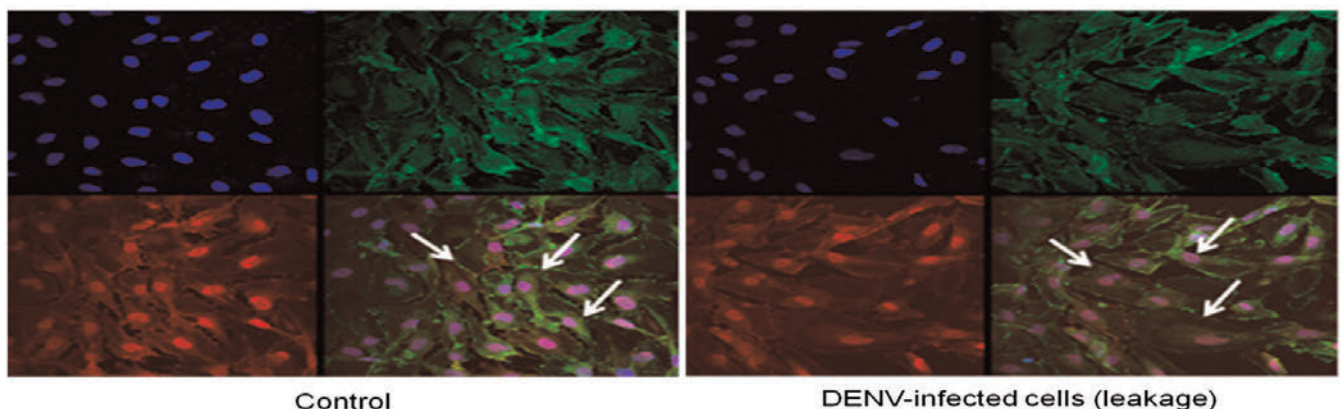
In addition to direct virus effect on the endothelial cells, viral infection can also cause oxidative stress which can also lead to endothelial damage. In dengue, serum NO in DF and DSS patients were observed to be much higher than in DHF and controls and an up regulation of intra-platelet L-arginine nitric oxide pathway was observed in dengue patients and this was associated with the decrease in platelet aggregation- a possible reason to why thrombocytopenia occurs in dengue. Currently, investigations into the role of oxidative stress in dengue infection of the microvascular ECs are being undertaken in collaboration with the University of Hong Kong.

Similarly, many other viruses have been known to target the endothelium leading enhanced permeability- hence we are also investigating endothelium dysfunctions caused by other viruses including respiratory viruses, influenza virus, and neuroviruses. By gaining an understanding of the mechanism of endothelium dysfunction in infectious disease, we hope to move on to finding therapeutic strategies to prevent/limit/reverse the disruption of the endothelial layer and thus minimise the severity of disease.

Antimicrobial/Antiviral Drug Development

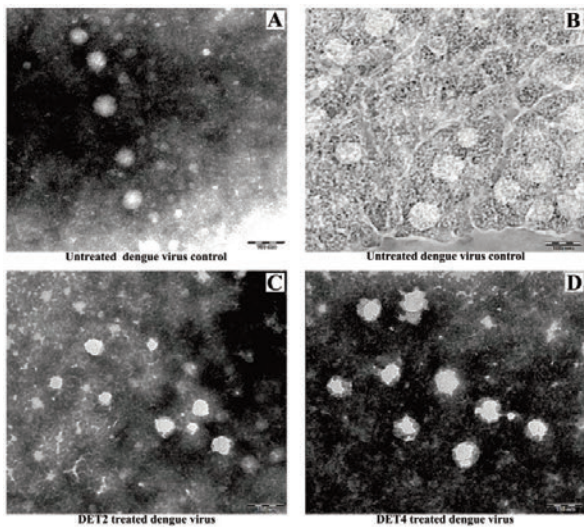
Dengue

The burden of dengue infection is consistently increasing as there is no licensed vaccine or approved antiviral drugs to combat DENV infection. At present, the management of this disease consists primarily of supportive care such as administration of antipyretics, analgesics and resuscitation with intravenous fluid to restore the lost fluid in patients manifesting vascular leakage. Considering the high mortality and morbidity rates due to DENV infection, several research groups in academia and industry are involved in the development and identification of specific vaccine candidates, and novel therapeutics to prevent dengue. The three major approaches being pursued to control dengue infections are vector control, dengue vaccines and antiviral therapy. Currently, the only method that is available to prevent this disease is vector control despite being expensive, ineffective and difficult to sustain. Several dengue vaccine candidates are being tested at different stages of pre-clinical or clinical development but, so far none have



Appanna R, Wang S M, Ponnampalavanar S A, Lum L C S, and Devi Sekaran S (2012)

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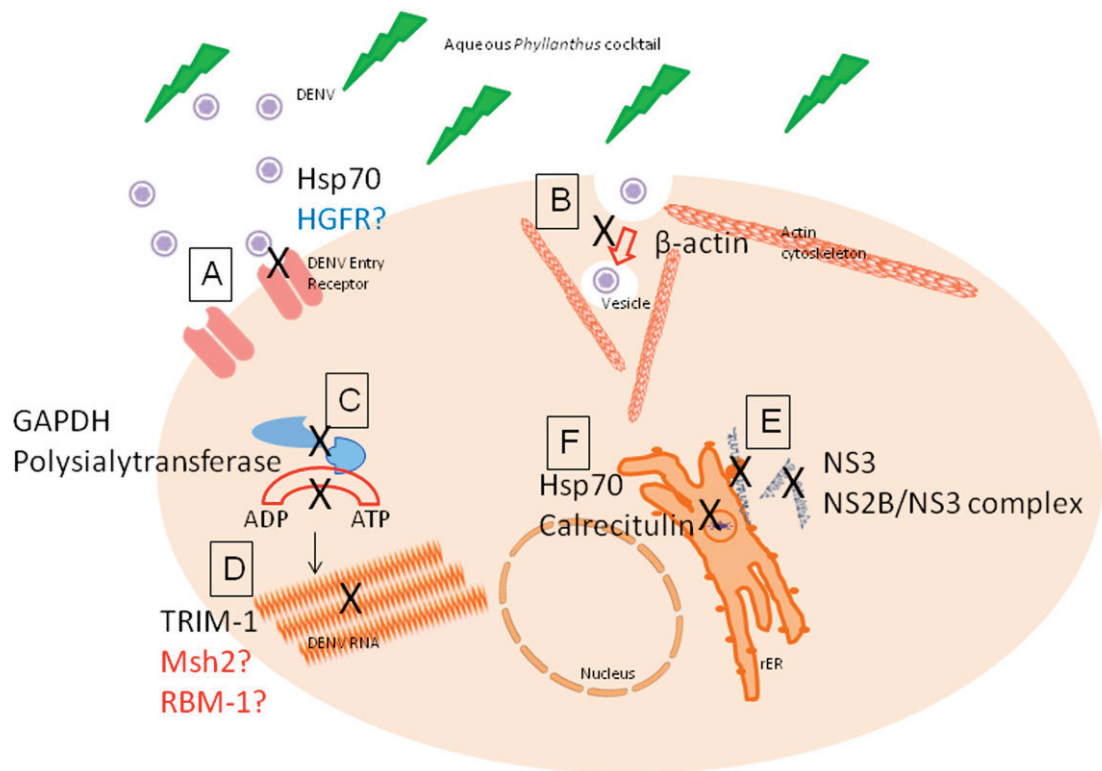
Alhoot M A, Rathinam A K, Wang S M, Manikam R, and Sekaran S D (2013)

been approved for clinical use. Hence, specific antiviral therapy needs to be developed for the treatment of DENV infection. Our past and current projects include (i) the application of siRNA to inhibit entry of dengue virus into target cells; (ii) use of natural products such as *Phyllanthus* plant species to inhibit dengue virus infections; and (iii) using combinatorial bioinformatics to design antimicrobial peptides (AMPs) against dengue virus.

Dengue virus-host cell interaction is initiated when the virus binds to the attachment receptors followed by endocytic internalisation of the virus particle. Successful entry into the cell is necessary for infection initiation; hence silencing of GRP78 and clathrin-mediated endocytosis was conducted to evaluate its effect on dengue virus entry and multiplication into HepG2 cells while silencing of CD-14 associated molecule and clathrin-mediated endocytosis was done to assess viral entry into monocytes. In both cell types, we showed that indeed silencing the attachment receptor, clathrin-mediated endocytosis genes, cell surface receptors and clathrin mediated endocytosis genes using RNA interference could inhibit dengue virus entry and multiplication into HepG2 cells – leading to reduction of infected cells as well as the viral load, which might function as a unique and promising therapeutic agent for attenuating dengue infection and prevent the development of dengue fever to the severe life-threatening DHF or DSS.

Besides the application of siRNAs, we also attempted to evaluate the possibility of natural products such as *Phyllanthus* (Euphorbiaceae), a local medicinal plant also known as Dukung Anak as a antiviral drug agent. In Ayurvedic medicine, *Phyllanthus* has a long tradition of use to treat jaundice, gonorrhoea, frequent menstruation, dysentery and diabetes as well as skin ulcers, sores, swelling, and itchiness. In traditional Chinese medicine, *Phyllanthus* has been used for generations to eliminate gallstones and kidney stones, as well as an immune system stimulator. We demonstrated that a cocktail of extracts consisting of four species of *Phyllanthus* (*P.amarus*, *P.niruri*, *P.urinaria*, and *P.watsonii*) had inhibitory activity against DENV2 with more than 90% of virus reduction in simultaneous treatment. Two-dimensional analysis revealed significantly altered levels of thirteen proteins which were involved in several biological processes, including viral entry, viral transcription and translation regulations, cytoskeletal assembly, and cellular metabolisms.

The design of novel therapeutic approaches in various studies has focused on targeting the different stages of the viral replication cycle. This has been accomplished in *in vitro* studies by the use of a number of inhibitory agents directed against a wide range of both cellular and viral targets. Small-molecule inhibitors such as Ivermectin, Mycophenolic acid and Brequinar, siRNAs and antiviral peptides have been designed and tested for their antiviral effects. The peptide- or protein-based therapeutics are of interest primarily because they bind with exquisite specificity to their *in vivo* targets, resulting in exceptionally high potencies of action and relative few off-target side-effects. Synthetic AMPs are believed to behave similarly with natural AMPs present in the body being potent and broad spectrum; however working mostly like immunomodulators. The application of AMPs is a promising future strategy against microbial infections especially with the augmented drug resistances in microbes. Despite the generic disadvantages of peptides such as their low systemic stability, high clearance, poor membrane permeability,



? Possible antiviral target of *Phyllanthus* (virus entry - HGFR; replication - Msh2 and RBM-1)

Lee S H, Tang Y Q, Rathkrishnan A, Wang S M, Ong K C, Manikam R, and Sekaran S D (2013)

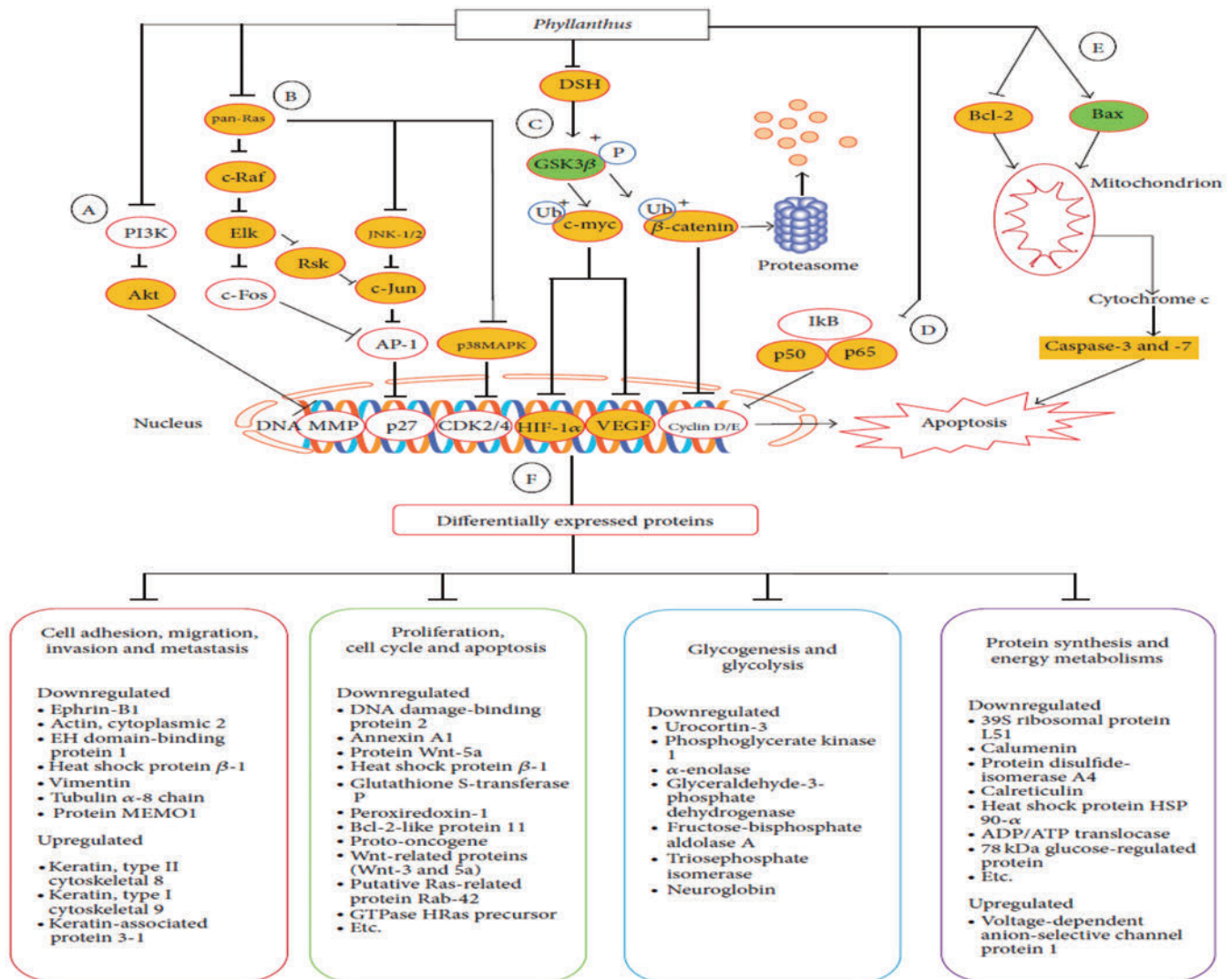
more than 100 peptide-based drugs are in clinical use for the treatment of diseases such as multiple sclerosis, breast cancer, prostate cancer and so on. One of our major interests is to develop novel peptides as antivirals against DENV. The peptides exhibiting antiviral effects by inducing conformational changes in the E protein or by inhibiting the E-PrM protein interactions have been reported by us as well as by others. Currently, the effect of peptides on DENV replication by targeting its non-structural proteins is being further examined. The non-structural proteins (NS1 to NS5) play crucial roles in the viral replication and hence, in the pathogenesis of dengue infection. Therefore, our interest lies in designing novel peptides targeting the non-structural proteins of DENV and testing their potencies in inhibiting DENV replication.

Pneumococcal diseases

Pneumococcal diseases represent a global threat heavily affecting children aged less than two and the elderly adults caused by *Streptococcus pneumoniae* or pneumococcus, a Gram-positive, alpha-hemolytic, aerotolerant anaerobic member of the genus *Streptococcus*. This pathogen was a major cause of pneumonia in the late 19th century and represents one of the major

etiological agents causing life-threatening diseases such as pneumonia, meningitis, and bacteremia. Although antimicrobial drugs such as penicillin have diminished the risk from pneumococcal disease, the proportion of strains that are resistant to antibiotics is steadily increasing. Moreover, the high prevalence of antibiotic-resistant pneumococci has greatly limited the choice of empirical therapy and thus the treatment outcomes. Therefore, an improved treatment and/or vaccine against *S. pneumoniae* are one of the top vaccine priorities in the world. Certain proteins or enzymes displayed on the surface of *S. pneumoniae* significantly contribute to pathogenesis and might be involved in the disease process caused by these pathogens.

Antimicrobial peptides (AMPs) represent an important part of the innate immune system and produced by virtually all living species to defend themselves against microbial pathogens. These peptides are typically short positively charged, amphiphilic, and have been isolated from single-celled microorganisms up to human beings. The primary role of these peptides is to kill of invading pathogenic organisms. It was formerly proposed that permeabilisation of the bacterial cell membrane was the only mode of action of antibacterial



Tang Y Q, Jaganath I, Manikam R, and Sekaran S D (2013)

peptides; however, several studies have revealed that some antibacterial peptides translocate into cells and do not cause membrane permeabilisation but rather mediate bacterial cell death by targeting essential intracellular processes. Because of their broad-spectrum activity, AMPs are considered to be very promising candidates as novel antimicrobial agents. Our main focus is to design novel synthetic antimicrobial peptides (AMPs) as potential antimicrobial candidates against *Streptococcus pneumoniae*. At the present stage, we have designed and tested a series of hybrid AMPs exhibiting strong antipneumococcal effects of which DM3 possessed promising *in vitro* and *in vivo* antipneumococcal activities and this has been patented. Based on this current experience, we have begun to investigate other hybrids generated based on Ranalexin and Indolicidin which showed potent antipneumococcal activity and the mechanisms of action of these AMPs will be evaluated alongside *in vivo* therapeutic efficacy.

Anti-cancer drug development

In conjunction with Professor Shamala's ambition in search of therapeutic agents against microbes, we had previously ventured into looking at the anti-cancer properties of the local plant known as *Phyllanthus*. We have demonstrated that *Phyllanthus* possessed broad spectrum anticancer properties as it exhibited anti-proliferative, anti-metastatic and anti-angiogenic effects as well as possesses capability to induce apoptosis in a number of human cancers cell lines including breast, lung, prostate and melanoma. Many of its anti-cancer actions were associated with disruption of multiple survival pathways and protein expression in *Phyllanthus* treated cancer cells. This project has resulted in 5 publications in peer-reviewed journals and 1 book chapter.



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