Analysis of Cellular Immune Response during Chikungunya Virus (CHIKV) Infection in Macrophage

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by **Mr. Tapas Kumar Nayak** entitled "**Analysis of Cellular Immune Response during Chikungunya Virus (CHIKV) Infection in Macrophage**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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sharing funny jokes during leisure time in the lab. Special thanks to my immediate junior Subhransu for sharing the luxury of healthy, hygienic and delicious homemade lunch in the NISER campus. I am indeed grateful to my collaborator Prabhudatta (ILS, Bhubaneswar) for his help and support while doing experiment at ILS, Bhubaneswar.

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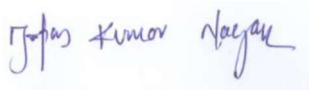

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*Dedicated to **my family**……..*

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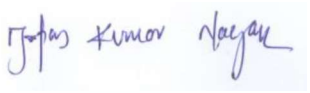

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Conference Proceedings:

1. **Tapas K. Nayak**, P Sanjai Kumar, Prabhudutta Mamidi, Subhransu S. Sahoo, Soma Chattopadhyay and Subhasis Chattopadhyay; 17-AAG mediated regulation of Chikungunya virus infection in macrophage; implications and future perspectives. National symposium on current research in biotechnology and annual meet of society for biotechnologist India (SBTI) organized by Jagadguru Sri Shivarathreeshwara University, Mysuru, India and JSS College of Pharmacy, Ooty, India, from 15th-16th Sep 2017.
2. **Tapas K. Nayak**, P. Sanjai Kumar, Subhransu S. Sahoo, Saumya Bandyopadhyay, Soma Chattopadhyay and Subhasis Chattopadhyay; Experimental Chikungunya virus infection and its implication towards anti-viral strategies with novel therapeutic approaches. National seminar on science and technology for national development in India organized by Indian Science Congress Association, Bhubaneswar chapter and KIIT University, Bhubaneswar, Odisha, India from 12th-13th Dec 2016.
3. **Tapas K. Nayak**, Subhransu S. Sahoo, B.M. Pratheek, Chandan Goswami, Sanjib Kar, Nrisingha Dey, Soma Chattopadhyay, Prasanta K. Maiti and Subhasis Chattopadhyay; Cellular Immuno-regulatory Responses: From Experiments towards

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Journal Publications:

1. *__Nayak, T.K.__[†]; Mamidi, P.[†]; Kumar, A.; Singh, L.P.; Sahoo, S.S.; Chattopadhyay, S.; Chattopadhyay, S. Regulation of viral replication, apoptosis and pro-inflammatory responses by 17-AAG during chikungunya virus infection in macrophages. *Viruses* **2017**, *9*.
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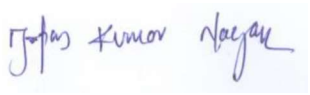
5. *Chattopadhyay, S.; Kumar, A.; Mamidi, P.; **Nayak, T.K.**; Das, I.; Chhatai, J.; Basantray, I.; Bramha, U.; Maiti, P.K.; Singh, S.*, et al.* Development and characterization of monoclonal antibody against non-structural protein-2 of chikungunya virus and its application. *Journal of virological methods* **2014**, *199*, 86-94.
6. Kumar, A.; Mamidi, P.; Das, I.; **Nayak, T.K.**; Kumar, S.; Chhatai, J.; Chattopadhyay, S.; Suryawanshi, A.R.; Chattopadhyay, S. A novel 2006 indian outbreak strain of

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.



Bhubaneswar
April, 2018

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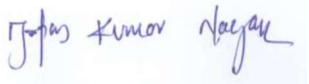

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4. Attended 83rd Annual meeting of society of Biological chemist (India), Haldane Memorial Symposium on Evolutionary Biology held at National Institute of Science Education and Research (NISER), India, on Dec 7th, 2014.
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6. National Symposium on “Emerging trends in Biotechnology: Present scenario and future dimensions” organized by P.G. Department of Biotechnology, Utkal University, Bhubaneswar, India, from Mar 29-30th, 2014.
7. National workshop on “Alternatives to Animal Use for Training Students in Science and Research” organized by Orissa University of Agriculture and Technology (OUAT), Odisha and People for the Ethical Treatment of Animals(PETA), India, on Nov 13 , 2013.
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1. **Tapas K. Nayak**, P. Sanjai Kumar, Subhransu S. Sahoo, Saumya Bandyopadhyay, Soma Chattopadhyay and Subhasis Chattopadhyay; Experimental Chikungunya virus infection and its implication towards anti-viral strategies with novel therapeutic approaches. National seminar on science and technology for national development in India organized by Indian Science Congress Association, Bhubaneswar chapter and KIIT University, Bhubaneswar, Odisha, India from 12^{th}-13^{th} Dec 2016
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1. ***Nayak, T.K**.; Mamidi, P.; Kumar, A.; Singh, L.P.; Sahoo, S.S.; Chattopadhyay, S.; Chattopadhyay, S. Regulation of viral replication, apoptosis and pro-inflammatory responses by 17-aag during chikungunya virus infection in macrophages. *Viruses* **2017**, *9*.
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altered macrophage cellular pathways towards apoptosis, induction of host cell immunity and pathogenesis for designing rationale therapeutic drugs against CHIKV.

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Firstly, we have found that the macrophages (Raw264.7, a mouse macrophage cell line) are susceptible for CHIKV infection at MOI 05. The nsp2 and E2 protein expression reached maximum at 8 hpi and then gradually reduced during later hpi. Furthermore, plaque assay result suggest that CHIKV infected macrophages released maximum infectious new viral progenies around 12 hpi suggesting that CHIKV complete one replication cycle in every 12 hours in host macrophage. It was also found that CHIKV infection induces apoptosis in the host macrophages via both extrinsic (caspase-8) and intrinsic (caspase-9) pathways. We also demonstrated that CHIKV induces robust TNF, IL-6 and MCP-1 expression in macrophage at different time post infection without any changes in the IL-12 and IL-10 [14]. Moreover, it was also observed that the phosphorylation of P38 but not JNK is involved in the induction of TNF (key inflammatory mediator) in the host macrophages, which has been confirmed by the use of specific MAPK inhibitors. Surface expression of MHC- I, II and CD86 also positively modulated during CHIKV infection in a time dependent manner indicating robust immune activation.

Previous studies from our laboratory and others have signified the use of HSP90 inhibitor to regulate CHIKV infection in host cells [15]. In this study we used 17-AAG, a potential HSP90 inhibitor on CHIKV-infected mouse macrophages, as the drug has been reported to have less toxic effect than its derivative. The results demonstrated that the 17-AAG has reduced both the number of live infectious viral particles and the level of viral protein expressions as compared to DMSO control. Interestingly, 17-AAG treatment abrogated CHIKV induced apoptosis without affecting the expression of MHCs and CD86. It could be possible that 17-AAG might preserve the expression of MHCs and co-stimulatory molecules in macrophages to strengthen the subsequent anti-viral adaptive immune responses. Moreover, up-regulated level of TNF, IL-6 and MCP-1 in CHIKV infected macrophage was down regulated by 17-AAG treatment [14]. Further studies are required to substantiate the mechanism of CHIKV infection associated to

4.6. 17-AAG Regulates Apoptosis and Cellular Immune Responses during CHIKV Infection in macrophages:

Since, CHIKV induces apoptosis in the host macrophages, 17-AAG was assessed whether it can regulate it in vitro. It was observed that both the Annexin V positive cells and induced cleaved caspase-3 were reduced by 17-AAG treatment as compared to DMSO control. As expected the induction of all the pro-inflammatory mediators (IL-6, TNF and MCP-1) were found to be down regulated by 17-AAG treatment significantly. Unlike pro-inflammatory cytokine and chemokine production, induction of MHC-I, MHC-II and CD86 by CHIKV were not abrogated upon 17-AAG treatment [14]. Taken together, the data indicate that 17-AAG may down regulate CHIKV induced apoptosis, pro-inflammatory cytokine/chemokine production of host macrophages, without altering the induced immune activation markers like MHCs and CD86 during infection probably to activate CHIKV specific T cell responses.

4.7. CHIKV infection induces TNF via MAP Kinase activation in macrophages

It has been reported previously that various mitogen activated protein kinases (MAPKs) are involved in the induction of TNF in the macrophages, though the type of MAPK involved depends on the type of host cells and kind of signal input [18]. Since CHIKV induces robust TNF (key mediator of inflammation) in macrophages, we sought to investigate the underlying mechanism associated with it. It was observed that CHIKV induces both p-P38 and p-JNK without any significant phosphorylation of ERK MAPK in a time dependent manner. Further studies revealed that P38 inhibitor (SB203580, 0.5, 1.0 μM), but not SP600125 reduces both CHIKV infection and associated TNF production in macrophages in a dose dependent manner. This suggests pro-CHIKV and pro-inflammatory role of P38 MAPK in CHIKV infected macrophages, which might have some therapeutic implications in future.

CHAPTER-5: DISCUSSION AND CONCLUSIONS

The surface expression of major histocompatibility complex (MHC)- I ,II and co-stimulatory molecule, CD86 were assessed in both mock and CHIKV infected samples at 8, 12 and 24 hpi by FC based analysis. In the CHIKV infected groups both MHC-I and CD86 surface expression were significantly up-regulated starting from 8, 12 and 24 hpi as compared to mock cells. Unlike MHC-I, the surface expression of the MHC-II was increased significantly only at late time point (24 hpi) in CHIKV infected groups than mock groups. This suggests that CHIKV infection in mouse macrophage positively modulate surface expression of MHC-I, and CD86 molecule from early time of infection, but MHC-II is induced by CHIKV only at late time point [14].

4.5. Regulation of CHIKV Infection by 17-AAG in macrophages:

Recent study showed that CHIKV nsP2 is stabilized by HSP90 during active stage of CHIKV replication in Vero cells which was abrogated by HSP90 inhibitor, GA [15]. Here, we used 17-AAG, a less toxic derivative of GA, to study its effect on CHIKV infection in mouse macrophages. The cytotoxic effects of different concentrations of 17-AAG (0.0265, 0.125, 0.25, 0.5 and 1.0 μM) were tested on Raw cells by MTT assay for 24 hours. We have selected 0.5 μM concentration of 17-AAG for our further study, as nearly 98% cells were found to be viable [14]. Viral plaque assay suggests that the treatment of 17-AAG on CHIKV infected macrophages, reduced the number of new viral progeny production significantly ($p<0.05$) as compared to DMSO. Next, FC analysis showed that 17-AAG treatment was found to inhibit both nsP2 and E2 protein expression as compared to DMSO control in a dose dependent manner. Furthermore, it was also found that 17-AAG treatment reduced the level of CHIKV RNA in macrophages. This suggests that 17-AAG may reduce the production of viral progeny by inhibiting both the levels of nsP2 and E2 proteins as well as RNA. Next, Western blot analysis showed that expression of HSP90 protein remained unchanged in the presence of 17-AAG, confirming that 17-AAG regulates CHIKV infection by abrogating HSP90 activity without modulating its protein expression level [14].

with 7-AAD at different hpi. The FC analysis showed a very small fraction of cells were positive for Annexin V in both mock and CHIKV infected cells at 8 hpi. However, a significant increment in the population of Annexin V positive cells was observed at 12 hpi and at 24 hpi (Mock; 5.3±0.06 versus CHIKV; 17.7±0.05, $p<0.001$). Interestingly, both Annexin V and 7-AAD dual positive cells were not increased significantly with time in CHIKV infected macrophages as compared to the mock, which confirms that CHIKV induces apoptotic marker, Annexin V without inducing necrosis. Western blot analysis was performed to observe apoptosis through cleaved caspase-3. Surprisingly, induction of cleaved caspase-3 was observed as early as 4 hpi in case of CHIKV infected macrophages. Furthermore, the expression of cleaved caspase-8 and 9 in CHIKV infected cells were significantly higher than the corresponding mock. Thus, our data suggest that CHIKV infection induces apoptosis via both extrinsic and intrinsic pathways in macrophages [14].

4.3. CHIKV infection in mouse macrophages modulates secretion of pro-inflammatory cytokine:

Mock and CHIKV infected cell culture supernatants at different time points (8, 12, and 24 hpi) were assessed for the quantitative measurement of released cytokines by BD ELISA kit as described previously in the materials and methods section. The level of both TNF and IL-6 was found to be increased significantly in CHIKV than the mock in all time points [14]. However, CHIKV infection positively modulated MCP-1 secretion only around 24 hpi as compared to mock, without any changes in the IL-10 and IL-12 production. This result suggests that CHIKV infection in macrophages up-regulate TNF, IL-6 and MCP-1 over time, while no such significant changes were observed for IL-10 and IL-12.

4.4. Modulation in the surface expression of MHC and co-stimulatory molecules in macrophages following CHIKV infection:

“656-ELVRAERTEHEYVYDVDQR-674” was predicted by Protein Hydroplotter, which works on the principle of hydrophobicity and antigenicity. The isotyping of the selected hybridoma clone was done by Pierce Rapid ELISA mouse mAb isotyping kit suggest the developed mAb was IgG2bκ. Then, the specific reactivity of the mAb was tested with the peptide by ELISA. It was observed that the optical density (OD) for isotype was below 0.2 as compared to nsP2 mAb (>2.0) indicating specific binding of the mAb to the nsP2 peptide. Further, CHIKV infected Vero cells were tested for nsP2 mAb reactivity by FC analysis. It was observed that there was positive shift in both percent positive cells (Mock; 0.35%, CHIKV; 10.82%) and mean fluorescence intensity (MFI), suggesting specific detection of nsP2 protein by the mAb [16].

The time kinetics experiment suggest that both nsp2 and E2 expression started at 4 hpi, reached maximum at 8 hpi and then gradually reduced towards later time point. Then, to determine the generation of the new viral progenies, cell culture supernatants were quantified by plaque assay. The result showed that maximum infectious new viral progenies were released around 12 hpi indicating that CHIKV complete one replication cycle in every 12hpi in the host macrophage. Additionally, our time course experiment showed frequency of nsP2 and E2 expression reached maximum at 8 hpi which was exactly correlated with the replication cycle of CHIKV in macrophage. Taken together our data suggest that CHIKV can successfully infect and actively replicate in mouse macrophage cell in vitro [14].

4.2. CHIKV Infection Induces Apoptosis in macrophages:

CHIKV infection has been recently reported to induce apoptosis in host epithelial cells [13,15,17]. However, apoptosis in host macrophages during CHIKV infection has not been reported yet. The bright field microscopic images showed the development of CPE at 8 hpi in CHIKV infected Raw cells. Those observed characteristic features of the cells indicate that the cells might have undergone apoptotic process after infection. In order to confirm apoptosis in macrophage during CHIKV infection, both mock and infected cells were stained with Annexin V

4. Plaque assay: For the determination of the virus titer, plaque assay was performed on the Vero cells as described previously [14,15].

5. Flow Cytometry (FC): For flow cytometric analysis both mock and CHIKV infected cells were harvested, and then fixed in 4% paraformaldehyde at RT. Then cells were further processed for intra cellular staining (ICS) and surface staining (CS) with various antibodies, followed by FC analysis as described elsewhere [14].

6. ELISA: Sandwich ELISA of macrophage cell culture supernatant was performed by using BD OptEIA for IL-10 and IL-12 according to the manufacturer instructions as described elsewhere [14].

7. Western blotting: Protein expression was assessed by Western blot analysis according to the protocol described elsewhere [14,15].

8. Annexin V staining: To detect the apoptotic cells, FC was carried out by using BD Annexin V Detection Kit I (BD Biosciences, USA) as described earlier [14,15].

9. RT-PCR: For quantitation of CHIKV RNA, inside the host cells, Raw264.7 cells were infected with CHIKV with and without 17-AAG and RT-PCR was performed as mentioned elsewhere [14].

10. Statistical Analysis: Statistical Analysis was performed by using the GraphPad Prism 5.0 software (GraphPad Software Inc. USA). Data are represented as Mean±SEM. $p < 0.05$ is considered as statistically significant difference between the groups.

CHAPTER-4: RESULTS

4.1. Standardization and establishment of CHIKV infection in mouse macrophage:

For the detection of infection, monoclonal antibody against CHIKV nsP2 was developed (by conventional hybridoma technology) and characterized. The 19-mer long amino acid sequence

CHAPTER-2: HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis: CHIKV infection may modulate host cell immunity by regulating MHC and associated immune responses in macrophages, which may reflect the respective outcome of innate and adaptive immunity

2.2. Specific Objectives:

1. To establish CHIKV infection in host macrophages.

2. To investigate signature cytokine milieu in the host cell during CHIKV infection.

3. To investigate whether, there is any alteration in the expression of MHC and co-stimulatory molecules during CHIKV infection.

4. To investigate immuno-regulatory candidate pharmaceutical drug for anti-CHIKV infection in macrophages

5. To investigate mechanism of pro-inflammatory response (e.g., TNF) induced during CHIKV infection in macrophages

CHAPTER-3: MATERIALS AND METHODS

1. Cell Culture: Mouse monocyte/macrophage cell line, Raw264.7 (ATCC® TIB-71™) was maintained in complete RPMI-1640 at 370C under a humidified atmosphere with 5% CO2 as mentioned earlier [14].

2. MTT Assay: According to the manufacturer's instructions, MTT assay was performed to assess cytotoxicity of 17-AAG and Z-VAD-FMK as described elsewhere in detail [14].

3. CHIKV Infection in Macrophage: The cells (both Raw274.7 and Vero cells) were seeded in six well plates before 18-20 h of infection. The cells were infected with CHIKV with different multiplicity of infection (MOI) as described earlier [14,15].

understand the immunobiology of CHIKV infection but also help us to design anti-CHIKV measures in future. Recently CHIKV infection has been suggested to mediate thorough host macrophages [2]. However, the role of host macrophage during CHIKV infection is not well established. There might be some yet unknown mechanism by which CHIKV may regulate host cell immunity and follow a concomitant flow of viral replication and infection for successive stages. Therefore, We hypothesize that there might be some immune regulation in terms of CHIKV-macrophage interface, where host cell immune response might be modulated during CHIKV infection.

1.2. Review of Literature:

Chikungunya fever (CHIKF) is very often characterized by abrupt appearance of high fever, nausea, vomiting, headache, rashes over skin followed by polyarthralgia and myalgia. CHIKV is generally transmitted to the vertebrate host by Aedes mosquitoes and maintained in two distinct transmission cycles; urban cycle between human and mosquitoes and sylvatic cycle within forest dwelling mosquitoes and non-human primates. The enveloped virus contains approximately 11.8 kb long single stranded positive sense RNA genome with two open reading frames (ORF). The 5' ORF codes for non-structural proteins, nsP1-4, mostly involved in viral replication and 3' ORF codes for three major structural proteins, capsid, E1 and E2 [3,4].

Acute CHIKV also induces many pro-inflammatory cytokines such as IFNs, IL-1β, IL-6, IL-12, TNF, IFN-γ [5-7], GM-CSF [8,9] and chemokines IL-8, MCP-1 [10-12] in the host. CHIKV infects a wide range host cells including some immune cells [13]. Among them, monocytes are known to be one of the major host cells for acute CHIKV infection and inflammation in humans. It has been shown that macrophages could be susceptible for CHIKV infection both in vivo [2] and in vitro [6]. This productive replication of virus in the host macrophage might be associated with CHIKV induced pathogenesis despite robust immune activation.

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SYNOPSIS OF Ph. D. THESIS

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2. **Name of the Constituent Institution: National Institute of Science Education and Research (NISER), Bhubaneswar**

3. **Enrolment No. : LIFE11201104004**

4. **Title of the Thesis: Analysis of Cellular Immune Response during Chikungunya Virus (CHIKV) Infection in Macrophage**

5. **Board of Studies: Life Sciences**

SYNOPSIS

(Limited to 10 pages in double spacing)

CHAPTER-1: INTRODUCTION AND REVIEW OF LITERATURE

1.1. Introduction:

Chikungunya virus (CHIKV), an Alphavirus belonging to the Togaviridae family, was first isolated during a Tanzanian (formerly Tanganyika) outbreak in 1952 [1]. Recently, CHIKV has re-emerged as one of the major concern in South-East Asia and the Pacific region, causing considerable morbidity with even some cases of fatality. It is now considered as a major public health problem without proper protective and preventive measures. Consequently, it is highly important to know the immunological basis of CHIKV infection, which will not only help us to

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SSC-H: Side Scatter-height

DRDE: Defence Research & Development Establishment

Nsp: Non-structural protein

OD: Optical Density

IgG: Immunoglobulin G

MΦ: Macrophage

MOI: Multiplicity of Infection

CPE: Cytopathic Effect

DMSO: Dimethyl Sulfoxide

SFM: Serum Free Media

PEC: Peritoneal Exudate Cells

TG: Thioglycolate

ip: Intraperitoneal

RBCs: Red Blood Cells

w/v: Weight/Volume

v/v: Volume/Volume

FC analysis: Flow Cytometry analysis

RT: Room Temperature

FACS: Fluorescence-Activated Cell Sorting

WCL: Whole Cell Lysate

DDT: Dithiothreitol

PVDF: Polyvinylidene Fluoride or Polyvinylidene Difluoride

cDNA: complementary DNA

SEM: Standard Error of the Mean

ANOVA: Analysis of Variance

PFU: Plaque Forming Units

MFI: Mean Fluorescence Intensity

Z-VAD-FMK: Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

FSC-H: Forward Scatter-height

EGTA: Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic Acid

HPLC: High-Performance Liquid Chromatography

PFA: Paraformaldehyde

TEMED: Tetramethylethylenediamine

APS: Ammonium Persulfate

TMB: 3,3',5,5'-Tetramethylbenzidine

FcR: Fragment Crystallizable Receptor

7-AAD: 7-Aminoactinomycin D

ELISA: Enzyme-Linked Immunosorbent Assay

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PCR: Polymerase Chain Reaction

RT-PCR: Reverse Transcription Polymerase Chain Reaction

PBS: Phosphate-Buffered Saline

ICS: Intracellular Staining

CS: Surface Staining

RIPA: Radio Immunoprecipitation Assay

mM: millimolar

TGS: Tris-Glycine-SDS

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBS: Tris-Buffered Saline

TBST: Tris-Buffered Saline Tween-20

hpi: Hours Post Infection

NCCS: National Centre for Cell Science

EBV: Epstein Barr Virus

HCMV: Human Cytomegalo Virus

HCV: Hepatitis C Virus

GA: Geldanamycin

17-AAG: 17-*N*-allylamino-17-demethoxygeldanamycin

RPMI-1640: Roswell Park Memorial Institute-1640

FBS: Fetal Bovine Serum

DMEM: Dulbecco's modified Eagle's Medium

IAEC: Institutional Animal Ethics Committee

PE: Phycoerythrin

APC: Allophycocyanin

MAPK: Mitogen-Activated Protein Kinase

mAb: monoclonal Antibodies

SAPK: Stress-Activated Protein Kinase

JNK: c-Jun N-terminal Kinase

ERK: Extracellular-signal-Regulated Kinase

IRF-3: Interferon Regulatory Factor 3

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HRP: Horseradish Peroxidase

AF488: Alexa Fluor 488

AF647: Alexa Fluor 647

SDS: Sodium Dodecyl Sulphate

EDTA: Ethylenediaminetetraacetic Acid

T_{EF}: Effector Memory T Cells

T_{RM}: Tissue Resident Memory T Cells

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic acid

EEEV: Eastern Equine Encephalitis Virus

CHIKV: Chikungunya Virus

VEEV: Venezuelan Equine Encephalitis Virus

ORF: Open Reading Frame

DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

L-SIGN: liver/lymph node-specific intracellular adhesion molecules-3 grabbing non-integrin

RRV: Ross River Virus

SINV: Sindbis Virus

CHIKF: Chikungunya Fever

SLE: Systemic Lupus Erythematosus

MIP: Macrophage Inflammatory Proteins

MCP-1: Monocyte Chemoattractant Protein-1

PAMP: Pathogen-Associated Molecular Patterns

TLR: Toll-like Receptor

RLR: Rig-1 like Receptor

SFV: Semliki Forest Virus

CHIKD: Chikungunya Disease

HSP: Heat Shock Protein

HSP90: Heat Shock Protein 90

AAM: Alternatively Activated Macrophages

CMI: Cell Mediated Immunity

TCR: T Cell Receptor

DN: Double Negative

TCRβ: T Cell Receptors β

DP: Double Positive

SP: Single Positive

T_H Cells: T helper Cells

Tc Cells: Cytotoxic T Cells

CTLA4: Cytotoxic T-Lymphocyte Associated Protein 4

GATA3: GATA Binding Protein 3

RORγt: RAR-related Orphan Receptor gamma t

Bcl-6: B-cell lymphoma-6

Tregs: Regulatory T cells

nTregs: natural Tregs

iTregs: induced Tregs

FOXP3: Forkhead box P3

GITR: Glucocorticoid-Induced TNFR-Related *protein*

TGF-β: Transforming Growth Factor beta

NKT cells: Natural Killer T cells

iNKT cells: invariant NKT cells

MAIT cells: Mucosal-Associated Invariant T Cells

T_{CM}: Central Memory T Cells

ER: Endoplasmic Reticulum

TAP: Transporters Associated with Antigen Processing

ATP: Adenosine Triphosphate

ERAP: Endoplasmic Reticulum Amino-peptidase

RER: Rough Endoplasmic Reticulum

CLIP: Class II-associated Invariant Chain Peptide

HLA-DM: Human Leukocyte Antigen DM

CTL: Cytotoxic T Lymphocytes

Ig: Immunoglobulin

Fab: Fragment Antigen Binding

Fc: Fragment Crystallizable

IL: Interleukin

ROS: Reactive Oxygen Species

RNS: Reactive Nitrogen Species

PHSC: Pluripotent Hematopoietic Stem Cell

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

Cx3CR: CX3C Chemokine Receptor

CCR: CC Chemokine Receptor

PMo: Patrolling Monocytes

IM: Inflammatory Monocytes

mDC: Myeloid Dendritic Cell

MPS: Mononuclear Phagocyte System

LPS: Lipopolysaccharide

ABBREVIATIONS

R-M System: Restriction Modification System

AMP: Antimicrobial Peptide

PRR: Pattern Recognition Receptor

RA: Rheumatoid Arthritis

SLE: Systemic Lupus Erythematosus

HIV: Human Immunodeficiency Virus

ACAID: Anterior Chamber Associated Immune Deviation

NK Cell: Natural Killer Cell

PLOs: Primary Lymphoid Organs

SLOs: Secondary Lymphoid Organs

MALT: Mucus Associated Lymphoid Tissues

NALT: Nasal Associated Lymphoid Tissues

BALT: Bronchus-Associated Lymphoid Tissues

HSCs: Hematopoietic Stem Cells

DC: Dendritic Cell

MHC: Major Histocompatibility Complex

CD: Cluster of Differentiation

APC: Antigen Presenting Cells

pAPCs: Professional Antigen Presenting Cells

IFN-γ: Interferon- γ

IFN: Interferon

TNF: Tumor Necrosis Factor

2.4. Role of Host HSPs during CHIKV infection and Pathogenesis:

The molecular chaperon heat shock proteins (HSPs) acts as an important hub in protein-protein interaction network involved mainly in protein homeostasis. They bind non-covalently to stabilize misfolded or unfolded polypeptide to ensure/guide proper folding and assembly with other polypeptide so that the normal protein function can be delivered [225-227]. A plethora of reports suggesting the induction of different HSPs during viral infection, however the types of HSPs involved depends on the kind of virus and host cells involved [228,229]. Recently it has been shown the functional requirement of HSP90 different viral infection such as Hepetitis E virus, Epstein Barr virus (EBV), Human Cytomegalo virus (HVMV), Hepatitis C virus (HCV), Human Immunodeficiency virus-1 (HIV-1) and Vaccinia virus [230-237]. Usually, the HSP90 protein express does not change in the host macrophages, however the antigen presentation, phagocytosis and inflammatory responses affected by the HSP90 functionality [238]. Recently, it has been reported that Geldanamycin (GA), HS-10 and SNX-2122 (newly synthesized HSP90 inhibitors) CHIKV infection and replication [239,240]. However, specific role of HSP90 in CHIKV infection associated immune modulation in macrophages has not been delineated. Accordingly 17-AAG (less toxic derivative of GA), a potential HSP90 functional inhibitor and a phase-2 clinical trial drug [241] was tested whether it has any role in CHIKV infection and associate immune modulation in the host macrophages.

Together it appears that CHIKV infection has re-emerged a key public health concern due to its recent worldwide epidemics and lack of control measures. Although CHIKV is known to infect macrophages, regulation of CHIKV replication, apoptosis and subsequent host cell immune response especially towards macrophages remains scanty. Moreover, there is a need to test of an anti-CHIKV drug candidate to investigate experimental CHIKV infection in macrophages associated to pathogenic inflammatory immune responses.

such as TNF, IL-1β, IL-6, IL-12, IFN-γ, IL-8, GM-CSF and MCP-1[206,211-218], most of which might be associated with arthritis like pathogenesis during CHIKV infection. In different in vivo system (both mouse and non-human primates), it has been observed that predominant cellular infiltration of macrophages, monocytes, NK cells and T cells to the site of inoculation and other tissues [191,219]. Immunohistochemistry and flow cytometry based analysis of muscles and synovial biopsies revealed that macrophages are major infiltrating cells among MPS [190,207]. A variety of both immune and non-immune cells are reported to be susceptible for CHIKV infection both in vitro and in vivo model system [111,178,188,189]. Blood monocytes and tissue macrophages are the major immune cells infected by the CHIKV [111,189,213], and in macaque, it has been identified that synovial macrophages are the major host for long term viral persistence [111]. This productive infection of CHIKV in the host macrophages might be associated with the arthritis like pathogenesis despite robust immune activation [189,220].

T cell immune responses specific to CHIKV is not clearly understood yet. Teo TH et al. have suggested that CD4+ T cells (but not CD8+ T cells) are essential for the development of CHIKV induced pathogenesis, which is independent of IFN-γ, without any effect on the virus infection and dissemination in mice [221]. Flow cytometry based analysis of circulating lymphocytes in patients confirms that there is both CD+ and CD+ T cell responses during early and late phase of infection respectively. Moreover, CD95 mediated apoptosis was also detected in CD+ T cells after 2 days of symptom appearance [222], which might be one of the strategy to evade host immunity. Purified T cells (both CD4+ and CD8+) from the chronic and recovered patients from 2005-06 La Reunion islands showed immune activation when challenged with synthetic CHIKV peptides and inactivated virus particles [220]. The DNA vaccine based on consensus sequence of E1/2 and capsid protein (with several modifications) of CHIKV resulted in robust IFN-γ and IgG production suggesting that CHIKV induces both T and B cell specific responses [223,224].

generally associated with high fever, skin rashes, headache, nausea, edema, vomiting and gastrointestinal complaints [84,170,193,194]. Clinical examinations revealed the hall mark feature of CHIKV infection such as polyarthralgia associated with synovitis and arthritis like disease also experienced by most of the patients [195,196]. Severe inflammation of periosteum, tenosynovitis and enthesitis of both Achillis tendons was also evidenced in several patients [195,197]. In most patients the acute symptoms disappears within 2 weeks of initial exposure, whereas chronic arthritis persists up to 2 years in about 12% of cases. High anti-CHIKV antibody titer in the synovial fluid of chronic patients was evidenced, which might be responsible for chronic arthritis like symptoms [198-200]. Furthermore, in some cases more complicated manifestations such as nephritis [201], myocarditis, pericarditis (Simon F et al., Am J Trop Med Hyg. 2008), retrobulbar neuritis [202,203], meningoencephalitis and death have also been reported [96,170,204,205].

2.3. Host immune responses to Chikungunya virus (CHIKV):

Current studies on CHIKV induced immune responses suggest that the host immune system is found to be both beneficiary in one hand by controlling viral infection/dissemination, whereas deleterious on the other hand by promoting severe inflammatory responses [185,206-209]. Like other microbial inoculations, CHIKV induces quick type I interferon (IFN) production to heighten anti-viral defences in the nearby cells. At first it has been reported that adult mice but not neonates are resistant to the CHIKV infection and the disease severity is age dependent in neonates. An adult mouse with partially or total knockout of IFN receptors develops mild to severe infection respectively. The pathogenesis and tissue tropism also vary among these two groups, suggesting an important role of IFN signaling to contain early CHIKV infection [185]. However, it has been suggested that CHIKV nsP2 is a potent suppressor of IFN mediated anti-viral signaling by inhibiting STAT1 phosphorylation/nuclear translocation [210]. Studies have shown that CHIKV induces heighten levels of different inflammatory cytokines/chemokines

pathogenesis varies among different strains and age groups of mice. There is no mouse model now a day to study the long lasting chronic arthralgia and arthritis like diseases, which is commonly associated with CHIKV infection [192]. In macaque model of CHIKV, it has been suggested that CHIKV targets liver, spleen, lymphoid tissues, CNS, joints and muscles during acute stage while infecting macrophages, DCs and some endothelial cells. In the later stages persistent CHIKV infection was found in liver, spleen, lymphoid tissues, joints and in macrophages after 3 months of initial inoculation [111].

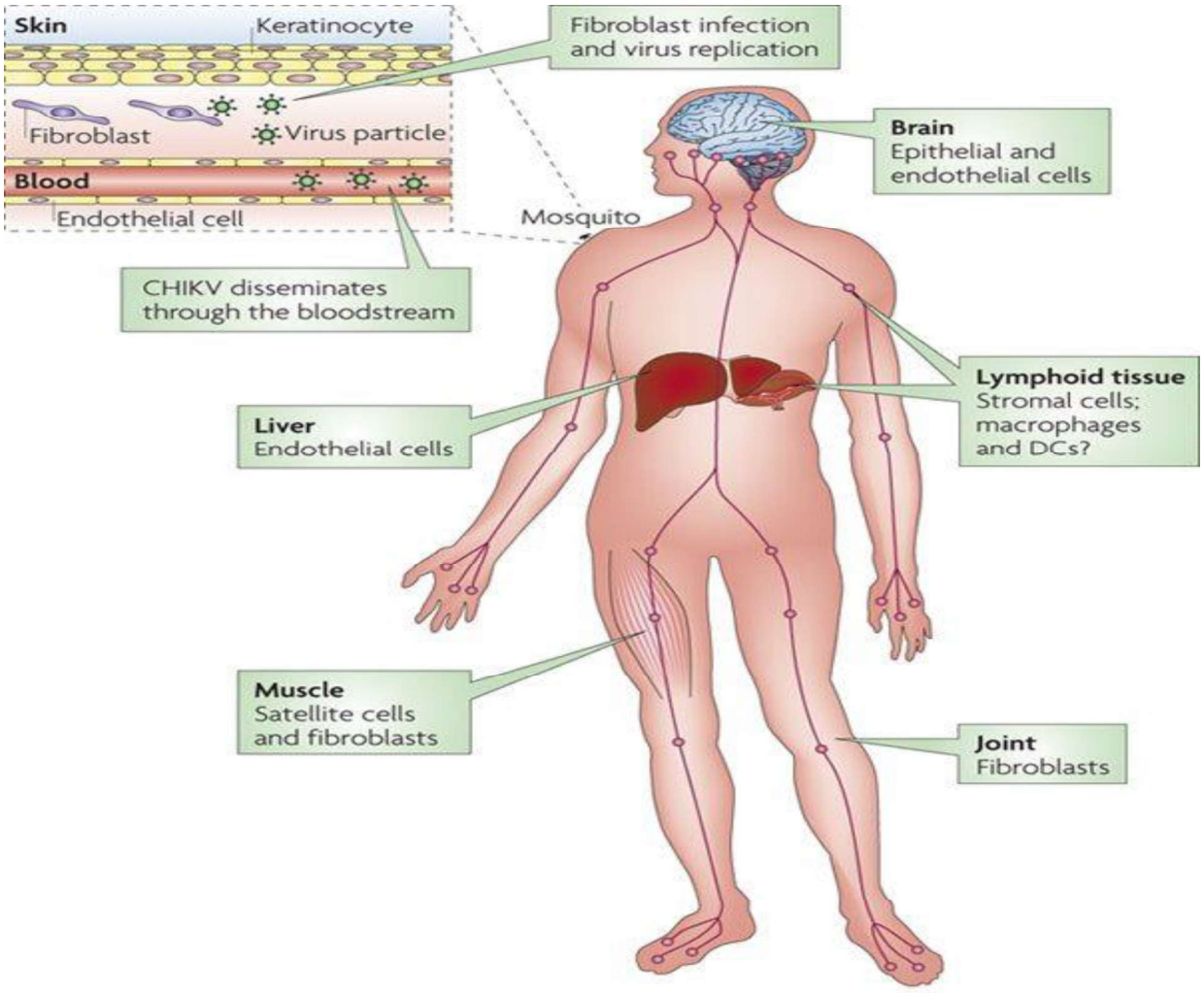

Figure 8. Dissemination Chikungunya virus in the vertebrate host *(Courtesy Ref: [84])*

CHIKV disease (CHIKD) associated symptoms usually develop 4-7 days after virus inoculation. Acute phase disease lasts for 1-10 days, whereas chronic symptoms may persists for a very long periods of time after a very short periods of initial incubation time. CHIKD is

antibodies, however CHIKV has never been isolated from those Malaysian wild monkeys. The non-human primates, monkeys, rodents, birds and others serve as a potential reservoir of CHIKV maintenance outside the epidemic periods (hence crucial for re-emergence of CHIKV), whereas humans serve as reservoir during epidemic periods [182]. Initially, the main vector for CHIKV transmission was considered to be the *Aedes aegypti,* which was well adapted to the urban conditions. However, studies showed that CHIKV has adopted *Aedes albopictus* as the principal vector for recent outbreaks in the different areas including Reunion island. Generally, *Aedes albopictus* is very versatile in terms of survival, aggressiveness, reproduction and life expectancy as compared to *Aedes aegypti*. The switch of this vector has been associated with a point mutation (A226V) in the E1 glycoprotein, which has increased the fitness of CHIKV to the *Aedes albopictus* to ensure better host transmissibility [183,184]. Thus, posing a risk of broader outbreak in the previously unaffected areas.

Following inoculation, CHIKV replicates locally in the skin fibroblasts , muscles and then disseminates in to the other sites such as liver, bones, lymphoid tissues and brains probably through the blood vascular system[185-187]. A large pile of effort has been made to study the cells and tissue tropism for the proper understanding of CHIKV mediated pathogenesis. CHIKV is known to infects a variety of adherent cells both *in vitro* such as Vero, CHEM, HEK293T, C6/36, HeLa, HepG2, macrophages [178,188] and *in vivo* such as macrophage, monocytes, muscle satellite cells, Whereas the non-adherant cells like T cells , B cells are not susceptible to CHIKV infection [111,189,190]. In the mouse model, the key difficulty is to mimic the pathogenesis induced by CHIKV in humans. Following infection, CHIKV replicates locally and then in muscles and joint tissues.. There is acute infiltration of NK cells, monocytes/macrophages, neutrophils and others to the musculoskeletal tissues. Typical inflammatory signs like swelling, edema were also observed in the muscles, though the magnitude is very less as compared to the inoculation site [191]. The peak viremia and

the period from February 2005 to June 2006 [174]. The complexity of CHIKV pathogenesis is increasing day by day with reported cases of fatality, which were never observed before [175-178]. Thus, severe panic was noticed among patients seeking the attention of physicians, scientists and other competent state authorities for help, support and suggestions. Currently, CHIKV is considered as one of the potential pathogens which spread almost throughout the globe. Hence, in order to understand the risk associated to this re-emerging Alphavirus, researchers around the world are emphasizing to dissect out the biology and associated pathophysiology in every possible aspect to develop effective anti-CHIKV therapeutic measures.

2.2. Chikungunya Virus transmission, clinical manifestations and pathogenesis:

CHIKV belongs to genus Alphavirus of family Togaviridae, under the group Semliki Forest virus (SFV), which comprises enveloped single stranded positive sense RNA virus. The enveloped virus contains approximately 11.8 kb long single stranded positive sense RNA genome with two open reading frames (ORF). The 5' ORF codes for non-structural proteins, nsP1-4, mostly involved in viral replication and 3' ORF codes for three major structural proteins, capsid, E1 and E2 [84]. The capsid of the CHIKV is icosahedral in shape and is approximately 50-70 nm in diameter [178]. The transmission of CHIKV to humans occurs through a bite by a invertebrate vector belong to Genus *Aedes*, although there are some maternal-fetal transmission case reports [179-181]. Transmission of the CHIKV occurs mainly through two different cycles, the urban cycle and sylvatic cycle depending on the type of host or vector involved. In the urban cycles (man-mosquito-man), the virus transmission involves urban mosquitoes and humans. Where as in the sylvatic cycle (animal-mosquitoes-man), which is observed mainly in Africa, the CHIKV circulated between forest dwelling mosquito species (e.g., *Ae. furcifer, Ae. taylori*) and non-human primates such as chimpanzees, and humans. Thus, the maintenance and transmission of the virus between the vertebrate host and invertebrate vector is different in Africa and Asia. It has been reported in mid 1960s that, the monkey's serum contains CHIKV neutralizing

diverting raw materials such as amino acids, which is supposed to be used by the host cell itself [155-158]. Very often viruses also shut-off host translational process, which might be a strategic decision to contain antiviral responses [159,160]. The integration of complex proteomics studies including in silico protein-protein interaction predictions keeps on unraveling the complex network of interaction with the host cell proteins. Throughout the course of replication these pathways rely heavily on the dynamic and temporarily regulated virus host protein-protein interactions which are crucial for the virus replication, pathogenesis, and viral subversion of host defence [152,158,161]. The identification and characterization of these interacting partners also helps in the delineation of the viral protein functions precisely and might be very helpful in designing rationale drugs for an effective treatment [152,161,162].

2. Review of Literature:

2.1. Chikungunya Disease; A potential re-emerging global threat:

Chikungunya virus a mosquito borne Alphavirus has re-emerged as a major public health concern due to unavailability of effective antiviral drugs or licensed vaccines for human use despite intense global research effort [163-165]. Though, there are several therapeutic candidate drugs and vaccines against CHIKV have been proposed, however they need rigorous testing and approval by the competent authority before final clinical trials or diagnostic use [166-168]. CHIKV is globally distributed and is responsible for massive endemics around the world. Like other viral and bacterial pathogens, CHIKV also regarded as one of the highly pathogenic arbovirus. Previously CHIKV was not considered as extremely pathogenic arbovirus, however, this misconception was challenged by the death of several CHIKV infected individuals in 2005-06 unprecedented outbreaks in the Reunion Island of Indian ocean, followed by explosion of cases in several other countries [108,169-173]. This re-emergence of CHIKV in India was witnessed after a gap of 32 years, which infects approximately 1.3 million people in different states [97]. Several other neighboring small islands of India also affected by this outbreak, during

where they differentiated in to tissue macrophages. The migration of the macrophage to the wound site is tightly regulated by a variety of chemokines (MIP, MCP-1, RANTES), which acts in a concentration dependent manner [129-131]. The neutrophils (arrived early), are then phagocytized by the macrophages along with the injected pathogens [132]. Macrophages detects PAMP of various pathogens by PRR, such as TLR, RLR. After activation it encounters almost all the pathogens by variety of pathways, such as they can engulf pathogenic bacteria, secretes anti-microbial peptides, produces ROS and NOS and activates specific adaptive immune system via antigen processing and presentation [132-138]. However, microbes have evolved in different directions to counter the host macrophages effector functions for the successful infection and replication. Microbes, especially viruses evade macrophage immune responses by a variety of pathways such as avoiding destruction in the lysosomes [133,139], interfere in macrophage infiltration by encoding chemokine homolog or receptors [140-143], interfere in antigen processing and presentation [144,145], killing of infected cells [139,146,147] and inducing immunosuppressive microenvironment for persistence [148,149].

1.7. Host protein utilization by viruses for viral infection, replication, and pathogenesis

Viruses are acellular and obligatory in nature, so they can only replicate inside a suitable host cell. Although, the viral genome is very small as compared to corresponding eukaryotes, it encodes a broad range of specific proteins. The viruses utilize key host proteins for its infection, replication, pathogenesis and persistence by exploiting either its expression or function [150-152]. The host cell enforces multiple barriers to prevent virus entry. However, viruses overcome this problem by exploiting normal cellular processes [153,154]. The viruses utilize more than one cell surface receptors and co-receptors for the virus attachment, uptake by receptor mediated endocytosis and finally cargo release to the cytosol. Both the DNA and RNA viruses exploit the host cell translational machinery to form virus specific proteins (structural and non-structural) by

arthritis, SLE, asthma, atherosclerosis etc. Inflammation generally classified in to two types (acute and chronic) on the basis of their nature of action and time taken for the completion of the resolution process. The initial inflammatory responses to any foreign antigen within very short periods of time (usually within hours or days) is acute inflammation. It is characterized by vasodilation, oedema, leukocyte migration by chemotaxis and production of inflammatory paracrine (IL-1β, TNF, histamines, IL6) [115-117]. Whereas prolonged inflammation is called chronic inflammation, often characterized by the infiltration of mononuclear cells, simultaneous tissue destruction (by inflammatory cells) and repair (with fibrosis and angiogenesis) [118,119]. Unlike acute inflammation, the chronic inflammation involves a variety of cells, cellular processes, cytokines/chemokines and other secretory products such as auto-antibodies [120-122]. Inflammation needs to be co-ordinated and controlled very tightly for the successful resolution from any antigenic insult, less inflammation might be fatal due to active tissue destructions by the pathogens and inadequate immune responses, whereas too high inflammation will cause un-necessary tissue damage, followed by chronic inflammatory conditions [112]. Obligate pathogens such as viruses also known to induce inflammatory responses along with multitude of immune activations during both acute and chronic stages of infection in the host [123,124]. Among viruses, Alphavirus infection is mainly associated with severe inflammatory responses in the host, which might be one of the crucial factors contributing towards pathogenesis [123-127].

1.6. Macrophages and microbes; An important interplay between the Immune system and the pathogens:

Macrophages are one of the professional APCs, which maintains host homeostasis by responding to both the external and the internal changes, by not only by phagocytosis of invading microbes, the dead/dying cells and other cellular debris, but also through trophic regulatory or repair functions [128]. Macrophages play a central role in host responses to a variety of pathogens. Monocytes are recruited to the site of infection very often preceded by neutrophils,

common secondary replication sites. In humans, joint tissues, skin (CHIKV, RRV) and nervous tissues that causes encephalitis (Tonate virus, VEEV) [84,86,87].

1.4. Chikungunya virus; A major global public health concern:

CHIKV, a mosquito burn Alphavirus belongs to Togaviridae family. It is transmitted through either *Aedes Aegypti* or *Aedes Albopictus* mosquito. CHIKV induced disease is one of the global challenge due to its rapid endemics in different parts of the globe (103 countries affected), such as Tanzania [90-92], Reunion island [93-96], India [97-102], Italy [103,104], Thailand and many other parts around the globe [105-108]. Currently, among Alphaviruses, CHIKV is considered as one of the most successfully evolved virus. The Arboviruses including CHIKV have been evolving and re-emerging from centuries, though the emergence and dispersion are more rapid and geographically extensive, may be due to increase in global communication, mass immigration, vector adaptation to urbanization and land perturbation [109]. Even though mortality due to CHIKV is very rare and restricted to children's (below 1 year), old age (above 65 years) or immune compromised patients, the pathogenesis (mainly inflammatory responses) may persist for very long periods of time both in humans and macaque model [110,111]. The non-availability of any effective anti-CHIKV drugs and vaccines makes CHIKV a stealthy winner, which might pose a serious socioeconomic threat in future, while considering the past few years of this virus success story.

1.5. Inflammation; a double-edged sword:

Inflammation is a complex immunological/pathophysiological process initiated mainly by white blood cells and associated components against harmful stimuli such as tissue injury, infections, foreign antigens, allergens, irritants and other types of insult [112,113]. In general, the inflammatory responses are associated with redness, swelling, heat, pain and loss of function [114]. It is good when it fight against foreign invaders and alert immune system for further action. It is bad and ugly when it ignites different inflammatory disorders such as rheumatoid

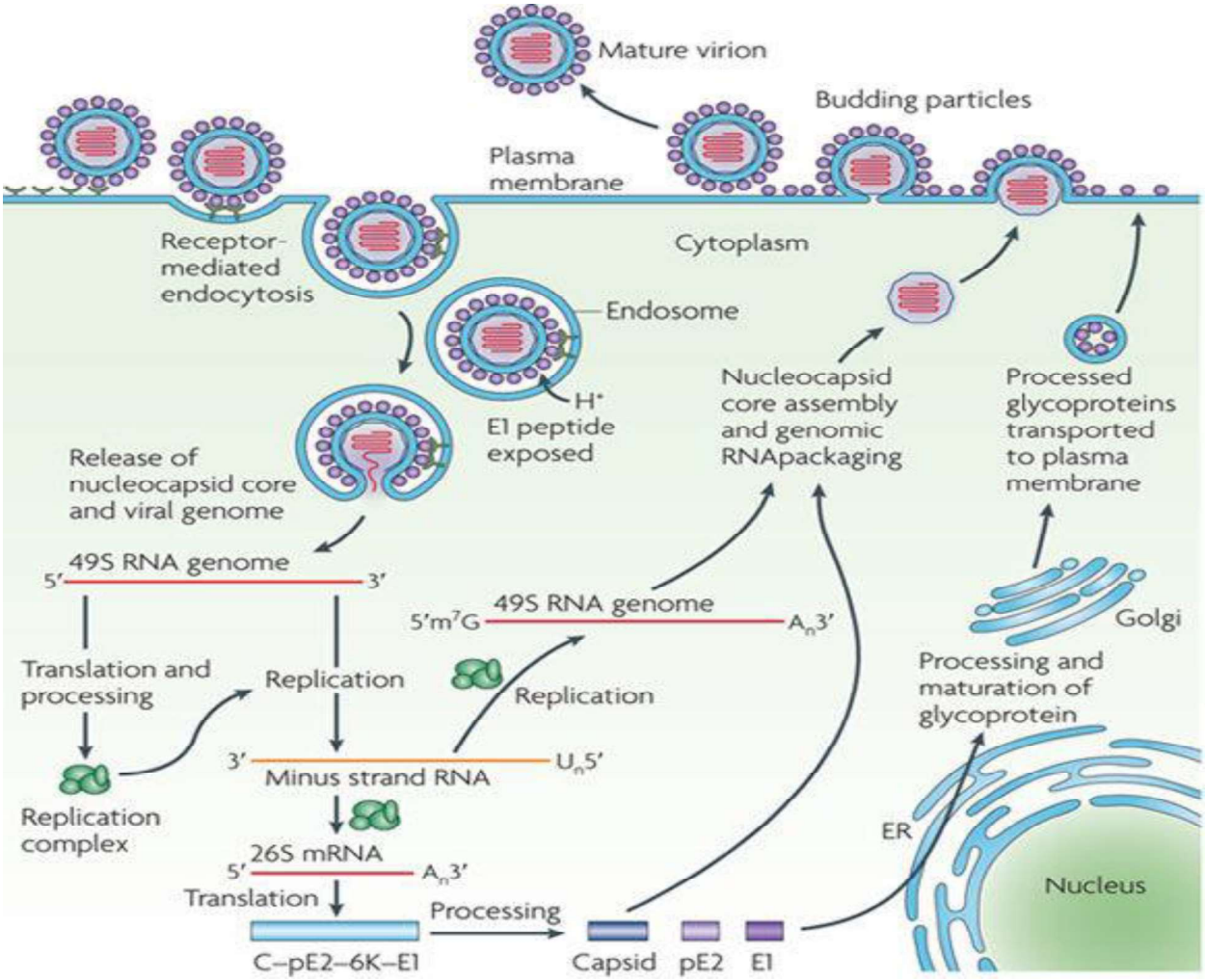

Figure 7. The Alphavirus lifecycle in the host cell *(Courtesy Ref: [84])*

1.3.2. Cell, tissue tropism and pathogenesis of Alphavirus infection:

The initial site of Alphavirus replication varies among different types virus and host cells. After natural inoculation, virus may infects local skeletal muscle cells (e.g., CHIKV, RRV, SINV) or infects DC of skin (Langerhans cells), subsequently transported to the local draining lymph nodes for further infection [81]. The increase in the viremia depends up on several factors including the rate of replication at the inoculation site and the viral clearance from the blood. Generally, large plaque viruses or virus strain shows early and intense viremia than that of small plaque viruses or virus strain. After initial replication at the site of inoculation, viruses spread in to other target tissues via blood. In mice brain, spinal cord, chondrocytes, osteoblasts are

1.3.1. Life cycle of an Alphavirus:

Alphaviruses are zoonotic arboviruses, transmitted through Aedes misquotes from one host to another. After entry in to a suitable host, viruses bind to specific receptor via E1 and E2 glycoproteins. The internalization of the virus particle is mediated through receptor mediated endocytosis. A very few receptors such as DC-SIGN, L-SIGN, heparin sulphate, lamin and integrins have been identified to be involved in this process, however their specific role has not been studied yet [84]. After endocytosis, the low pH of endosome compartment facilitates conformational changes in the envelope, exposing E1, which mediates viral and host cell membrane fusion, as a result the viral genome released in to the host cell cytoplasm. The viral mRNA utilizes host cell translational machinery to form non-structutal proteins (nsP1-4), from their respective precursor proteins after proteolytic cleavage. All the non-structutal proteins assemble to form main replication complex, for the synthesis of full length negative sense RNA intermediate from the existing positive sense RNA. From this negative sense RNA both 26S sub-genomic and 49S genomic RNA is synthesized. The sub-genomic RNA translated to produce C–pE2–6K–E1 polyprotein precursor, which is further cleaved by serine autoproteolytic activity. Then the capsid protein is released followed by post-translational modification of both the pE2 E1 occurs via the ER/trans-golgi network. Then all the proteins transported on to the plasma membrane where pE2 converted to E2 and the E3 protein mediate proper folding of the E2 and association with the E1 protein. Then the viral nucleopsid binds to the viral RNA followed by recruitment of the membrane associated envelope glycoproteins. The completely assembled virus particle then egress from the host plasma membrane for further infection [84,87-89].

encephalitis virus subgroup (e.g., VEEV) and contains approximately 30 members, which diverged few thousand years ago [82]. All alphaviruses contains single stranded positive sense RNA as genome with an outer envelope. They infect a wide of animals such as humans, cats, horses, fish, birds and some invertebrates and all are Arboviruses or arthropod-borne viruses as viral transmission between the hosts occurs via mosquitoes. The Alphaviruses are divided in to two main categories, old world virus, involved mainly in rheumatic diseases, whereas, the new world viruses associated with fatal encephalitic diseases [83-85]. The enveloped Alphavirus is approximately 60-70 µm in diameter with slightly spherical in shape. The genome present inside the nucleocapsid is ranges from 11k to 12 k in length starts with 5′ cap ends with 3′ poly-A tail. There are open reading frame (ORF) in the genome of an Alphavirus' ORF-1 and ORF-2. The 5′ ORF-1 codes for viral non-structural proteins, whereas the 3′ ORF-2 codes for structural proteins. The structural proteins are the main constituents of the virus particles along with RNA and some lipids in the envelope, whereas the non-structural proteins expressed only inside the host cells suggesting crucial role in replication [84,86,87].

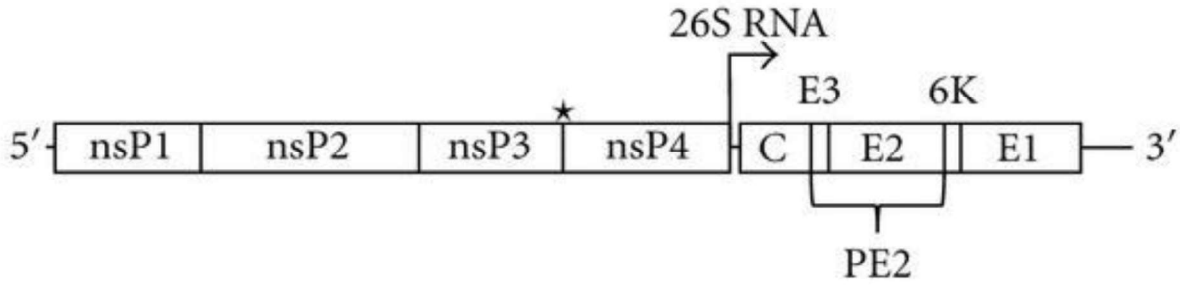

Figure 6. Genome organization of Alphavirus. * denotes leaky stop codon, *(Courtesy Ref: [83])*

1.2. Introduction to the viruses

Viruses are small obligatory intracellular pathogens and infect almost all the groups of living organisms including animals, plants, bacteria and archaea. Viruses do not possess any cellular structures of their own, so very often called acellular in nature. Outside the host virus particles remain in dormant state (inert), up on entry in to a suitable host cell, they utilize the metabolic pathways of the host for replication. A complete virus particle is called as virion consist of the genetic materials (either DNA or RNA), protected by protein coat, called capsid and in some viruses the whole structure is surrounded by an outer layer of capsule. The capsid is made up of identical protein subunits called capsomeres. Individual capsomere is encoded by the viral genome during replication and subjected to self-assembly by non-covalent interactions to form complete capsid surrounds genome. Sometimes some enzymatic proteins associated with the viral genome, called nucleoproteins (e.g., HIV), whereas the association of the viral genome with the capsid proteins is called nucleocapsid. The viruses surrounded by an outer capsules or envelope contain main bulk of lipids, carbohydrates and some glycoproteins are called enveloped viruses. The envelope is generally derived from the host (last infected) hence share significant resemblance with the host plasma membrane. It is the shape of viral capsid, which determines the morphology of the virus particle. There are four basic morphological types of the virus, such as icosahedral (e.g. piconavirus), helical (e.g. tubulovirus), envelope (e.g. paramyxovirus) and complex (e.g. vaccinia virus). The exceptionally simple architecture of the viruses makes them one of the most adaptable and rapidly evolving pathogens, by acquiring and inheriting point mutations rapidly [81].

1.3. Alphavirus- An emerging global threat:

The genus Alphavirus belongs to Togaviridae family of group IV according to the Baltimore classification. It has three subgroups, such as the eastern equine encephalitis virus subgroup (e.g., EEEV), the Semliki Forest virus subgroup (e.g., CHIKV) and Venezuelan equine

polymorphic CD1d molecule. NKT cells acts like both helper T cell and killer NK cells (predominantly via Fas ligand). They are either $CD4^+$ or $CD4^-$ cells or express most of the NK cell specific markers [70-73]. NKT cells has been classified in to three main sub types as follows

- Type 1 NKT or Classical NKT or invariant NKT (iNKT) cells
- Type 2 NKT or non- classical NKT or diverse NKT cells
- NKT-like (CD1a restricted, CD1b restricted, CD1c restricted and MR1 restricted or MAIT cells) [74]

- Memory T cells: The memory T cells are differentiated helper T cells, which are generated against specific antigens during infections and tumors formation. These cells remain in dormant conditions for years, up on recognition of its cognate antigens, it activate and proliferates, thereby gives immediate and effective immune responses for the clearance of the foreign antigens. Until now the memory T cell populations comprises long lived central memory T cells (T^{CM}), which localizes and resides in the secondary lymphoid organs, and effector memory T cells (T_{EF}), that circulates between peripheral non-lymphoid tissues, blood and spleen. Besides this, a third type of cells called tissue resident memory T cells (T_{RM}) has also been identified. T_{RM} occupy in different tissues without circulating to the blood or spleen, mainly responsible for the activation of CMI against pathogens [75-77].

- Gamma delta T cells: T cells expressing TCR composed of γ and δ chains are called γδ T cells, responds to infections, cellular transformations (cancer) and tissue damage. Un like αβ TCR which recognizes processed antigens presented by MHC, the γδ T cells damaged tissues, cells and microbes directly. Although, a very few reports suggests γδ T cells can recognize self MHC molecule without cargo and to some MHC class I like proteins [78-80].

CTL also induce apoptosis of the target cells by engaging Fas ligands-receptor interaction. Like T_H cells, CTLs also differentiated in to two sub groups, Tc1 and Tc2. Tc1 produces high level of IFN-γ and kills target cells more efficiently by both perforin and Fas mediated pathways. Whereas, Tc2 secretes high amount of IL-4, IL-5, IL-10, IL-13 and low level of IFN-γ, kills target cells only via perforin mediated pathway [61,62].

- Regulatory T cell: Regulatory T cell (Tregs) generally immunosuppressive in nature maintains self-immune tolerance and regulates autoimmunity. It suppresses activation and proliferation of the effector T cells, and thereby reduces inflammatory responses. Some Tregs are naturally induced in the thymus called natural Tregs (nTregs) express $CD4^+$, $CD25^+$ and $FOXP3^+$, whereas others induced from naïve T cells as a consequence of specific antigen exposure or cytokine milieu called induced Tregs where the FOXP3 is induced in the periphery [63-67]. There are several other specific surface markers have been reported, which are associated with Tregs such as CD62L, CD103, CTLA-4, GITR [68]. However some other kinds of T cells also show immunosuppressive phenotypes such as Tr1 and Tr3 by producing IL-10 and THF- β respectively. There are four basic mechanisms by which Tregs suppress immune responses, those are by secreting inhibitory cytokines, granzyme mediated cytolysis, inhibition of DC maturation/function and metabolic disruption [69]. Other T cell populations such as NK T cells, CD8+ T cells and gamma delta T cells also have potent suppressive effect under certain defined circumstances. Although CD8+ T cells can be induced to express FOXP3, the suppressive phenotype of these cells needs to be studied.

- Natural Killer T cells: The Natural Killer T cells or NKT cells are heterogeneous populations of regulatory immune cells that possess properties of both NK cells and T cells, that promote cell mediated immunity to tumors and infectious diseases. These cells express both αβ TCR and NK cell markers (CD161) on the surface and helps in bridging (cross-talk) innate and adaptive immunity. NKT cells recognize and bind to the glycolipid presented by a non-

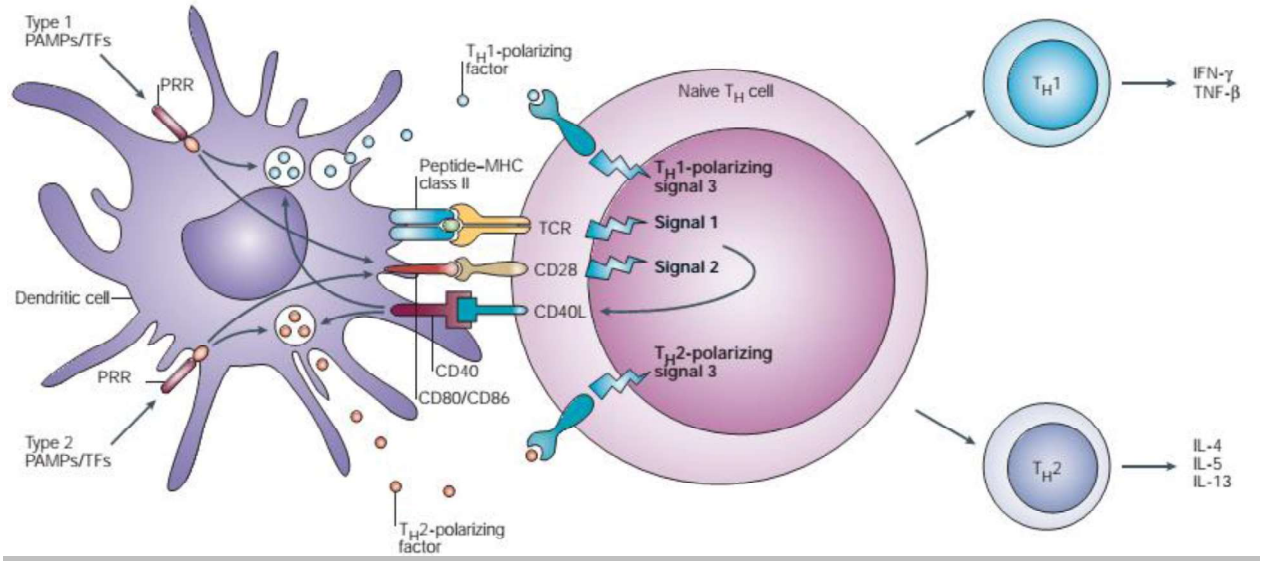

Figure 5. T cell stimulation, activation and polarization requires three consecutive signals from APCs (DC) *(Courtesy Ref: [60])*

As far as co-receptor of TCR and MHC class restriction is concern there are two major types of T cells. The $CD4^+$ T cells, which recognize peptide presented by MHC class II, whereas the $CD8^+$ T cells recognizes peptide presented by MHC class I. Moreover, on the basis of specific functions T cells have been classified in to following types.

- Helper T cells: T_H cells assist proper functioning of the other immune cells such as B cells, plasma cells, memory B cells, activation of CTL response and macrophages. These types of cells are also called $CD4^+$ T cells. The helper T cells further differentiated in to several sub populations such as T_H1 (t-bet+), T_H2 (GATA3+), T_H17 (RORγt+) and T_{FH} (Bcl-6+) in the presence of specific polarizing cytokines.

- Cytotoxic T lymphocytes: These cells are also called killer T cells or CD8+ T cells. These types of cells generally targets self-altered cells such as cancerous cells, virus infected cells and foreign tissue grafts. After formation of the immunological synapse through MHC I-TCR and co-stimulatory molecules activated CTL differentiated in to functional effector CTL, releases several cytotoxic granules perforins, which in turn kill the target cells. Moreover,

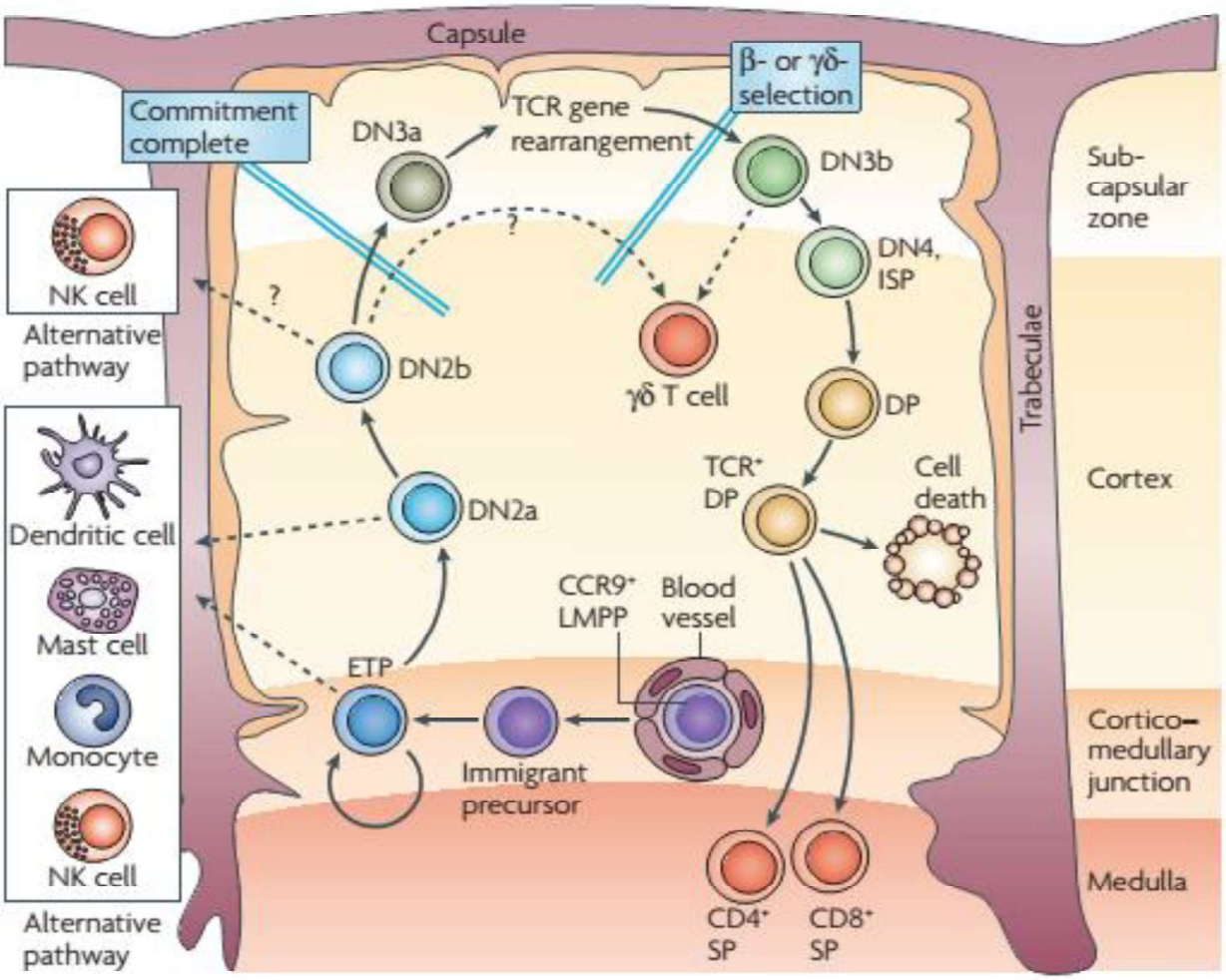

Figure 4. Stages of early T cell development in an adult thymic lobule depicting the migration path of different T cell precursors *(Courtesy Ref: [56])*

In the periphery, the activation of specific clone of T cell requires three sequential and consecutive signals from APCs. The signal 1 is antigen specific signal mediated through TCR with MHC antigen complex. Signal 2 is the co-stimulatory signal between B7 (CD80/CD86) molecule and CD28 on T cell. Signal 3 is polarizing signal mediated via various soluble cytokines such as IL-12, CCL2 to promote either T_H1 or T_H2 phenotype respectively. However, T cells will be in immunosuppressive state, if B7 molecules interact with CTLA4 on T cells [59,60].

T lymphocytes are a type of white blood cells responsible for giving protection against antigenic insult via cell mediated immunity (CMI). The hallmark of T cell is T cell Receptors (TCR) which interacts with MHC peptide complex for T cell activation (signal 1). Majority of the T cells contain αβ chains of the TCR so called αβ T cells. A very few T cells rearrange γδ chains of the TCR to become γδ T cell. The αβ TCR are highly diverse and only recognize MHC-antigenic peptide complex, whereas the less diverse γδ TCR believed to recognize lipid antigens (e.g., phospholipids) presented by CD1a, CD1c and CD1d [53,54]. All T cells derived from PHSC in bone marrow through common lymphoid progenitor cell, which migrated to the thymus via blood where it undergoes several stages of development and then exported to the periphery for activation and differentiation. In the thymic cortex, the mature thymocytes (T cell precursor) are CD4-CD8- so called DN1 (double negative1), undergoes TCRβ locus rearrangement and proliferation to form DN2 cell type. The DN2 cell either enters less diverse γδ T cell pathway (few further changes in the surface phenotype) or it differentiates in to DN3 type. The β-selection occurs between the transitions from DN3 to DN4, followed by TCR α locus rearrangement to form CD4+CD8+ double positive cells (DP). Then these DP thymocytes undergoes positive selection for self-MHC restriction in the thymic cortex followed negative selection for self-tolerance in the thymic medulla, where they become SP or single positive (CD4+ or CD8+) T lymphocytes. Mature T cells (express either CD4 or CD8) leaves the thymus and for further journey to peripheral tissues through bloodstream. While majority of the DP cells differentiates in to SP cells, a very few population differentiated in to Tregs, T_H17, IEL and NK1.1 T lymphocytes [55-58].

Macrophages (MΦ)	Tissue	Functions	Pathology
Microglia	Brain	Brian development (121), immune surveillance, synaptic remodeling (122)	Neurodegeneration (123)
Osteoclasts	Bone	Bone modeling and remodeling, bone resorption (124), support to hematopoiesis (125)	Osteoporosis, osteopetrosis, arthritis (126)
Heart MΦ	Heart and vasculature	Surveillance	Atherosclerosis (127)
Kupffer cells	Liver	Toxin removal, lipid metabolism, iron recycling, erythrocyte clearance, clearance of microbes, and cell debris from blood (128, 129)	Fibrosis (130), impaired erythrocyte clearance (131)
Alveolar MΦ	Lung	Surfactant clearance, surveillance for inhaled pathogens (132)	Alveolar proteinosis (133)
Adipose tissue-associated MΦ	Adipose tissue	Metabolism, adipogenesis, adaptive thermogenesis (134)	Obesity, diabetes, insulin resistance, loss of adaptive thermogenesis (131)
Bone marrow MΦ	Bone marrow	Reservoir of monocytes, waste disposal (131)	Disruption of hematopoiesis (131)
Intestinal MΦ	Gut	Tolerance to microbiota, defense against pathogens, intestinal homeostasis (135)	Inflammatory bowel disease (136)
Langerhans cells	Skin	Immune surveillance (137)	Insufficient healing, fibrosis (138)
Marginal zone MΦ, red pulp MΦ	Spleen	Erythrocyte clearance, iron processing, capture of microbes from blood (139)	Impaired iron recycling and erythrocyte clearance (140)
Inflammatory MΦ[a]	All tissues	Defense against pathogens, protection against dangerous stimuli (141)	Chronic inflammation, tissue damage, autoimmunity (91)
Healing MΦ[b]	All tissues	Branched morphology, angiogenesis (142)	Cancer, fibrosis, epithelial hyperplasia (91)

[a]Also known as inflammatory macrophages or M1 macrophages.
[b]Also known as deactivated or M2 macrophages.

Table 4. Tissue specific macrophage distribution, their functions and associated pathological conditions up on anomalous activation in the main tissues *(Courtesy Ref: [48])*

The alteration in the normal physiology such as stress, injury, infection and other homeostatic imbalance activates macrophages. The type of macrophage activation to various endogenous stimuli is patio-temporal dependent. The classical immune activation of macrophage is called M1 type, which is activated by bacterial lipopolysaccharide (LPS) and IFN-γ. It expresses high MHC class II, CD86, TNF, IL-12 and low level of IL-10. Whereas, the M2 macrophage (also called as alternatively activated macrophages; AAM), is responsible for wound healing, tissue repair and it promotes immune-suppression mainly via IL-10 production [32,35,49-52]. AAM is generally induced in the presence of IL-13 and IL-4 and some other immunosuppressive pharmaceutical drugs.

1.1.3.8. T Cells

monocytes (IM) recruited quickly upon tissue damage or infection, where they differentiated in to either macrophages (tissue specific) or DC for further immune activation [40]. Together the monocyte precursor cells in the bone marrow, blood monocytes, tissue macrophages and mDC grouped as mononuclear phagocyte system (MPS) or mononuclear phagocytic system (formerly called reticuloendothelial system) [34,41-46].

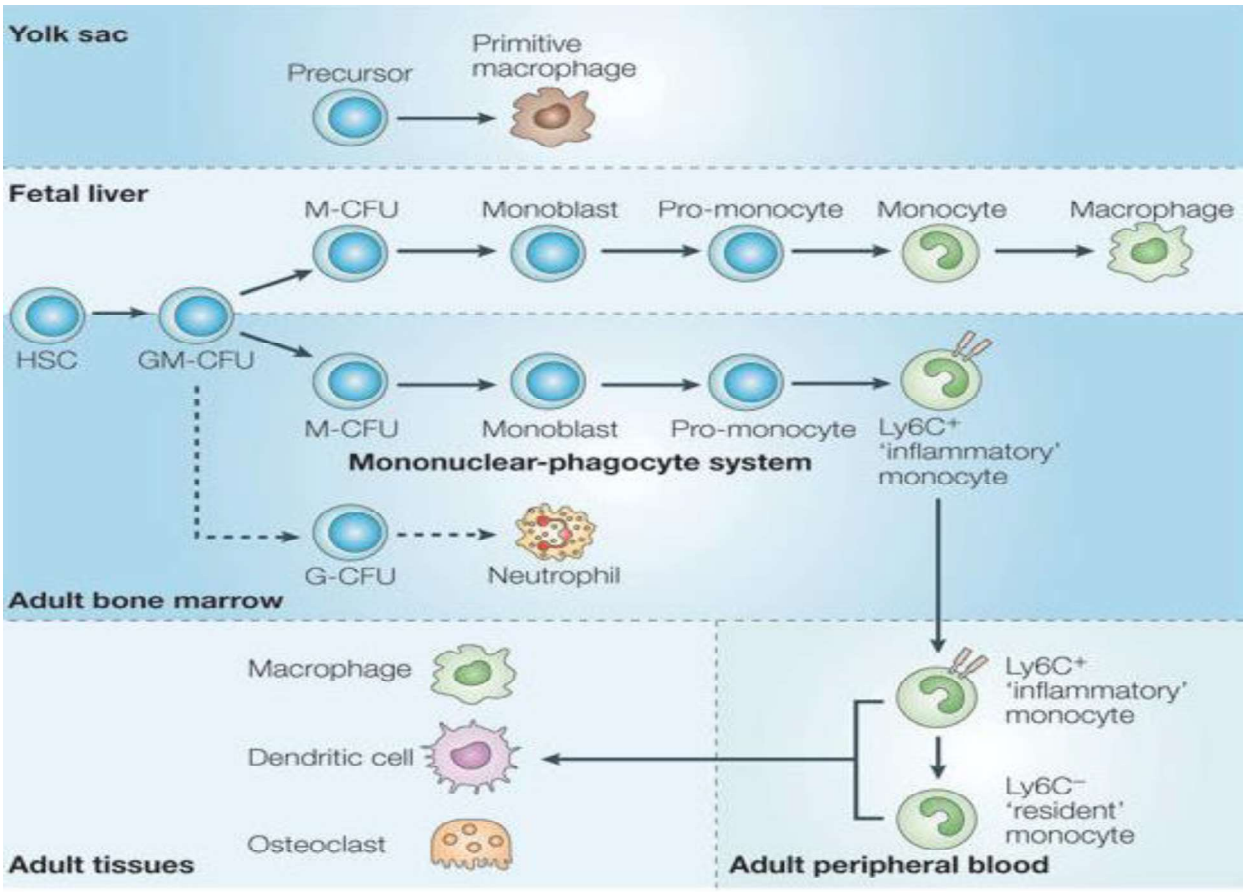

Figure 3. Development and maturation of the mononuclear phagocyte system (MPS) and its lineage *(Courtesy Ref: [34])*

Tissue macrophages are heterogeneous and highly versatile found strategically in almost all tissue types to encounter any foreign intruder. They may represent up to 10-15% of total cells in the specific tissues and the number increases significantly up on any infection or injury. They have been identified and renamed according to their tissue distribution as mentioned in the table below. Although their transcriptome profiles vary [47], they still perform the same function [48].

(3) The intermediate monocytes shows high expression of CD14 and low level expression of CD16 ($CD14^{++}$ and $CD16^{+}$)

While in mouse there are only two different kinds of monocytes as follows

(1) Inflammatory monocytes ($CX3CR1^{low}$, $CCR2^{+}$, $Ly6C^{high}$)

(2) Resident monocytes ($CX3CR1^{high}$, $CCR2^{-}$, $Ly6C^{low}$), also known as patrolling monocytes

Species	Subset[a]	% In WB	% In blood monocytes	Half-life	Markers	Chemokine receptors	Other surface markers	Main functions
Human being	Classical	~10%	85%	1–2 days	$CD14^{++}CD16^{-}$	$CCR2^{+}CX3CR1^{-}$	$CD62L^{+}$, $CD64^{-}$, MHC class II^{+}, $CD163^{+}$	Phagocytosis, inflammatory effectors
	Intermediate		5%	–	$CD14^{++}CD16^{+}$	$CCR2^{-}CX3CR1^{+}$	$CD62L^{+}$, $CD64^{-}$, MHC class II^{++}, $CD163^{+}$	Inflammatory effectors
	Non-classical		10%	–	$CD14^{+}CD16^{++}$	$CCR2^{-}CX3CR1^{+}$	$CD62L^{-}$, $CD64^{+}$, MHC class II^{++}, $CD163^{-}$	Patrolling, antiviral role
Mouse	$Ly6C^{low}$	4%	~60%	18–20 h	$CD11b^{+}CD115^{+}$ $Ly6C^{+}$	$CCR2^{+}CX3CR1^{-}$	$F4/80^{+}$, $CD62L^{-}$, MHC class II^{b}, $CD43^{+}$	Phagocytosis, Inflammatory effectors
	$Ly6C^{high}$		~40%	5–7 days	$CD11b^{+}CD115^{+}$ $Ly6C^{-}$	$CCR2^{-}CX3CR1^{+}$	$F4/80^{+}$, $CD62L^{+}$, MHC class II^{b}, $CD43^{-}$	Patrolling, tissue repair

[a] *Work by Sunderkötter et al. (47) characterized a population of $Ly6C^{med}$ monocytes with intermediate features between $Ly6C^{+}$ and $Ly6C^{-}$. These are not included in the table, because this population remains poorly characterized in terms of both phenotype and function.*

[b] *Inducible.*

WB, whole blood; Ly6C, lymphocyte antigen 6 complex; CCR2, chemokine (C-C motif) receptor 2; CX3CR1, CX3C-chemokine receptor 1.

Table 3. Different monocytes subsets of humans and murine with specific markers *(Courtesy Ref: [48])*

The small group non-classical patrolling monocytes (PMo) crawl along blood vessel and actively patrol the vascular endothelium during homeostatic and inflammatory conditions. They may act as a reservoir for tissue resident monocytes in the absence of an inflammatory insult. The PMo also involved in the wound healing and the resolution of inflammation in damaged tissues by removing damaged tissues and cells through phagocytosis [36-39]. The inflammatory

effectively among themselves, chemokines are those cytokines that induces mobilization of the immune cells from one part to the other part of the body against concentration gradient for a specific purpose, a process called chemotaxis [2,24-27].

1.1.3.7. Macrophages:

Macrophages (Greek; *macros* meaning large, *phagin* meaning to eat) are big (around 20 μm in diameter) white blood cells that ingest and digest a broad spectrum of foreign materials including microbes, self-altered cells, and infected cells by a process called phagocytosis. It was first described by Elie Metchnikoff, a Russian zoologist in the year 1883. He noted that certain white blood cells ingested microbes and other foreign substances, and thereby contributing to the immune state of an organism. He termed it as phagocytes [28,29]. Besides this macrophages activate non-specific innate immune responses through PRR signaling and help induce specific adaptive immune responses by activation of T cells, thereby a major player of innate and adaptive cross-talk. Other than this macrophages are also involved in other important cellular process such as production of ROS, RNS, tissue repair, tissue remodeling and embryo developmental process [30-32].

In the bone marrow, the PHSC differentiated in to common myeloid progenitor cells in the presence of IL3, GM-CSF and M-CSF, which is further differentiated in to pro-monocytes through granulocyte-macrophage progenitor cells [33-35]. The pro-monocytes leave the bone marrow and converted in to monocytes in the blood circulation. In humans monocytes can be classified in to three different types.

(1) The classical monocytes, expresses high CD14 ($CD14^{++}$ and $CD16^{-}$)

(2) The non-classical monocytes characterized by low surface expression of CD14 with high level expression of CD16 ($CD14^{+}$ and $CD16^{++}$), also called patrolling monocytes

confers humoral immunity, which includes agglutinations, complement activation, neutralization, precipitation and lysis of foreign cells, whereas the immune response other than that is called cell mediated immunity [2].

1.1.3.6. Cytokines and Chemokines:

Cytokines and chemokines are small (5 to 20 kDa) signaling proteins secreted (some are membrane bound) by both immune and non-immune cells. Most of them acts by either autocrine or paracrine manner, though a very few reported to have some endocrine functions. These soluble proteins regulate a host of physiological processes including immune responses such as cell activation, proliferation, differentiation, apoptosis, cell migration and neuro-immune interaction. Some of the cytokines are known to induce antimicrobial effector functions (e.g., IFN) to reduce or contain infections. Whereas, others play a crucial role in the cross-talk signaling between innate and adaptive immune responses, while impaired regulation in the production of cytokines results in the severe pathophysiological repercussion. The interaction of cytokines with the specific receptors on the target cells induces intracellular signaling cascades as a result changes in the cell behaviour and effector functions. However, some cytokines are compatible for multiple receptors (homo and hetero dimers), which in turn fine tune the cellular responses. The susceptibility of cells to respond a particular cytokine depends on the presence of specific receptor, concentration of the secreted cytokine and extent of the exposure time etc. The cytokine that mediates distinct biological effector function on different cell types are called pleotropic effect, while two or more cytokine perform same function are called redundant. Auto induction or cascade induction of cytokines occurs when a specific cytokine induces its own production on the same target cell. When two or more cytokines augment cellular process significantly higher than the effect confers by either of the cytokine alone is called synergy. When two cytokine inhibit each other's effect is called antagonize action (e.g., IFN-γ or IL-2 and IL-10). Although, cytokine refers to all the secreted proteins helps immune cells to communicate

cell activation is called cross-priming. The first evidence of antigenic cross-presentation was reported by Michael J. Bevan in 1976 and later vividly explained by Peter Cresswell group [21-23].

1.1.3.5. Antibodies and soluble Factors:

Antibody is a γ-globular protein (so called immunoglobulin) produced mainly by terminally differentiated B cells called plasma cells, which can recognize enormous types of antigens. The incredible diversity of antibodies are created by specific type of genetic rearrangements in the germ line called V(D)J recombination. All antibodies are glycosylated belongs to immunoglobulin superfamily, which is crucial for its structure and function. Antibody possesses multiple immunoglobulin domains made up of anti-parallel β strands with loops. It has both variable domains (binds to antigens) and constant domains. All antibodies are made up of two identical heavy (H) chains (approx. 55 kDa) and light (L) chains (approx. 22 kDa) joined by disulphide bonds as well as non-covalent interactions. There are five different types of heavy chains and two types of light chains found in the placental mammals. These are referred as class such as γ (G), α (A), μ (M), ε (E), and δ (D) with one of the light chain κ or λ. Each clone of antibody recognize a unique molecular pattern on antigens called epitope to neutralize pathogens such as bacteria, viruses etc. However, sometimes auto-reactive antibodies are produced due to the failure of self-tolerance called autoimmunity (e.g., systemic lupus erythematosus). Antibodies works either in monomeric form (IgG, IgE), dimeric form (IgA) or pentameric form (IgM). The variable region of the antibody is called Fab (fragment antigen binding) which binds to the epitope, whereas the constant region is called Fc (fragment crystallizable) responsible for communication and signal transduction with other components of the immune system. The antibodies secreted freely in to the blood or body fluids are called secreted immunoglobulins (IgG, IgA and IgE), whereas those bound to the plasma membrane of B cells or memory cells are called membrane or surface immunoglobulins (IgM and IgD). The production of antibodies

prevented to binds to peptides designed for MHC class I molecule by invariant chain (li, CD74) in the RER. It has been reported that in the li is required for proper folding and transport of MHC class II molecule from RER to cis-Golgi to endosomal compartments through trans-Golgi networks. In the late endosome, MHC class II- compartments (MIIC), the li is trimmed to short CLIP (Class II-associated invariant chain peptide), which is attached to the antigen binding groove. HLA-DM, a non-classical MHC class II molecule mediate exchange of CLIP with antigenic peptide in the MIIC, followed by transport of the resulting MHC class II antigenic peptide complex to the plasma membrane for presentation [2,14-16].

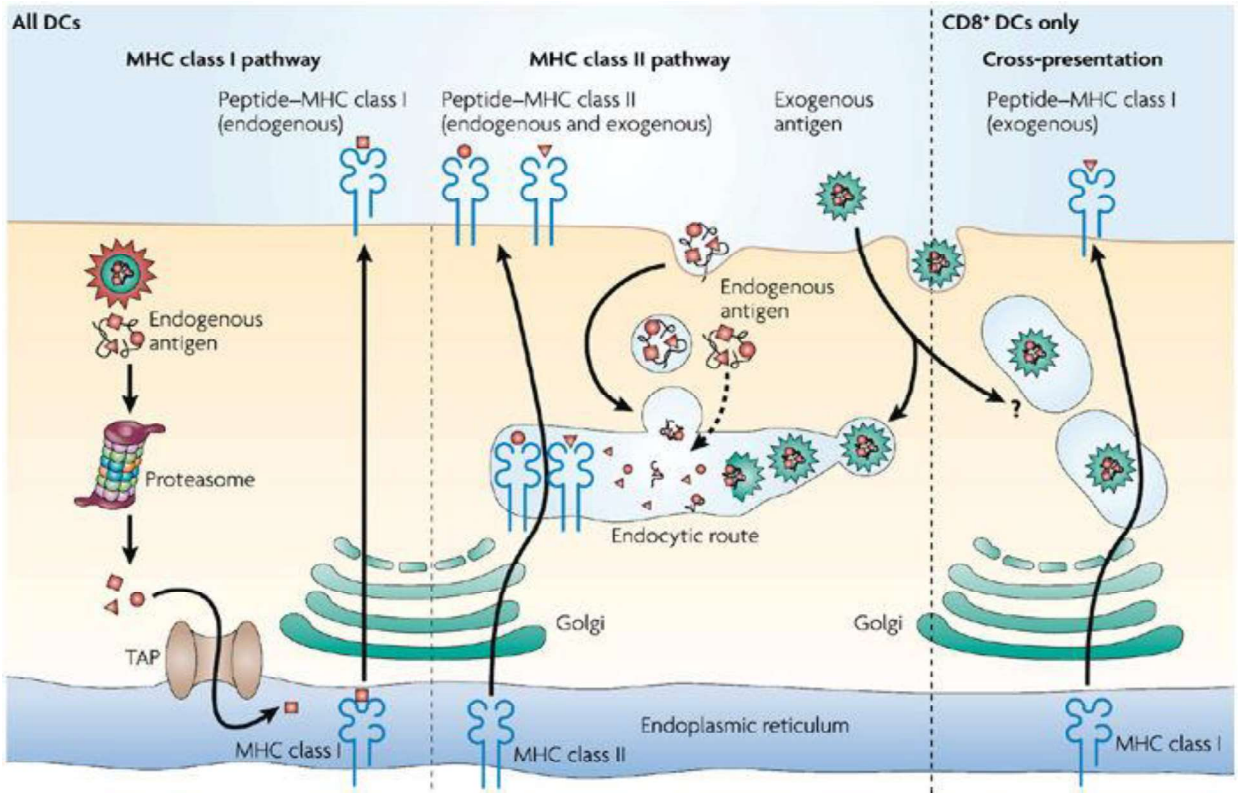

Figure 2. The antigen processing and presentation pathways in dendritic cells (DC) *(Courtesy Ref: [15])*

Sometimes, professional APCs (specialized subset of DC) presents exogenous antigens through MHC class I pathway (instead normal MHC class II pathway) to elicit effector CD8+ T cell responses (CTL responses) called antigen cross presentation [17-20]. This form of CD8+ T

cells contain a distinct proteasome celled immunoproteasome, which can be activated by IFN-γ and TNF. The turnover and protease catalytic activity of immunoproteasome is much higher than normal 20S proteasome, which might be associated with autoimmunity due to over processing of the self-antigens, thereby reducing self-tolerance. The peptides then transported in to the ER lumen via TAP (transporters associated with antigen processing). It is a heterodimer consists of TAP1 and TAP2 protein with cytosolic ATP binding domain, which facilitates transport of peptides from cytosol to ER lumen in a ATP dependent manner. It belongs to ATP-binding cassettes proteins found in many cells including bacteria. Ideal peptide length for MHC class I molecule is around 9 amino acids so longer peptides transported by TAP1 and TAP2 are trimmed in the ER lumen by ERAP (endoplasmic reticulum amino-peptidase). Moreover, TAP preferentially binds to the peptides with hydrophobic or basic carboxyl-terminal amino acids (favourable anchor residues for class I MHC). Thus, TAP is optimized to transport antigenic peptides which is more likely binds to the MHC class I. Once inside the lumen of the rough ER, class I MHC α-chain binds to calnexin and ERp57 promote its proper folding. When β2 microglobulin binds to α-chain, the calnexin released, followed by binding of calreticulin and tapasin (TAP associated proteins). The processed antigenic peptide binds to the class I MHC molecule followed by release of chaperon proteins. Then MHC class I with peptide proceeds to the plasma membrane via trans-Golgi network for antigen presentation.

Exogenous antigens can either internalize by phagocytosis (e.g., macrophages and DCs) or endocytosis (both receptor mediated and pinocytosis) for the exogenous pathway of antigen processing and presentation to the T cells. After internalization of the antigens passes through different endosomal compartments such as early endosomes (pH 6.0-6.5), late endosomes or endolysomes (pH 4.5-5.0) and finally lysomes (pH 4.5). Throughout the process the antigens are cleaved by different pH dependent proteolytic enzymes in to 13-18 residues for MHC class II compatibility. Since MHC class II molecules supposed to express exogenous antigens, so it is

professional antigen presenting cells acquires antigens via phagocytosis or receptor mediated endocytosis and display to the T cells via MHC class II to the CD4+ T cells (though they do express MHC class I). Except professional antigen presenting cells all other nucleated cells are known as non-professional antigen presenting cells which presents antigens via only MHC class I molecules to CD8+ T cells.

Si No.	Attributes	Professional antigen presenting cells	Non- professional antigen presenting cells
1	MHC type	Both MHC class I and II	Only MHC class I
2	Cell type	Macrophage, DC and B cells	All nucleated cells, Self-altered cells or virus infected cells
3	Ag presents to	Both CD8+ T cells and CD4+ T cells	CD8+ T cells
4	Ag type	Generally foreign	Self-antigens
5	Pathway	Exogenous pathway	Cytosolic pathway

Table 2. Basic difference between professional and non-professional APCs *(Courtesy Ref: [2])*

1.1.3.4. Antigen processing and presentation:

The antigens are processed via two different pathways, exogenous and cytosolic pathways. Endogenous antigens are processed through cytosolic or endogenous pathways and presented by MHC class I molecules. Whereas, exogenous antigens processed through exogenous or endocytic pathway by MHC class II molecule. The endogenous pathway of antigen presentation tags various endogenous proteins by ubiquitination, followed by proteasome mediated degradation in to small peptides. In addition to the regular proteasome (20S) pAPCs and infected

differentiated in to DCs, natural killer cells (NK cells), T cells (from T cell progenitor) and B cells (B cell progenitor) [11-13].

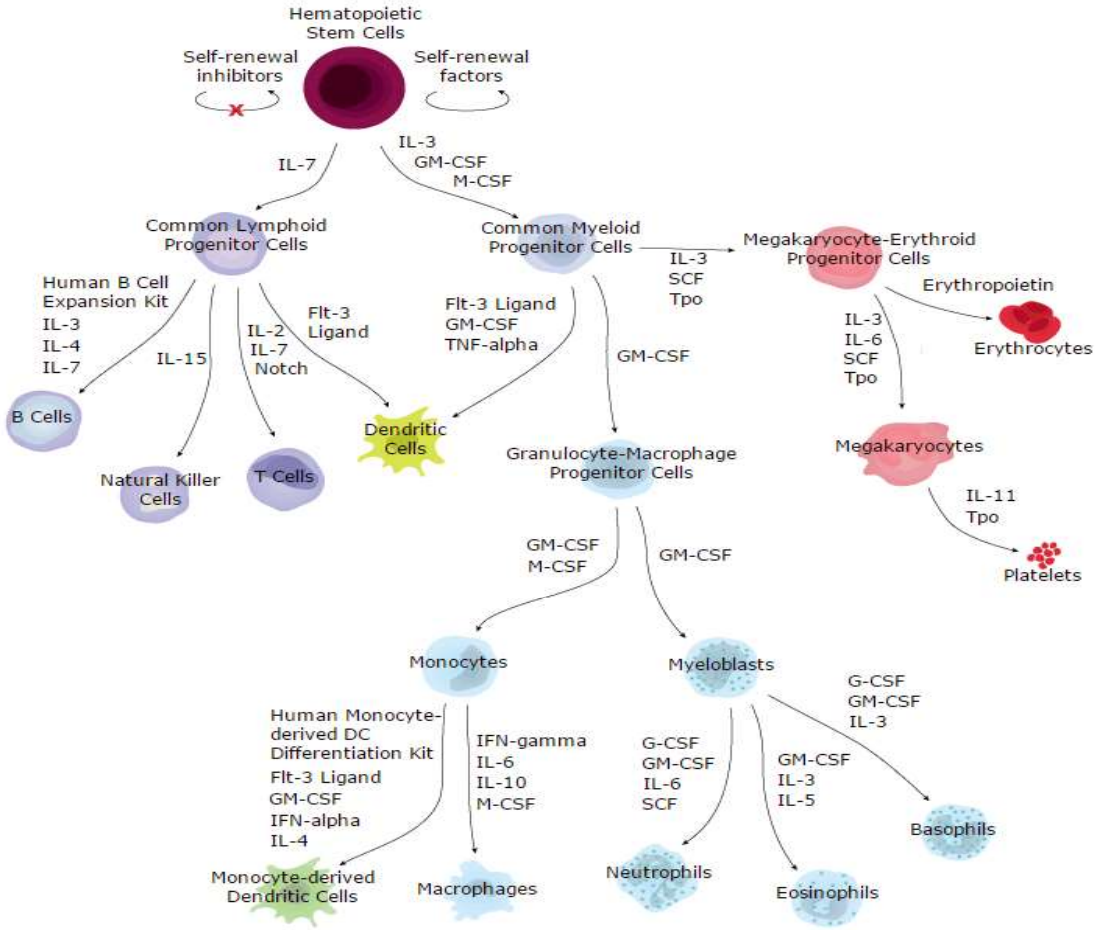

Figure 1. Pluripotent hematopoietic stem cell (PHSC) differentiation pathways and lineage specific markers (Source; https://www.rndsystems.com/pathways/hematopoietic-stem-cell-differentiation-pathways-lineage-specific-markers)

1.1.3.3. Antigen Presenting Cells (APCs)

Stimulation and activation of naïve T cells generally requires presentation of the processed antigenic peptides/lipids via major histocompatibility complex (MHC) or CD1 to the T cells. All nucleated cells can present peptides to the T cells via MHCs. As far as antigen presentation is concern, nucleated cells are grouped in to two categories, professional antigen presenting cells (macrophage, DC and B cells) and non- professional antigen presenting cells (all other cells). A

For an effective activation and mounting of the immune system against a pathogen, immune system brought an array of different types of cells with effector molecules in a precisely coordinated manner. For this, proper growth and development of those cells are crucial which occurs at specialized organs or sub-anatomical compartments. Those organs are mainly two types

(1) primary lymphoid organs and,

(2) secondary lymphoid organs

The primary lymphoid organs (PLOs) comprises bone marrow and thymus, which regulates the origin and development of the immune cells from their immature precursor cells. Whereas in the secondary lymphoid organs (SLOs) antigen specific responses are generated and maintained in order to activate immune system against any pathogens and the development of immune tolerance for self and non-self antigenic detection. The SLOs includes lymph nodes, tonsils, appendix, Peyer's patches, MALT (mucus associated lymphoid tissues), GALT (gut associated lymphoid tissues), and NALT (nasal associated lymphoid tissues) BALT (bronchus-associated lymphoid tissues). All SLOs develops in the embryonic stages (pre-natal) except NALT and BALT which development is post-natal [10].

All the blood cells including RBCs are generated from a single pluripotent cell (self-renewing) lineage celled hematopoietic stem cells (HSC) by a series of positive and negative selection process called hematopoiesis. All the process of hematopoiesis starts in the bone marrows, followed by further development in destined organs according to the cell types. Hematopoietic stem cells give rise to two main types of cells, myeloid progenitor cells and lymphoid progenitor cells. The myeloid progenitor cells further differentiated in to dendritic cells (DC), monocytes/macrophages, neutrophils (through granulocyte monocyte progenitor), eosinophil (eosinophil progenitor), basophil/mast cells (basophil progenitor), platelets (from megakaryocytes) and finally RBCs (from erythroid progenitor). The lymphoid progenitor cells

7	Major cell types	Phagocytes (monocytes, macrophages neutrophils), natural killer (NK) cells, other leukocytes, epithelial and endothelial cells	T cells, B cells, antigen-presenting cells

Table 1. Comparison of innate and adaptive immune system *(Courtesy Ref: [2])*

1.1.3. Components of the immune system

The components of the immune system are diverse and works in both specific and non-specific manner. Together they form an invincible natural barrier between the good and the bad. However, sometimes they either fails to do so or being manipulated by pathogens resulting in various diseases.

1.1.3.1. Physical and Chemical barriers

It provides body's first line of defence to foreign invaders. The physical barriers possesses naturally by the host such as thick epithelial lining of our body. The highly keratinized external epidermis and specialized tissue layers encircles different systems; mucus layers of gastrointestinal tract, respiratory tract, ducts of secretory exocrine glands and urogenital tracts etc. prevents entry of pathogens. Moreover, specialized structures (such as cilia on respiratory tract) and body's secretions (urine, tears, saliva, mucus and milk) also helps in sweeping out pathogens entered during respiration. The chemical barriers include gastric pH, vaginal fluid pH, anti-microbial proteins and peptides in different body's secretions also prevent microbial activity.

1.1.3.2. Cells and Tissues of the immune system

activation' typically within 5-6 days after initial pathogenic exposure to the innate immune system (immediate activation) or breach in the barriers. This is because the adaptive immune system requires initial activation of innate immune responses and there is very few number of cells having specific receptors to recognize the pathogens. After this T and B lymphocytes undergo clonal expansion and proliferation for subsequent effector functions and final resolution. Throughout these processes, immunologic memory is generated for a possible antigenic encounter in future. However, the life span of the memory varies from antigen to antigen.

Basic differences between innate and adaptive immune systems are given below

Si no	Characteristics	Innate	Adaptive
1	Response time	Minutes/hours	Days
2	Diversity	Limited to certain pre-determined receptors encoded in the germ line	Highly diverse generated through genetic recombination
3	Specificity	Specific to certain molecular patterns	Highly specific, even minute change in the antigen can be distinguished
4	Memory	No memory (limited memory observed in invertebrates and NK cells)	Persistent memory with fast response in greater magnitude after first exposure
5	Self/non-self-discrimination	Perfect	Very good; occasional failures of discrimination resulted in autoimmune disorders
6	Soluble components of blood	Many antimicrobial peptides, proteins, and others	Antibodies and cytokines

innate immune systems are evolutionarily more primitive (dominant immune system present in almost all plants, insects and animals) and generic in nature since they are directly transmitted from one generation to the next via germ line to recognize and eliminate foreign invaders immediately. The cellular, physical, chemical immune barriers and specific highly conserved receptors (e.g., PRRs) are the main components of the innate immune system which acts non-specifically throughout the life span of an organism. Unlike adaptive immune system, the recognition systems/receptors of the innate immune system are very rapid (within seconds of the antigenic encounter) and non-specific in nature, therefore, it fails to distinguish minute differences in the antigens. Some important purposes of the innate immune systems are

- It provides the first line of defense via physical and chemical barriers
- It eliminates pathogens, dead cells and cell debris by phagocytosis effectively
- It prime and therefore activates specific adaptive immune response
- Recruits immune cells such as neutrophils and blood monocytes/tissue macrophages to the site of inflammation and infection for effective immune activation
- Helps in tissue repair and remodeling

1.1.2. The adaptive immune system

The adaptive immune system, also known as specific or acquired immune system, which is precisely developed by antigenic encounter during the life cycle of an organism. Unlike innate immunity, the adaptive immunity is antigen specific and takes more time for activation followed by generation of either transient or permanent (depends on the types of the antigen) immunologic memory. However, activation of the adaptive immune system solely requires antigen processing and presentation via innate immune activation. These types of the immune system comprise mainly B cells (matured in bone marrow) and T cell (matured in the thymus). The T cells give cell mediated immunity whereas B cell gives humoral immunity through the generation of antibodies. The specificity of the adaptive immune response comes with a price ‘sluggish initial

sophisticated and unique immune system to recognize same pathogens upon second encounter throughout its life cycle with an immensely overwhelming response. This is called immunological memory which is the basis of vaccination.

Immunological balance is a key for an effective defense against a foreign intruder. Over activation of the immune system causes life threatening autoimmune disorders (self against self) such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) etc., where severe inflammatory responses are induced followed by damage or destruction of tissues (detected as foreign), altered organ growth and/or functions [5,6]. Whereas, immunodeficiency is caused when the immune system fails to mount effectively on foreign antigens or pathogens. This may be caused by either consumption of some immunosuppressive drugs (e.g., prednisone, steroids) or by some infections (e.g., HIV), as a result of which host might be susceptible to some common opportunistic infections [7-9]. Moreover, in humans, there are certain immune privileged sites comprising mainly vital organs (e.g., eye, brain) where the immune system displays less activation or inflammation to protect vital organs from severe inflammation (e.g., ACAID). This physical barrier is maintained by specific tissue architectures such as blood-CSF barrier, the blood-brain barrier to separate peripheral immune system from the vital organs.

Deadly pathogens such as bacteria, viruses, fungi and some protozoans have been evolving continuously so that they can evade immune surveillance for completion of their life cycle in the host. However, our host defense system is equipped with multiple layers of check points for pathogen recognition and subsequent neutralization by a variety of mechanisms and is broadly categorized into two separate but interdependent defense system; the innate and adaptive immune system.

1.1.1. The Innate immune system

The innate immune system also known as inborn immunity system comprises inbuilt cellular, chemical and physical barriers to the foreign antigens or pathogens. The components of the

1. Introduction

1.1. Introduction to the Immune System

Immunology is the branch of science that deals with the study of different structural and functional aspect of the immune system. It is one of the eldest branch of medicine and physiology which can be traced back at the times of plaque pandemic of Athens in 430 BC, when Thucydides mentioned that plaque recovered people could nurse the sick without contracting the illness a second time. Although the phenomenon was unexplained at that time, it took approximately next 2000 years for medically effective practice as vaccine.

The immune system (noun) is a multicellular host defense system within an organism which is made up of a multitude of biological structures and synchronized processes. It acts in a coordinated/balanced and controlled manner to give adequate defense against different diseases, infections or any undesirable foreign invasion by anticipating that it might pose a severe threat to host existence while providing enough immune tolerance to avoid common allergies and autoimmune disorders. The latter is known as immunity (verb) meaning 'exempt', a state of protection from infectious diseases, which was derived from Latin term '*immunis*'.

Protecting self is one of the in-born basic instincts of all living organisms. Even single cell organisms such as bacteria have its own defense system to protect itself from bacteriophages. This primitive immune system of bacteria encounters bacteriophage in almost every major stages of its life cycle such as preventing phage attachment, blocking bacteriophage DNA entry, activating restriction modification (R-M) system, abortive infection and finally interference during assembly phase [1,2]. Besides this invertebrates, lower vertebrates (jawless fishes, super Class Agatha) and plants also possesses their own multilayered immune system, such as phagocytosis, antimicrobial peptide (AMP) production, pattern recognition receptors (PRR), polymorphic NB-LRR (nucleotide binding and leucine rich repeat) proteins, lectins and complement system [3,4]. However, vertebrates (higher than Class Agnatha) evolved with more

CHAPTER#1

Introduction

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Review of Literature

2.1. Hypothesis: CHIKV infection may modulate host cell immunity by regulating MHC and associated immune responses in macrophages, which may reflect the respective outcome of innate and adaptive immunity

2.2. Specific Objectives:

1. To establish CHIKV infection in host macrophages.

2. To investigate signature cytokine milieu in the host cell during CHIKV infection.

3. To investigate whether, there is any alteration in the expression of MHC and co-stimulatory molecules during CHIKV infection.

4. To investigate immuno-regulatory candidate pharmaceutical drug for anti-CHIKV infection in macrophages

5. To investigate mechanism of pro-inflammatory response (e.g., TNF) induced during CHIKV infection in macrophages

CHAPTER#2

Hypothesis

&

Specific Objectives

For T cell macrophage co-culture experiment, mouse peritoneal macrophages were isolated as described above and seeded in 24 well plate at a density of 0.2 x 10^6 per well before 10-12h of infection. Then, the cells were infected with CHIKV with MOI 5 as described in the above section. After infection, purified T cells were added over both mock and CHIKV infected peritoneal macrophages for co-culture as described earlier [248,249] at a ratio of 1:5 for 48h. Then, the T cells were harvested for subsequent flow cytometric analysis and macrophages for detection of CHIKV infection. The macrophage-T cell co-culture supernatants were collected at the time of cell harvesting aliquoted and stored at -80 ˚C for cytokine ELISA analysis.

3.2.13. Statistical Analysis

Statistical Analysis was performed by using the GraphPad Prism 5.0 software (GraphPad Software Inc. USA). Data are represented as Mean±SEM. The comparison between the groups was performed by the 2 way ANOVA with Bonferroni post-hoc test unless otherwise mentioned. The comparison between the groups with only one parameter was performed by 1 way ANOVA with Bonferroni post-hoc test. Data presented here are representative of at least three independent experiments. $p< 0.05$ is considered as statistically significant difference between the groups. (ns, non-significant; *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

MTT assay (cell viability assay) was performed to assess cytotoxicity of different drugs used according to the manufacturer's instructions. Briefly, Raw264.7 cells were seeded in 96 well plate at a density of $5X10^3$ per well before drug treatment. Then, cells were washed in 1x PBS and different concentrations of respective drug were treated in triplicate in the incubator. DMSO was taken as solvent control. After 24h of treatment, cells were incubated in the MTT reagent to a final concentration of 10% of total volume. Then cells were incubated for 2h in the incubator for the formation of visible crystals. Later, the media were removed carefully and 100 µl of solubilization solution was added per well followed by incubation for 15 minutes at RT. The absorbance of the solution was taken at 550 nm by iMark™ Microplate Reader (Bio-Rad). The percent viable cells were calculated with the help of percentage of control cells (solvent control) in triplicate.

3.2.11. Splenocytes isolation and T cell purification

The splenocytes were isolated from 6-8 weeks old BALB/c mice as described previously [246,247]. In brief, the isolated spleens were ruptured and passed through a 70µm cell strainer to remove cell debris. RBCs were lysed by 1x RBC lysis buffer and cells were washed with 1x PBS followed by suspended in complete RPMI-1640. T cell purification was done by using untouched mouse T cell isolation kit from Invitrogen according to the manufacturer's instructions. In brief, the splenocytes were re-suspended in isolation buffer and incubated with biotinylated antibody for 20 min. Then the cells were washed in excess isolation buffer and incubated with streptavidin conjugated magnetic beads for 15min and then placed on magnet for 2 minutes. The resulting enriched T cells were evaluated by flow cytometry and found to be around ≥95%. The isolated T cells were cultured in complete RPMI-1640 and incubated in the incubator till co-culture with primary macrophage.

3.2.12. T cell: Macrophage Co-culture *in vitro*

PowerPac Basic. Then the transferred membranes were blocked with 3% BSA followed by overnight incubation with the different primary antibodies at 4°C. Then the membranes were thoroughly washed with TBST for five times and incubated with the HRP conjugated secondary antibodies for 2h at RT with continuous rocking on a gel rocker. After washing with TBST for three times, the membranes were subjected to either Immobilon Western chemiluminescent HRP substrate (Millipore) or SuperSignal West Femto trial kit (Thermo Scientific, MA, USA) chemiluminescence detection, by Bio-Rad gel doc with Quantity one software (Bio Rad, CA, USA). Loading control (GAPDH) was used as standard for Western blot band intensity quantitation.

3.2.9. RT-PCR

For quantitation of CHIKV RNA inside the host cells, Raw264.7 cells were infected with DRDE-06 strain of CHIKV either in presence of DMSO or 17-AAG as mentioned previously. Cells were harvested at 8 hpi and total RNA was isolated using Trizol reagent (Invitrogen, MD, USA). RT was performed with equal amount of RNA (1µg) by using Maxima H minus First Strand cDNA synthesis kit (Thermo Scientific, MA, USA) as per the manufacturer's instructions. This cDNA was used to amplify viral non-structural (NSP2) using primers (F)-5′CGAGGATCCACTGAATGAAATATGC-3′ and (R)-5′CGACTCGAGTTAACATCCTGCTCGGGTGG-3′; structural genes (E1) using primers (F)-5′TGCCGTCACAGTTAAGGACG3′ and (R)-5′CCTCGCATGACATGTCCG3′ through RT-PCR, along with GAPDH as housekeeping gene in Eppendorf master cycler pro S. The RT-PCR products were subjected to 1.5% agarose gel electrophoresis and GAPDH served as internal amplification control [239]. Relative band intensities were calculated with the help of Image J software for the respective amplification [245].

3.2.10. MTT Assay

both the mock and CHIKV infected cells were detached from the cell culture dishes by trypsin-EDTA treatment. Cells were washed twice in ice cold 1x PBS and then re-suspended in 100 µl of 1x Annexin V binding buffer at a density of $1x10^6$ cells/ml. Then, 2.5 µl of APC conjugated Annexin V and 7-AAD per sample was added, gently mixed by vortexing and incubated at RT for 15 min in the dark. After that, 400 µl of 1x Annexin V binding buffer was added per tube and samples were acquired immediately by the BD FACS Calibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed by the CellQuest™ Pro software (BD Biosciences, San Jose, CA, USA). A total of approximately more than five thousands cells/sample was acquired.

3.2.8. Western Blot Analysis

Protein expression was assessed by Western blot analysis according to the protocol described earlier. In brief, both the mock and CHIKV infected cells were washed once with ice cold 1x PBS and the whole cell lysate (WCL) was prepared by Radio Immuno Precipation Assay (RIPA) lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, supplemented with protease inhibitor cocktail) and centrifuged at 13,000 rpm for 15 min at 4°C. After this supernatants were collected carefully in fresh 1.5 ml microfuge tube and stored at -80°C until further use. The protein concentration in the WCL samples was quantified by the Bradford reagent (Sigma-Aldrich, MO, USA) with different concentrations of bovine serum albumin fraction V as standard. Equal amount of protein (20 µg/well) was loaded in the 10% SDS-PAGE after mixing with 2X sample buffer (130 mM Tris-Cl, pH 8.0, 20% (v/v) Glycerol, 4.6% (w/v) SDS, 0.02% Bromophenol blue, 2% DTT) at a ratio of 1:1 along with pre-stained protein marker in one well. After completion of SDS-PAGE, gel was washed in distilled water to remove excess of salt followed by incubation in 1x transfer buffer until transfer assembly ready. Then proteins (in the gels) were blotted on to a methanol activated PVDF membrane (Millipore, MA, USA) at 80 volt (constant) for 2.5hr at 4°C with the help of Bio-Rad

3.2.6. Sandwich ELISA for Cytokine Analysis

Cytokine production by the macrophages was analyzed from the cell culture supernatants by the BD OptEIA™ sandwich ELISA kit (BD Biosciences, CA, USA) according to the manufacturer's instructions. Briefly, micro-wells of SPL medibinding strip immunoplates were coated with ELISA capture antibodies diluted in coating buffer at 100 µl per well. For optimum antibody coating, strip immunoplates were sealed by plate sealer followed by incubated over night at 4°C. Next day, individual wells were aspirated and washed 3 times with 300 µl/well ELISA wash buffer. After last wash, plates were inverted on absorbent paper to remove any residual wash buffer. Plates were then blocked with 300 µl/well ELISA assay diluent and incubated for 1h at room temperature (RT). After washing once, both samples and standards (serially diluted) were pipetted at 100 µl/well, incubated for 2h at RT with plate sealer. After that, plates were washed for 5 times and incubated with working detector (detection antibody+ streptavidin HRP reagent) incubated for 1h at RT with plate sealer. Then working detector were aspirated and washed 7 times (in the final wash plates were soak for 0.5-1 minute in wash buffer), followed by incubation in the substrate solution (TMB/H_20_2) at dark without plate sealer. After development of blue color (usually 5-30 min) HRP-TMB/H_20_2 reaction was stopped by stop solution (2 N H_2S0_4) and reading was taken at 450 nm within 30 minutes by Bio-Rad iMark^{TM} Microplate absorbance reader. The cytokine concentrations in the test samples were calculated in comparison with the corresponding standard curve.

3.2.7. Annexin V Binding Assay

The mock and CHIKV infected Raw264.7 cells were harvested by trypsin-EDTA treatment at different time post infection. For the detection of the Annexin V positive cells in mock and CHIKV infected samples, flow cytometry was carried out by using BD Annexin V Detection Kit I (BD Biosciences, USA) according to the manufacturer's instructions. Briefly,

suspended in FACS buffer (1X PBS, 1% BSA, 0.01% NaN_3) and stored at 4°C until staining (both ICS and CS). For intra cellular staining (ICS) of CHIKV antigens, the cells were permeabilized in 1x permeabilization buffer (1x PBS + 0.5% BSA + 0.1% Saponin + 0.01% NaN_3) followed by blocking in 1% BSA (in permeabilization buffer) for 30 min at room temperature. After washing with 1x permeabilization buffer, cells were incubated for 30 minutes with primary antibodies such as anti-CHIKV-nsP2 and E2 antibodies [242]. Cells were subjected to gentle tapping (manually) at an interval of 10 minutes during the incubation period to ensure proper distribution of the antibodies inside the cells. Then cells were washed 3 times in 1x permeabilization buffer to remove unbound primary antibodies, followed by incubation in Alexa Fluor® 488 or Alexa Fluor® 647 conjugated IgG (H+L) secondary antibodies. Both the primary and secondary antibodies were diluted in 1x permeabilization buffer. The normal mouse/rabbit IgG were taken as isotype control during ICS. For the surface staining (CS), the fixed cells were washed with ice cold FACS buffer and then, fluorophore conjugated antibodies against different immune markers were added (diluted in FACS buffer). The mock and CHIKV infected cells were incubated with antibodies for 30 min on ice and washed twice with ice cold FACS buffer to remove un-bound antibodies. The fluorophore compatible isotype control antibodies were used as isotype control for CS. The FcR blocking reagent was used at a dilution of 1:20 prior to the primary antibody incubation to prevent non-specific binding of antibodies to the Fc receptors on macrophages. After staining (both CS and ICS) cells were re-suspended in FACS buffer containing 1% paraformaldehyde (w/v) and stored at 4°C until sample acquisition. Then, the cells were acquired (within 1-2 days of staining) by the BD FACS Calibur™ flow cytometer (BD Biosciences, CA, USA) and analyzed by the CellQuest Pro software (BD Biosciences, CA, USA). A total of approximately ten thousand cells were acquired per sample. For analysis dot plot quadrants were set on the basis of isotype control.

cells were collected by peritoneal lavage with 5 ml of ice cold sterile isolation solution (1x HPBS + 3% FBS) and washed twice with the same solution by centrifugation at 800 x g for 10 min at 4 ˚C. Isolated cells then treated with sterile 1X RBC lysis buffer solution for 2-3 min followed by neutralization in complete RPMI-1460 media. Cells were then centrifuged at 800 x g for 10 min at 4 ˚C and isolated cells were re-suspended in complete RPMI-1640 (supplemented with 2.0 mM L-glutamine, Penicillin 100 U/ml, Streptomycin 0.1 mg/ml, 10% FBS), counted by trypan blue exclusion method and plated in six well plate with approximate seeding density of 1.5 X 10^6 cells per well. Cells were incubated for 8-12h in humidified incubator at 37 ˚C with 5% $C0_2$ followed by washing in sterile 1x HPBS twice to remove non adherent cells. Then cells were cultured in complete RPMI-1640 for 1-2 days according to the assay procedure.

3.2.4. Plaque assay

For the determination of the virus titer in the cell culture supernatant, plaque assay was performed on the monolayer of Vero cells as described previously [244]. Briefly, collected cell culture supernatants were subjected to serial diluted in serum free DMEM. Then after infecting the Vero cells using the appropriate diluted cell culture supernatants collected from CHIKV infected Raw cells, the cells were overlaid with complete DMEM containing 2% (w/v) methyl cellulose and maintained in 37°C incubator. After development of visible plaques (usually 4-5 days), the cells were fixed in 8% (v/v) formaldehyde for approximately 6-8hr at room temperature. After fixation, cells were washed gently in distilled water and stained with crystal violet solution for 2-4 h at room temperature. Then, the cells were washed gently by distilled water and the numbers of plaques were counted manually under white light.

3.2.5. Flow Cytometry (FC) Analysis

CHIKV infected and mock Raw264.7 cells were harvested by scraping, fixed in 4% paraformaldehyde for 10 min at RT and were washed twice (centrifuged at 2000 rpm for 5 minutes at 4°C) in ice cold 1x PBS to remove excess paraformaldehyde. Then the cells were re-

maintained in fresh complete RPMI-1640 media at 37°C in the humidified incubator. The virus infected cells and the supernatants were collected at different time points (hpi) and used for further processing according to the assay procedure. Furthermore, the CHIKV infected and the mock (uninfected) cells were observed inverted light under microscope at a magnification of 20X and cell culture pictures were taken at different hpi to detect the cytopathic effect (CPE).

3.2.2. Drugs and modulators treatment

For 17-AAG (less toxic derivative of geldanamycin and a functional HSP90 inhibitor) treatment, the infection was carried out for 2h in the presence of solvent control, DMSO or 17-AAG at dark. The cells were washed thoroughly with 1x PBS after 2h and cultured in serum free media (SFM) containing drugs for 3h. Then, fetal bovine serum was added to the cells and maintained in the incubator until harvesting.

For the treatment of SB203580 (p-P38 inhibitor) and SP600125 (p-SAPK/p-JNK inhibitor), cells were seeded in six well cell culture plates before 18-20h of treatment with around 60-70% confluency. Then cells were washed 3 times in 1x PBS followed by drug treatment (pre-treatment) in SFM for 2h. Then cells were inoculated with appropriate MOI in the presence of both SB and SP. After that normal infection procedure followed (as described previously), followed by post-infection drug treatment in serum free media. After 3h fetal bovine serum was added to the cells and cultured in the incubator till harvesting.

3.2.3. Isolation of Mouse Peritoneal Exudate Cells (PEC) and Macrophage preparation

BALB/c mice (4-6 weeks old) were maintained according to the Institutional Animal Ethics Committee (IAEC) guidelines with food and drink ad libitum. 4% w/v brewer thioglycolate (TG) medium was injected intraperitoneally (i.p.) at 1.0ml per mouse. After 4 days of injection, peritoneal exudate cells were isolated as described earlier [244]. Peritoneal exudate

	blotting	
10	Tris-buffered saline (TBS)	0.0153 M Trizma HCl, 0.147 M NaCl in ultrapure water (Milli Q), pH adjusted to 7.6 by HCl
11	Tris-buffered saline Tween-20 (TBST)	0.05% (v/v) Tween-20 in 1x TBS

3.2. Methods

3.2.1. Chikungunya virus infection

3.2.1.1. In Vero cell line

Vero cells with at least 90% confluency were seeded in 35 mm^2 cell culture dishes in complete DMEM media. After 24h of the seeding, cells were washed in 1x PBS three times followed by inoculated with different strains of CHIKV (s 27 and DRDE-06 strains) for 1.5h as described previously [243]. After infection, cells were washed again in 1x PBS three times to remove any excess non-bound virus particles in the cell culture dishes. Then cells were cultured in complete DMEM in the incubator till harvest. Cells and supernatants were collected and processed at different hours post infection (hpi) and according to the assay(s) requirements.

3.2.1.2. In Raw264.7 cell line and murine peritoneal macrophage

The Raw264.7 cells (obtained from NCCS, Pune, India) of low passage number were seeded in six well cell culture plates before 18-20h of infection with around 60-70% confluency. The cells were infected with different strain(s) of CHIKV with different multiplicity of infection (MOI) as described earlier [243]. In brief, the cells were washed in 1x PBS and the virus was laid over the confluent monolayer of the cells for 2h in the incubator with manual shaking at an interval of 15 min. Then, the virus inoculum was washed in 1x PBS and the cells were

3.1.7. Buffers and other reagents composition

Table 7. Details of different buffers used

Si no	Buffers and other reagents	Composition
1	Permeabilization buffer	1x PBS (pH 7.4-7.6), 0.01% NaN3 (w/v), 0.5% BSA fraction-V (w/v), 0.1% saponin (w/v)
2	Blocking buffer for ICS	1x PBS (pH 7.4-7.6), 0.01% NaN_3 (w/v), 1% BSA fraction-V (w/v), 0.1% saponin (w/v)
3	RIPA(Radio Immunoprecipitation Assay buffer) lysis buffer	150 mM sodium chloride, 1.0% NP-40 or Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (sodium dodecyl sulphate) (w/v), 50 mM Tris, pH 8.0
4	2x Laemmli buffer	4% SDS (w/v), 10% 2-mercaptoehtanol (v/v), 20% glycerol (v/v), 0.004% bromophenol blue (w/v), 0.125 M Tris HCl check the pH and bring it to pH 6.8
5	1x TGS or SDS-PAGE running buffer	25 mM Tris base, 190 mM glycine, 0.1% SDS (w/v)
6	1x Transfer buffer	25 mM Tris base, 190 mM glycine, 20% HPLC grade methanol (v/v)
7	Methylcellulose media for plaque assay	Complete DMEM media, 2% Methylcellulose (w/v)
8	4% Paraformaldehyde (PFA)	1x PBS (pH 7.4-7.6), 4% paraformaldehyde (w/v)
9	Blocking reagent Western	3% BSA fraction-V in TBST

35	20x TMB/H202	Bangalore Genei, Bangalore, India	62160118010A
36	FcR blocking reagent, mouse	Macs Miltenyi Biotech, Gladbach, Germany	130-092-575
37	7-AAD	BD Biosciences, CA, USA	559925
38	10x Annexin V binding buffer	BD Biosciences, CA, USA	556454
39	Thioglycollate Glycolate (TG) medium brewer modified	BD Biosciences, CA, USA	211716
40	SP600125	Merck Millipore, MA, USA	420119
41	17-Allylaminogeldanamycin (17-AAG)	Merck Millipore, MA, USA	100068
42	SB203580	Merck Millipore, MA, USA	559389
43	Trypan blue	Himedia Laboratories Pvt. Ltd., Mumbai, India	TC193

3.1.5. Kits

Annexin V apoptosis detection kit I, OptEIA™ ELISA kits for IL-2, IFN-γ, TNF, IL-12, IL-10, IL-6 and MCP-1 were purchased from BD biosciences (CA, USA). First Strand cDNA synthesis kit was purchased from Thermo Scientific (MA, USA). EZcountTM MTT Cell Assay Kit was purchased from Himedia Laboratories Pvt. Ltd, (Mumbai, India).

3.1.6. Primers for PCR amplifications

Chikungunya virus nsP2 (F)-5′CGAGGATCCACTGAATGAAATATGC-3′ and (R)-5′CGACTCGAGTTAACATCCTGCTCGGGTGG-3′; structural genes (E1) (F)-5′TGCCGTCACAGTTAAGGACG3′ and (R)-5′CCTCGCATGACATGTCCG3′.

		Ltd., Mumbai, India	500G
23	10x RBC lysis buffer	Himedia Laboratories Pvt. Ltd., Mumbai, India	R075-100ML
24	HPLC grade Methanol	Himedia Laboratories Pvt. Ltd., Mumbai, India	AS061-2.5L
25	Paraformaldehyde	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM-3660-500GM
26	HiGlutaXL™ RPM-1640	Himedia Laboratories Pvt. Ltd., Mumbai, India	AL060G
27	Acrylamide	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB068-1KG
28	Trypsin-EDTA	Himedia Laboratories Pvt. Ltd., Mumbai, India	TCL014-5x100ML
29	Antibiotic solution 100x liquid (10000 U Penicillin+ 10 mg Streptomycin)	Himedia Laboratories Pvt. Ltd., Mumbai, India	A001A-5x100ML
30	10x Phosphate Buffered Saline	Himedia Laboratories Pvt. Ltd., Mumbai, India	TL1032-500ML
31	TEMED	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB026-100ML
32	Fetal Bovine Serum (FBS), Australia origin	PAN Biotech, Aidenbach, Germany	P30-1302
33	DMEM	PAN Biotech, Aidenbach, Germany	P04-01550
34	Ammonium persulfate (APS)	Bio-Rad, CA, USA	161-0700

8	Bromophenol blue	Sigma Aldrich, MO, USA	114391
9	Crystal violet	Sigma Aldrich, MO, USA	C6158
10	EGTA	Sigma Aldrich, MO, USA	E3889
11	Methyl cellulose	Sigma Aldrich, MO, USA	M0387-250g
12	Gentamycin	Sigma Aldrich, MO, USA	G1272
13	Bis-Acrylamide	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB005-250G
14	Glycine	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB013-1KG
15	Sodium chloride	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM031-1 kg
16	Tris base	Himedia Laboratories Pvt. Ltd., Mumbai, India	TC072-1KG
17	Bovine serum albumin fraction-V	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM105-100G
18	Sodium azide	Himedia Laboratories Pvt. Ltd., Mumbai, India	GRM123-100G
19	EDTA	Himedia Laboratories Pvt. Ltd., Mumbai, India	R066-500ML
20	Glycerol	Himedia Laboratories Pvt. Ltd., Mumbai, India	MB060-500ML
21	Sulphuric acid (H_2S0_4)	Himedia Laboratories Pvt. Ltd., Mumbai, India	AS016-500ML
22	Tween-20	Himedia Laboratories Pvt.	GRM156-

24	Monoclonal Antibody to GAPDH	Abgenex India Pvt. Ltd. BBS, India	10-10011/ ABM22C5
25	Chicken anti-mouse AF488	Invitrogen, CA, USA	A21200/NA
26	Goat anti-rabbit AF647	Invitrogen, CA, USA	A21244/NA
27	HRP-goat anti-mouse IgG	BD Biosciences, CA, USA	554002/NA
28	HRP-goat anti-rabbit IgG	BD Biosciences, CA, USA	554021/NA

The mouse anti-CHIKV-nsP2 monoclonal antibody used in this study was developed as described earlier [242]. The anti-CHIKV-E2 monoclonal antibody was a kind gift from Dr. M. M. Parida, DRDO, Mysore, India.

3.1.4. Chemicals, reagents and modulators

Table 6. Details of chemicals used

Si no	Chemicals	Company	Catalog no
1	Triton X-100	Sigma Aldrich, MO, USA	MB031-500ML
2	Sodium deoxycholate	Sigma Aldrich, MO, USA	D6750-25G
3	SDS (sodium dodecyl sulphate)	Sigma Aldrich, MO, USA	L6026
4	Saponin	Sigma Aldrich, MO, USA	47036-50GM
5	2-mercaptoehtanol	Sigma Aldrich, MO, USA	63689
6	PhosStop™ (phosphatase inhibitors cocktail)	Sigma Aldrich, MO, USA	04906837001
7	Complete EDTA-free protease inhibitor	Sigma Aldrich, MO, USA	05892970001

12	p38 MAPK Antibody	Cell Signaling Technology, MA, USA	9212
13	Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP®Rabbit mAb	Cell Signaling Technology, MA, USA	4511
14	SAPK/JNK (56G8) Rabbit mAb	Cell Signaling Technology, MA, USA	9258
15	Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb	Cell Signaling Technology, MA, USA	4668
16	p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell Signaling Technology, MA, USA	4695
17	Phospho-p44/42 MAPK (Erk1/2) (Thr202/ Tyr204) (D13.14.4E) XP®Rabbit mAb	Cell Signaling Technology, MA, USA	4370
18	Phospho-Stat1 (Ser727) (D3B7) Rabbit mAb	Cell Signaling Technology, MA, USA	8826
19	Phospho-Stat1 (Tyr701) (D4A7) Rabbit mAb	Cell Signaling Technology, MA, USA	7649
20	Phospho-IRF-3 (Ser396) (4D4G) Rabbit mAb	Cell Signaling Technology, MA, USA	4947
21	Phospho-Stat3 (Tyr705) Antibody	Cell Signaling Technology, MA, USA	9131
22	Mouse IgG1 Isotype Control	Abgenex India Pvt. Ltd. BBS, India	10-101/ MOPC31C
23	Beta-actin antibody pAb	Abgenex India Pvt. Ltd. BBS, India	IMG-5142A/NA

3.1.3. Antibodies

Table 5. Details of antibodies used

Si No.	Antibodies	Company	Catalog no/clone no
1	PE mouse anti-mouseH-2kd, Clone SF1-1.1	BD Biosciences, CA, USA	553566/SF1-1.1
2	PE Rat ant-mouse I-Ad/I-Ed, Clone 2G9	BD Biosciences, CA, USA	558593/2G9
3	PE Rat IgG2c, k isotype control, clone A23-1	BD Biosciences, CA, USA	559841/A23-1
4	APC Rat IgG2a k isotype control	BD Biosciences, CA, USA	553932/R35-95
5	Purified mouse anti-HSP90, clone 68/HSP90	BD Biosciences, CA, USA	610418/R35-95
6	APC Annexin V	BD Biosciences, CA, USA	550474/G155-178
7	CD80 (B7-1) mAb APC, clone 16-10A1	eBiosciences, CA, USA	17-0801-82/16-10A1
8	CD80 (B7-2) mAb APC, clone GL1	eBiosciences, CA, USA	17-086282/GL1
9	Cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb	Cell Signaling Technology, MA, USA	9664
10	Cleaved caspase-8 (Asp387) (D5B2) XP® Rabbit mAb (mouse specific)	Cell Signaling Technology, MA, USA	8592
11	Cleaved caspase-9 (C9) Mouse mAb	Cell Signaling Technology, MA, USA	9508

3.1. Materials

3.1.1. Cells and Viruses

The Indian outbreak strain of CHIKV, DRDE-06 (accession no. EF210157.2), CHIKV prototype strain S-27 (accession no. AF369024.2) and Vero cells (African green monkey kidney epithelial cell line) were kind gifts from Dr. M. M. Parida, DRDO, Mysore, India. The mouse monocyte/macrophage cell line, Raw264.7 (ATCC® TIB-71™) was maintained and cultured in RPMI-1640 (HiGlutaXL™ RPMI-1640) supplemented with 2.0 mM L-glutamine, Penicillin 100 U/ml, Streptomycin 0.1 mg/ml (Himedia Laboratories Pvt. Ltd, Mumbai, India), 10% Fetal bovine serum (FBS; PAN Biotech, Germany) at 37°C under a humidified atmosphere with 5% CO_2 concentration. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM; PAN Biotech, Germany) supplemented with 5% FBS, Gentamycin, and Penicillin-Streptomycin (Sigma, USA). The enzyme free cell dissociation reagent (ZymeFree™; Himedia Laboratories, Pvt. Ltd, Mumbai, India) was used for sub-culturing the cells.

3.1.2. Animals

BALB/c mice of both sexes ware obtained from the Institutional animal breeding facility. Animals were maintained in 12 h light/dark alternate cycles with food and water provided adlibitum. Institutional Animal Ethics Committee (IAEC) approval (V-186-MISC/2009-10/2973/ILS) was taken prior use of animals for the isolation of the macrophage. Six to ten weeks old mice were used for the isolation of peritoneal macrophage.

CHAPTER#3

Materials

&

Methods

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inhibitor of HSP90, to regulate CHIKV infection, pro-inflammatory cytokine/chemokine production and apoptosis during viral infection explained in Figure 32. Further, the macrophage-T cell co-culture suggested that CHIKV infected macrophages could stimulate and activates T cells in vitro. In depth analysis revealed pro-viral as well as anti-viral role of P38 MAP kinase, whereas inflammatory and antiviral role of JNK MAP kinase during CHIKV infection in the host macrophages (Figure 33). However, further studies are required to substantiate the mechanism of CHIKV infection in macrophage associated cellular pathways towards apoptosis, induction of host cell immunity and pathogenesis for designing rationale therapeutic drugs against CHIKV infection.

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ignored, as it might be a contributing factor for SB mediated suppression in the TNF production, which is induced by CHIKV.

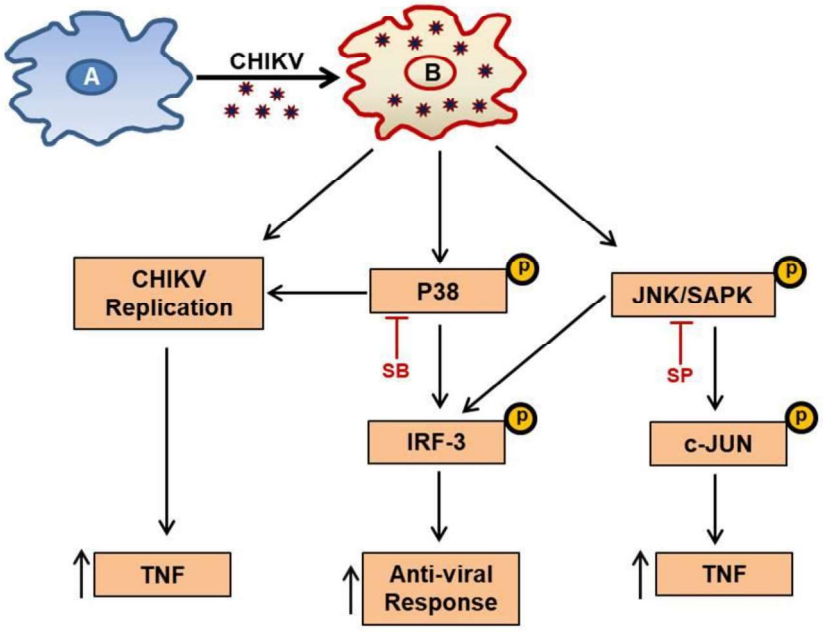


Figure 33. Proposed working model showing involvement of MAPK pathways in the induction of TNF and anit-viral responses via different transcription factors during CHIKV infection in macrophages. Uninfected macrophage **(A)**; and CHIKV infected macrophage **(B)** depicting induction of p-P38 and p-JNK, followed by the activation of p-IRF and p-c-JUN in the host macrophages.

In the current study it has been suggested that CHIKV completes one replication cycle in the Raw264.7 macrophage cell line in every 12 hours post infection at MOI 05. The susceptibility of Vero cell line for CHIKV infection is much higher as compared to the Raw cells, which is dose dependent. For the first time it has been showed that CHIKV infection induces apoptosis via both extrinsic and extrinsic pathways in the host macrophages. CHIKV also enhances MHCs and co-stimulatory molecule expression along with TNF, IL-6 and MCP-1 production in mouse macrophages, *in vitro*. It has also been identified that an important role of 17-AAG, a potential

Uninfected macrophage **(A)**; and CHIKV infected macrophage **(B)** showing induction of MHC I/II, CD86 molecules as immune activation markers along with inflammatory cytokines/chemokine (TNF, IL-6 and MCP-1) production and apoptosis by phosphatidylserine (PS) and cleaved caspase-3 expression. **(C)** 17-AAG has been found to regulate the viral infection, apoptosis and inflammatory responses (TNF, IL-6 and MCP-1), suggesting its therapeutic implication in CHIKV infection (Nayak TK et al., Viruses 2017).

Except few cases, CHIKV is not fatal, however, the long term polyarthralgia, arthritis like symptoms along with severe inflammation remains a concern for most of the chronic patients [110,171,172,176,207,294]. It has been reported previously that TNF is one of the key mediator for arthritis or arthritis like diseases in humans by inducing severe inflammation. Despite elevation of several other inflammatory cytokines in RA, anti-TNF therapy remain holds a promise for the effective treatment against it [276,277]. Since, CHIKV induces TNF via P38/JNK MAP kinase pathways, and p-c-JUN, which is reported to be upstream of TNF production in other models [261]. Western blot analysis suggests that p-c-JUN expression reduced around 1.5 fold in the presence of SP, whereas SB treatment does not affect p-c-JUN at all. However, both SB and SP treatment suppresses p-IRF-3 expression induced by CHIKV as compared to the DMSO control. Further, it has been reported that c-JUN gets activated when phosphorylation by upstream JNK MAPK) as result of TNF induction [261]. Taken together, the current data depicts that CHIKV induces p-c-JUN via JNK pathway whereas induction of p-IRF-3 is dependent on both P38 and JNK MAP kinase. Therefore, it is quite possible that JNK pathway induction by CHIKV is involved in the activation of antiviral response via p-IRF-3 and pro-inflammatory responses (TNF) via p-c-JUN pathways. The P38 pathway involved in both pro-viral pathways, since SB treatment reduces CHIKV infection and anti-viral pathways via induction of p-IRF-3 in the host macrophages. Though the proinflammatory TNF induced by CHIKV is reduced in the presence of SB, the inhibition of viral infection by SB cannot be

significantly at both the time points as compared to the corresponding DMSO control, suggesting inflammatory role of both p-P38 and p-JNK during infection in the macrophages. Moreover, an antiviral alkaloid barbarine has been studied to reduce both CHIKV infection and induced MAPK in the host cells [269].

Very often viral infection is associated with the activation and localization of several transcription factors (e.g., IRFs, c-JUN, P53), which in turn regulates host responses to the viruses such as anti-viral, inflammatory cytokines productions [269-275]. In this study, the expression of key transcription factors involved mainly in antiviral responses (p-IRF3) and TNF production (p-c-JUN) were assed. Western blot analysis suggests that both p-IRF3 and p-c-JUN were induced significantly in the CHIKV infected macrophages as compared to the corresponding mock, which indicates that CHIKV infection in the Raw macrophage cell line induces phosphorylation (activation) of key antiviral and inflammatory transcription factors.

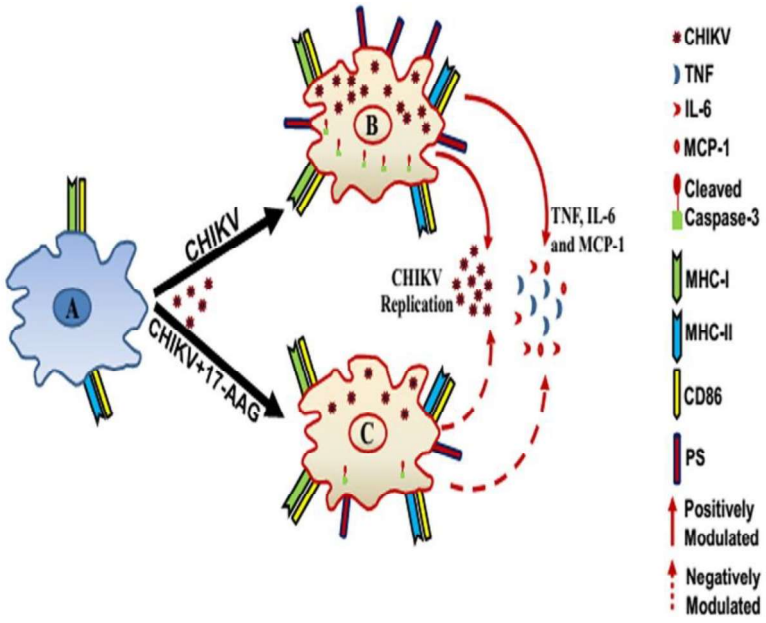


Figure 32. Proposed working model depicting CHIKV infection in macrophages and 17-AAG mediated possible regulation of its altered immune responses by inhibition of HSP90.

significant increments toward 12 hpi as compared to the corresponding mock. Likewise, the expression of the p-JNK was found to be increased rapidly around 2 fold during early hours (3 and 6 hpi), whereas, it went to around 3 folds with respect to the mock. Though total P38 and JNK (t-P38 and t-JNK) expression remains unaffected in both the groups. However, p-ERK1/2, t-ERK1/2 (total-ERK1/2) expression remains unchanged, throughout all the time points as compared to the corresponding mock. This investigation suggests that CHIKV induces activation of both P38 and JNK by phosphorylation in the specific residues in a time dependent manner in macrophages without any alteration in the p-ERK1/2. Since, CHIKV induces both P38 and JNK MAPK activation in the host macrophages, next it was assessed whether these two MAPKs are crucial for the viral infection and replication in the macrophages. For this Raw cells were inoculated with the DRDE-06 strain of CHIKV as mentioned above in the presence of either SB203580 (p-P38 inhibitor) or SP600125 (p-JNK inhibitor) or DMSO (solvent control) and nsP2 expression was assessed by Flow cytometry. It was found that the percent positive cells for nsP2 reduced around 1.8 fold in the presence of SB without any significant changes after SP treatment, despite significant reduction in the p-JNK expression. Further, plaque assay of the cell culture supernatant revealed that SB but not SP treatment reduces the number of new viral progenies release around 1.5 fold as compared to the corresponding DMSO control. This result indicates that activation of P38 but not JNK MAPK is crucial for the CHIKV infection and replication in the host macrophages, suggesting its pro-viral role, which has also been observed in other viral infections [269,293].

Activation of MAP kinases by different pathogens and other inflammatory diseases has been shown to induce pro-inflammatory cytokines such as TNF in the host cells [259-262,265]. Since, CHIKV triggers robust TNF production (a key mediator of inflammation) in the host macrophages, it was investigated whether, any MAPKs are involved in the induction of TNF. It was demonstrated that both SB and SP treatment could suppress CHIKV induced TNF

Annexin V during viral infection cannot be ruled out. Furthermore, it was reported that several HSP90 inhibitors promote anti-cancer T cell responses without interfering the immune activation state such as MHC expression [291]. Our current observation also suggest that upregulated level of pro-inflammatory cytokines (TNF and IL-6) and chemokine (MCP-1) in CHIKV infected macrophage could be down regulated by 17-AAG treatment, while preserving the expression of MHC and co-stimulatory molecules in macrophages to strengthen the subsequent anti-viral immune responses. The activation of T cells during CHIKV has been reported earlier, however the role of host macrophages in this activation is still not studied [221,292]. Although, CHIKV infects macrophages and induces both MHC and co-stimulatory molecules, it remained unclear whether infected macrophage could modulate the effector function of the T cells *in vivo*. Co-culture experiment of mouse primary peritoneal macrophages and T cells were performed to mimic the *in vivo* CHIKV infection condition. It was observed that there was an increase in the surface expression of the T cell activation marker CD25 (late activation marker) and CD69 (early activation marker) in T cells co-cultured with CHIKV infected macrophages. Further it has been demonstrated that there was upregulation of IL-2 and IFN-γ from the supernatant, which indicates that CHIKV infection in macrophage stimulates robust activation of T cells possibly by up regulation of MHC and stimulatory molecules.

The mitogen activated protein kinase (MAPK) pathways is known to affect multiple cellular pathways such as cell proliferation, activation, inflammation, cytokine and chemokine productions and other pathological conditions [27,258-263]. The MAPKs have been shown to be activated by phosphorylation in a specific positions (Ser/Tyr/Thr) by several viral infections, such as corona virus type 2, Hepatitis C virus, Rhinovirus and Epstein-Barr virus [264-268]. The Western blot analysis of time course experiments suggests that CHIKV induces both p-P38 and p-JNK significantly as compared to the corresponding mock. The p-P38 MAPK expression was found to be increased around 1.5 fold as early as 3 and 6 hpi, followed by approximately 3 fold

Host derived endogenous HSP90 has been identified to play a critical role in the pathogenesis of CHIKV. Previous studies from our laboratory and others have signified the use of HSP90 inhibitor to regulate CHIKV infection in host cells [239,240]. However, such effect of HSP90 inhibitor has not been evaluated to words CHIKV infection in host macrophages. In this study 17-AAG, a potential HSP90 inhibitor was used on CHIKV-infected mouse macrophages, as this drug has been reported to have less toxic effect than its derivative [241,257]. The results demonstrated that the treatment of 17-AAG along with CHIKV infection has reduced the number of live infectious viral particles around 3-fold. Moreover, it was also observed that the level of viral proteins, nsP2 and E2 were decreased by ~2 fold in 17-AAG treated macrophages as compared to DMSO control. Further analysis revealed that 17-AAG also reduced CHIKV RNA expression around 50% for nsP2 and around 15% for E2 at 0.5 μM concentration indicating it's regulatory role during CHIKV infection in macrophages. Taken together our result suggests that 17-AAG could inhibit viral RNA as well as viral protein levels, however it reduces the protein level more effectively.

Apoptosis is the predominant form of cell death in response to various cellular stimuli, which in turn affect the physiology of an organism. It also plays an important role towards viral replication, dissemination of virus particle to the neighboring host cells as well as antigen presentation [250-252]. In case of CHIKV infection it was observed that 17-AAG treatment abrogated CHIKV induced apoptosis (Annexin V binding) without affecting the expression of cleaved caspase-3, which was not significantly reduced in presence of 17-AAG as compared to the CHIKV+DMSO control It is quite possible that induction of the cell death by CHIKV might be mediated partly via caspase independent pathway too. Although, 17-AAG did not suppress the expression of cleaved caspase-3 that is induced during infection, however it did reduce the binding of Annexin V to the infected macrophages. Since, blocking of phosphatidylserine by recombinant Annexin V was found to reduce robust CHIKV infection ([252], the role of 17-AAG in suppressing induced

infection is not clearly evaluated. In this study, it has been assessed that the expression of macrophage pro-inflammatory cytokines likes IL-12, TNF, IL-6 and MCP-1 along with IL-10, an anti-inflammatory cytokine during CHIKV infection at various time intervals. It was observed that there is a time-dependent increase in the secreted TNF, IL-6 and MCP-1 during CHIKV infection in macrophages, while the production of IL-10 and IL-12 remain unchanged during infection as compared to mock infected cells. Pathogenic inflammatory TNF responses during CHIKV infection has been recently reported [213]. Moreover, TNF may act as an apoptotic mediator in many viral infections [250]. It might be plausible that CHIKV infection driven pro-inflammatory TNF induction may facilitate apoptosis in macrophages, which need further investigation. Viral infection in APCs like macrophages are known to enhance antigen processing and presentation via MHC class I and class II to $CD8^+$ and $CD4^+$ T cells respectively to elicit adaptive immune responses. However, some viruses are known to manipulate the expressions of MHC and co-stimulatory molecules to evade activation of immune response against them [145,254,256,278,289]. Accordingly, the level of MHC and co-stimulatory molecules on CHIKV infected macrophages were studied. It was observed that the levels of MHC-I, MHC-II and CD86 activation markers were elevated in the CHIKV infected macrophages at various time intervals. Moreover, our recent *in silico* analysis has identified several highly conserved CHIKV specific immunodominant MHC-I restricted peptide epitopes which may elicit strong anti-CHIKV $CD8^+$ T cell responses [290]. The current observations suggest that CHIKV infected macrophages may up-regulate expression of both MHC-I and MHC-II to present CHIKV specific immunodominant peptides and also induce CD86 expression for possible successive adaptive immune responses. Further investigations are required to understand the mechanism of CHIKV infection induced MHC expression which is associated with viral immunodominant epitopes reactive to T cells for effective anti-CHIKV immune responses.

differences in the observations might be due to different MOIs of viruses that were used in the experiments. Apoptosis is an important anti-viral mechanism and is generally considered to have a protective role in macrophages to ward off the viruses, thereby impairing viral propagation [281,282]. Arguably, our study may highlight the importance of viral-induced apoptosis in increasing the viremia and propagation of infection to the nearby host cells. Moreover, the application of caspase inhibitors have been shown to reduce the number of viral progenies in CHIKV infected Hela cells [252]. Interestingly, the treatment of Z-VAD-FMK was found to reduce new viral progeny release of CHIKV and infection, without altering the frequency of E2 percent positive host macrophages. The importance of Z-VAD-FMK on CHIKV infection was also studied earlier in HeLa cells, suggesting the reduction of both CHIKV replication and infection *in vitro* [252]. Moreover, Z-VAD-FMK was also found to differentially regulate other viral replication and infection [282-285]. Arguably, our study in macrophages may highlight the importance of CHIKV-induced apoptosis in increasing the viremia and propagation of infection to the nearby host cells. Further investigations are required to understand the mechanism of CHIKV induced apoptosis in the host macrophages in details.

CHIKV infection associated inflammatory response involves production and secretion of several pro-inflammatory cytokines and antiviral molecules in both circulatory and tissue system. However, pre-dominance of anti-inflammatory responses might also prevail under such conditions, which are against a common description and notion of CHIKV infection [209]. The cytokines IL-12 and TNF are known to possess potent effector antiviral activity and may act synergistically to promote macrophage activation [286-288], while IL-10 plays an important cross-regulatory role during infection associated inflammation [149]. Moreover, IL-6 and MCP-1 were also found to be induced during CHIKV infection in animals and patient serum samples as an indicator of inflammatory as well as heightened cellular immune responses [125,209]. However, such induction of IL-6 and MCP-1 responses from host macrophages during CHIKV

[278]. CHIKV has been known to infect a wide range of cells including monocyte/macrophage, both *in vivo* and *in vitro* [178,188,189,213]. However, an in depth understanding of CHIKV infection and the immune regulation in macrophage is still warranted. Here it has been studied the altered immune responses of mouse macrophages (Raw cell line) comprising MHCs, inducible co-stimulatory molecule (CD86), major pro-inflammatory cytokines/chemokine production and host cell apoptosis during CHIKV infection *in vitro*. Moreover, using 17-AAG, a potential HSP90 inhibitor, an investigation was carried out to assess the possible regulation of CHIKV infection and its effect on host macrophages towards altered immune responses and apoptosis, if any. Acute phase of CHIKV infection in human blood monocytes was found to induce a robust and rapid innate immune response [189]. Studies on non-human primates further indicated that macrophage could act as the main cellular reservoir for long term CHIKV infection and persistence [111]. In the current study, the macrophages (Raw cell lines) incubated with CHIKV showed an increased levels of viral proteins (both nsP2 and E2) at different time intervals which peaked at 8 hpi and gradually declined at a later time point. Subsequently, plaque assay showed the highest release of new viral progenies from the infected cells at 12 hpi. Like other alphaviruses, it was also reported that CHIKV infection induces apoptosis and cytopathic effect (CPE) in the infected cells [99,216,280]. However, induction of apoptosis in CHIKV infected macrophages is yet to be defined. Here it was found that the surface binding of Annexin V was increased in CHIKV infected mouse macrophages as compared to mock. Furthermore, Western blot analysis revealed that the expression of cleaved caspase-3 was induced in CHIKV infected macrophages starting from 4 hpi with peak expression at 12 hpi as compared to mock, suggesting an induction of apoptotic cell death after viral infection. Furthermore, it was demonstrated for the first time that CHIKV induces apoptosis in macrophages by both intrinsic and extrinsic pathway, as induced expression of the cleaved caspase-9 and -8 were observed during infection. However, Kumar et.al., could not detect apoptosis in mouse macrophages infected with CHIKV at MOI 1. The

significant difference between the groups. (ns, non-significant; * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Discussion and Conclusion:

The recent epidemics of Chikungunya viruses (CHIKV) with unprecedented magnitude and unusual clinical severity have raised a great public health concern worldwide, due to absence of vaccine or specific anti-CHIKV therapy. This emphasizes the need to understand the biological processes of this virus in details along with macrophage associated pathogenesis. Although CHIKV associated research has been initiated long back, the availability of CHIKV specific basic reagents such as antibodies for in-depth investigation of viral infection and replication are scanty. For Alphavirus replication, non-structural protein 2 (nsP2) is known to play a key regulatory role among all other non-structural proteins. The current study describes the development and characterization of nsP2 specific monoclonal antibody (mAb) against a synthetic peptide of CHIKV. Reactivity and efficacy of this mAb have been demonstrated by various techniques such as ELISA, Western blot, Flow cytometry and Immunofluorescence assay . Moreover, homology analysis of the selected epitope sequence reveals that it is conserved among all the CHIKV strains of different genotypes, while analysis with other Alphavirus sequences shows that none of them are 100% identical to the epitope sequence [242].

Various viral infection models demonstrate the importance of host cell immunity to suppress virus propagation and dissemination [278]. CHIKV infection has also been shown to elicit strong innate immune response which involve production of anti-viral IFNs as well as many pro-inflammatory cytokines, chemokines and growth factors [206,209,211,213,216-218,279]. Macrophages act as one of the major host immune cells that respond to many types of pathogens and activate not only host innate immunity but also may facilitate adaptive immune responses

expression induced by CHIKV as compared to the DMSO control (Figure 31A, B, C, D). Taken together, the current data depicts that CHIKV induces p-c-JUN via JNK pathway whereas induction of p-IRF-3 is dependent on both P38 and JNK MAP kinase.

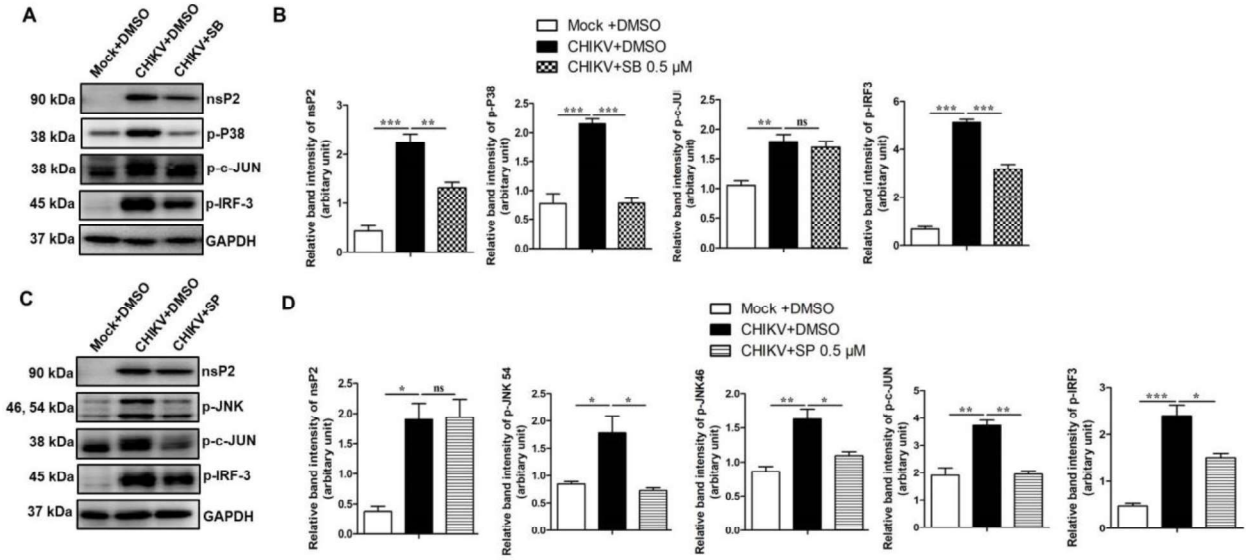


Figure 31. CHIKV induces p-c-JUN via JNK MAPK activation in macrophages. Raw264.7 cells were infected with CHIKV at MOI 5. The cells were treated with either DMSO or SB (0.5 µM) or SP (5.0 µM) as described earlier. Both mock and CHIKV infected Raw264.7 cells were harvested at different time intervals followed by Western blot based analysis. **(A)** Western blot analysis depicting nsP2, p-P38, p-c-JUN and p-IRF-3 protein expression at different time post infection for mock+DMSO, CHIKV+DMSO and CHIKV+SB. **(B)** Bar diagram showing relative band intensity of nsP2, p-P38, p-c-JUN and p-IRF3 for mock+DMSO, CHIKV+DMSO and CHIKV+SB at 12 hpi. **(C)** Western blot analysis depicting nsP2, p-JNK, p-c-JUN and p-IRF-3 protein expression at different time post infection for mock+DMSO, CHIKV+DMSO and CHIKV+SP. **(D)** Bar diagram showing relative band intensity of nsP2, p-JNK, p-c-JUN and p-IRF3 for mock+DMSO, CHIKV+DMSO and CHIKV+SB at 12 hpi. GAPDH serves as loading control. Data represented here at least from three independent experiments. Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically

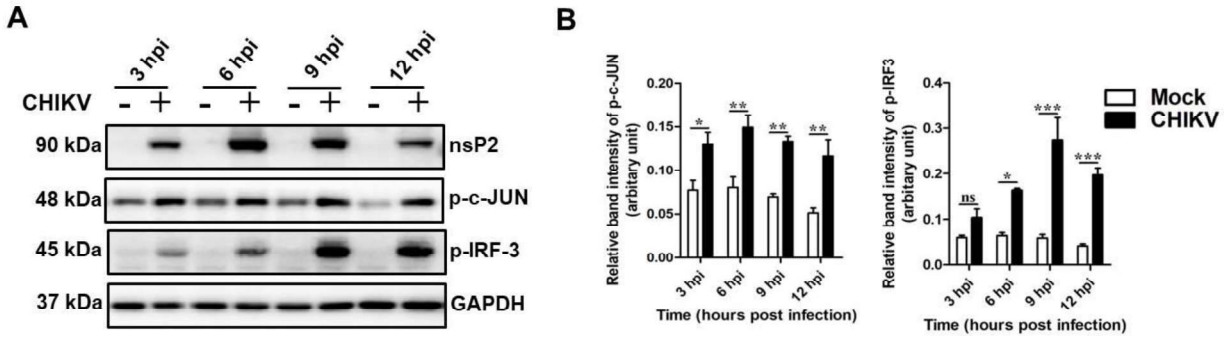


Figure 30. Induction of key transcription factors by CHIKV in macrophages. CHIKV infected Raw264.7 cells were harvested at different time intervals followed by Western blot based analysis. **(A)** Western blot analysis depicting p-c-JUN and p-IRF-3 protein expression at different time post infection. GAPDH serves as loading control. **(B)** Bar diagram showing relative band intensity of p-c-JUN and p-IRF3 at different time post infection. Data represented here at least from three independent experiments. Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

4.6.5. CHIKV induces p-c-JUN via JNK MAPK activation in macrophages

In general, CHIKV is not fatal, however, the long term polyarthralgia, arthritis like symptoms along with severe inflammation remains a concern for most of the chronic patients. It has been reported previously that TNF is one of the key mediator for arthritis or arthritis like diseases in humans by inducing severe inflammation. Despite elevation of several other inflammatory cytokines in RA, anti-TNF therapy remain holds a promise for the effective treatment against it [276,277]. Since, CHIKV induces TNF via P38/JNK MAP kinase pathways (Figure 29), and phosphorylation of c-JUN, which is reported to be associated with TNF production in other models [261]. The phosphorylation of c-JUN was assessed by Western blot analysis. The expression of p-c-JUN went down around 1.5 fold in the presence of SP, whereas SB treatment does not affect p-c-JUN. However, both SB and SP treatment suppresses p-IRF-3

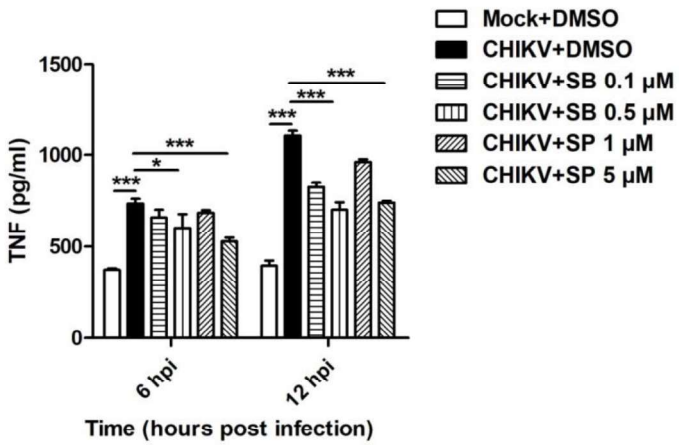


Figure 29. Pharmaceutical inhibitors specific to p-P38 and p-JNK abolishes CHIKV induced TNF in the host macrophages. Raw264.7 cells were infected with CHIKV at MOI 5. The cells were treated with either DMSO or SB or SP at different concentrations as described earlier. Both cells and cell culture supernatants were collected and processed further according to the assay procedure. Bar diagram depicting production of TNF (pg/ml) at 6 and 12 hpi with mock+DMSO, CHIKV+DMSO, CHIKV+SB (0.1, 0.5 µM) and CHIKV+SP (1.0, 5.0 µM). Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; *** $p \leq 0.001$).

4.6.4. Time dependent induction of key transcription factors by CHIKV in macrophages

Often, viral infection is associated with the activation and localization of several transcription factors (e.g., IRFs, c-JUN, P53), which in turn regulates host responses to the viruses such as anti-viral, inflammatory cytokines productions [270-275]. Here, expression of key transcriptional factors involved mainly in antiviral responses (p-IRF3) and TNF production (p-c-JUN) were assed at different hpi by western blot analysis. It was observed that both p-IRF3 and p-c-JUN were induced significantly in the CHIKV infected macrophages as compared to the corresponding mock (Figure 30A, B). This data suggests that CHIKV infection in the Raw cell line might associated with the elevation of key antiviral and inflammatory transcription factors.

p-JNK (lower panel). **(H)** MFI of nsP2 (upper panel) and p-JNK (lower panel) for mock+DMSO (purple filled), CHIKV+DMSO (solid red) and CHIKV+SP (solid green). **(I)** Bar diagram showing CHIKV titer as pfu/mL in CHIKV, CHIKV + DMSO and CHIKV + SB and SP at 12 hpi. Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; *** $p \leq 0.001$).

4.6.3. Pharmaceutical inhibitors specific to p-P38 and p-JNK abolishes CHIKV induced TNF in the host macrophages

Activation of MAP kinases by different pathogens has been shown to induce pro-inflammatory cytokines such as TNF in the host cells [261,265]. Since, CHIKV triggers robust TNF production (a key mediator of inflammation) in the host macrophages, it was interesting to investigate whether, any MAPKs are involved in this pathway. For these macrophages were treated with both the SB and SP and infected with CHIKV as explained elsewhere. The cell culture supernatants were subjected to sandwich ELISA for the detection TNF production at early (6 hpi) and late (12 hpi). It was observed that both SB and SP could suppress induced TNF significantly at both the time points as compared to the corresponding DMSO control. At 6 hpi the TNF level for CHIKV+DMSO was found to be 737±27 pg/ml (mean±SEM), reduced to 604±75 pg/ml (mean±SEM, $p<0.05$) and 528±20 pg/ml (mean±SEM, $p<0.05$) in the presence of SB (0.5 μM) and SP (5.0 μM) respectively. Similarly, at 12 hpi, the TNF production was 1104±29 pg/ml (mean±SEM) in CHIKV+DMSO sample, whereas 703±41 pg/ml (mean±SEM, $p<0.05$) for SB and 741±10 pg/ml (mean±SEM, $p<0.05$) after SP treatment (Figure 29). Taken together, this result suggests that CHIKV induces TNF in the host macrophages via both P38 and JNK mediated pathways.

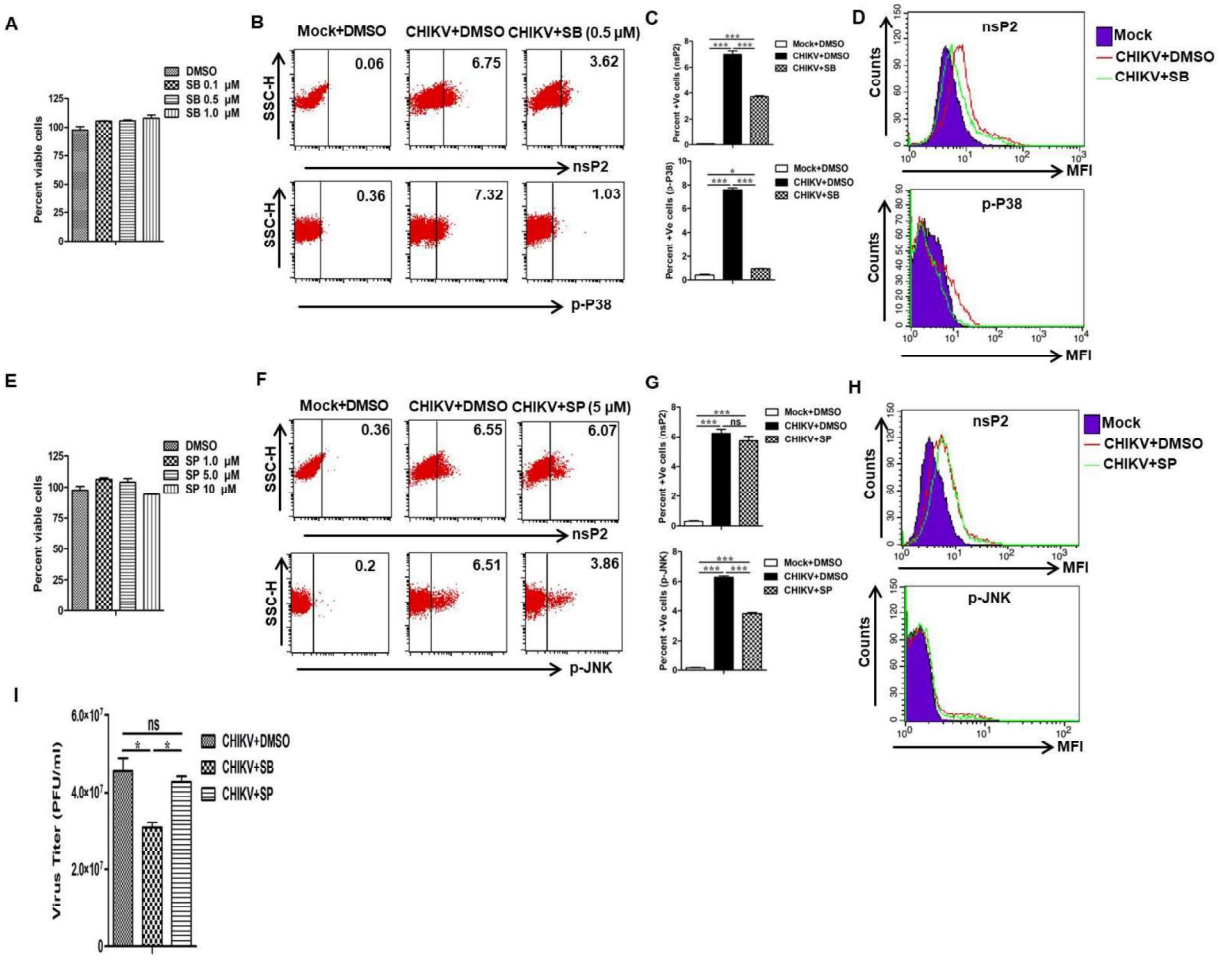


Figure 28. Treatment of SB (p-P38 inhibitor) reduces CHIKV infection in macrophages. CHIKV infected Raw264.7 cells were harvested at 12 hpi with either DMSO or SB or SP treatment followed by flow cytometry and plaque assay based analysis. **(A)** MTT assay showing cytotoxicity of SB in Raw cell line. **(B)** Dot plot analysis showing expression of nsP2 (upper panel) and p-P38 (lower panel) for mock+DMSO (left), CHIKV+DMSO (middle) and CHIKV+SB (right). **(C)** Bar diagram showing percent positive cells for nsP2 (upper panel) and p-P38 (lower panel). **(D)** MFI of nsP2 (upper panel) and p-P38 (lower panel) for mock+DMSO (purple filled), CHIKV+DMSO (solid red) and CHIKV+SB (solid green). **(E)** MTT assay showing cytotoxicity of SP in Raw cell line. **(F)** Dot plot analysis showing expression of nsP2 (upper panel) and p-JNK (lower panel) for mock+DMSO (left), CHIKV+DMSO (middle) and CHIKV+SB (right). **(G)** Bar diagram showing percent positive cells for nsP2 (upper panel) and

E) as described earlier [269]. At 12 hpi both mock and CHIKV infected cells were harvested and the expression of nsP2, p-P38 and p-JNK were assessed by Flow cytometry. It was observed that the percent positive cells for nsP2 went from 6.98±.28 (CHIKV+DMSO) to 3.72± 0.07 (CHIKV+SB), whereas the percent positive cells for p-P38 went from 7.6± 0.16 (CHIKV+DMSO) to 1.0±0.02 (CHIKV+SB) at 0.5 μM concentration (Figure 28B, C). Likewise, the MFI for both the p-P38 and nsP2 were reduced at 12 hpi in the SB treated cells as compared to the corresponding DMSO control cells (Figure 28D). However, inhibition of p-JNK by SP (5.0 μM) did not affect nsP2 expression in the macrophages as compared to the DMSO control (CHIKV+DMSO; 6.24±0.28, CHIKV+SP; 5.70±0.33, $p>0.05$) despite significant reduction in the p-JNK percent positive cells (CHIKV+DMSO; 6.28±0.11, CHIKV+SP; 3.82±0.05, $p<0.05$) and MFI (Figure 28F, G, H). Further, plaque assay of the cell culture supernatant revealed that SB but not SP treatment reduces the number of new viral progenies release around 1.5 fold as compared to the corresponding DMSO control (Figure 28I). This result indicates that activation of P38 but not JNK MAPK is crucial for the CHIKV infection and replication in the host macrophages.

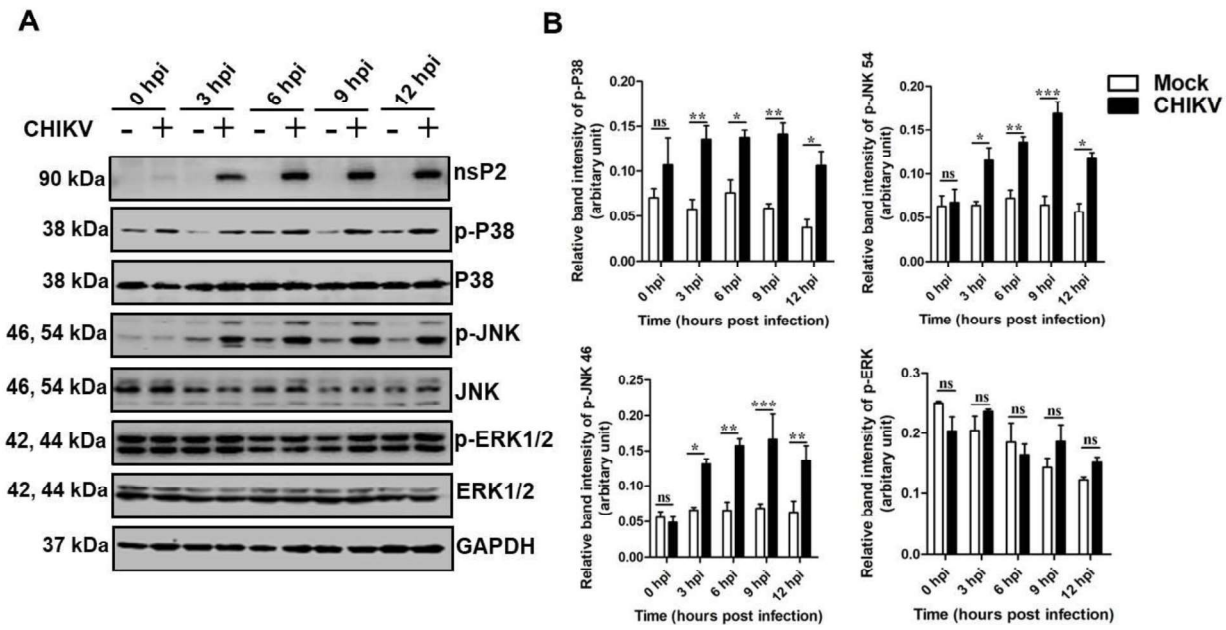


Figure 27. Induction of p-P38 and p-JNK MAPK during CHIKV infection in macrophages. CHIKV infected Raw264.7 cells were harvested at different time intervals followed by Western blot based analysis. **(A)** The protein expression of nsP2, p-P38, P38, p-JNK, JNK, p-ERK1/2, ERK1/2 were assessed by Western blot analysis. GAPDH was used as loading control. Data represented here at least from three independent experiments. **(B)** Bar diagram showing relative band intensity of p-P38, p-JNK and p-ERK1/2 at different time post infection. Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

4.6.2. Inhibition of P38 activation reduces CHIKV infection in macrophages

Since, CHIKV induces both P38 and JNK MAPK activation in the host macrophages, we next wanted to assess the whether these two MAPKs are crucial for the viral infection and replication in the macrophages. For this Raw cells were inoculated with the DRDE-06 strain of CHIKV as mentioned above in the presence of either SB203580 (p-P38 inhibitor) or SP600125 (p-JNK inhibitor) or solvent control DMSO, after performing MTT assay for cytotoxicity (Figure 28A,

represents means±SEM of at least three independent experiments. $p<0.05$ was considered as statistically significant difference between the groups. (*$p < 0.05$; **$p \leq 0.01$).

4.6. Mechanism of CHIKV induced inflammatory cytokine (TNF) production in the host macrophage *in vitro*

4.6.1. CHIKV induces both P38 and JNK phosphorylation in macrophages in a time dependent manner

The mitogen activated protein kinase (MAPK) pathways is known to affect multiple cellular pathways such as cell proliferation, activation, inflammation, cytokine and chemokine productions and other pathological conditions [27,258-263]. The MAPKs have been shown to be activated by phosphorylation in a specific positions (Ser/Tyr/Thr) by several viral infections [264-268]. To determine whether any MAPK (P38, JNK and ERK) is activated during CHIKV infection in macrophages, Raw cells were infected with virus at MOI 5 *in vitro* and harvested at different time points (0 to 12 hpi). Both the cells and cell culture supernatants were subjected to various assay procedures according to the experimental plans. The Western blot analysis of time course experiments suggests that CHIKV induces both p-P38 and p-JNK significantly as compared to the corresponding mock cells. The p-P38 MAPK expression was found to be increased around 1.5 fold as early as 3 and 6 hpi, followed by approximately 3 fold significant increments toward 12 hpi as compared to the corresponding mock cells (Figure 27). Similarly, the expression of the p-JNK was found to be increased rapidly around 2 fold during early hours (3 and 6 hpi), whereas, it went to around 3 folds with respect to the mock. Though total P38 and JNK (t-P38 and t-JNK) expression remains unaffected in both the groups. However, p-ERK1/2, t-ERK1/2 (total-ERK1/2) expression remains unchanged, throughout all the time points as compared to the corresponding mock (Figure 27A, B). This data suggests that CHIKV induces activation of both P38 and JNK by phosphorylation in the specific residues in a time dependent manner in macrophages.

represents means±SEM of at least three independent experiments. $p<0.05$ was considered as statistically significant difference between the groups. (**$p \leq 0.01$).

As mentioned earlier, among different kinds of T cells, the Th1 and Th2 are more popular because they drive either proinflammatory or anti-inflammatory responses up on activation respectively. Since, CHIKV induces pro-inflammatory responses predominately, the Th1 specific cytokines (IL-2 and IFN-γ) were assessed from the MΦ+T cell co-culture supernatant, to address if CHIKV infected macrophages drives Th1 biased T cell responses. As expected the IL-2 level was increased significantly from 256±1.13 pg/ml (mock-MΦ+T cell) to 266±1.37 (CHIKV-MΦ+T cell, $p<0.05$). Similarly, the IFN-γ level was also increased from 577±41.1 pg/ml (mock-MΦ+T cell) to 2263±120.9 (CHIKV-MΦ+T cell, $p<0.05$) (Figure 26A, B). Thus, the above results suggests that, exposure to CHIKV infected mouse primary macrophage activated T cell immune response *in vitro* presumably via upregulation of MHCs and co-stimulatory molecules.

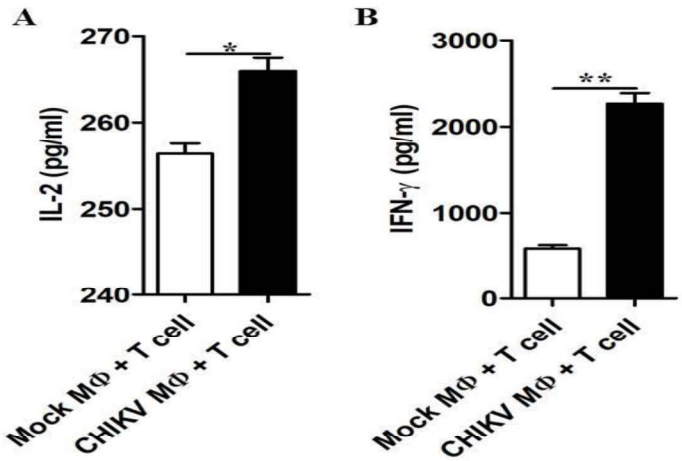


Figure 26. CHIKV infected mouse primary macrophage induces IL-2 and IFN-γ in the co-cultured T cells *in vitro*. Mouse peritoneal macrophages were infected with CHIKV at MOI 05 followed by co-culture with syngeneic purified T cells. The co-cultured cell culture supernatants were collected and analyzed by ELISA. Bar diagram showing level of secreted IL-2 **(A)** and IFN-γ **(B)** in primary macrophage and T cells co-cultured supernatants collected at 48 hr. Data

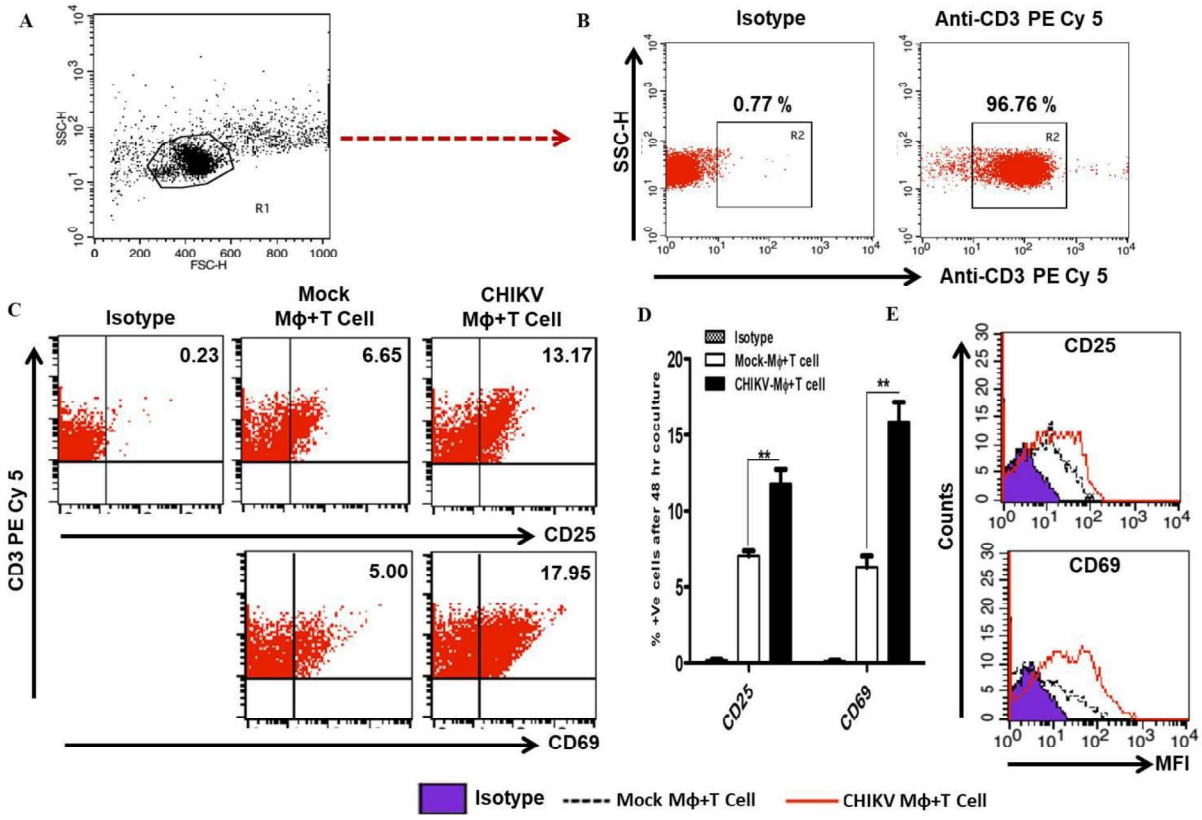


Figure 25. CHIKV infected mouse primary macrophage mediated-T cell activation *in vitro*. Mouse peritoneal macrophages were infected with CHIKV at MOI 05 followed by co-culture with syngeneic purified T cells. co-cultured T cells were harvested and processed for flow cytometry analysis while cell culture supernatants were collected for ELISA assay. **(A)** FSC-H vs SSC-H of co-cultured T cells. **(B)** Dot plot analysis of purified T cells with isotype control (left) and anti-CD3 PE-cy 5 (right). **(C)** Dot blot analysis depicting CD25 (upper row) and CD69 (lower row) positive T-cell after 48 hours of co-culture with CHIKV infected macrophages representing isotype (left), mock (middle) and CHIKV (right). **(D)** Bar diagram showing percent positive T cells for CD25 and CD69 after 48 hours of co-culture with CHIKV infected macrophages representing isotype (black crossed), mock (white bar) and CHIKV (dark bar). **(E)** Mean fluorescence intensity (MFI) of CD25 and CD69 in T-cell after 48 hours of co-culture with Isotype (purple line), mock (dashed line) and CHIKV infected macrophage (red line). Data

followed by cells were infected with CHIKV at MOI 05 and assessed for FACS analysis. **(A)** FSC-H vs SSC-H dot plot analysis of mouse PEC by flow cytometry. **(B)** Dot plot analysis depicting CD14 percent positive cells in enriched mouse peritoneal macrophages. **(C)** Flow cytometry dot plot analysis of CD14 gated macrophages showing percent positive cells for E2 against FSC-H. Data represented here at least from three independent experiments.

It was observed that approximately 15% peritoneal macrophages were positive for CHIKV, suggesting the susceptibility and infectivity of PEC to the virus *in vitro* (Figure 24C). After 48 hours of co-culture, T cell specific early (CD69) and late (CD25) activation markers were detected flow cytometry based assay, while gating only CD3(purity approximately 96% achieved) positive cells (Figure 25A, B). Dot plot analysis showed that the percent positive cells of early activation marker (CD69) was 6.39±0.62 (mean±SEM) for mock-MΦ+T cell, which was increased up to 11.78±0.84 for CHIKV-MΦ+T cell. Similarly, percent positive cells for late activation marker (CD25) was found to be 6.39±0.62 for mock-MΦ+T cell, which was amplified significantly up to 15.84±1.15 in CHIKV-MΦ+T cell. Further the MFI of both CD69 and CD25 in the T cells were also found to be increased by the CHIKV infected peritoneal macrophages as compared to the mock cells (Figure 25C, D, E). This data indicates that CHIKV infected macrophages induces both early (CD69) and late (CD25) T cell activation markers in a co-culture *in vitro* set-up.

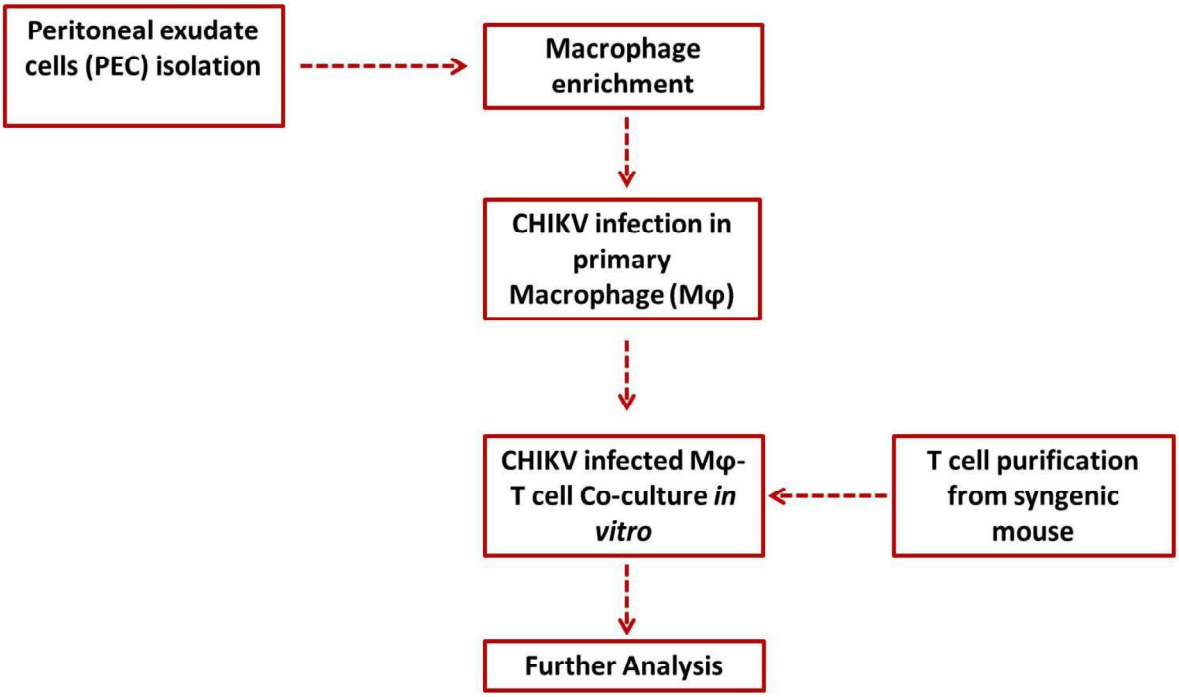


Figure 23. Schematic representation showing different steps followed for the mouse peritoneal macrophages and purified syngeneic T cell co-culture *in vitro*.

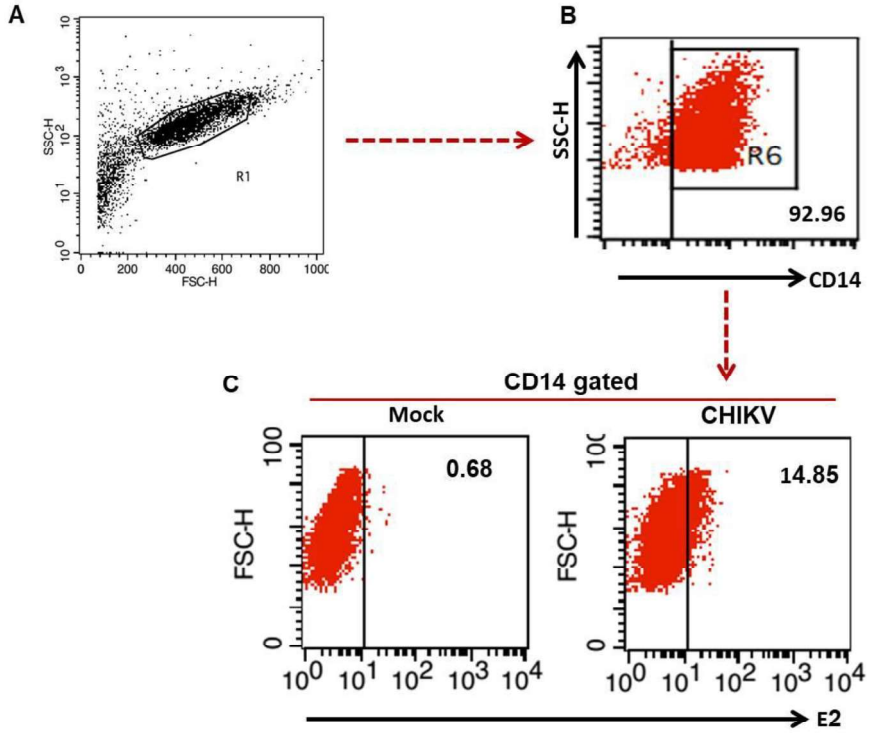


Figure 24. Susceptibility of mouse peritoneal macrophages to the CHIKV infection. Mouse peritoneal macrophages were isolated and enriched from the peritoneal execute cells (PEC),

4.5. CHIKV infection in mouse primary peritoneal macrophage activates T cell immune response *in vitro*

Since CHIKV infection in mouse macrophage induced the surface expression of MHCs and co-stimulatory molecules, we further checked the effect on T cell activation *in vitro*. The schematic representation of the CHIKV infected macrophages and T cell co-culture at a ratio of 1:5 (macrophage: T cell) was mentioned in the Figure 23. Briefly, mouse peritoneal macrophage (MΦ) cells cultured in 24 well plates were infected with CHIKV at MOI 05. Cells were washed and incubated with syngeneic purified T cells for further analysis. After 48 hours peritoneal macrophages were harvested and processed for dual staining, CS followed by ICS (as mentioned in the materials and methods section) to detect percent positive cells for CHIKV. Before doing co-culture experiments, the macrophages from the PEC ware enriched by using adherent property of the peritoneal macrophages. The macrophage/monocyte mainly expresses specific marker such as CD14 in both normal and activated stages, so CD14 was used to assess the purity of peritoneal macrophages from the whole PEC. It was found that approximately 93% PEC showing positive for CD14, which was used for the co-culture experiments with the syngeneic purified T cells (Figure 24A, B).

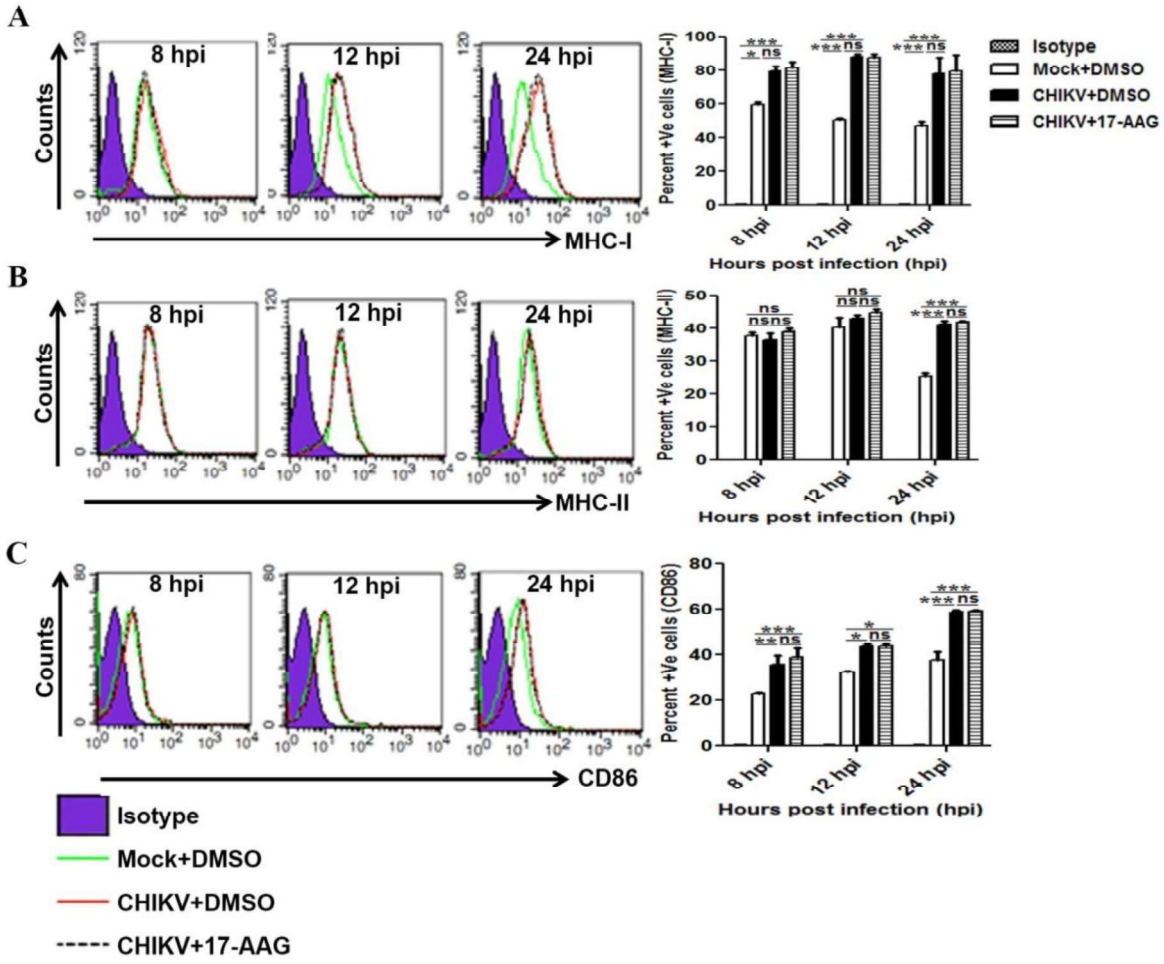


Figure 22. Expression level of MHC and CD86 in CHIKV infected macrophages after 17-AAG treatment. CHIKV infected Raw cells were harvested at different time intervals followed by FC based analysis: **(A)** MFI (left) and percent positive cells (right) of MHC-I expression; **(B)** MFI (left) and percent positive cells (right) of MHC-II expression; and **(C)** MFI (left) and percent positive cells (right) of CD86 expression at 8, 12 and 24 hpi. Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) [243].

for mock + DMSO, CHIKV + DMSO and CHIKV + 17-AAG at 8, 12 and 24 hpi. **(B)** Western blot analysis showing the expression of the cleaved caspase-3, E2 and GAPDH in the presence of 17-AAG during CHIKV infection in macrophage at 12 hpi (left). Bar diagram showing relative band intensity of cleaved caspase-3 of mock + DMSO, CHIKV + DMSO and CHIKV + 17-AAG analyzed by the Quantity One 1-D analysis software (right). Cell culture supernatants were collected and were assessed for TNF **(C)**, IL-6 **(D)** and MCP-1 **(E)** secretion for mock + DMSO, CHIKV + DMSO and CHIKV + 17-AAG by sandwich ELISA at 8, 12 and 24 hpi. Data represent mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; ** p) [243].

Moreover, the modulation of MCP-1 production in CHIKV+17-AAG was found to be 641±7 pg/ml as compared to 1399±15 pg/ml in CHIKV+DMSO control at 24 hpi (Figure 21E). Although, significant upregulation of MCP-1 was observed only at 24 hpi, treatment of 17-AAG reduced MCP-1 production below basal levels at all the time points. Unlike pro-inflammatory cytokine and chemokine production, inductions of MHC-I, MHC-II and CD86 by CHIKV were not suppressed upon 17-AAG treatment in all the time points (Figure 22A, B, C). Together, the data indicate that 17-AAG may down regulate pro-inflammatory cytokine/chemokine production of host macrophages, without altering the induced immune activation markers like MHCs and CD86 during infection.

TNF level was 683±26 pg/ml and 838±18 pg/ml in CHIKV+DMSO at 12 and 24 hpi respectively, whereas, the levels were reduced to 304±12 pg/ml and 385±10 pg/ml in the presence of 17-AAG (Figure 21C). Similarly, the IL-6 levels were 85±6 pg/ml and 476±15 pg/ml in CHIKV+DMSO at 12 and 24 hpi respectively, whereas, the levels were reduced to 44±3 pg/ml and 98±2 pg/ml in the presence of 17-AAG (Figure 21D). The present data showed that TNF was reduced significantly (around 50%) upon 17-AAG treatment as compared to DMSO control. Similarly, the reduction of IL-6 after 17-AAG treatment was found to be around 50% at 8 and 12 hpi, whereas at 24 hpi it reached up to 80% as compared to the corresponding DMSO control.

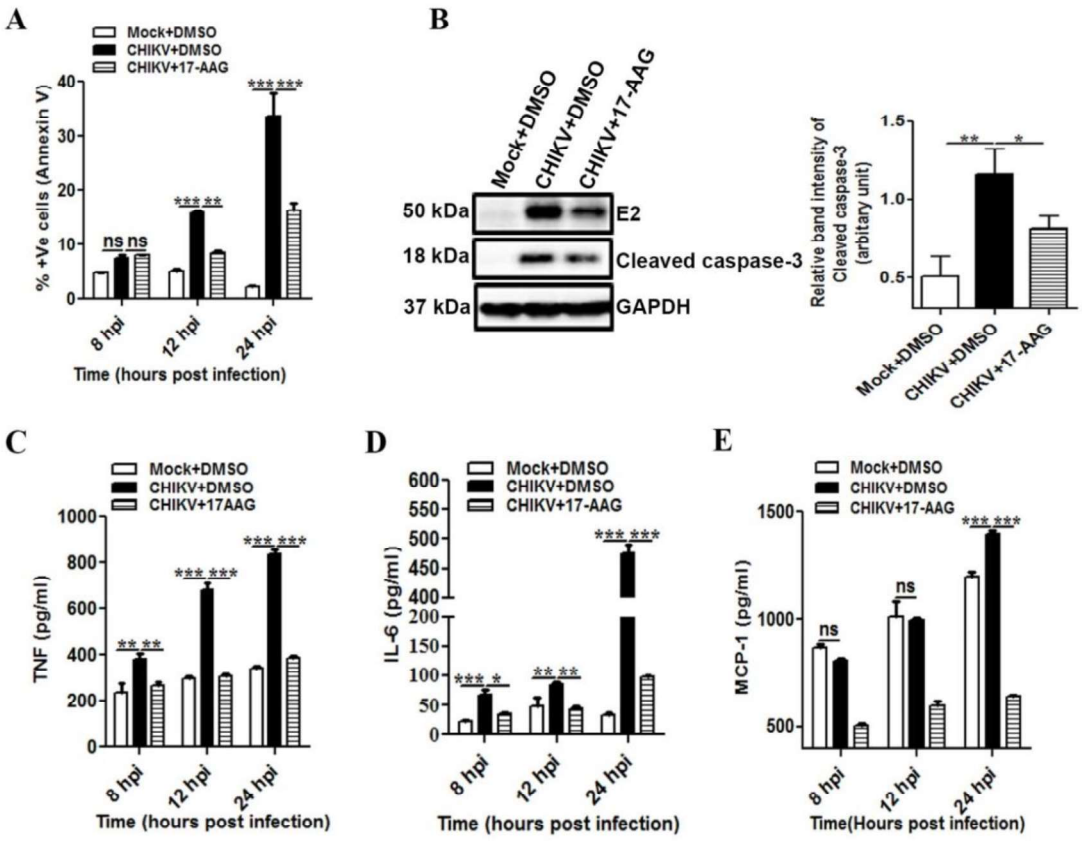


Figure 21. Suppression of CHIKV induced apoptosis and pro-inflammatory responses in macrophages by 17-AAG. Raw cells were infected with CHIKV at MOI 5. The cells were treated with either DMSO or 17-AAG as described earlier. **(A)** Percent Annexin V positive cells

(upper panel) and E2 (lower panel) in different samples at 8 hpi. **(D)** MFI representing expression of nsP2 and E2 in isotype (purple filled), mock + DMSO (green solid line), CHIKV + DMSO (red solid line) and CHIKV + 17AAG (black dashed line) at 8 hpi. **(E)** Graphical representation showing percent positive cells for nsP2 (left) and E2 (right) at 0.1 μM, 0.3 μM and 0.5 μM 17-AAG treatments at 8 hpi. **(F)** Agarose gel pictures showing RT-PCR of nsP2 and E2 (left) and bar diagram (right) showing reduction in the CHIKV RNA synthesis in Raw264.7 cells after 0.5 μM 17-AAG treatment at 8 hpi. **(G)** Representative Western blots showing HSP90 level for mock + DMSO, CHIKV + DMSO and CHIKV + 17-AAG at 8 hpi. Data represent mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) [243].

4.4.2. 17-AAG regulates host cell apoptosis and cellular immune responses during CHIKV infection in macrophages

Since, apoptosis was detected by Annexin V binding during CHIKV infection (Figure 15), thus experiments were performed to test whether 17-AAG can regulate CHIKV induced apoptosis and cellular immune responses in macrophages. It was observed that the Annexin V positive cells were 33.68±4.15% after CHIKV infection, which was reduced to 18.43±1.52% with 17-AAG ($p<0.05$) at 24 hpi (Figure 21A). Furthermore, quantitative Western blot analysis showed that CHIKV induced cleaved caspase-3 upregulation was found to be reduced by around 30% with 17-AAG treatment as compared to the DMSO control at 12 hpi (Figure 21B). Together, it appears that 17-AAG treatment may regulate CHIKV induced apoptosis of host macrophage.

Since, pro-inflammatory responses such as IL-6, TNF and MCP-1 were significantly induced in CHIKV infected macrophages (Figure 18), experiments were carried out to assess the efficacy of 17-AAG to suppress the cytokine and chemokine induction. The data showed that

affecting its level of expression. The Western blot analysis showed that expression of HSP90 protein remained unchanged in CHIKV+DMSO and CHIKV+17-AAG treated macrophages as compared to Mock+DMSO macrophages. This confirms that 17-AAG regulates CHIKV infection by abrogating HSP90 function without modulating it's expression level (Figure 20G).

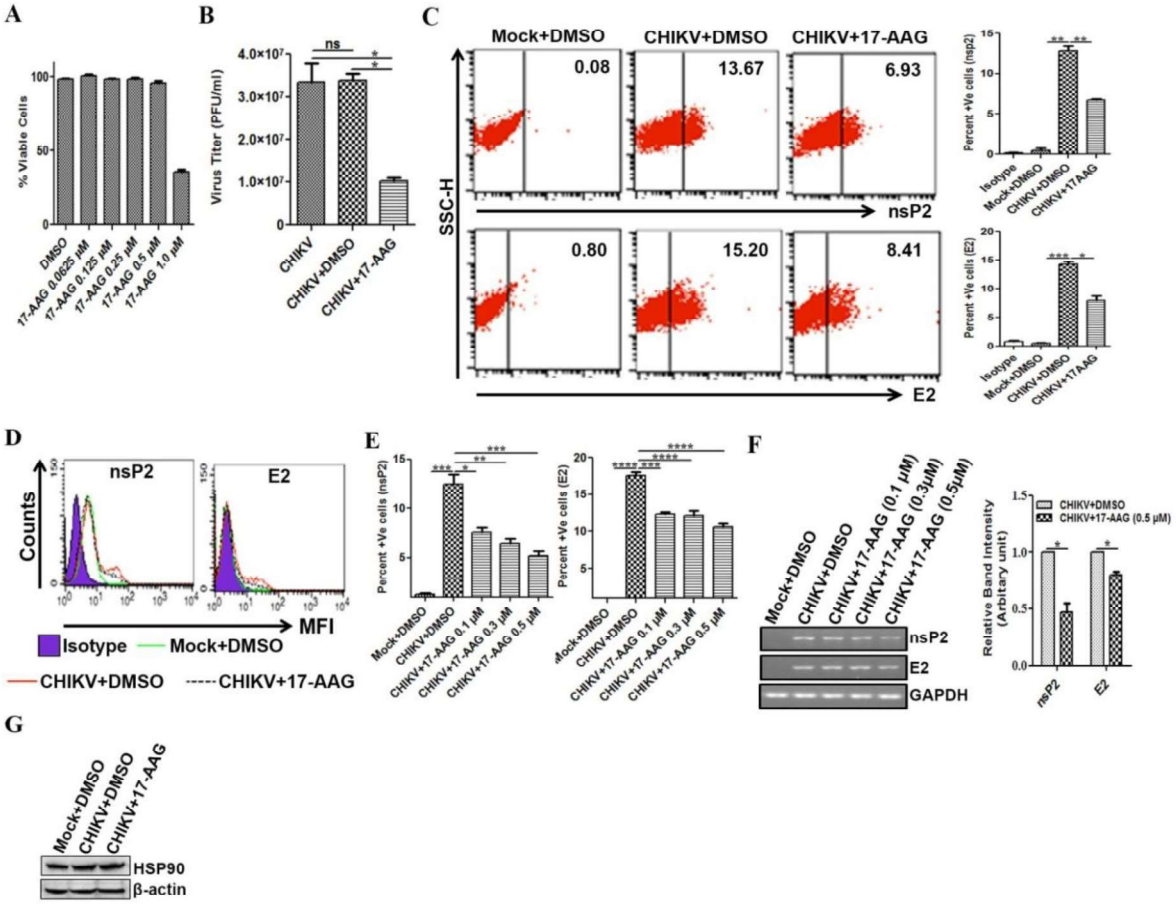


Figure 20. Modulation of CHIKV infection by 17-AAG in macrophages. Raw264.7 cells were infected with DRDE-06 strain of CHIKV at MOI 5. The cells were treated with either DMSO or 17-AAG as described earlier. **(A)** Percent viable cells treated with different concentrations of 17-AAG with respect to the solvent control (DMSO) as determined by MTT assay. **(B)** Bar diagram showing CHIKV titer (pfu/mL) in CHIKV, CHIKV + DMSO and CHIKV + 17-AAG (0.5 µM) at 12 hpi. **(C)** Representative dot plot analysis depicting percent positive cells for nsP2 and E2 at 8 hpi. Bar diagram showing percent positive cells for nsP2

The Raw264.7 cells were infected with DRDE-06 strain of CHIKV in the presence of either DMSO (solvent control) or 0.5 μM concentration of 17-AAG and the cells were maintained along with the drugs until harvesting. Then, the cells and supernatants were collected at 8, 12 and 24 hpi for different assays. Viral plaque assay was performed using supernatants of DMSO control and 17-AAG treated CHIKV infected macrophages to determine the number of newly synthesized infective virus particles. It was observed that the treatment of 17-AAG reduced the number of new viral progeny production by 3.3 fold ($p<0.05$) as compared to DMSO control (Figure 20B). Subsequently, the harvested macrophages were processed to estimate the nsP2 and E2 protein levels. The flow cytometry analysis showed that 17-AAG treatment (0.5 μM) was found to inhibit the level of both the viral proteins by 50% as compared to DMSO control (Figure 20C, D). Furthermore, 17-AAG dose kinetics were performed to show the efficacy of different concentrations of 17-AAG to suppress CHIKV infection in macrophages. Figure 20E depicts that 17-AAG inhibit nsP2 expression around 39% at 0.1 μM whereas it was further reduced to 58% with 0.5 μM of 17-AAG. In case of the E2 protein, the expression was reduced 30% at 0.1 μM, however it was further reduced to 40% with 0.5 μM of 17-AAG. This observation confirm that 17-AAG inhibit CHIKV specific protein synthesis and new viral progeny production in macrophages. Moreover, in this study we also explored whether 17-AAG treatment reduces the level of CHIKV RNA. It was observed that the RNA level was reduced by 2 fold ($p<0.05$) for nsP2 and 1.25 fold ($p<0.05$) for E2 in presence of 0.5 μM of 17-AAG (Figure 20F). This suggests that 17-AAG may reduce the production of viral progeny by inhibiting both the level of nsP2 and E2 proteins as well as RNA. The result also indicates that all the concentrations of 17-AAG were able to suppress the viral protein levels remarkably, whereas the RNA levels were reduced significantly only at 0.5 μM concentration.

Next, Raw264.7 cells were lysed to assess the level of HSP90 in CHIKV infected and 17-AAG treated macrophages to confirm that 17-AAG is a functional inhibitor of HSP90 without

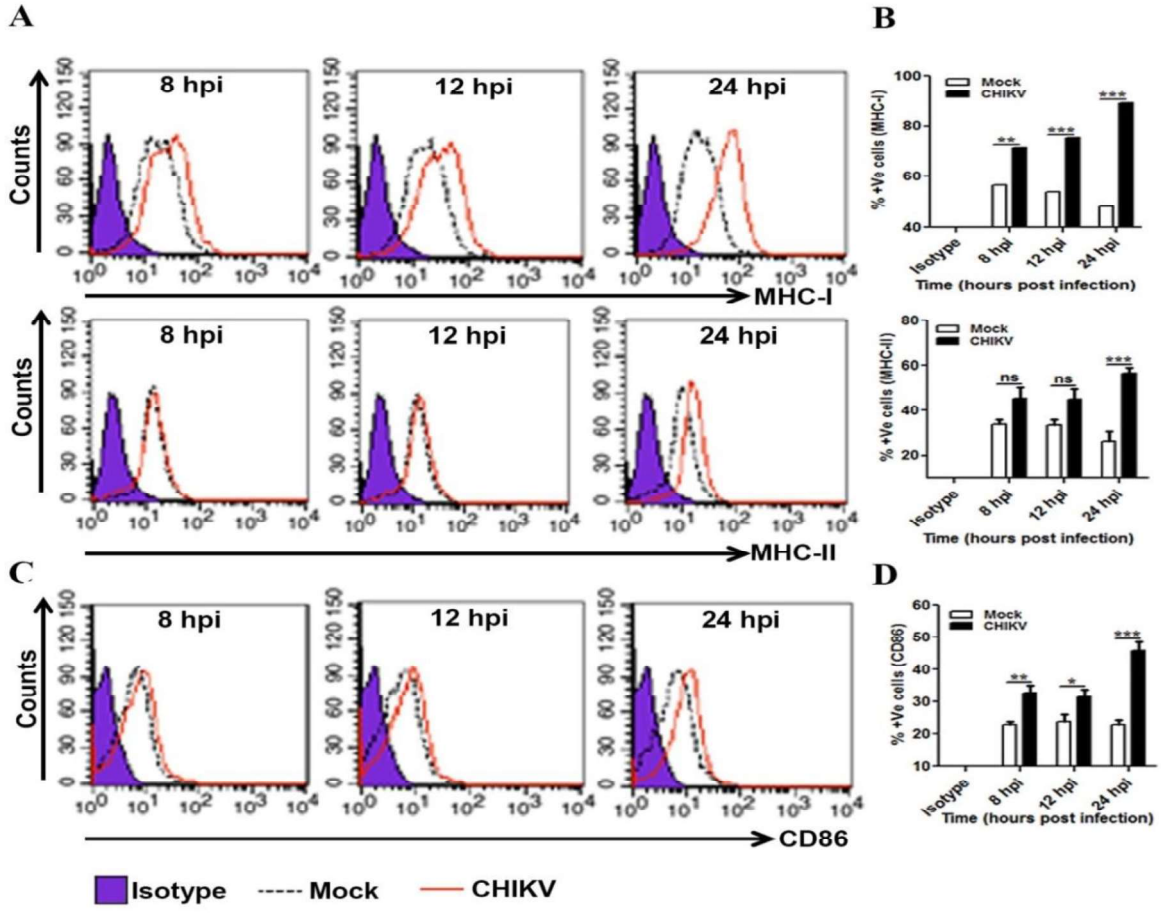
Figure 19. Expression pattern of MHC and co-stimulatory molecules during CHIKV infection in macrophages. CHIKV infected Raw264.7 cells were harvested at different time intervals followed by flow cytometry based analysis. **(A)** MFI representing MHC-I (upper panel) and MHC-II (lower panel) expressions in isotype (purple filled), mock (black dashed line) and CHIKV infected macrophage (red solid line). **(B)** Bar diagram showing percent positive cells for MHC-I (upper panel) and MHC-II (lower panel) in mock (white bar) and CHIKV infected samples (dark bar) at 8, 12 and 24 hpi. **(C)** MFI depicting CD86 expression in isotype (purple filled), mock (black dashed line) and CHIKV infected macrophage (red solid line). **(D)** Bar diagram showing percent positive cells for CD86 in mock (white bar) and CHIKV infected samples (dark bar) at 8, 12 and 24 hpi. Data represent Mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p \leq 0.05$; ** $p \leq 0$ [243].

4.4. Regulation of CHIKV infection and immune responses by 17-AAG in the host macrophages

4.4.1. Regulation of CHIKV infection by 17-AAG in macrophages

Recent study showed that CHIKV nsP2 is stabilized by HSP90 while interacting physically during active stage of CHIKV replication in Vero cells which was abrogated by HSP90 inhibitor, GA [239]. Herein, 17-AAG, a less toxic derivative of GA [257], was employed to study its effect on CHIKV infection in mouse macrophages, if any. The cytotoxic effects of different concentrations of 17-AAG (0.0265, 0.125, 0.25, 0.5 and 1.0 μM) were tested on Raw264.7 cells by MTT assay (as described in the methods section) for 24 hours. It was noticed that around 98% cells were viable up to 0.5 μM concentration of 17-AAG (Figure 20A). However, with 1μM of 17-AAG, approximately 50% cells were found to be viable. Hence, 0.5 μM concentration of 17-AAG was selected for further study.

expression was significantly up-regulated at 8, 12 and 24 hpi (Figure 19A, B). The percent positive cells for MHC class-I was found to be 71.58±2.88. (mock 56.67±0.8, $p<0.05$), 75.56±1.85 (mock 53.86±1.69 , $p<0.05$) and 89.21±9.32 (mock 48.11±4.77 , $p<0.05$), at 8, 12 and 24 hpi respectively. Unlike MHC-I, the surface expression of the MHC-II was increased significantly only at the later time point (24 hpi) and was found to be 56.46±3.20 (mock 26.28±2.9, $p<0.01$), after CHIKV infection (Figure 19A, B). Similar to MHC-I, the expression level of CD86 was found to be upregulated at different hpi (Figure 19C, D). The percent positive cells for CD86 was found to be 32.68±2.85 (mock 22.82±1.23, $p<0.01$), 31.59±2.53 (mock 23.88±2.58, $p<0.01$) and 45.79±3.26 (mock 22.74±1.92, $p<0.01$), at 8, 12 and 24 hpi respectively. This result indicates that CHIKV infection may significantly induce MHCs and co-stimulatory molecules in mouse macrophages.



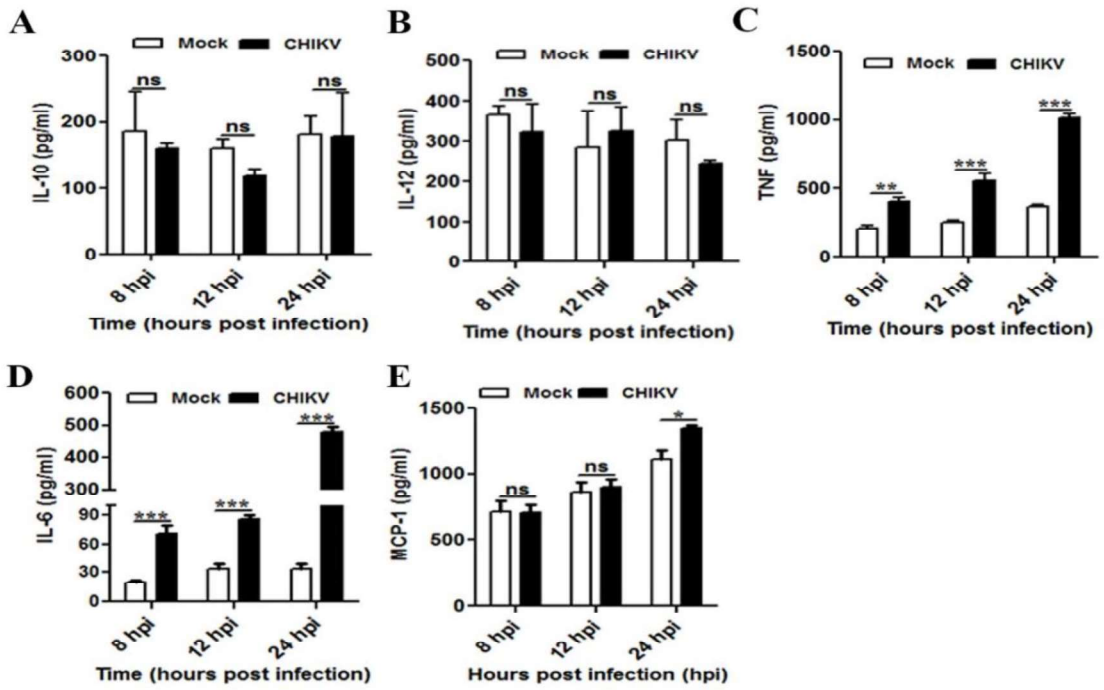


Figure 18. Modulation of macrophage derived cytokines/chemokines during CHIKV infection. Raw264.7 cells were infected with CHIKV at MOI 5. The cell culture supernatants were collected at different time points and the levels of secreted cytokines in the samples were quantified. Graphical representation showing the amount of secreted: IL-10 **(A)**, IL-12 **(B)**, TNF **(C)**, IL-6 **(D)** and MCP-1 **(E)** in mock and CHIKV infected macrophage as quantified using sandwich ELISA. Data represent mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; ** $p \leq 0.01$; *** $p \leq 0.001$) [243].

4.3.2. Modulation of MHC and co-stimulatory molecules in CHIKV infected macrophages

APCs like macrophages are known to show altered expression of MHCs and co-stimulatory molecules (e.g., B7 molecules) during pathogenic encounter including viral infection [145,254-256]. Flow cytometry based investigations were carried out to detect the surface expression of MHC-I, MHC-II and inducible co-stimulatory molecule, CD86 (B7.2) in the CHIKV infected macrophages at different time points. It was found that the MHC-I surface

mock in time dependent manner (Figure 18D). Production of IL-6 was upregulated as early as 8 hpi (mock 20±2 pg/ml, CHIKV 71±8 pg/ml, $p \leq 0.001$), 12 hpi (mock 34±5 pg/ml, CHIKV 86±5 pg/ml, $p \leq 0.001$) followed by peaked at 24 hpi (mock 34±6 pg/ml, CHIKV 480±17 pg/ml, $p \leq 0.001$).

The role of CC chemokine MCP-1 has been shown as an important mediator of inflammation in a variety of diseases, which recruit other immune cells to the site of impact to induce inflammatory responses [125]. Monocytes and macrophages are one of the major sources of MCP-1 production. Accordingly, we also assessed whether CHIKV infection induces MCP-1 production in macrophages. It was observed that there was no significant difference in MCP-1 production during CHIKV infection at early time points (8 and 12 hpi) as compared to mock (Figure 18E). However, CHIKV infection positively modulate MCP-1 secretion around 24 hpi (CHIKV 1354±19 pg/ml, $p<0.05$) as compared to mock (mock 1114±64 pg/ml). This result indicates that CHIKV infection in macrophages upregulated cytokines TNF, IL-6 and chemokine MCP-1 production over time, while no such significant changes were observed for IL-10 and IL-12.

CHIKV + DMSO and CHIKV + Z-VAD-FMK at 12 hpi **(E)** Raw cells were treated with 25 ng/mL concentration of Anisomycin for 3 h to use as a positive control for cleaved caspase-8 and -9 and Western blot was performed with the whole cell lysate along with the CHIKV infected cells. **(F)** Representative Western blot analysis showing expression of cleaved caspase-3, -9 and -8 in Mock + DMSO, CHIKV + DMSO and CHIKV + Z-VAD-FMK at 12 hpi (left), representative bar diagram depicting relative band intensities of cleaved caspase-3, -9 and -8 (right). Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) [243].

4.3. Innate immune modulation of host macrophages by CHIKV

4.3.1. CHIKV infection up-regulates pro-inflammatory responses in the macrophages

To determine the physiological and functional relevance of macrophages during viral infection, the mock and CHIKV infected cell culture supernatants were collected at different time post infection (8, 12, and 24 hpi) and further quantified for the secreted cytokines (e. g., IL-10, IL-12, TNF, and IL-6) and chemokine, macrophage chemoattractant protein-1 (MCP-1) using sandwich ELISA. We have found that there were no significant changes in both IL-10 and IL-12 cytokines in CHIKV infected cells compared to the mock infected in all the time points (Figure 18A, B). In contrast, the level of TNF was significantly upregulated in CHIKV infected macrophages (Figure 18C) and interestingly, it was observed that there was a time dependent increase in the level of TNF in the CHIKV infected cells as compared to the respective mock samples, 8 hpi 405±33 pg/ml (mock 211±20 pg/ml, $p \leq 0.01$), 12 hpi 555±51 pg/ml (mock 259±15 pg/ml, $p \leq 0.001$) and 24 hpi 1016±30 pg/ml (mock 367±15 pg/ml, $p \leq 0.001$). Similarly, the production of IL-6 was also increased during CHIKV infection in Raw264.7 cells as compared to

macrophages (Figure 15A, B), the effect of Z-VAD-FMK on Annexin V binding was also assessed. It was found that, Z-VAD-FMK reduced the Annexin V binding by 35% (Figure 17D) with 40 μM concentration. Moreover, the treatment of Z-VAD-FMK was found to reduce the inductions of the cleaved caspase-3, cleaved caspases-8 and cleaved caspase-9 (Anisomycin was used as a positive control foe cleaved caspases-9 and 8 [253] during CHIKV infection in the host macrophages (Figure 17E, F). Taken together, the result suggests that inhibition of apoptosis by Z-VAD-FMK significantly affects CHIKV infection in macrophages, without altering the level of virus protein expression.

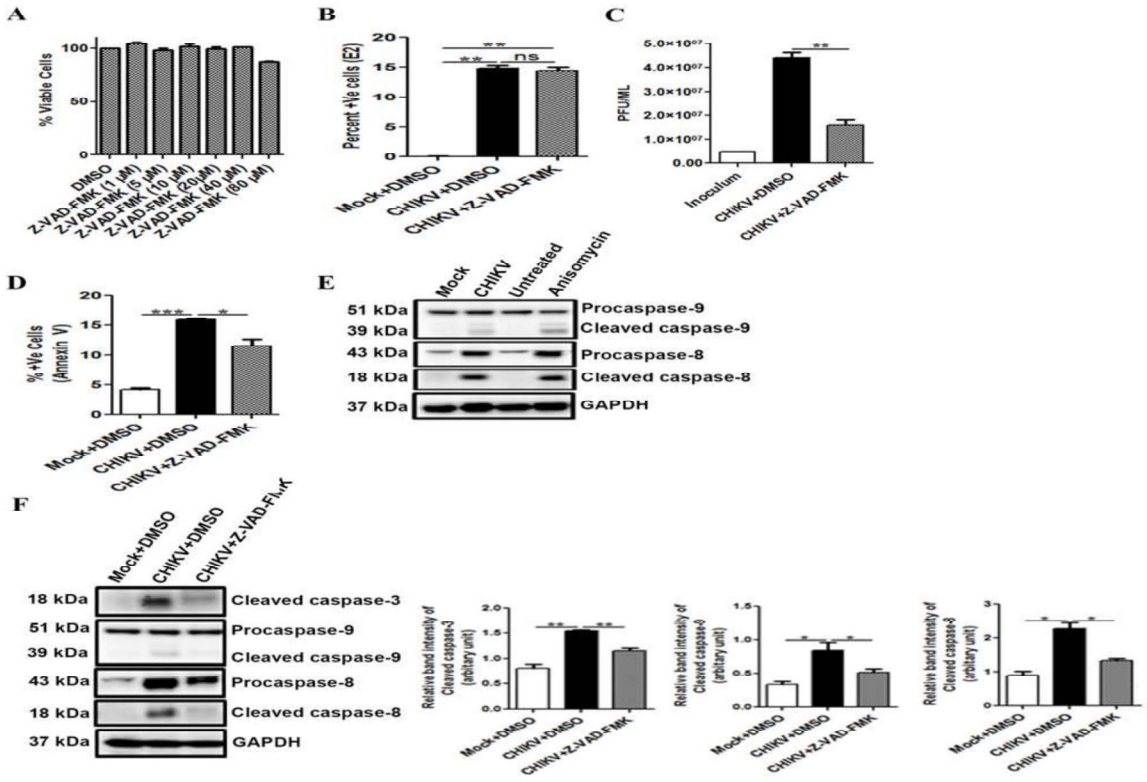


Figure 17. Effect of Z-VAD-FMK on CHIKV infection and apoptosis in macrophages. **(A)** Bar diagram showing percent viable cells in the presence of different concentrations of Z-VAD-FMK by MTT assay. **(B)** Cells were infected with DRDE-06 strain of CHIKV with MOI 5 and expression of E2 was assessed by FC analysis at 8 hpi. **(C)** Graphical representation showing virus titer (pfu/ml) in inoculum, CHIKV + DMSO and CHIKV + Z-VAD-FMK at 12 hpi in macrophages. **(D)** Bar diagram showing percent Annexin V positive cells in Mock + DMSO,

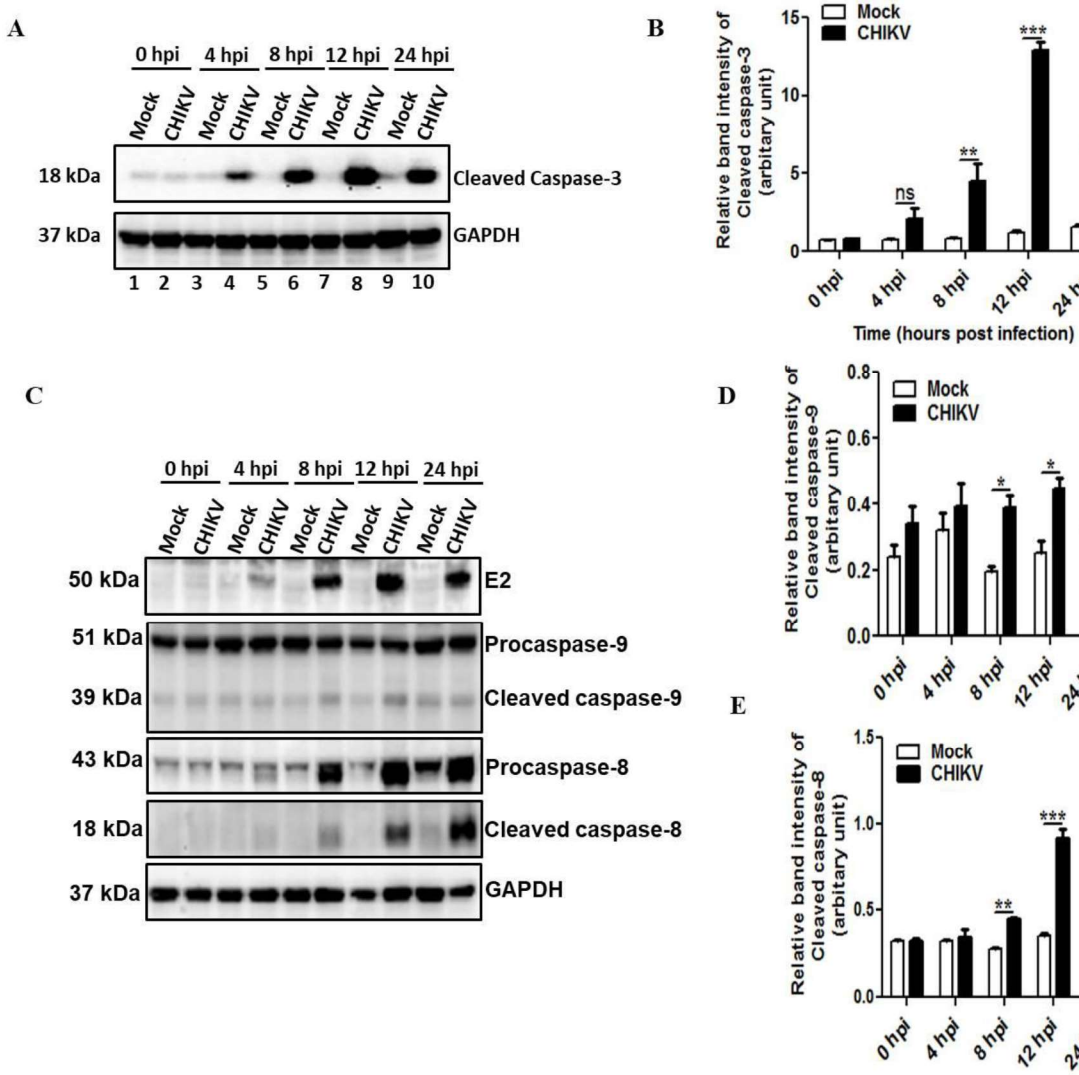
Figure 16. Induction of apoptosis by cleaved caspases during CHIKV infection in the host macrophages. CHIKV infected Raw264.7 cells were harvested, lysed and separated by SDS-PAGE. Western blot was performed and expression pattern of cleaved caspases were assessed. **(A)** Western blot analysis showing modulation in the cleaved caspases-3 expression at different time post infection. **(B)** Graphical representation depicting relative band intensity of cleaved caspases-3 with respect to the loading control. **(C)** Western blot analysis showing modulation in the cleaved caspases-9 and -8 expressions at different time post infection with bar diagram showing relative band intensity of cleaved caspases-9 **(D)** and cleaved caspases-8 **(E)** with respect to the loading control. Data represent mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; ** $p \leq 0.01$; *** $p \leq 0.001$) [243].

4.2.3. Inhibition of apoptosis by Z-VAD-FMK reduces CHIKV replication and cleaved caspases in the macrophages

Since CHIKV infection induces apoptosis in the host macrophages, investigation was carried out to assess the importance apoptosis by using a pan caspase inhibitor, Z-VAD-FMK. First, the cytotoxicity of Z-VAD-FMK was assessed by MTT assay with different concentrations for 24 hours. As shown in Figure 17A, around 100% and 90% cells were viable after 24 h with 40 µM and 80 µM concentrations of Z-VAD-FMK respectively. So, 40 µM concentration of Z-VAD-FMK was chosen for further experiments. The CHIKV infection was assessed in RAW264.7 cells in the presence of Z-VAD-FMK by flow cytometry based analysis. It was observed that, Z-VAD-FMK did not suppress the CHIKV E2 percent positive cells (Figure 17B). Surprisingly, treatment of Z-VAD-FMK did reduce the newly synthesized CHIKV progenies around 2.5 fold (Figure 17C). This suggests that Z-VAD-FMK might not restrict the CHIKV protein expression but it can reduce CHIKV infection by reducing the release of new virus particles from the host macrophages. Since, CHIKV induced phosphatidyl serine in the host

mock macrophages. Moreover, the expression of cleaved caspase-3 at 8 and 12 hpi in CHIKV infected cells were significantly higher than the corresponding mock (Figure 16A, B). Hence, our data suggest that CHIKV infection induces apoptosis in macrophages in a time dependent manner.

To investigate the in-depth pathway of cleaved caspase-3 mediated apoptosis by CHIKV in macrophages, expression of both cleaved caspase-9 and cleaved caspase-8 was assessed by Western blot analysis. It was observed that both cleaved caspase-9 and cleaved caspases-8 were induced during CHIKV infection in the macrophages in a time dependent manner (Figure 16C, D, E). This suggests that CHIKV might induces apoptosis in macrophages through both intrinsic and intrinsic pathways.



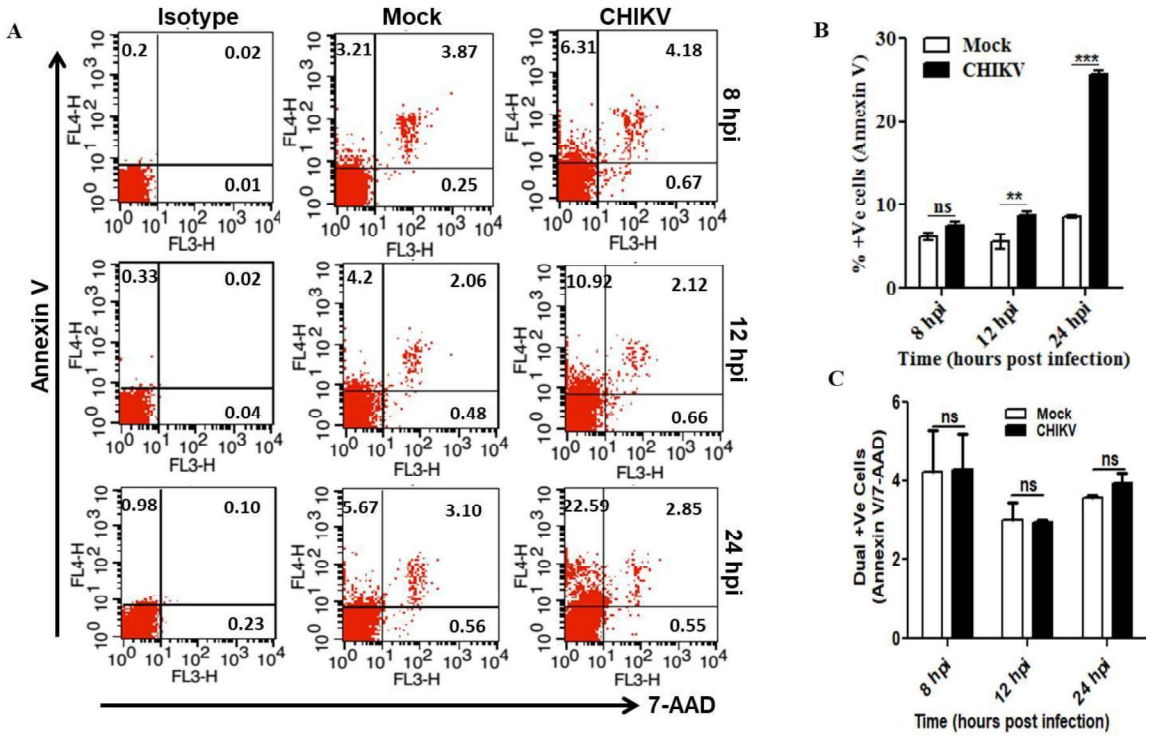


Figure 15. Detection of apoptosis during CHIKV infection in the macrophages by Annexin V and 7-AAD. Raw264.7 cells were infected with DRDE-06 strain of CHIKV at MOI 5 and cells were harvested at different time points and processed for Annexin V and 7-AAD staining. **(A)** Dot plot analysis depicting percent positive cells for AnnexinV/7-AAD for isotype (left panel), mock (middle panel) and CHIKV infected (right panel) macrophages at different time post infection. **(B)** Bar diagram showing Annexin V percent positive cells at 8, 12 and 24 hpi. **(C)** Graphical representation of percent dual (both Annexin V and 7-AAD) positive cells at 8, 12 and 24 hpi. Data represent mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; ** $p \leq 0.01$; *** $p \leq 0.001$) [243].

To further confirm the induction of apoptosis, infected cells were harvested at different time intervals (0, 4, 8, 12 and 24 hpi) and Western blot analysis was performed to observe apoptosis through executioner caspases cleaved caspase-3. Surprisingly, induction of cleaved caspase-3 was observed as early as 4 hpi in case of CHIKV infected samples as compared to the

different time points as described earlier. Bright field microscopic images taken at 8, 12 and 24 hpi with 20X magnification. White arrows indicate observed morphological changes in the infected cells [243].

4.2.2. CHIKV infection promotes host cell apoptosis in macrophages

In order to confirm apoptosis in macrophage cells during CHIKV infection, the mock and the infected cells were processed for Annexin V with 7-AAD staining at different hpi according to the protocol explained in the methods section. The flow cytometry based analysis depicted that a very small fraction of macrophage cells were found to be positive for Annexin V in both mock (6.2±0.5) and CHIKV (7.55±0.36, $p>0.05$) infected cells at 8 hpi (Figure 15A, B, upper panel). However, a significant increment in the frequency of Annexin V percent positive cells was observed at 12 hpi (Mock; 5.6±0.9 versus CHIKV; 8.67±0.49, $p<0.01$) and maximum at 24 hpi (Mock; 5.3±0.06 versus CHIKV; 17.7±0.05, $p<0.001$) (Figure 15A, B, middle and lower panel). Interestingly, both Annexin V/7-AAD dual positive cells were not found to be increased significantly ($p>0.05$) at different time post infection as compared to the corresponding mock cells, which confirms that CHIKV induces apoptotic marker, Annexin V without inducing necrotic cells in the host macrophages (Figure 15C).

as antigen presentation [250-252]. CHIKV infection has been recently reported to induce apoptosis in host epithelial cells [178,239,252]. However, apoptosis in host macrophages during CHIKV infection has not been studied yet. To assess whether mouse macrophages undergo apoptosis following CHIKV infection, the Raw274.7 cells were inoculated with CHIKV at MOI 05 and processed at different time intervals for subsequent analysis. The bright field microscopic images showed the development of cytopathic effect (CPE) at 8 hpi in CHIKV infected Raw cells (Figure 14). Furthermore, prominent tiny cell blebbings were observed at 12 hpi followed by few rounding and detachment of cells at 24 hpi (Figure 14, right bottom). However, no such morphological changes were observed in mock macrophages (Figure 14, upper panel). The observed characteristic features of the cells indicate that the cells might have undergone apoptotic process after CHIKV infection *in vitro*.

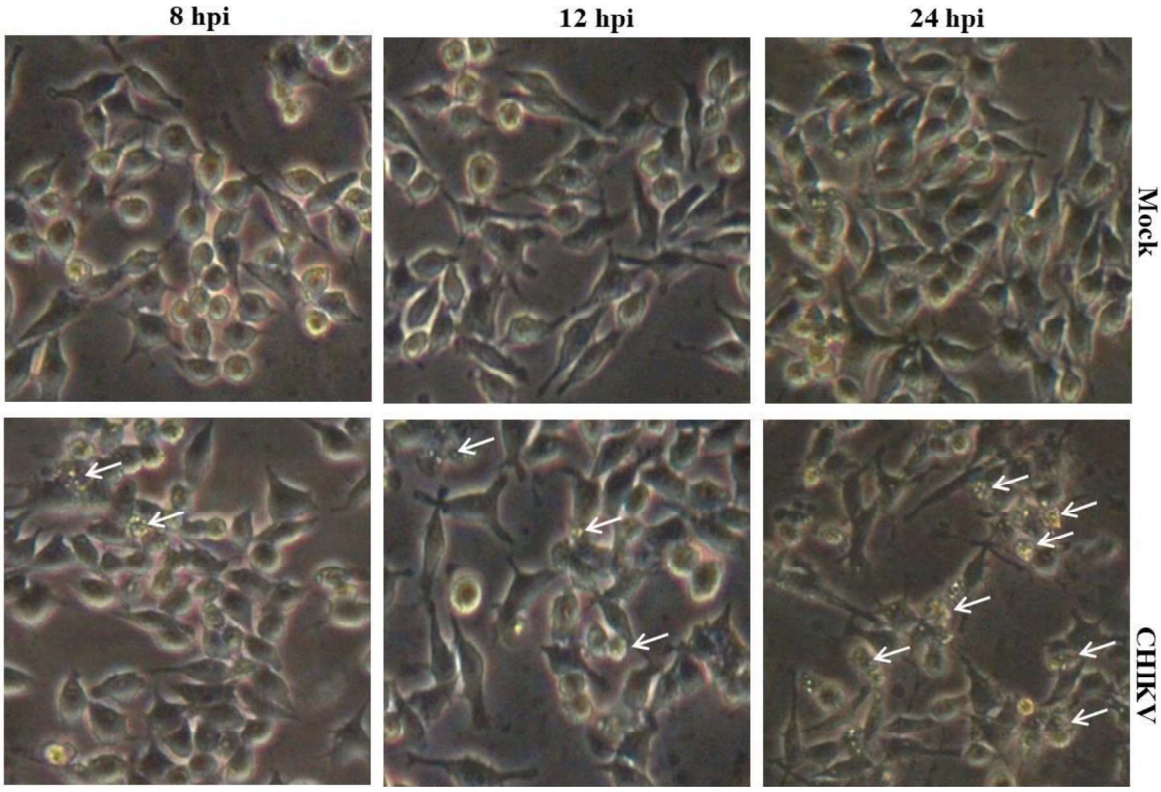


Figure 14. Induction of cytopathic effect (CPE) following CHIKV infection in macrophages. Raw264.7 cells were infected with DRDE-06 strain of CHIKV at MOI 5 for

of CHIKV could successfully infect and actively replicate in mouse macrophage cells *in vitro* and Raw264.7 cells are less permissible than Vero cells.

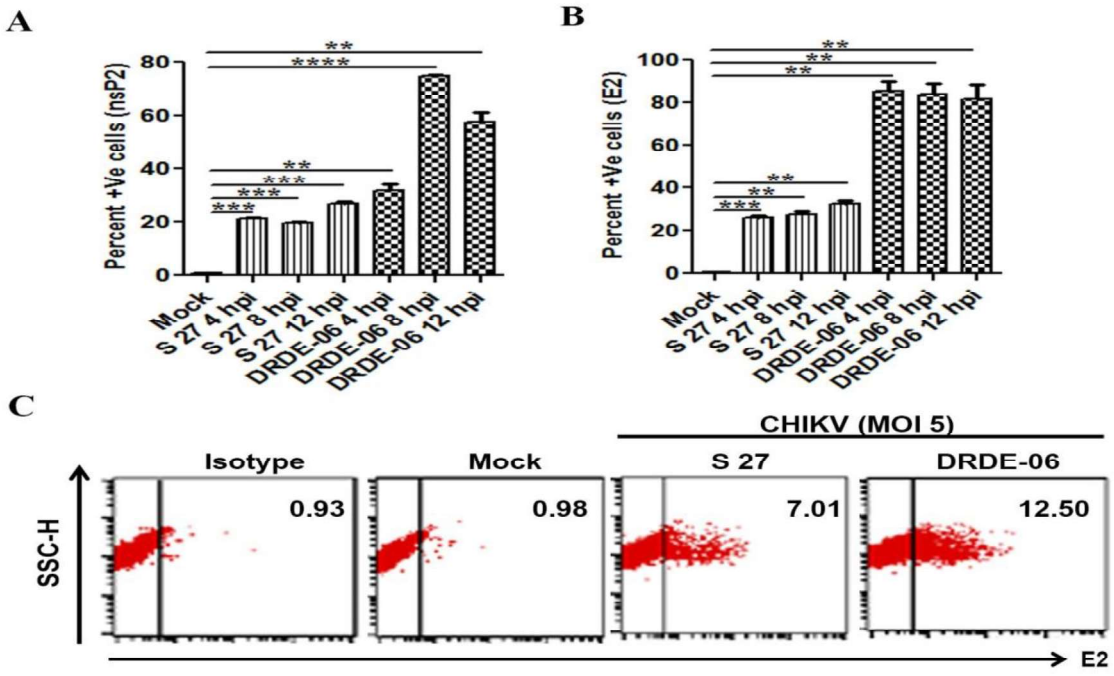


Figure 13. Infection pattern of S 27 and DRDE-06 strains of CHIKV in Vero and Raw cell lines. Vero cells were infected with both S 27 and DRDE-06 strains of CHIKV with MOI 5 and expression pattern of nsP2 **(A)** and E2 **(B)** were assessed by FC at 4, 8 and 12 hpi. **(C)** Raw264.7 cells were infected with both S 27 and DRDE-06 strains of CHIKV with MOI 5 and expression pattern of E2 was observed by FC analysis at 8 hpi. Data represent mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$) [243].

4.2. Induction of host cell Apoptosis during CHIKV infection in macrophages

4.2.1. CHIKV induces mild characteristic CPE in the host macrophages

Apoptosis of virus infected cell is known to bear one of the important consequences towards viral replication, dissemination of the virus particle to the neighboring host cells as well

Figure 12. Characterization of DRDE-06 strain of CHIKV in Raw264.7 cells at different MOIs. Raw264.7 cells were infected with DRDE-06 strain of CHIKV with different MOIs (0.1, 1, 5 and 10) and expression of E2 was assessed by flow cytometry based analysis at 8 hpi. **(A)** Representative dot plot analysis showing the expression (percent positive cells) of E2 protein with mock and CHIKV infected macrophages with 0.1, 1, 5 and 10 MOIs at 8 hpi. **(B)** Graphical representation showing percent positive cells of E2 with 0.1, 1, 5 and 10 MOIs at 8 hpi. **(C)** MFI representing expression of E2 in isotype (purple filled), mock (black dashed line) and CHIKV infected macrophages with different MOIs; 0.1 (blue solid line), 1 (yellow solid line), 5 (green solid line) and 10 (red solid line). Data represent mean ± SEM of at least three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups. (ns, non-significant; *** $p \leq 0.001$; **** $p \leq 0.0001$) [243].

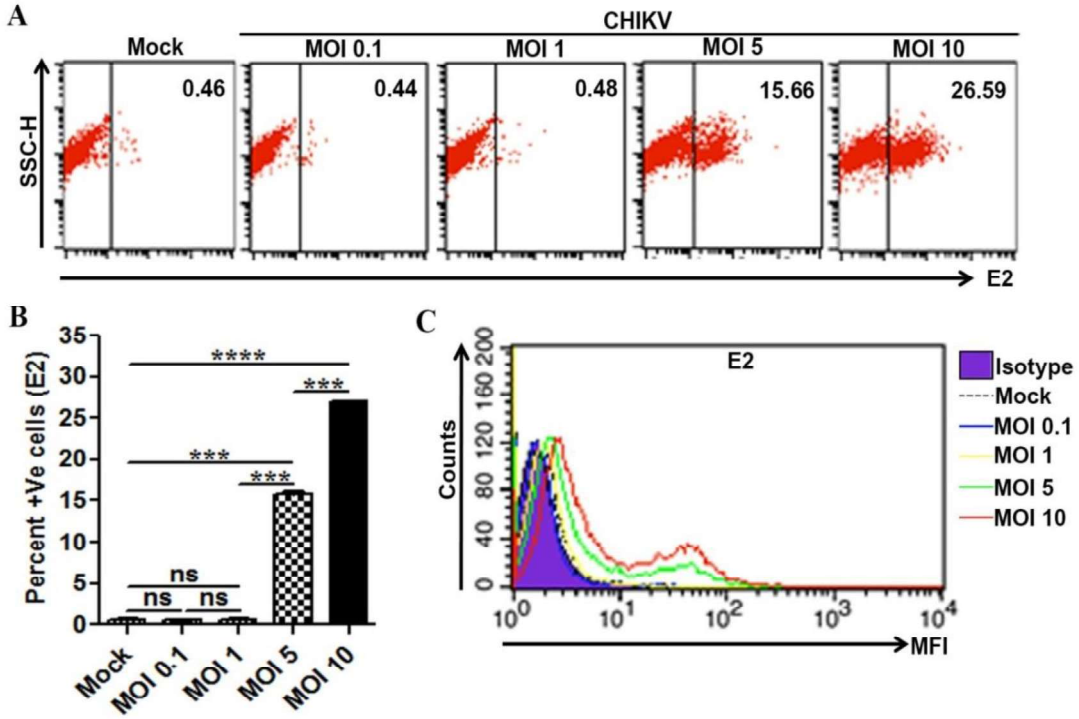
4.1.6. CHIKV infects macrophages in a strain independent manner

Generally, immune cells such as monocytes/macrophages, DCs, NK cells are resistant to viral infections as compared to other somatic cells, such as epithelial cells [178]. Thus, here the permissiveness of DRDE-06 strain was compared in Vero cell line. The expression pattern of CHIKV proteins was assessed by flow cytometry analysis. It was observed that both nsP2 and E2 were detected as early as 4 hpi (DRDE-06 nsP2: 31.79±2.30 and E2: 85.62±4.67), while the highest level of proteins were noticed at 8 hpi (DRDE-06 nsP2: 74.83±0.52 and E2: 83.84±5.39) followed by the gradual decrease at later time points (Figure 13A, B). Earlier it was reported that DRDE-06 strain of CHIKV replicates much earlier than S 27 strain in the Vero cell line [99]. Thus, the infectivity of the S 27 strain in the Raw264.7 cell line was tested, to demonstrate that the observation of CHIKV infection in Raw264.7 cells are not strain specific. It was observed that S 27 also infects and replicates in Raw264.7 cells with less infectivity (around 50% less) as compared to DRDE-6 strain (Figure 13C). Taken together, our data showed that different strains

infected macrophage cell culture supernatants collected at different time points. Data represent mean ± SEM of three independent experiments. $p < 0.05$ was considered as statistically significant difference between the groups [243].

4.1.5. CHIKV infects macrophages in a dose dependent manner

It has been reported previously that susceptibility of CHIKV infection varies among cell lines with different MOIs *in vitro* [178] so, the efficacy of the DRDE-06 strain of CHIKV to infect Raw264.7 cell was assessed at different MOIs (0.1, 1, 5 and 10) at 8 hpi. It was found that Raw264.7 cell was not susceptible to CHIKV infection at the MOI 0.1 and 1 *in vitro* as no significant E2 percent positive cells (0.47±0.02, mean±SEM, $p>0.05$) were detected with respect to the mock (0.61±0.13, mean±SEM, $p>0.05$). However, at the MOI 5 and 10, E2 positive cells was found to be around 15% (15.82±0.29, mean±SEM, $p<0.05$) and 30% (26.90±0.16, mean±SEM, $p<0.05$) respectively as compared to the corresponding mock cells (Figure 12A, B, C). Therefore, MOI 05 was used for rest of the experiments.



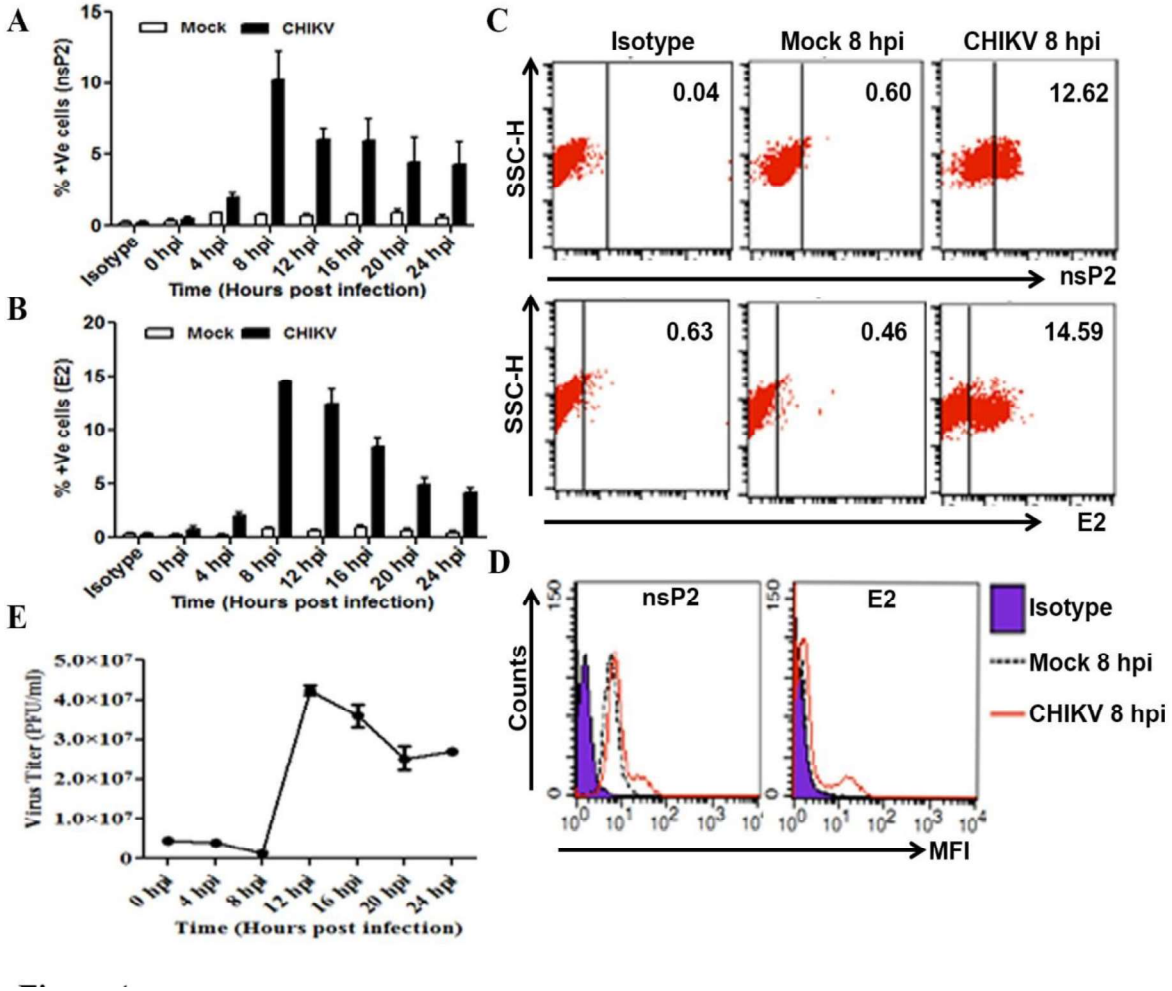


Figure 11. Chikungunya virus infection in host macrophages. The Raw264.7 cells were infected with CHIKV at MOI 5 for different time points as described in the materials and methods section. The infected cells were harvested from the 6 well plates and stained with respective antibodies against the viral proteins (nsP2 and E2) followed by flow cytometry based analysis. Representative bar diagram showing the time kinetics of percent positive cells for nsP2 **(A)** and E2 **(B)** in mock and CHIKV infected Raw cells at an interval of 4 hpi. **(C)** Representative dot plot analysis showing the expression of nsP2 (upper) and E2 (lower), along with isotype control (left), mock (middle) and CHIKV infected (right) macrophages at 8 hpi. **(D)** Mean fluorescence intensity (MFI) of nsP2 and E2 at 8 hpi representing the isotype (purple filled), mock (black dashed line) and CHIKV infected (red solid line) macrophage cells. **(E)** Line diagram of viral plaque forming units (PFU/mL), determined by plaque assay from CHIKV

4.1.4. Determination of CHIKV infection in macrophages

In order to establish CHIKV infection in host macrophages, Raw264.7 cells and CHIKV strain, DRDE-06 was used at MOI 05. The infected cells and the cell culture supernatants were harvested at different time post infection (an interval of 4 h from 0 to 24 hpi). The cells were fixed in 4% PFA as mentioned in the materials and methods section followed by processed further for intracellular staining to detect CHIKV specific proteins such as nsP2 and E2 at different time post infection. Next, the expression pattern of these two CHIKV proteins were assessed by flow cytometry and it was noticed that, both nsP2 and E2 were detected as early as 4 hpi (CHIKV; nsP2: 2.0±0.24 and E2: 2.05±0.22, Mock; nsP2: 0.88±0.06 and E2: 0.26±0.05), while the highest level of proteins were observed at 8 hpi (CHIKV; nsP2:10.19±2.04 and E2:14.47±0.17, Mock; nsP2: 0.77±0.09 and E2: 0.82±0.13) followed by the gradual decline towards the later time points (Figure 11A, B). Since, the nsP2 protein is synthesized during active replication stages of CHIKV infection [99], the current observation indicates that the CHIKV could infect and replicate actively in the host macrophages (Raw264.7 cells), with a peak of nsP2 level around 8 hpi (Figure 11C, D). Accordingly, Raw 264.7 cells were harvested only at 8 hpi to assess CHIKV infection for subsequent experiments.

Next, to determine the release of new infectious virus particles, viral plaque assay was performed using the supernatants collected at different time post infection from the above experiments. Almost no virus particle was found to be released at 8 hpi, while the viral titer increased significantly to $42.5x10^6 \pm 1.2x10^6$ pfu/ml at 12 hpi indicating active replication of CHIKV and release of newly synthesized virus particles (Figure 11E). Subsequently, the viral count went down slowly at 16, 20 and 24 hpi. Since, the supernatant was collected at every 4 h interval, the viral titer reflects the newly generated virus particles which were released in that particular time period. Taken together, our data shows that CHIKV could successfully infect and actively replicate in mouse macrophage cells *in vitro*.

developed mAb reacts only to the CHIKV nsP2 without any cross-reactivity with other host proteins. Further the specific reactivity of the newly purified mAb was evaluated by flow cytometry. The mock and CHIKV infected Vero cells were processed and analyzed for percent positive cells of nsP2 at 16-18 hpi. It was observed that around 7.75±1.89% of Vero cells were showing positive for nsP2 as compared to 0.57±0.15 ($p<0.05$) in corresponding mock (Figure 10A, B). Furthermore, there was positive shift in terms of MFI for nsP2 was also observed in CHIKV infected Vero cells as compared to the mock (Figure 10C). Taken together, it appears that the newly synthesized mAb against nsP2 was found to be very specific and can be used for the detection of nsP2 by Western blotting and immunofluorescence (IF) techniques.

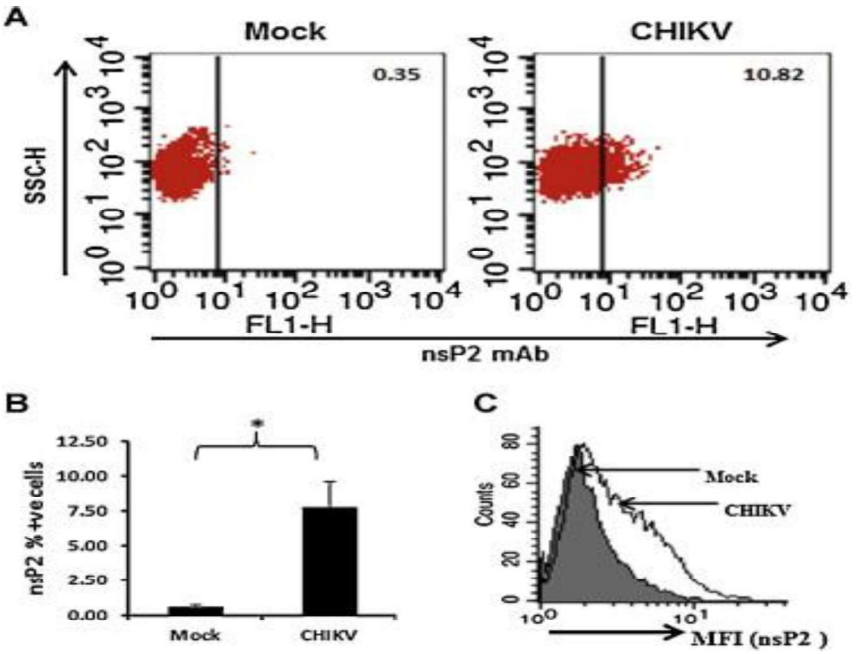


Figure 10. Detection of nsP2 in CHIKV infected Vero cell line by flow cytometry. (A) Dot plot analysis showing nsP2 percent positive cells at 16–18 hpi. Right panel of the dot plot depicting nsP2 percent positive cells during CHIKV infection (MOI 1) and left panel shows the mock uninfected cells along with SSC-H. **(B)** Graphical representation showing percent positive cells for nsP2 of at least three independent experiments. **(C)** MFI of nsP2 expression in the CHIKV infected Vero cell line at 16-18 hpi [242].

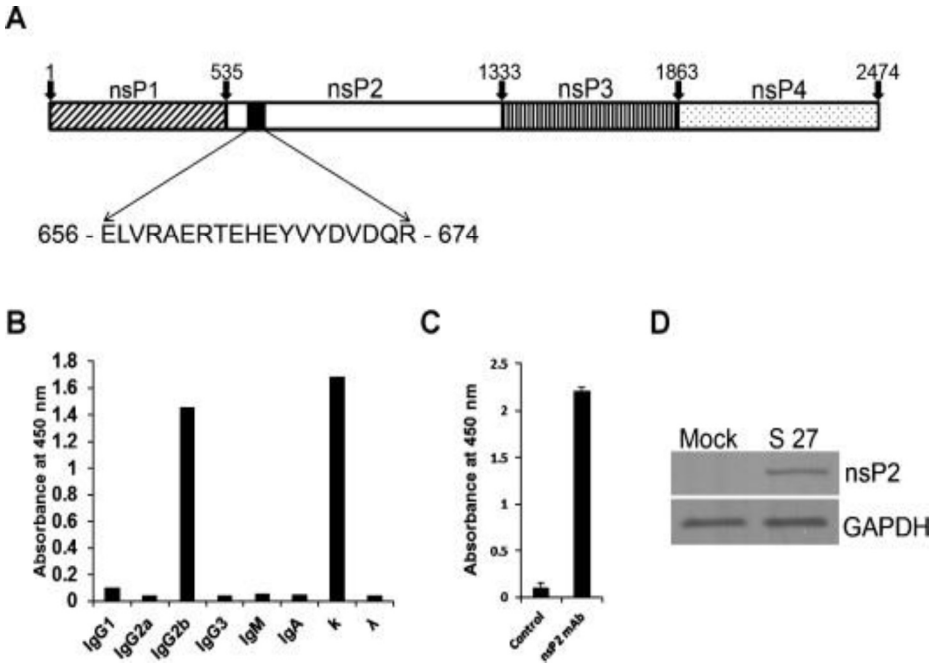


Figure 9. Generation and evaluation of CHIKV anti-nsP2 mAb. (A) Schematic representation showing non-structural polyprotein of CHIKV. The gradient bars showing nsP1, nsP3, nsP4 and the white band showing nsP2.The dark band in the nsP2 towards N-terminal region showing the peptide sequence from 656-676, against which mAb was developed. **(B)** Isotyping of 3F3.2E10 clone with the help of Pierce Rapid ELISA mouse mAb isotyping kit. **(C)** Determination of reactivity of anti-nsP2 mAb clone 3F3.2E10 hybridoma cell with the peptide by ELISA. Data are representative of at least three independent experiments. **(D)** Western blot analysis showing specific reactivity of nsP2 mAb to the CHIKV infected Vero whole cell lysate. GAPDH was used as loading control [242].

4.1.3. Evaluation and application of the mAb

The specificity of the nsP2 mAb was determined by Western blot analysis by using CHIKV infected Vero cell whole cell lysate at 18 hpi. The purified mAb showed strong reactivity only with the CHIKV infected cell (MOI 1) lysate as a single band was observed around 90 kDa position, which is the predicted size of nsP2. Moreover, no such bands were observed in the corresponding mock whole cell lysate (Figure 9D). This suggests that the

4.1. Establishment and characterization of Chikungunya virus infection in macrophages *in vitro*

4.1.1. CHIKV nsP2 epitope designing

The 19-mer amino acid sequence peptide from 656-674 of CHIKV nsP2 was chosen for raising mAb on the basis of hydrophobicity, hydrophilicity and antigenicity indexes are -1.129, 0.557 and 1.078 respectively (Figure 9A). The peptide homology was compared with several other CHIKV nsP2 sequences randomly chosen from GenBank database with different genotypes of virus strain isolated worldwide. The results indicated that the peptide was fully conserved with 100% sequence identity among all the CHIKV strains. Moreover, similar studies with other Alphavirus nsP2 sequence revealed that the sequence identities range from 68-31%. This suggests that the predicted epitope is very specific for different strains of CHIKV and may not cross react with the nsP2 of other Alphaviruses [242].

4.1.2. Monoclonal generation and characterization

By using the conventional hybridoma technology, different clones were screened for reactivity by ELISA against the predicted peptide. The clone 3F3.2E10 was selected on the basis of consistent higher ELISA OD value of 2.2±0.04 by using peptide (Figure 9B). Then the IgG heavy and light chain were determined by isotyping and it was found to be IgG2bk (Figure 9C). This clone was cultured and the resulting cell culture supernatant was purified by affinity chromatography for further use. The above procedure repeated for at least three times and the results suggest that the developed mAb against nsP2 binds very strongly with CHIKV peptide.

CHAPTER#4

Results and Discussion

1. **Nayak, T.K**.; Mamidi, P.; Kumar, A.; Singh, L.P.; Sahoo, S.S.; Chattopadhyay, S.; Chattopadhyay, S. Regulation of viral replication, apoptosis and pro-inflammatory responses by 17-aag during chikungunya virus infection in macrophages. *Viruses* 2017, *9*.

2. Chattopadhyay, S.; Kumar, A.; Mamidi, P.; **Nayak, T.K**.; Das, I.; Chhatai, J.; Basantray, I.; Bramha, U.; Maiti, P.K.; Singh, S.*, et al.* Development and characterization of monoclonal antibody against non-structural protein-2 of chikungunya virus and its application. *Journal of virological methods* 2014, *199*, 86-94.