‘Status of Siglecs in Chikungunya infection’

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by

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CERTIFICATE FROM THE SUPERVISORS

This is to certify that the thesis entitled "**Status of Siglecs in Chikungunya Infection**" submitted by Sri **Nilotpal Banerjee** who got his name registered on **28th August 2015** for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Sumi Mukhopadhyay** and **Prof. (Dr.) Bibhuti Saha** and that neither this thesis nor any part of it has been submitted for either any degree or diploma or any other academic award anywhere before.

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Declaration

I, Nilotpal Banerjee declare that the thesis entitled "**Status of Siglecs in Chikungunya Infection**" submitted by me is completely based on my own work under the supervision of **Dr. Sumi Mukhopadhyay** and **Prof. (Dr.) Bibhuti Saha**. Neither this thesis nor any part of it has been submitted for either any degree or diploma or any other academic award anywhere before.

Nilotpal Banerjee 17/06/2022

Nilotpal Banerjee

Index No. 150/15/Life Sc./24

Dedicated in the loving memory of my father

Advocate Nirmal Kumar Banerjee, B.Com., LL.B.

and

my motherly mentor

Prof. (Dr.) Indrani Bhattacharyya MBBS, MD (Microbiology)

ॐ सह नाववतु ।
सह नौ भुनक्तु ।
सह वीर्यं करवावहै ।
तेजस्वि नावधीतमस्तु मा विद्विषावहै ।
ॐ शान्तिः शान्तिः शान्तिः ॥

Om Saha Naav[au]-Avatu |
Saha Nau Bhunaktu |
Saha Viiryam Karavaavahai |
Tejasvi Naav[au]-Adhiitam-Astu Maa Vidvissaavahai |
Om Shaantih Shaantih Shaantih ||

Krishna YajurVeda, Taittiriya Upanishad (2.2.2)

Om, Together may we two Move (*in our Studies, the Teacher and the Student*), Together may we two Relish (*our Studies, the Teacher and the Student*), Together may we perform (*our Studies*) with Vigour (*with deep Concentration*), May what has been Studied by us be filled with the Brilliance (*of Understanding, leading to Knowledge*); May it Not give rise to Hostility (*due to lack of Understanding*),
Om Peace, Peace, Peace.

পরমব্রহ্ম আমাদের উভয়কে (আচার্য ও শিষ্যকে) একসঙ্গে পালন করুন। সমভাবে উভয়কে বিদ্যাফল ভোগ করান। সমভাবে দৈবশক্তিতে বলীয়ান হয়ে আমরা যেন সকল কর্ম সুষ্ঠুভাবে সম্পাদন করি। আমাদের অধ্যয়ন যেন দীপ্ত ও ফলপ্রসূ হয়। আমরা যেন পরস্পরকে বিদ্বেষ না করি। ওঁ শান্তিঃ শান্তিঃ শান্তিঃ ।।

এই বৈদিক প্রার্থনা ঘোষণা করছে শিক্ষার মহান নীতিসমূহকে; যেমন প্রেম ও সৌভ্রাতৃত্ব; পারস্পরিক সমঝোতা ও সহায়তা; শান্তি ও ঐক্য। উপনিষদের প্রতিটি পাঠ শিক্ষার পূর্বে ছাত্রকে গুরু উপদেশ দিতেন যে বিদ্যা হ'ল সমভাবে উপলব্ধি করবার বিষয়, তা করতে গিয়ে যদি শিক্ষক বা শিক্ষার্থীর মধ্যে ভুল বোঝাবুঝি হয় কিংবা সামান্যতম ক্রোধ বা অসহিষ্ণুতা দেখা দেয় তবে দাতা, গ্রহীতা ও দান - সমস্তই কলুষিত হয়ে যায়।
ওঁ ত্রিবিধ বিঘ্নের শান্তি হউক।।

‘गुरु बिन भव निधि तरइ न कोई।
जौ बिरंचि संकर सम होई।।’

Guru vina vaba nidhi taraha na koi |
Jo viranchi shankara sama hoi ||

Shri Ramacharitamanas by Goswami Tulasidasa

Without Guru’s guidance, no one can swim across the vast ocean of life.
One may have the power to create or destroy like Brahma or Shiva but to rise above one’s mind, feelings and beliefs; one needs a Guru to be directed in the right path.

ব্রহ্মা এবং শঙ্করের মতন ক্ষমতাবান হলেও, গুরুর কৃপা ছাড়া জীবনসাগর পাড়ি দেওয়া অসম্ভব।

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(*This is always incomplete...*)

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अनुकुलस्य सङ्कल्पः प्रतिकुलस्य वर्जनम्
राक्षसीति विश्वसो गोप्तर्त्वे वरणं तथा
आत्म-निक्सेप-कर्पणेसद्-विधा सरनागतिः

"ānukūlyasya saṅkalpaḥ prātikūlyasya varjanam
rakṣiṣyatīti viśvāso goptṛtve varaṇaṁ tathā
ātma-nikṣepa-kārpaṇye ṣaḍ-vidhā śaraṇāgatiḥ"

Sri Caitanya Caritamrita, Madhyalleela (22.100) by Krishnadaas Kaviraaj

The six divisions of surrender are:
1) To desire only in accordance with the desire of God.
2) Reject what is not in favor of devotion.
3) The faith that God will surely protect me.
4) To maintain an attitude of gratitude toward God.
5) To see everything we possess as belonging to God.
6) To give up the pride of having surrendered.

শরণাগতির ছয় লক্ষণ। ঈশ্বরেচ্ছাই পরম, এটি অনুভব। পরমতত্ত্ব লাভে যা বাধা, তা বর্জন। পরমাশক্তি সাধককে অবশ্যই রক্ষা করেন। সর্বদা পরমশক্তির প্রতিকৃতজ্ঞ থাকা ও কৃতজ্ঞচিত্তে তাঁকে স্মরণ করা। যা কিছু জ্ঞান ও অর্জন, তা সবই যে ঈশ্বরময় তা অনুভব করা। শরণাগতির অহংকারটুকুও ত্যাগ করা।

List of Abbreviations

CHIKV : Chikungunya Virus

DENV : Dengue Virus

SIGLEC : Sialic acid binding Immunoglobulin like lectins

ChikWoPP : Chikungunya Patients Without Persisting polyarthralgia

ChikWPP : Chikungunya Patients With Persisting polyarthralgia

WHO : World health Organization

ROS : Reactive Oxygen Species

EP : Envelope protein

ER : Endoplasmic reticulum

HLA : Human leukocyte antigen

TNF : Tumour necrosis factor

CRP : C-reactive protein

IL-6 : Interleukin-6

IFN : Interferon-gamma

TGF- β : Transforming growth factor- β

IL-10 : Interleukin-10

IL-12 : Interleukin-12

CXCL10 : C-X-C motif chemokine ligand-10

CXCL9 : C-X-C motif chemokine ligand-9

RANTES : Regulated on activation normal T cell expressed and secreted

OD : Optical density

NO : Nitric oxide

PBMC : Peripheral blood Mononuclear cells

WBC : White blood cell

RBC : Red blood cell

HB : Hemoglobin

HCT : Hematocrit

PLT : Platelet

PBS : Phosphate buffered saline

TCA : Trichloroacetic acid

ELISA : Enzyme-linked immunosorbent assay

SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophorosis

PAS : Periodic acid solution

FACS : Flourescence-activated cell sorting

DAB : Diaminobenzidine

DCFDA : 2'-7'-dichlorodihydrofluorescein diacetate

MDA : Malondialdehyde

SOD : Superoxide dismutase

TBA : Thiobarbituric acid

HD : Healthy Donor

SGOT : Serum Glutamic Oxaloacetic Transaminase

SGPT : Serum Glutamic Pyruvic Transaminase

IgM : Immunoglobulin

Tregs : Regulatory T cells

INDEX

CHAPTER FOUR

v) Results& Discussion

a) Basic pathological parameters of study subjects

b) Status of Siglecs on PBMC Surface

c) Status of Siglec Gene expression in PBMC

d) Status of Intracellular IFN-Gamma production

e) Status of CD4, CD8 cells

f) Status of Tregs

g) Status of Degranulation

h) Status of Intracellular ROS

i) Status of Inflammatory Cytokines

j) Status of Inflammatory Chemokines

k) Status of MDA

l) Status of SOD activity

m) Status of Nitric Oxide

n) Status of protein carbonyls

o) Status of oxygen radical scavenging activity

p) Status of DTNB activity

vi) Bibliography

vii) Publications

Chapter One

Introduction

Bengali Folklore and Chikungunya

Acute and Chronic manifestations of fever with joint pain, rash and restlessness are described in folklore of Bengal. Oral folklore was documented and compiled by famous Bengali writer Dakshinaranjan Mitra Majumder in his iconic book Thakumar Jhuli. Before the molecular and genetic establishment of Chikungunya as a distinct virus from Dengue,bone-breaking fever was the common name of Dengue and the same is documented by American soldiers travelled in South Asia and Africa. Persisting Polyarthralgia is common in Chikungunya mainly which is the actual bone-breaking fever. This bone breaking fever is documented in Thakumar Jhuli as 'HarrmurrmurrI Byaraam'. So, it was a common disease of Bengal which can be managed by spreading smoke regularly at the patients room, as described in the story. This use of smoke to treat the patient relates with mosquito repellent which eventually decreases the disease in the area. There was account of rash, fever and restlessness of patient, who is imitating the signs & symptoms of a patient suffering from the HarrmurrmurrI Byaram or the bone breaking fever. Small joint pain in the body extremities was described in elderly person after whole day workout, which is distinct from rheumatic involvements as there was no morning stiffness.

The figures are from the book Thakumar Jhuli published by MITRA & GHOSH PUBLISHERS, ISBN: 978-81-7293-045-5.

Chikungunya Virus is present in Human-Mosquito reservoir for long time as deduced by ancient history of fevers with acute joint pain, but finally discovered in Southern Tanzania and also named in their Makonde language which means “that which bends up”[1]. Unlike Dengue, 20% of Chikungunya patients suffer from recurrent polyarthralgia within a year of the infection. As the infection causes severe arthralgia, it has been reported that, 13 million Man hours along with USD 6 Billion have been lost by Chikungunya epidemic in Jamaica[1,165,182]. In India, the 2006-2009 outbreaks prove the debilitating potential of Chikungunya among Indian population. The four southern states along with Maharashtra suffered most during the epidemic. However, the Eastern states like West Bengal and Bihar were also affected notably [2, 3, 185].

Presently, there is no rapid, cheap, user friendly antigen based Chikungunya diagnostic system. Further, there is no chronic arthralgia predictive marker [171]. Considering the huge global burden of Chikungunya, it thus becomes imperative to identify novel biomarkers with diagnostic and prognostic potential [141].

Siglecs are Sialic acid (Sia) binding immunoglobulin like lectins, found on the surface of cells of the immune system [4]. These Siglecs are I (Ig) type lectins having an N terminal V set Ig like extracellular domain which binds with Sia [4]. Sialic acids are terminally expressed nine-carbon sugars widely expressed on cell surface [4]. Siglecs possess Immunoreceptor Tyrosine based inhibition motifs (ITIM) in their cytosolic tails which activates the immune system [4]. NK cell and Macrophage which are the key cells of innate immunity holds Siglecs on their surface which binds to pathogen or host cell membrane expressing Sialic acid.

When Siglecs bind with Sialylated glycoconjugates, they undertake several immunological functions including NK cell dysfunction [5,195]. As innate immune system is the first line of defense, which has evolved for many years to clear the invading pathogens, the modulation of innate immunity means a lot for the pathogen itself to proliferate within the host. So it is necessary to characterize the exact roles of Siglecs in microbial pathogenesis and also to make Siglec biomarkers for different microbial diseases [142].

Siglecs are found to have important roles in both bacterial and viral infection [6]. In HIV infection it has been reported that decreased expression of Siglec 7 leads to dysfunctional NK cells with high levels of HIV-1 viremia [7, 56, 184]. However, the status of Siglecs in Chikungunya infection is still not known. The current study aims to identify and characterize novel Siglec markers in Chikungunya infection as well as decipher their role in pathogenesis [132].

Siglecs are promising immune molecules and their characterization will certainly be beneficial in the field of Chikungunya immunodiagnostics [57].

Aims & Objectives

A] Characterization of Siglecs in patients with Chikungunya infection.

B] Study of Siglec-Sialic acid interaction in patients with Chikungunya infection.

C] Deciphering the biological roles of Siglec-Sialic acid interaction in Chikungunya infection.

Chapter TWO

Review of Literature

अथवा कृतवाग्द्वारे वंशेऽस्मिन्पूर्वसूरिभिः ।
मणौ वज्रसमुत्कीर्णे सूत्रस्येवास्ति मे गतिः ॥

athavā kṛtavāgdvāre vaṁśe'sminpūrvasūribhiḥ |
maṇau vajrasamutkīrṇe sūtrasyevāsti me gatiḥ ||

RaghuvaMsham (1-4), by Kalidaasa

Or else, my course in depicting this dynasty might as well be easy through the idiomatic gateway already crafted by the earlier poets, like a diamond boreholed by a diamond-edged tool for an easy passage of thread.

'Though dunce, I have a way in through the epics already rendered by vAlmIki, vyAsa et al, which will hold the thread of my narration tight, like the thread that easily moves in and out of the bored diamonds, tightly holding many diamonds...' whereby my work will not be sva kapola kalpitam... not concocted by me This indicates that the threads and strings of his narrations are drawn from the earlier poets, but they are not his whims or fancies, hence they are authentic.

সূত্র যেমন বজ্রবিদ্ধ রত্নের মধ্যে সহজেই প্রবেশ করে, আমিও তেমন পূর্বপণ্ডিতগণের নির্মিত বাক্যদ্বার দিয়ে এই বংশে প্রবেশ করব।

অর্থাৎ, আমার নিরাশ হবার কোনও কারণ নেই। বাল্মীকি, ব্যাস প্রমুখ পণ্ডিতগণ এই সূর্য্যবংশের বর্ণনা আগেই করে গেছেন। তাঁদের দেখানো পথ ধরেই আমিও আমার যাত্রা শুরু করছি। সুতো যেমন নিজে রত্নের ভিতর প্রবেশ করতে পারে না কিন্তু মণিবেধক বজ্রদ্বারা যখন সেই রত্নে ছিদ্র প্রস্তুত হয়, তখন দুর্বল সুতো অনায়াসেই সেই পথে প্রবেশ করে অনেক মণিগ্রন্থিত করে রত্নমালা প্রস্তুত করে। সেইরূপ আমার কাব্যরচনার পথও পূর্ব হতেই প্রস্তুত রয়েছে।

Viral Glycoproteins

The viruses that infect humans cause a huge global disease burden and produce immense challenge towards healthcare system. Glycoproteins are one of the major components of human pathogenic viruses[188]. They have been demonstrated to have important role(s) in infection and immunity [145,176]. Concomitantly high titres of antibodies against these antigenic viral glycoproteins have paved the way for development of novel diagnostics [60,61,181]. Availability of appropriate biomarkers is necessary for advance diagnosis of infectious diseases especially in case of outbreaks [175]. As human mobilization has increased manifold nowadays, dissemination of infectious agents became quicker that paves the need of rapid diagnostic system [58,59, 147, 151, 152]. In case of viral infection it is an emergency as virus spreads and mutates very fast.

Glycans are major components of the outermost surface of viruses. Thus, majority of the interactions of viral pathogens with their hosts are influenced by the pattern of glycans and glycan-binding receptors that each expresses. [8, 9, 10] Glycans are most complex biomolecules due to extensive branching of carbohydrates, and a variety of glycoproteins have been identified in human viral pathogens [146,187]. These pathogenic glycans either virus encoded or host derived usually elicit high humoral responses in human body [11]. These virus specific high levels of glycan specific antibodies have been exploited to develop novel diagnostic assays [163].

The Chikungunya Virus are known to produce 4 Non structural glycoproteins (nsP1-4) [12] these nsPs have been demonstrated to have important role in keeping the replicase

complex of the virus intact in the host as well as to circumvent important host immune responses. Chikungunya Virus has two envelope proteins, namely E1 and E2 [13]. Thus, viral glycoproteins have diverse structure and function. Taken together, glycoproteins are important components of the virus structure and each have unique role to establish pathogenesis. [14].

Viral glycoproteins have a definite role in their pathogenesis. The primary goal of viral infection is to identify a receptor on the host cell surface and binding with it [137]. Subsequently this will pave the way of viral entry into the host cell. In most cases, the first attachment site of the virus is a glycan, either a glycoprotein or a glycolipid [64]. So, glycoproteins play a crucial role in viral pathogenesis. The study of glycoproteins in viral infection is most important to know the disease process as well as to develop antiviral treatments [62,63].Glycoprotein–receptor interactions also play important roles in pathogen pattern recognition and in the regulatory signals that control the activities of cells of the immune system [170,189]. The most important cause behind viral infection is that it has evolved to present its own sugars and receptors in a manner that mimics or interferes with host glycan-based immune functions [65,164].

Siglecs

Sialic acids are abundant acidic sugars decorating the cell surfaces of vertebrates and their close relatives[66]. Siglecs are a family of sialic acid recognizing immunoglobulin-like receptors in mammals that mediate a variety of functions in different biological processes. The CD33-related Siglecs (CD33rSiglecs) are prominent on immune cells and play a role in distinguishing self and non-self by recognizing sialoglycans as “self-associated

molecular patterns."Some pathogenic viruses exploit this self-recognition system by molecular mimicry of ligands or by direct binding, thus averting detection and elimination by the innate immune system [67, 172]. Several enveloped viruses have been reported to interact with Siglecs, with overall end results in favour of the viruses.

Siglecs can be grouped into two subsets on the basis of their sequence similarity and evolutionary conservation: (i) Siglecs common to mammals, including sialoadhesin or Siglec 1, Siglec-2, -4 and -15 and (ii) the CD33-related Siglecs (CD33rSiglecs), most of which possess a cytoplasmic domain containing both a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) and a membrane-distal ITIM-like motif. Negative regulation of immune functions by Siglecs with ITIMs has been reported in the realms of cell expansion, cytokine production, cellular activation and induction of apoptosis [4]. Sialic acid can act as self-associated molecular patterns (Varki 2011), which are recognized by the inhibitory CD33rSiglecs and serve to maintain a baseline non-activated state of innate immune cells, and help to counter-regulate inflammatory responses activated upon sensing of danger-associated molecular patterns or pathogen-associated molecular patterns. Unlike most of Siglecs which have one or more ITIMs in their cytosolic tails, sialoadhesin or Siglec 1 lacks known signaling domains and possesses a unique extended 17 Ig-like extracellular domain structure. The extended extracellular length of sialoadhesin makes it standing out of the surface glycocalyx to prevent potential cis-ligand masking of its Sia-binding pocket [160]. Therefore, sialoadhesin is believed to mediate critical initial contacts with sialylated pathogens through direct phagocytosis/endocytosis, or in coordination with other pattern recognition receptors to promote efficient uptake or activate responses to counteract sialylated pathogen infection [55].

Siglecs are Sialic acid binding immunoglobulin like lectins expressed on hematopoietic cells. These are transmembrane proteins consisting of an extracellular N-terminal V-set immunoglobulin domain responsible for sialic acid recognition, followed by variable numbers (from 1 to 16) of C2-type Ig repeats. The V-set domain includes an essential arginine residue, which is essential for sialic acid binding. Most of the Siglecs contain combinations of tyrosine motifs, including one or more membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) and a membrane-distal ITIM-like motif containing Grb2-binding or Fyn kinase phosphorylation sites. These motifs are involved in inhibitory signal transduction through recruitment of SHP-1, SHP-2 tyrosine phosphatases as well as other Src-Homology 2 (SH2)-domain containing effector proteins [158].

Siglecs have established roles in infection and inflammatory diseases as evident from recent publications. Pathogenesis of infectious diseases like HIV and *Leishmania* are strongly dependent on Siglecs. Similarly, the manifestation of inflammatory disease like SLE, Rheumatoid arthritis [74], Systemic sclerosis is also mediated by Siglecs [68,69,112]. Siglecs are reported to control various intracellular pathways and the cell surface expression of those PRRs is also manipulated by different metabolic pathways [71, 72, 143]. Disease specific altered expression of Siglecs is well established.

A recent study shows differential metabolite expression with relation to another glycoprotein GM-CSF [55, 114, 139]. This cytokine is able to single handedly control the metabolomics profile in healthy donors. So, it can be apprehended that Siglecs can also have similar effects with the host intracellular environment [70,113,162].

It has been described that along with Toll like receptors, it plays crucial role in infection and inflammation. Toll-like receptors or TLRs are a type of protein that recognizes

patterns found in different types of pathogen, known as pathogen-associated molecular patterns or PAMPs [115]. Injured cells also release proteins that are also recognized by these toll-like receptors and are called danger associated molecular patterns or DAMPs. An immune response is triggered when PAMPs and DAMPs are recognized, but the response must be properly controlled [155,156]. If it goes uncontrolled, it can result in an over-activation of the immune cells that leads to production of neo antigens. Following Chikungunya infection, free radicals are generated within the host to combat the viral infection and leads to the generation of auto antibodies, cytokines, immune complexes, cytotoxic T lymphocytes [190]. As the virus migrates towards synovial joints, immune complexes trigger excessive ROS within the chondrocytes and osteoblasts and induce osteoclastogenesis [75]. Infiltrating CD 8 T cells further destroys cells and all these available free radicals further generates neoantigens and triggers an inflammatory cycle 'Immune complex-ROS-neoantigen-Immune complex' [73,79]. Those patients suffering with polyarthralgia possibly are more prone to produce higher amount of immune complexes [168]. Literatures suggest IgG1 and IgG2 present in those soluble immune complexes mediate inflammatory signals to the cells they attach [157]. However, it was unclear how Siglecs interact directly with toll-like receptors. Chen et al. show that most (although not all) Siglecs bind to TLRs, and that deleting the gene for a Siglec protein that can bind to multiple TLRs boosted the response of the immune cells to a range of microbial PAMPs. Deleting the gene for another Siglec that did not bind to any TLRs had no effect on the immune response [76].

Chen et al. suggest that the Siglec proteins that interact with toll-like receptors act a bit like a brake that slows down the activation of the receptors. However, when an immune cell detects a foreign molecule through a TLR, an enzyme called Neu1 is relocated from the inside of the cell to the cell's surface, where it removes the sugar molecules from the TLRs

[77]. This disrupts the interaction between the TLRs and the Siglecs, thus activating the receptors and triggering an immune response against the invading pathogen or damaged cells. This represents a newly discovered mechanism that can regulate the signaling of TLRs [78].

NK cells and Siglecs in Immunology

Human Natural Killer cells are categorized into two populations according to the degree of CD56 [neural cell adhesion molecule (NCAM)] surface expression, as well as expression of CD16, the FcγRIII. CD56dimCD16bright comprises nearly 90% of circulating Natural Killer cells, whereas CD56brightCD16−/dim constitutes the remaining 10%. Contrastingly, CD56bright Natural Killer cells predominate in lymph nodes and sites of inflammation. CD56bright Natural Killer cells secrete sample cytokines (as for example IFN-γ) and possess immuno-regulatory function , whereas CD56dim cells play an important role in natural and Antibody-mediated cell cytotoxicity. The detailed phylogeny as well as ontogeny of human Natural Killer cell subsets are poorly understood [202].

The principle functions of the Natural Killer (NK) cells are either in viral infection and/or tumour development in case of cancer, which are controlled by several activation and inhibition cellular receptors expressed over the surface of NK cells, and sialic acid-bind immunoglobulin-like lectins (Siglecs) work as very important inhibitory proteins which are expressed on the surface of these Natural Killer cells [203]. Several reports have shown that Siglec7 and Siglec9 are expressed on the surfaces of Natural Killer cells, and these proteins modulates the function of Natural Killer cells and controls the immune response by the interactions of Siglecs [204]. Siglec7 and Siglec9 are quite analogous in

distribution, ligand affinity, genetic makeup, protein composition and functions in controlling the mammalian immune-system against viruses and cancers. There are differences also between Siglec 7 and 9 [205].

Natural killer cells or NK cells are part of the mammalian innate immune system which can directly take part in killing the 'diseased-own' cells and those may be the so called Virus affected cells.As the MHC-I is down-regulated in viral infection, those cells can escape from the attack of T-cells [206]. Fortunately, Natural Killer cell mediated cytotoxic action does not dependent on specific antigenic stimulus and MHC-I molecules can not control them.

Several receptors like NKp30, NKp44, NKp46, CD16, NKG2C, and NKG2E, are key players of theNatural Killer cell mediated cyto-toxicity [207]. These actions include both activation and inhibition. The immune-harmony is very crucial for the interplay in viral infections [215].

Siglecs are those crucial receptors, which can control the signal transmission of Natural Killer cells in human body. Siglecs are very crucial cellular receptors of Sialic acid. They can support inter-cellular recognition and can also modulate the cytotoxic effects of Natural Killer cell in case of viral infection through the attachment with the Sialic acid residues present in the glyco-conjugates present over the target cells.

Here comes an evolutionary concept [207]. It has been reported that, Siglecs can broadly be classified among two different sub-groups. The evolution-conserved Siglecs include

sialoadhesin (Siglec1), CD22 (Siglec2), MAG (Siglec4), and Siglec15. Whereas, rapidly evolving Siglecs are CD33-related Siglecs. In human, CD33-related Siglecs are CD33 (Siglec3), Siglec5, Siglec6, Siglec7, Siglec8, Siglec9, Siglec10, Siglec11, Siglec14, and Siglec16 namely [208].

Among them, Siglec7 and Siglec9 are chiefly expressed on the surfaces of human immune cells. Siglec7 is found to be primarily expressed on the Natural Killer cells, particularly on $CD56^{bright}$ cells.

On the other hand, Siglec9 is present widely among all major mammalian leukocytes namely, the monocytes, neutrophils, B cells, Natural Killer cells, and in some minor sub-sets of T cells. It has been found that, in the human peripheral blood, the surface expression of Siglec9 is primarily on the neutrophils and the degree of expression decreases in NK cells, B cells, and monocytes accordingly. In some studies, Siglec9 has also been found on the tissue resident macrophages but significantly at low level [209].

Established roles of Siglec 7 and Siglec 9 in Viral infections

It has been reported that Siglec7 has active role in HIV-1 infection, which is reported as the changes in Siglec7 surface expression in Natural Killer cells but without altering the total number of Natural Killer cells in the bloodstream. Brunetta et al.'s study has been reported that the Siglec7 can be used as a biomarker for the disordered functional sub-sets of Natural Killer cells and HIV-1 infection, as they identified that the decreased number of Siglec7 positive Natural Killer cell sub-group has certain relation with the higher levels of

HIV-1 viral replication. They also reported that the down-regulation of Siglec7 over the surfaces of Natural Killer cells was because of the decreased magnitude of the Siglec7 positive Natural Killer cell sub-population and increased number of the Siglec7 negative Natural Killer cells, but interestingly, the overall number of Natural Killer cells were unchanged in peripheral blood [209]. However, relying on the studies of Siglec's endocytic activity, the same research group furnished another concept that Siglec7 may possibly attach to the HIV-1envelope glycoprotein 120 which can initiate the endocytic process of Siglec7.

Another study by Zulu et al.stated that the decreased expression of Siglec7 on Natural Killer cell surface in maximum HIV-1 samples obtained from healthy donors, chronic viral infection survivors, long-term non-progressor (LTNP), and early viremic HIV-1infected individuals [210]. This group also reported that Siglec7 expression on the $CD56^{dim}$ Natural Killer cells in patients suffering from viral infections have decreased in comparison with healthy donors. Interestingly, antiretroviral therapy reverses the surface expression of Siglec7. this has been proved by in-vitro studies also [211].

The expression of Siglec7 over the Natural Killer cell surface was thought to be disguised by virus, and the higher levels of serum sSiglec7 can be due to the secretion from the apoptotic Natural Killer cells [212].

The role of Siglec7 has also established in Hepatitis C and Hepatitis B viral infections. As similar to expression profile in case of HIV-1 patients, the manifestation of Siglec7 on Natural Killer cells were decreased in both Hepatitis C and Hepatitis B infected patients, however the serum sSiglec7 level increased in these cases. The serum sSiglec7 level has

good association with the HBV and HCV viral level and has negative correlation with the numbers of Siglec7 positive NK cells [213].

On the other hand, Siglec9 takes part in the Hepatitis B viral replication and is engaged in the dysfunctioning of the Natural Killer cells. A study reported the decreased expression of Siglec 9 on the natural Killer cells in HBV positive blood samples. And this down-regulation has negative correlation with viraemia [214].

If the Siglec 9 receptors are blocked in-vitro, there is a significant increase in the levels of cytokines like Interferron gamma and TNF alpha as well as CD 107a degranulation [209].

All these studies invoked the investigation of expressional differences along with functinal roles of variable Siglec 7 and Siglec 9 expression on Natural Killer cells in Chikungunya infection. This also suggested them as possible candidate biomarkers of the manifestation of Chikungunya.

Biological manifestation of altered Immune-regulation in Viral infection

Redox homoeostasis is an integral part of the maintenance of living organisms. Viral infection like Chikungunya manifests into small joint inflammation and a debilitating polyarthralgia affecting the life-style of the patient directly. The patho-physiology of this viral infection is not understood well and there is paucity of definitive therapeutics. The pathogenic function of free-radicals in joint inflammation and arthritis is well known [223].

The mediators of joint inflammation include immunological,genetic, biochemical and environmental factors which together evolve into joint inflammation and painful sensation. Different studies suggested that there is an immunological turmoil within the host due to any viral infection but there is no such report which directly studied the status of oxidative stress experimentally in chikungunya infection but it is known that this definitely plays a pivotal role in the manifestation of post viral infection arthralgia. Different studies proved that there is increased production of soluble immune-complexes or ICs along with pro-inflammatory cytokines, chemokines and auto-antibodies in the chikungunya patients. This is eventual that all these changes must alter the redox homeostasis of the patient [223]. So, it is inevitable to study the same in Chikungunya infection and the same was done in this study.

It is known that cartilage destruction is there in case of chikungunya infection and the level of oxidative stress in peripheral blood could correspond the level of the damages in patients. Host has the natural procedure to fight the altered oxidative stress and that have to study in depth. Siglec-9 manages the oxidative stress as per some previous studies [207]. This study that's why aimed to evaluate the magnitude of reactive oxygen species (ROS), different oxidative-stress markers along with the antioxidant potential and cytokines in peripheral blood to get the idea of oxidative-stress generated in the patient.

Oxidative Stress Markers

Malondialdehyde or MDA is a secondary outcome of lipid-peroxidation, whereas protein carbonyls are recognised marker of protein oxidation and have been shown to be more

vulnerable for cross-linking and cleavage due to instantaneous proteolysis. Protein-bound sulphhydryl groups or thiol groups are also very important in redox study [225].

Sulfhydryl groups are excitedby the leaving group 5-thio-2-nitrobenzoic acid that can be utilized to couple free-thiols by disulfide exchange. A TNB–thiol-activated species can be developed by the reaction of existing sulfhydryl group with the Ellman's reagent, that is 5,5′-dithio-*bis*(2-nitrobenzoic acid) or the DTNB, which is a compound helpful for the quantitative analysis of sulfhydryls in aquassolution [216]. The disulfide linkage of Ellman's reagent promptly undergoes disulfide transaction with a free-sulfhydryl group to create a mixed disulfide compound with incidental liberation of the chromogenic molecule 5-sulfido-2-nitrobenzoate or the 5-thio-2-nitrobenzoic acid that is TNB. The TNB–thiol group can now experience exchange with a sulfhydryl group bearing target molecule to produce a disulfide crosslink. After coupling with a sulfhydryl molecule, the TNB group is discharged. The deep yellow color developed by the TNB anion is measured by its absorbance at 412 nm (ε=1.36×104 M−1 cm−1 at pH 8.0). As the sulfhydryl group is coupled and generates single molecule of TNB per molecule of Ellman's reagent, it becames easier to enumerate Sulfhydryl group in peripheral blood and the same is executed in this study [216].

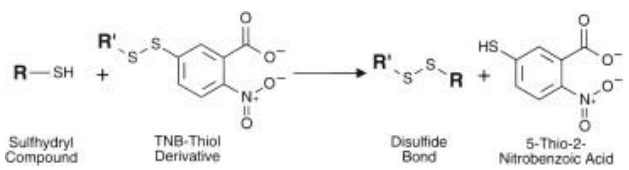


Figure 1: *Chemical reaction of Thiol Production*

Superoxide dimutase activity

Superoxide dismutase enzyme or SODs are general enzymes of aerobic living organisms. These catalyze the transition of superoxide ion into molecular oxygen and hydrogen peroxide. Superoxide anions are the product of cell signaling enzymes and also the by-product of different complex metabolic pathwayslike mitochondrial respiration. By its activity SOD enzymes control the amount of reactive oxygen species or ROS and reactive nitrogen species that is RNS, and eventually decreases the possible toxicity of these reactive compounds and modulates the cellular activity [217].

That is why MDA/SOD ratio has very importance according to its clinical aspect. It concludes higher oxidative damage in the patients. MDA level reports the destruction of lipid bilayer present in cells on the other hand SOD reports the amount of damage in the cartilaginous tissue. So the higher MDA/SOD ratio means that there is damage in the tissue and studied in chikungunya for the first time.

Chemokines and Cytokines

The complex interplays between cytokines and chemokines are emerging as key communication signals in the shaping of innate and adaptive immune responses against foreign pathogens, including viruses. In particular, the virus-induced expression of cytokine and chemokine profiles drives the recruitment and activation of immune effector cells to sites of tissue infection [218].

Interleukin 6 (IL-6) is a pleiotropic cytokine which is produced in response to viral infections and tissue injuries and takes part in the host defense by the activity of acute phase responses and hematopoiesis. Though its manifestation is rigorously harnessed by

transcriptional as well as post-transcriptional modifications, production of IL-6 plays a pathological effect on chronic inflammation as well as autoimmunity. It becomes one of the primary cytokine to be studied in chikungunya infection [219].

CXCL 9 and 10 chemokines are also reported to express in alternative manner in chikungunya. So it was inevitable to study the same in the patient population and to correlate with new possible markers with the established markers of inflammation [226].

RANTES : This chemokine is known to interact with the P-selectin protein in mediating the monocyte and or macrophage infiltration towards inflammatory areas and need to be studied in chikungunya infection also [227].

TGF beta: Due of its established role in immuneregulation, it is a highly studied cytokine in the fields of auto-immunity and infectious disease. So as a standard marker this also needs to be studied in chikungunya [225].

Some other oxidative stress markers like NO and quenching of DPPH radicals and protein carbonyls were also studied as standard ROS markers in this study.

This study incorporated for the first time to elucidate the status of different common oxidative-stress biomarkers and tried to study their correlation in chikungunya patients suffering with polyarthralgia by statistical analysis.

Finding possible source for ameliorating ROS in Chikungunya patients

As Chikungunya is a tropical disease, it was identified by molecular means in the twentieth century. But there was indication of arboviral fevers with debilitating polyarthralgia and skin rash in the folklore of Ancient and Medieval Bengal. So, this has been reviewed from existing literature and published as a popular science article in daily newspaper [220].

In South India, there were also similar incidences of arboviral fevers with join pain and the same was treated with extracts of *Tinospora cordifolia* leaf extract since ancient times in the 'Siddha' method of local medicine which exists in Kerala and Tamilnadu.

Similar practices has been observed by the author in rural Bangladesh to apply leaf extract of *Tinospora* which has been grown in Mango Tree (*Mangifera indica***)** to treat Chikungunya induced chronic polyarthralgia. The same has been documented also [221]. It is very interesting that Mango is rich in Vitamin C, which has established roles as antioxidant and a creeper like *Tinospora*, if grown on the mango tree, and then it definitely contains more Vitamin C, which is a potential antioxidant [222].

For this reason to indicate a therapeutic agent to decrease the debilitating effects of ROS in Chikungunya patients, *Tinospora cordifolia* was studied.

Free radical equilibrium is essential to maintain the living processes. Human immune-system involves the occurrence and action of thousands of cells within the body and create ROS in a regular manner [223]. Evolutionary modifications have proved improved physiologic pathways to ameliorate these free-radicals inside the human immune-system. It has been proved that some immunoglobulin-like (Ig-like) lectins are there which are found to be alter-expressed in managing production of exuberant ROS and interestingly, their manifestation associates well with the life-span of the organism [224].

Tinospora cordifolia is an herbaceous vascular plant of Menispermaceae family native to the Indian sub-continent. *Tinospora* is mentioned in the ancient texts like Ayurveda- the Indian system of medicine as Guduchi [223].

Gulancha is the Bangla name of the vine. In south-Indian terminology, this climber is mentioned as Amrita balli or the climber which gives immortality which indicates its medicative possibility [223].

This word Amrita; linguistically related to the Greek word Ambrosia and have the same interpretation. *Tinospora* is known as a supplier of alkaloids, lactones and glycosides with immuno-modulatory potential as described by Dr. R. N. Chopra from Calcutta School of Tropical Medicine in 1958 [223].

The ministry of AYUSH, Government of India declared that *Tinospor*a extract can be used for the treatment of arboviral fevers with joint pain or 'Sandhijwara' [223].

The anti-oxidant potential of *Tinospora cordifolia* leaf extract has recently been demonstrated in many studies [223]. But, till date, there is no scientific study explaining the potentiality of ameliorating the intra-cellular ROS by *Tinospora cordifolia* leaf extract on the living cells in ex-vivo condition. So, this study started investigating this to indicate any possibilities of using the same for therapeutic purpose.

Epidemiology, Demography and Disease burden

Viruses are dynamic players in the ecology of the planet. Worldwide, arthropod-borne virus infections are progressively more common causes of severe febrile disease that can

progress to long-term physical or cognitive impairment or result in early death [15,16,80].The concomitant ***co-circulation of Dengue, Chikungunya and Zika represents a major modern public health and biomedical challenge*** throughout the world [17,18,81,82]. There are more than 500 known arboviruses and more than 130 arboviruses among them are known to cause human disease, and are responsible for some of the most explosive epidemics of emerging infectious diseases over the past decade [19,186]. Tropical arboviral infections, like other Neglected Tropical Diseases (NTDs), occur in poor urban and rural environments and disproportionately affect low-income populations [20,83,177]. Like other NTDs, viral NTDs can lead to debilitating chronic sequelae that impact individual work productivity and family income. Although the neglected tropical viral diseases are part of poverty in tropical nations, their full impact is likely not to be determined in isolation, as arboviruses constitute a part of the larger health problem in resource-poor settings [140]. ***More research, more insight, and more discussions regarding these important NTDs and the populations that suffer from them will be necessary to fully define the impact of neglected tropical viruses in the regions where they persist*** [21,179].

Dengue, a *Flavivirus* and Chikungunya, an *Alphavirus*, transmitted by *Aedes* mosquitoes, are a cause of great concern to public health in India. Every year, thousands of individuals are affected and contribute to the burden of health care [22,84]. Disability adjusted life years (DALY) is an appropriate scientific measure of population health to express epidemiological burden of diseases [2]. In the 2006 epidemic of Chikungunya in India, 25,588 DALYs were lost with overall burden 45.26 DALYs per million which varies from state to state having a range of 0.01 to as high as 265.62 per million [2]. The productivity loss in terms of income due to Chikungunya associated joint pain was estimated Rs. 391

million in the year of 2006 and up to 1.98 million loss in DALY due to dengue in 2013 [23].

West Bengal, especially Kolkata has become an endemic region for Dengue and cases are happening throughout the year with surge of cases in post monsoon season [85]. As per the Dept of Health & Family Welfare, Government of West Bengal, there is almost 5.79 fold increased Dengue cases in West Bengal in 2016 as compared to that in 2014. NVBDCP reports about 3.81 fold higher dengue cases throughout India in 2016 with respect to 2014.Unfortunately, in West Bengal there is 8.42 fold increased number of Chikungunya patients in 2016 as compared to 2014. NVBDCP reports almost 3.99 fold increased Chikungunya infections throughout India [24]. As West Bengal is an endemic zone for Dengue and Chikungunya, addressing these neglected tropical diseases are extremely necessary for both Health services and Health research.

Challenges of Arboviral infections

Chikungunya and Dengue viruses belong to different families but share common mammalian host and arthropod vectors [134].Diagnosis of these two infections is difficult owing to overlapping clinical presentation leading to gross misdiagnosis and patient mismanagement [25,89,91]. Recent studies have shown that almost ten percent of suspected dengue cases are in fact co-infected with chikungunya virus making it imperative to distinguish the difference between the two infections for better patient management [153].

Importantly severe dengue cases if left untreated, carries a mortality rate of 20% that if properly managed can be reduced to less than 1% [26,90,178]. Thus, early recognition is crucial. Development of early diagnostic and prognostic marker for fatal Dengue is thus an

immediate need for the public health care system in India and other tropical countries[86,87].

Between 20% and 50% of all chikungunya -infected patients may develop chronic arthralgia [105]. To date there is no marker that could be used to predict chronic chikungunya infection. It is difficult to predict who will develop chronic disease and therefore finding biomarkers associated with disease is an important step in understanding pathogenesis and aiding the therapeutic decision-making to control the inflammation process early enough before any chronic arthritis occurs [27].Development of early biomarker for Chikungunya induced arthralgia appears yet another challenging area which needs immediate attention.

During the past 20 years many factors have converged to cause a dramatic resurgence or emergence of epidemic arboviral diseases affecting both humans and domestic animals [92,93,180]. Some of these factors include demographics, social changes, urban sprawl, changes in agricultural practices, genetic changes in pathogens and global climate changes. To successfully develop prophylactic and therapeutic interventions to lessen the toll on human and animal health, key interactions between these viruses, their invertebrate vectors and their vertebrate hosts must be understood [94]. Pathogenic viruses interface with a susceptible host at many points including viral entry, pathogen recognition by the host and engagement of effector molecules of the innate and adaptive immune systems [95,105]. Glycan components of enveloped viruses have been shown to facilitate many of these pathogen-host interactions, making viral glycan-mediated interactions rational targets for therapeutic intervention [106].

The interaction of virus with its host cell receptor is a critical factor in determining host and tissue tropism [104]. Since arboviruses transmit between arthropods and vertebrates in nature, these viruses must either adapt to receptors conserved in both hosts or diversify to interact with multiple receptors in different hosts [135ev]. Many arboviruses target dendritic cells (DCs) for viral replication after transmission from the mosquito vector [28,96]. In particular, immature DCs express a large variety of C-type Lectins *Receptors* (CLRs) which can function as antigen uptake receptors, including DC-SIGN and mannose receptor [97]. In addition to their role as antigen receptors, CLRs that facilitate productive infection by increasing the efficiency of virus binding, but whose presence is not absolutely required for viral entry, are often referred to as attachment factors [29,107]. DC-SIGN represents a common attachment factor for multiple viral pathogens, including HIV, Ebola, and arboviruses within the *Flaviviridae* and *Togaviridae* family [99,100,101,183].

West Bengal is an endemic zone for arboviral infections like Dengue and Chikungunya. During last three years there is a rapid rise in both Dengue & Chikungunya cases in this state especially in and around the metropolitan city of Kolkata [136,150]. Dengue has high mortality rate and Chikungunya induced polyarthralgia affects the life style and economy of both the patients and the State badly [102,104]. There is an immediate need for proper diagnostics against this debilitating joint pain post Chikungunya infection.

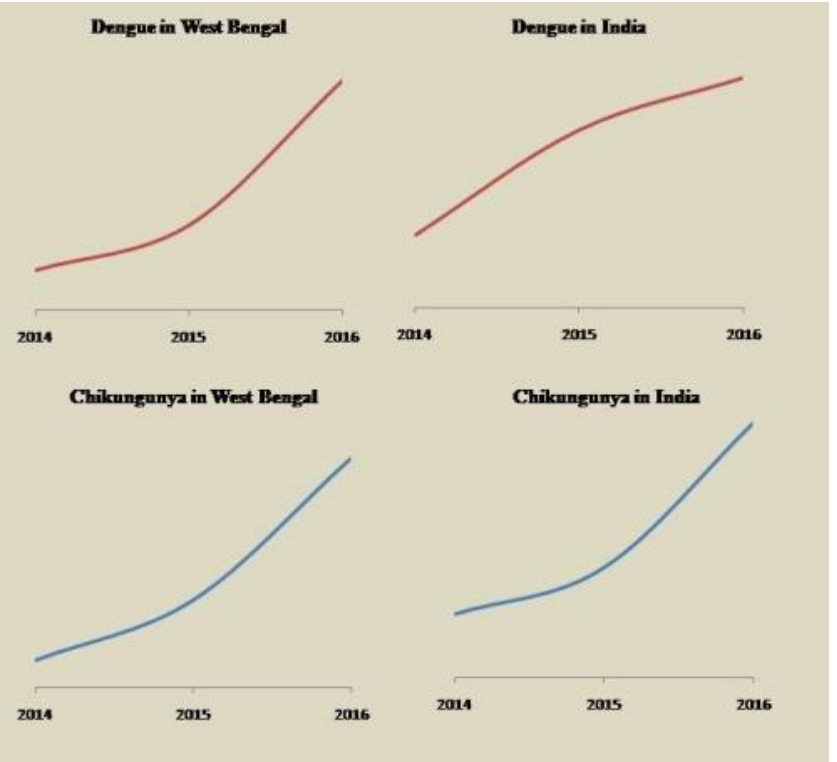


Figure 2: *Increase of Dengue and Chikungunya cases with time in West Bengal as well as in India*

These graphs depict Dengue & Chikungunya cases from the state of West Bengal and the whole country India. Interestingly, both the curves are similar and shows steep rise of both the cases from 2014 to 2016 [103]. West Bengal Chikungunya cases data are collected from the report published by Dept. of Health and Family Welfare, Govt. of West Bengal and India data are from NVBDCP [138].

Dengue & Chikungunya cases were also increased similarly in the neighbouring states of Bihar and Jharkhand.

From a published report of 2016 from Kolkata, it is clear that 95% of the Chikungunya infected patients suffer from Persisting polyarthralgia [30] thus affecting the life style of

the patient directly and state economy indirectly. Dengue deaths are increasing year by year significantly. Only measure is to manage those infected patients from a very early stage.

So it is clear that proper measures should be taken for early severity diagnosis of Dengue & Chikungunya patients to improve their life style along with the state economy'[110,111].

Chapter Three

MATERIALS & METHODS

बुद्धियुक्तो जहातीह उभे सुकृतदुष्कृते |
तस्माद्योगाय युज्यस्व योगः कर्मसु कौशलम् ||

buddhi-yukto jahātīha ubhe sukṛita-duṣhkṛite
tasmād yogāya yujyasva yogaḥ karmasu kauśhalam

Shrimad Bhagavad Gita, Chapter Two, Sānkhya Yog, verse 50
(The Yog of Analytical Knowledge)

One who prudently practices the science of work without attachment can get rid of both good and bad reactions in this life itself. Therefore, strive for Yog, which is the art of working skillfully (in proper consciousness).

সর্বভূতে সমান বুদ্ধিসম্পন্ন নিষ্কাম কর্মী ইহজাগতিক কর্মে পাপ ও পুণ্যের বিচার থেকে মুক্ত হন যোগের দ্বারা। এই যোগই হল (নিষ্কাম) কর্মের কৌশল।

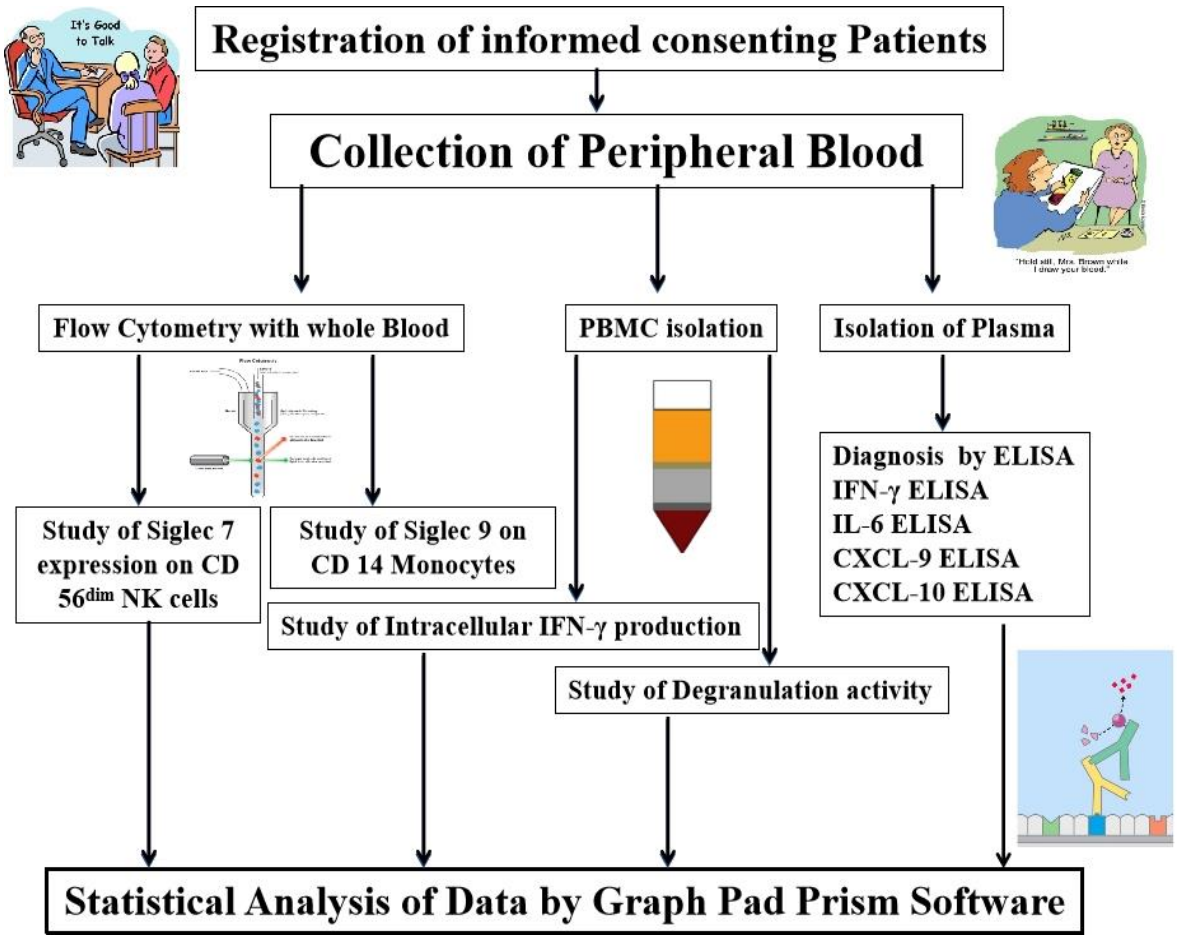


Figure 3: *Methods followed in this study*

This figure pictorially describes the basic methods and procedures followed in this study.

Study population

This study was done at the Carmichael Hospital for Tropical Disease, School of Tropical Medicine, Government of West Bengal, Kolkata, India. The patients were enrolled at the outpatients department of Tropical Medicine at the Institute. Within the period of April 2015 to December 2018 with prior approval from Institutional Human Ethics Committee, peripheral blood was collected from only informed and written consented patients following the Helsinki guidelines [116]. Clinical study was done by clinician and detailed history was taken by completing a pre-determined questionnaire from all patients. Chikungunya infection condition was confirmed by a WHO recommended MAC-ELISA kit provided by Indian Council for Medical Research-National Institute of Virology, Pune, Maharashtra and/or by Real Time PCR with genesig® Chikungunya standard Kit obtained from Primerdesign®Ltd UK [118,148,169].

Ethical Clearance

Adhering to the Helsinki protocol, this study was approved by the Institutional ethical committee[117,159]. Ethical clearance of the study was obtained through the letter issued by Clinical research Ethics Committee (*CREC-STM*) at School of Tropical Medicine, Government of West Bengal, for the *Study No. 32* at the meeting held on 30th August 2013 in favour of *Dr. Sumi Mukhopadhyay*, who supervised and guided this scientific work.

Methods

Demographic study

Patients were grouped as Chikungunya patients without persisting polyarthralgia or ChikWoPP and Chikungunya patients with persisting polyarthralgia or ChikWPP. Persisting polyarthralgia was determined by clinician from 10 to 30 days of infection. Healthy donors include age and sex matched non-smoking, non alcoholic fellow researchers, scientists, clinicians of the institute without any history of fever and/or joint pain in last three months.

Collection of peripheral blood

Peripheral blood was collected in EDTA coated vials from informed and written consented individuals. Peripheral Blood Mononuclear cells were obtained by layering cells from whole blood using Lymphoprep^{TM} obtained from Axis-Shield, Oslo, Norway. Blood collection was done by Government employees who are trained phlebotomists working at the Department of Laboratory Medicine, School of Tropical Medicine, Kolkata, India.

Multicolour Flow Cytometry with whole blood

Siglec-7 expression on $CD56^{dim}$ NK cells was studied by Multicolor Flow Cytometry. Initially, peripheral blood was collected from Chikungunya patients(CHIK) confirmed by MAC-ELISA and/or qPCR along with Healthy donors(HD) and incubated with Anti-human CD3 PE/Cy5, CD 4 FITC, CD 8 FITC, CD56 FITC, CD328 PE(Siglec-7), CD335 PE Cy7(NKp46), CD337 APC(NKp30), CD 14 mAbs (Biolegend). After incubation, blood was treated with Pharm Lyse Buffer (BD Bioscience) and washed with PBS followed by flow cytometry through FACSVerse Cytometer (BD Bioscience) and analyzed with BD FACSuite software.

Real Time RT-PCR

Siglec 7 mRNA levels were studied in NK cells sorted by MojoSort magnetic cell separation system (Biolegend) using ABI 7500 Fast PCR machine [173].

ELISA

Plasma Siglec-7 titers were measured by Sandwich ELISA kit obtained from RayBio. Other different immune mediators were also studied by ELISA, following the protocols mentioned in the brochure provided by respective company (RayBio, USA). Sandwich ELISA was performed by using standard kits obtained from RayBiotech, USA for each of Siglec-9, IL-6, TGFβ1, IL-10, IFN-γ, CXCL-9 according to manufacturer's protocol.

Degranulation assay by Flow cytometry

To analyze functionality of those cells degranulation assay of magnetically sorted NK cells was done using Anti-human CD107a mAbs. Intracellular IFN-γ was measured by kit from BD Bioscience. Plasma IFN-γ was measured using ELISA kit from RayBio. Statistical analysis was done using GraphPad Prism 5 software [144].

Degranulation was enumerated by the detection of LAMP1 or CD107a, on PBMC cultured in the absence or presence of 1000 IU/ml of Proleukin-2 (Chiron) for 48 hours as described earlier [228], against HeLa cell lines. PBMC/sorted NK cells were resuspended in the presence of anti-CD107a mAb (H4A3, Becton Dickinson) with target cells at an Effector : Target (E:T) cell ratio of 1:1. After 1 hour of incubation, Monensin beads were added at 2 mM concentration for 4 hours at incubation.

To induce IFN-γ production, similar treatment was done as described earlier [228]. Cells were fixed and permeabilized with a cytofix/cytoperm kit (Becton Dickinson) and stained with anti-IFN-γ mAb (B27; Becton Dickinson), as represented earlier [228].

Siglec-9 expressions on Monocytes were assessed by both flow Cytometry and semi-quantitative PCR techniques [119].

Whole blood collected in EDTA vial was incubated with FITC conjugated CD14 (M5E2) and PE conjugated CD329 (K8) (Siglec-9) and treated with BD Pharm Lyse. Further acquisition was done using a FACSCalibur Flow Cytometer and the data was analyzed using BD Cell Quest Pro software provided by company.

$CD14^+$ cells were sorted from whole blood using Mojosort Magnetic cell separation kit obtained from Biolegend, USA. Further mRNA was extracted using a Quiagen RNeasy mini kit followed by SYBRGreen based semi-quantitaitave PCR in an Applied Biosystem 7500 Fast platform. Gene expression level was determined with respect to β-actin expression following ΔΔCT method.

Magnetic isolation of cells

Within one hour of collection of peripheral blood, PBMC or Peripheral Blood Mononuclear cells were layered by Lymphoprep™ obtained from Axis-Shield, Oslo, Norway and washed with Phosphate buffered saline or PBS. Further, cells were sorted by a Mojosort™ Magnet and CD14 selection kit obtained from Biolegend, California, USA to isolate $CD14^+$ monocytes.

Determination of Intracellular ROS by FACS

Intracellular ROS was determined using 2′,7′-Dichlorofluorescin diacetate or DCFDA obtained from Sigma-Aldrich, Missouri, USA by Flow Cytometry as described earlier [31]. For monocytes, 10,000 gated events were acquired through BD FACSCalibur™ and analyzed using BD CellQuestPro™ software. Cells treated with H_2O_2 were considered as positive control.

Basic biochemical assays to determine Oxidative stress

Malondialdehyde (MDA) was estimated in plasma of all study subjects by following Pasha and Sadasivudu's method [32]. MDA reacts with Thiobarbituric acid (TBA) to produce a

colored compound, which is measured spectrophotometrically at 530nm. TBA detects only free MDA in peroxidising lipid system. In this study TBA was obtained from Sigma-Aldrich®.

Nitric Oxide or NO present in the plasma sample was measured by using Griess reagent (modified) obtained from Sigma-Aldrich®. The method followed in this study was earlier described by Griess [33]. Sample (n=65) used for determination of NO level were collected in EDTA coated vial as heparin produces precipitate with Griess reagent [33] Instruction from Sigma-Aldrich®.

Thiol content in the plasma samples of both patients and healthy controls were determined using Ellman's reagent or 5,5′-Dithiobis(2-nitrobenzoic acid) obtained from Sigma-Aldrich® following the description elsewhere [34].

Protein carbonylation status was estimated by derivatization with 2,4-dinitrophenylhydrazine as described previously[35].

DPPH or 2,2-Diphenyl-1-picrylhydrazyl was obtained from Sigma-Aldrich® and the assay was performed following method described by Janaszewaska and Bartosz earlier [36].

Superoxide Dismutase enzymatic activity with respect to inhibition of auto oxidation of pyrogallol in the plasma was determined by following the method of Marklund as described by P Jyothi et al [37].

Sandwich ELISA was performed by using standard kits obtained from RayBiotech, USA for each of Siglec-9, IL-6, TGFβ1, IL-10, IFN-γ, and CXCL-9 according to manufacturer's protocol.

DCFDA Assay

To determine the optimal concentration of DCFDA, PBMC of 10^6 order was mixed with various concentrations of DCFDA at 37^0 C for 30 min in dark condition. The intracellular ROS measurement assay was performed within 2 h of collection of the peripheral blood sample. PBMC of 10^6 orders collected from study subjects were suspended in Phosphate buffered saline of physiological pH and treated with 1 M 2,7-Dichlorofluorescin diacetate obtained from Sigma-Aldrich in polystyrene tubes suitable for flow cytometry maintaining dark condition at room temperature for 30 min [120].

Free radical scavenging assay

To determine the free radical scavenging potential of *T. cordifolia* leaf extract, peripheral blood mononuclear cells isolated from the enrolled subjects were briefly incubated with the plant formulation [121,123]. PBMC of 10^6 order was treated with ethanolic extract of *T. cordifolia* leaves in concentrations of 0.5 and 1 lg/mL for 1 h at room temperature. The analysis was done, and the presence of ROS was documented. $P = 0.05$ was taken significantly in all cases [122]. T-test was done to determine statistical significance among two groups of data and one-way ANOVA was done for the same within three groups.

Statistical Analyses

At least three sets of experiments were performed and results were expressed as mean ± S.E.M. for individual set of experiments. Statistical analysis was performed using the Graph-Pad Prism statistics software (Graph-Pad Software Inc., San Diego, CA). All variables were individually assessed with the W test of normality. For comparisons of levels of antibody responses amongst the different groups, Students t test and analysis of variance (ANOVA) or the Mann-Whitney test was applied depending on the normality of the sample. Significance levels were set at less than 5% probability of Alfa error. Receiver operator characteristic (ROC) curves were constructed to summarize the sensitivity and specificity estimates.

ODDS Ratio and Degree of Association analysis

Prognostic Adjusted ODDS ratio of different variables were calculated using Microsoft Excel 7 software along with XLSTAT (ADDINSOFT, New York) statistics Software available in between two study groups namely CHIK patients at presentation and CHIK patients at follow up. Adjusted OR (ODDS RATIO), 95% Confidence Interval (CI) was determined. Degree of Association was determined by chi-square test and Fischer's exact test for clinical variables of CHIK patients to expression levels of all the studied immune mediators (Siglec 9, RANTES, CXCL 10, CXCL 9, IL-7 and IL-6) and the P value is presented.

Chapter Four

Results and Discussion

कर्मण्येवाधिकारस्ते मा फलेषु कदाचन।
मा कर्मफलहेतुर्भूर्मा ते सङ्गोऽस्त्वकर्मणि॥

Karmaṇye-vādhikāras te mā phaleṣhu kadāchana |
mā karma-phala-hetur bhūr mā te saṅgo 'stvakarmaṇi ||

Shrimad Bhagavad Gita, Chapter Two, Sānkhya Yog, verse 47
(The Yog of Analytical Knowledge)

You have a right to perform your prescribed duties, but you are not entitled to the fruits of your actions. Never consider yourself to be the cause of the results of your activities, nor be attached to inaction.

স্বধর্ম বিহিত কর্মে তোমার অধিকার আছে কিন্তু কোনও কর্মফলে তোমার অধিকার নাই। কখনো নিজেকে কর্মফলের হেতু মনে করোনা এবং কখনো স্বধর্ম আচরণ থেকে বিরত হয়োনা।

योगस्थः कुरुकर्माणि सङ्गंत्यक्त्वा धनञ्जय |
सिद्ध्यसिद्ध्योः समोभूत्वा समत्वं योग उच्यते ||

yoga-sthaḥ kuru karmāṇi saṅgaṁ tyaktvā dhanañjaya |
siddhy-asiddhyoḥ samo bhūtvā samatvaṁ yoga uchyate ||

Shrimad Bhagavad Gita, Chapter Two, Sānkhya Yog, verse 48
(The Yog of Analytical Knowledge)

Be steadfast in the performance of your duty, O Arjun, abandoning attachment to success and failure. Such equanimity is called Yog.

হে অর্জুন! ফলভোগের কামনা পরিত্যাগ করে ভক্তিযোগস্থ হয়ে স্বধর্ম-বিহিত কর্ম আচরণ কর। কর্মের সিদ্ধি ও অসিদ্ধি সম্বন্ধে যে সমবুদ্ধি, তাকেই যোগ বলা হয়।

Study Period	**: April 2015 To December 2018**
Study Site	**: School of Tropical Medicine, OPD and Indoor wards**
Study Population	**: Total population Screened >2000**
	: Total study subjects 88 Chikungunya, 55 Dengue, 88 Healthy donors, 40 Rheumatoid Arthritis Patients
Severity Criteria	**: Fever and ≥6 localizations of arthralgia.***

Table 1: *Population Data*

***** Gérardin P, Fianu A, Michault A, et al. Predictors of Chikungunya rheumatism: a prognostic survey ancillary to the TELECHIK cohort study. Arthritis Research & Therapy. 2013;15(1):R9. doi:10.1186/ar4137.

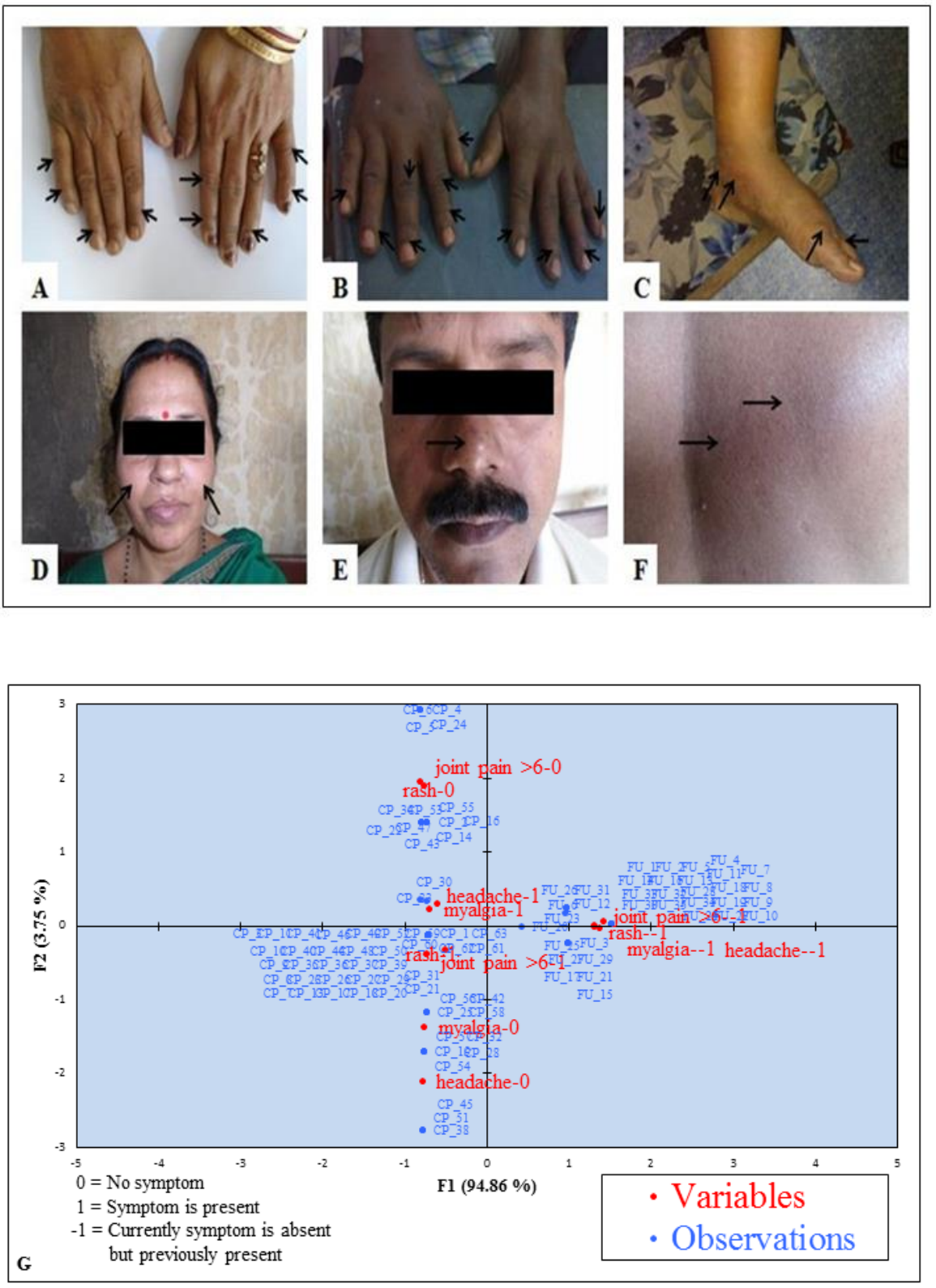


Figure 4: *Patients and the Multiple Correspondence Analyses of Signs & Symptoms*

A-C shows polyarthralgia in patients with CHIK infection. Black arrows point the area of inflammation. D-F shows rashes on face, nose and trunk respectively. G is a plot generated

by Multiple Correspondence Analysis (MCA) using XLSTAT Add-in software of Microsoft Excel 7. Patients as observations were plotted against clinical manifestations as variables namely Rash, Headache, Myalgia and Joint Pain > 6 locations.

These analyses depict the manifestation of the disease during Chikungunya infection and follow up.

Study Parameter	Healthy Donors	CHIK Patients at presentation (5 days to 15 days after onset of fever)	CHIK Follow up patients (>15 to 90 days after onset of fever)
Age(in Years) [Median with range]	27 (19-55)	36 (5-90)	52 (33-78)
Male	n=13	n=11	n=10
Female	n=12	n=14	n=5
People residing at Kolkata metropolitan Area	n=20	n=16	n=11
People residing at Municipal Town	n=3	n=05	n=03
People residing at Village	n=2	n=04	n=01

Table 2: *Demographic Data*

Study Parameter	Healthy Donors	CHIK Patients at presentation (5 days to 15 days after onset of fever)	CHIK Follow up patients (>15 to 90 days after onset of fever)
Total count of WBC* (X 10^3 /µL)	6.3 ± 2.56	6.82 ± 3.38	5.8 ± 2.15
Lymphocyte count* (%)	32 ± 10.58	28.64 ± 7.98	34.21 ± 4.68
Monocyte Count*(%)	6 .1 ± 1.76	7.13 ± 3.98	6.54 ± 1.58
Total Bilirubin* (mg/dL)	0.8 ± 0.67	1.22 ±0.13	0.9 ± 0.54
Total Protein*(gm/dL)	6.8 ± 1.5	7.70 ±0.20	7.1 ± 1.3
AST(SGOT)* (IU/L)	31.89 ± 3.5	33.42 ±2.31	32.95 ± 1.9
ALT(SGPT)*(IU/L)	33.25 ± 2.54	35.04 ±2.53	33.97 ± 1.29
Alkaline Phosphatase*(IU/L)	72.64 ± 3.98	145.3 ±1.21	82.17 ± 2.35

Table 3: *Hematological Data [n = 88]*

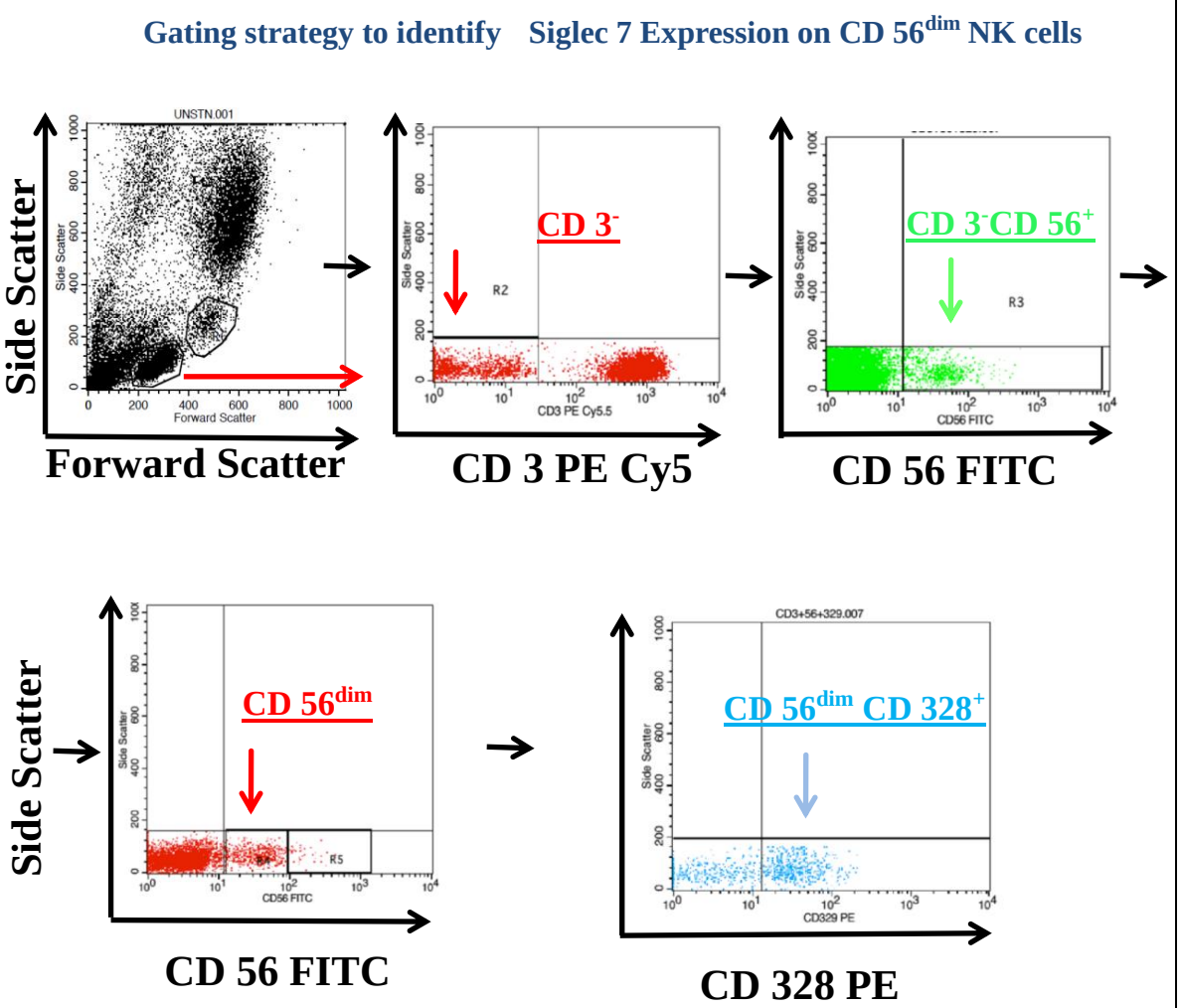


Figure 4: *FACS Gating strategy of NK cells*

Gating strategy to identify Siglec 9 Expression on CD 14 Monocytes

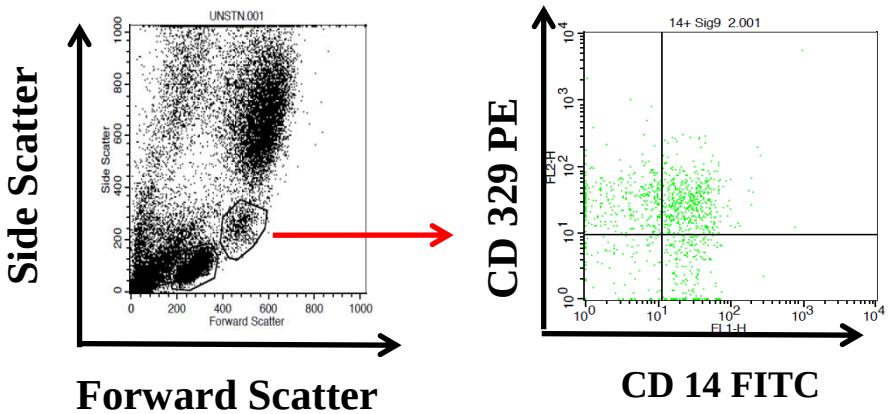


Figure 5: *FACS Gating strategy of Monocytes*

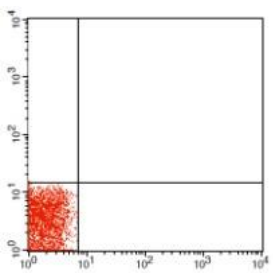
Gating strategy is the foremost part of any cellular analysis through flow cytometry. In this study multicolor flow cytometry was one of the key methods to elucidate the surface expression of siglecs.

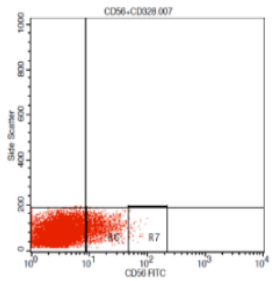
Two different populations were studied in this study. $CD3^{-}CD56^{dim}CD328^{+}$ population were identified to know the surface expression of CD 328 on CD 56 dim cell surface. First, Lymphocyte population were identified using Forward and Side Scatter. The $CD\ 3^{-}$ population was selected because $CD\ 3^{+}$ population contains NKT which are functionally different. So, CD 3- CD 56+ population were screened. Then CD 56dim were identified using gating strategy. After that CD 328 expression were studied in the said population.

Some representative figures

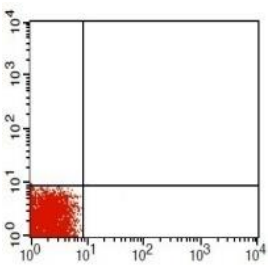
CHIK Patients with Mild/No Polyarthralgia

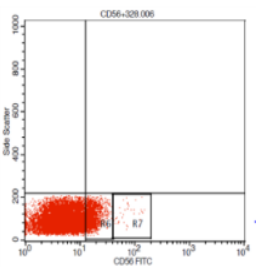
Control | **FITC Positivity**



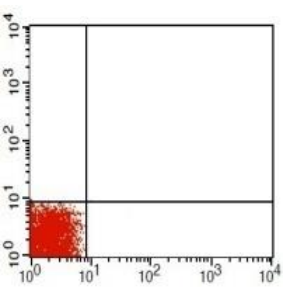


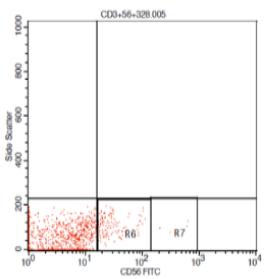
CD 56^{dim}	CD 56^{bright}
1.04%	0.01%





CD 56^{dim}	CD 56^{bright}
3.05%	0.09%





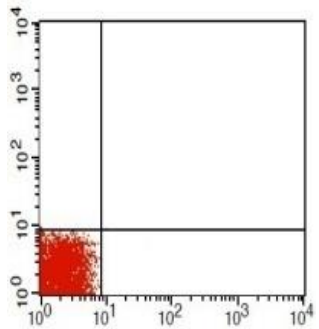
CD 56^{dim}	CD 56^{bright}
3.65%	0.05%

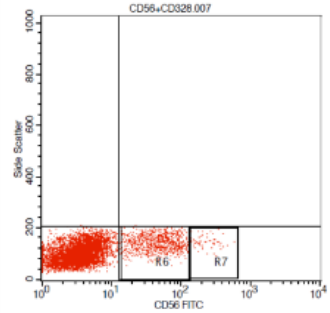
Figure 6: *Representative Dataset 1*

CHIK Patients with Severe Polyarthralgia

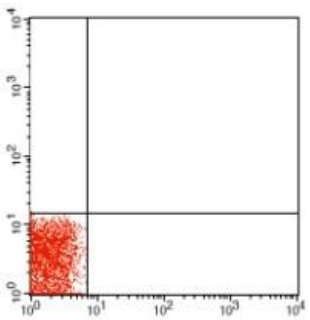
Control

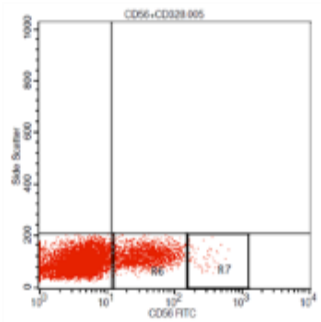
FITC Positivity



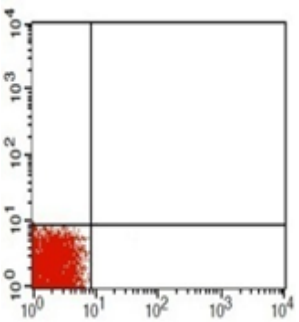


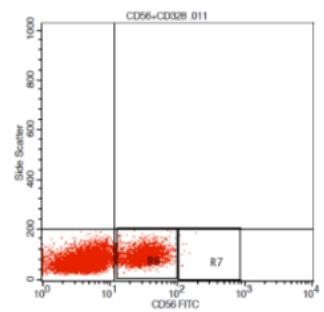
CD 56^{dim}	CD 56^{bright}
7.56%	0.05%





CD 56^{dim}	CD 56^{bright}
14.08%	0.01%





CD 56^{dim}	CD 56^{bright}
7.56%	0.05%

Figure 7: *Representative Dataset 2*

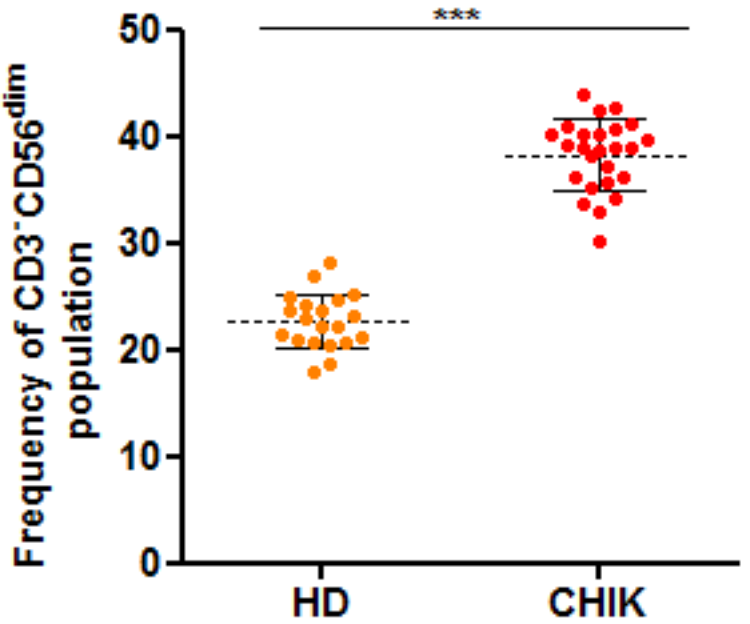


Figure 8A

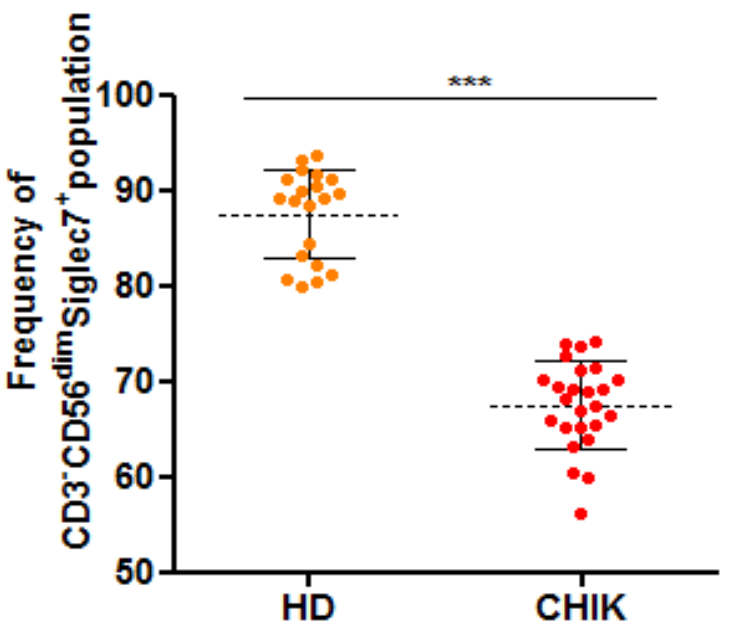


Figure 8B

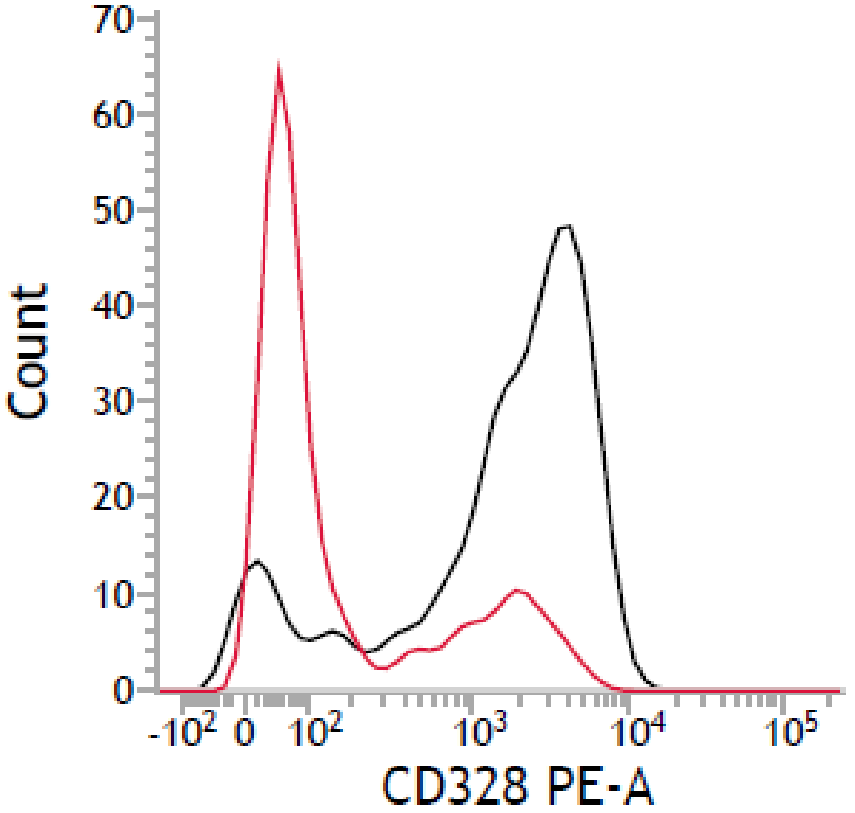


Figure 8C

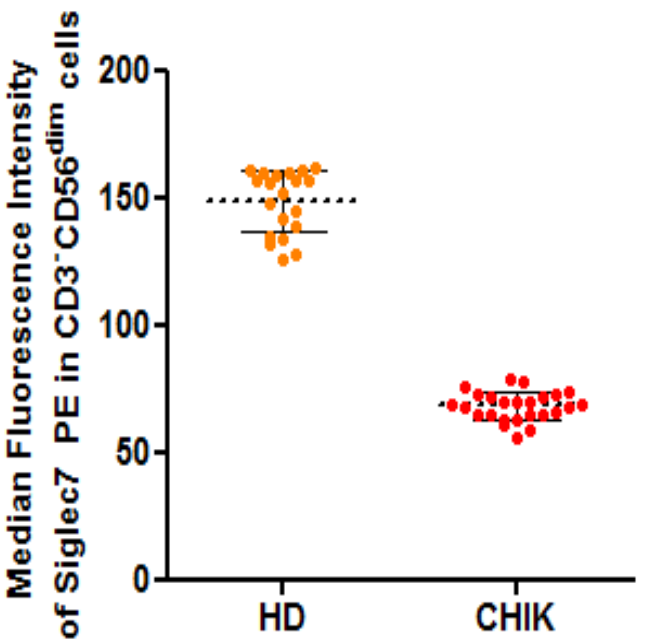


Figure 8D

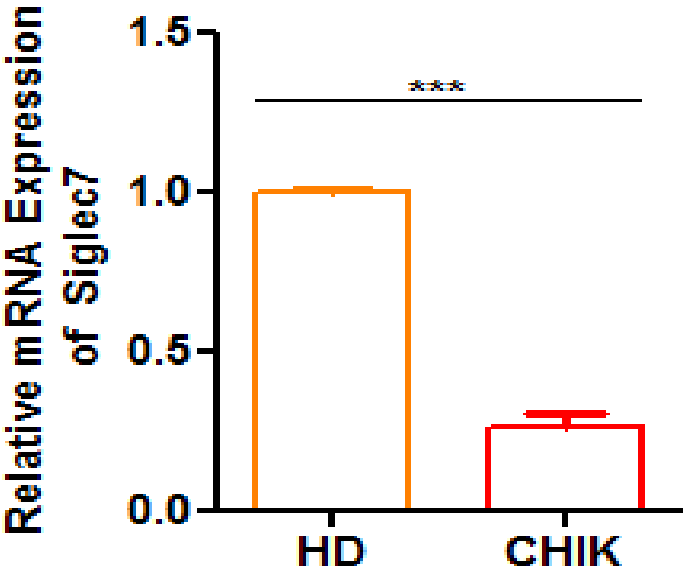


Figure 8E

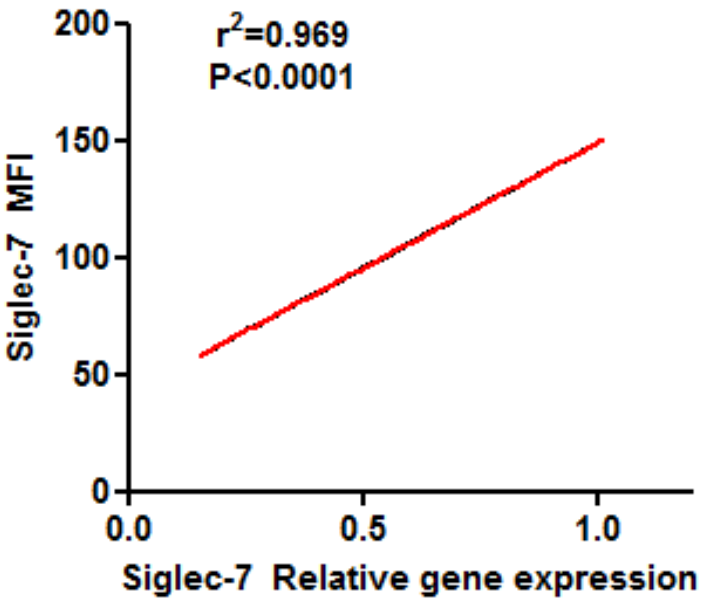


Figure 8F

Figure 8 A-F: *Siglec-7 expression is decreased in CD3-CD56^{dim}NK cell population among Chikungunya subjects (n =25)*

$CD3^{-}CD56^{dim}$ NK cells are increased in CHIK than HD as evident from Figure 8A. These changes are statistically significant (P<0.0001) Median Fluorescence Intensity of Siglec-7 PE is significantly low in CHIK $CD3^{-}CD56^{dim}$ NK cells (8B). Frequency of $Siglec7^{+}$ cells within $CD3^{-}CD56^{dim}$ NK cells is also significantly lower in CHIK than HD as described in Figure 8C. Figure 8D is a representative histogram of Siglec 7 expression in CHIK (Red) and HD (Black). Relative mRNA expression of Siglec7 in sorted $CD56^{+}$NK cells was normalized with respect to β-actin following $2^{-\Delta\Delta CT}$ method and represented in 8E. Figure 8F Describes correlation between relative gene expression and MFI of Siglec-7. ***P<0.0001.

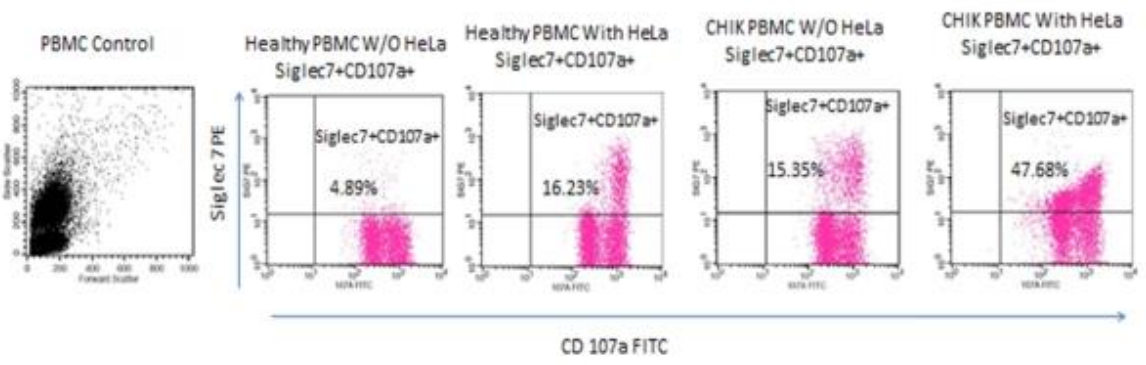


Figure 9: *Degranulation study with or without interaction with HeLa cells*

More degranulation implies more cellular damage. As small joint tissue possesses sialic acid so a similar model has been chosen in this study, as the HeLa cell line. Being a cancer cell line, these cells are rich in surface sialic acid. So, this can mimic the small joints suffering from inflammation. CD107 is the mAB which binds with granulation proteins secreted from lysosomes. After interaction with Sialic acid reach HeLa cells, NK cells of patient reacts with then through Siglec receptors and produces Interferons and degranulation. That is different in Healthy individuals, chikungunya patients with or without persisting polyarthralgia.

It has been found that Inter-cellular Interferon gamma production is 12 fold lower in severe Chikungunya patients than non severe patients and 20 fold lower than healthy donors. This prohibits the involvement of adaptive immunity.

Intra-cellular Interferon Gamma Production

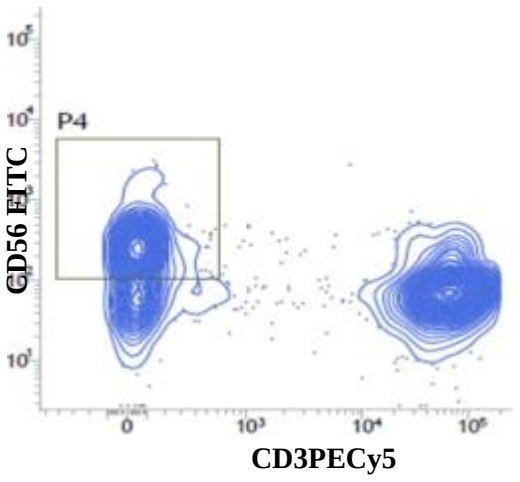


Figure 10A

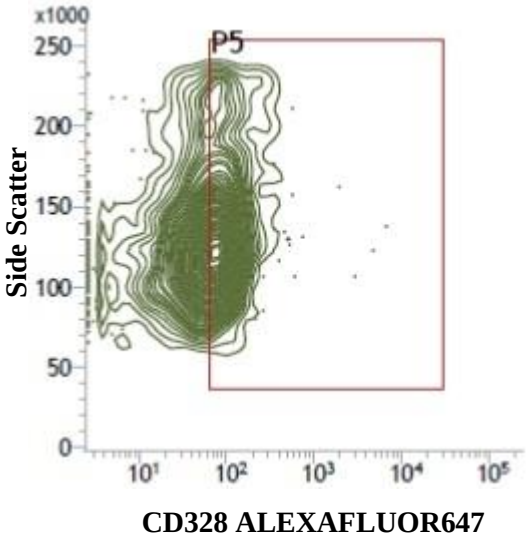


Figure 10B

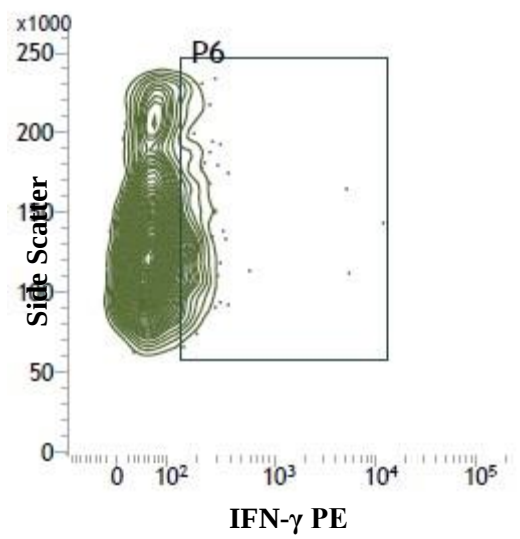


Figure 10C

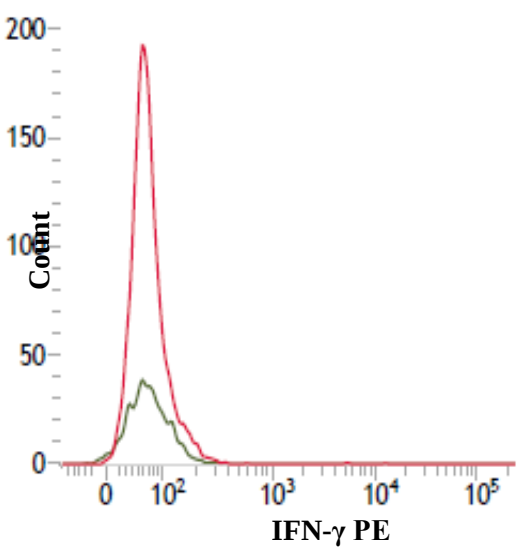


Figure 10D

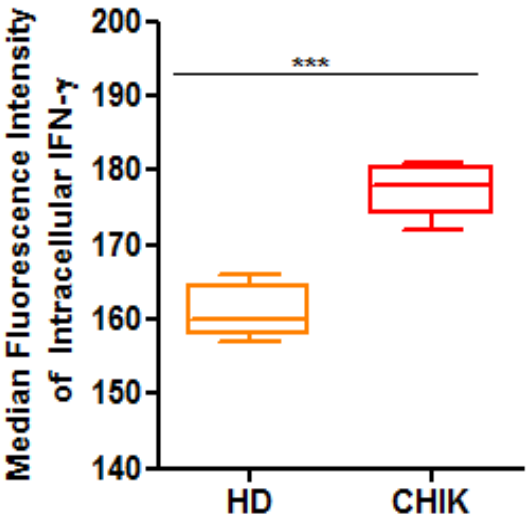


Figure 10E

Figure 10: *Representative figure of IFN gamma secretion. CD3^{-}CD56^{dim}Siglec7^{+} cells produce higher interferon gamma in CHIK*

Intracellular IFN-γ levels were determined by MFI of IFN- γ PE within CD3-CD56dimSiglec-7+ cells. Fig. 9A-C represents gating strategy and 9D&E describes overlay histogram plot and statistical analysis of Box and Whiskers plot respectively. ***P<0.0001.

CD56^{dim} NK cells were found to be 1.6 fold increased (P<0.0001) in CHIK than HD but Siglec-7 surface expression was decreased 1.14 fold (P<0.0001) in CHIK compared to HD. Siglec-7 mRNA levels were decreased in NK cells with overall decreased titers in CHIK plasma [125]. Interestingly natural cytotoxic receptors (NCR) like NKp30 & NKp46 were also found to be decreased in CD56^{dim} NK cells. However, Degranulation capacity of NK

cells was found to be increased in CHIK than HD. Both intracellular and Plasma IFN-γ levels are found to be higher in CHIK [124].

Balance between activating and inhibitory receptors is necessary to maintain healthy immune system. Our study reveals that Siglec-7 expression is significantly decreased in $CD56^{dim}$ NK cells [126].

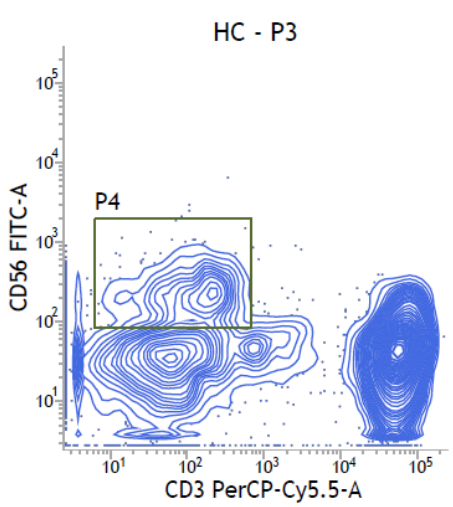


Figure 11A

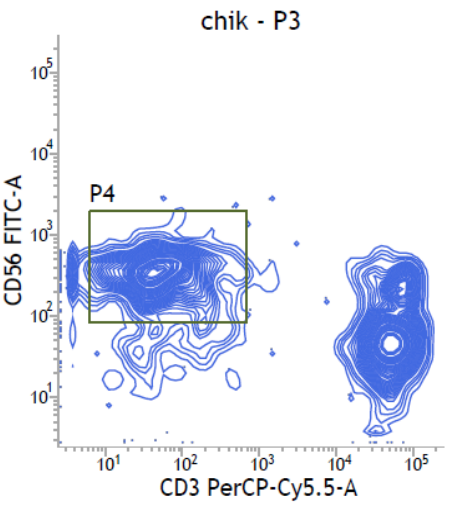


Figure 11B

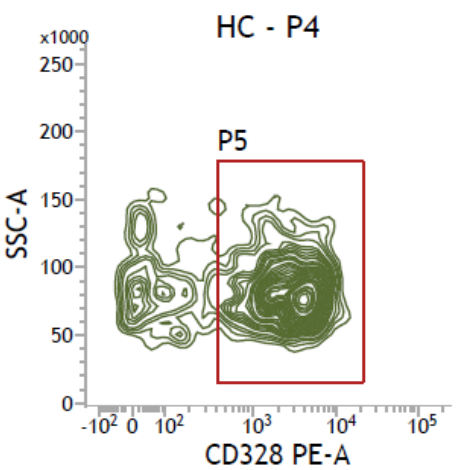


Figure 11C

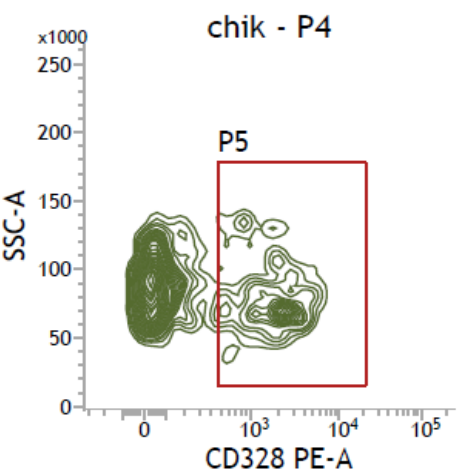


Figure 11D

Figure 11: *Gating strategy to study NCRs in NK cell*

A-D: gating strategy followed for further phenotypic study of NKP30, NKP46 and both NKP30 and NKP46 expression on $CD3^{-}CD56^{dim}$Siglec 7^{+} cells. P5 denotes $CD3^{-}CD56^{dim}$ Siglec 7^{+} population in both Healthy control and Chikungunya patients.

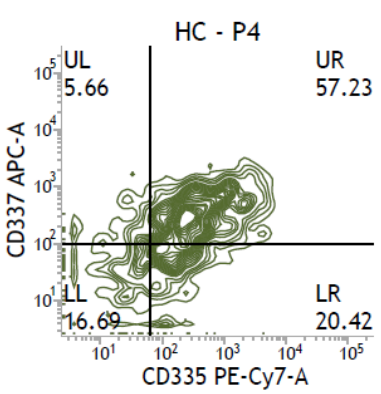


Figure 12A

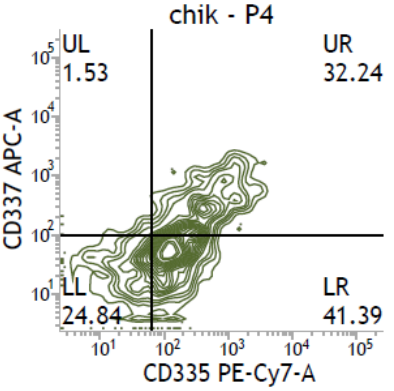


Figure 12B

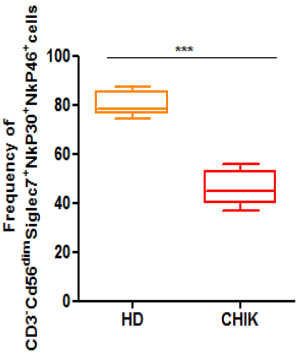


Figure 12C

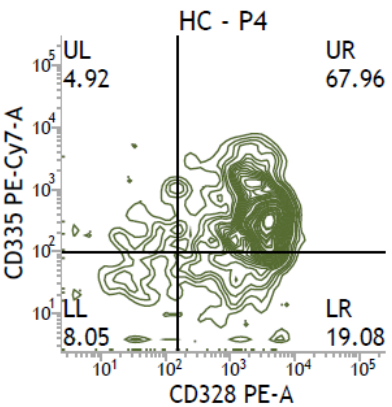


Figure 12D

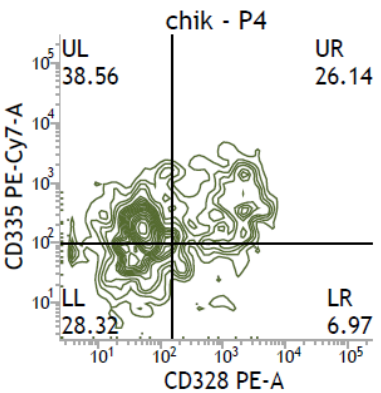


Figure 12E

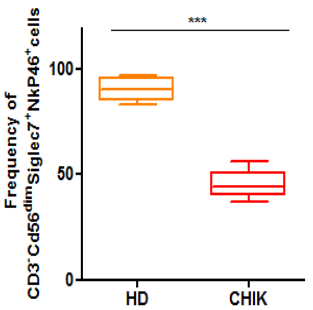


Figure 12F

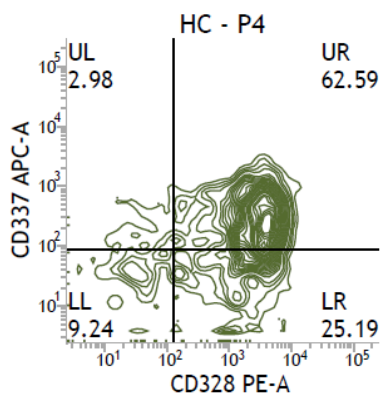


Figure 12G

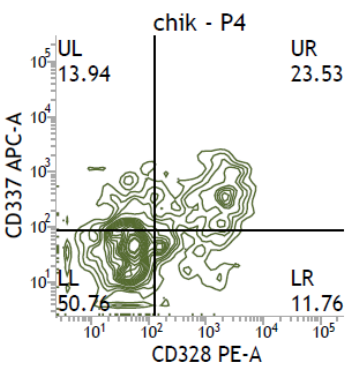


Figure 12H

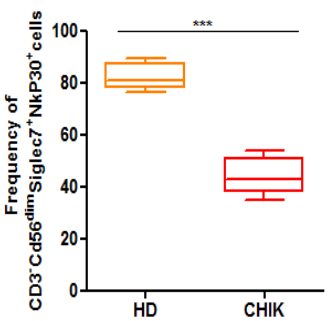


Figure 12I

Figure 12: *Immunophenotypic studies of NCRs in NK cell*

A-J: Siglec7 is down regulated along with decreased NCR (natural cytotoxicity receptors) expression

$CD3^{-}CD56^{dim}$ gated NK cells were further analyzed for CD 335, CD337 expression along with CD328. Fig 12A-C describes representative contour plots in HD and CHIK subjects depicting $CD335^{+}CD337^{+}$ population. 12C describes Box n Whiskers plot data for 10 individuals in each group. $P<0.05$ was assumed as significant. 12D, 12E & 12F describes the frequency of $CD335^{+}CD328^{+}$ cells and their statistical analysis for all study subjects. $CD328^{+}CD337^{+}$ cell frequencies in the same gated population along with statistical analysis is demonstrated in Fig 12G-I. ***$P<0.0001$

Additionally it was found that other natural cytotoxicity receptors (NCRs) are also decreased in those cells. However these cells have higher functional capacities like degranulation and intracellular cytokine production. We report a functionally distinct NK cell population having lower Siglec-7 and NCRs. These receptors are reported to be alter-expressed in some other diseases, but for the first time we report their status on the surface of $CD56^{dim}$ NK cells in Chikungunya infected individuals. Functionally hyperactive $CD56^{dim}$ NK cells having skewed Siglec-7, NKp30 and NKp46 expression might have role in Chikungunya pathogenesis [13]. So, it means that the patient have hyperactive cells which ultimately causes cellular destruction.

Recent report in mouse model shows that the involvements of Chikungunya virus-specific CD4+ but not CD8+ T cells are indispensable for the manifestation of joint swelling. The role of regulatory T cells or Tregs has also been shown in Chikungunya pathogenesis in mice model [229]. So, immunology of T cell has a crucial role in Chikungunya associated arthralgia.

T lymphocytes are basically innate-immune cells having crucial role in cell-mediated immune reactions throughout viral infections. CD 3 is the key marker of T lymphocytes

and enumeration of such CD 3+ population in peripheral blood is a regular method in viral infections like HIV [230]. This CD 3+ population can be further well characterized by the demonstration of various CD markers which identifies different sub-populations of T lymphocytes like CD 4+ and CD 8+ T cell. Natural Killer T-cells or NKT cells are CD 3+CD 56+ cell population which are functionally different from other CD 3+ cells which possess both the characteristics of T lymphocytes as well as natural Killer cells. CD 3+CD4+CD 25+ cell population is best-known as regulatory T cells which play critical role in case of inflammatory as well as autoimmune diseases. Regulatory T cells are also called as suppressor T cells and those are well known to maintain the self-nonself balance in immune system by cascading various signaling pathways to stamp down other immune cells. So, Treg homeostasis is essential for the immune system and any modification in their frequency can manifest into destruction of healthy human cells. The CD 4: CD 8 ratio in peripheral blood always shows the tendency of our immune system towards the development of inflammation [231]. These crucial parameters are very significant to find out the status of the disease manifestation at any given point of time. So, they have importance in prognosis. These NKT cells can regulate the immune response as well as have an effect on the production of inflammatory That's why, the status of both NKT cells and Tregs are all important to comment on the pathophysiology of joint inflammation and pain due to Chikungunya viraemia. To the best of our knowledge, there is no such previous study about the status of those cells in Chikungunya infection in the human subjects. So, Tregs and NKT cells were studied in this work.

	Healthy Controls (N=25)	CHIK Patients without polyarthralgia (N=20)	CHIK patients with polyarthralgia (N=25)	P value (one way ANOVA)
Lymphocyte count	31 ±10.25	33 ±7.56	34.26 ±4.69	0.0023
% of CD3+ cells (among lymphocytes)	57.55±1.21	61.15±1.05	62.42±1.23	0.0573
CD 4: CD8	1.61±0.15	1.9 ±0.11	2.2 ±0.13	0.0216
% of CD3+CD56+ cells (among lymphocytes)	4.47±1.06	6.6 ±3.63	12.07±2.70	0.0013
% of CD3+CD4+CD 25+ cells (among lymphocytes)	23.85±2.76	18.24 ±4.65	2.52±5.61	<0.0001

Table 4: *Different study parameters of the subjects in the Treg and NKT cell study represented as Mean ± S.E.M. P<0.05is significant.*

T regulatory cells are positive and found to have decreased in Chikungunya patients with polyarthralgia. In general Chikungunya patients it is not decreased significantly. So, it is evident that decrease of Tregs or Suppressor T cells is found in patients with polyarthralgia after CHIK infection. Naturally the CD 4: CD 8 is also altered significantly. The overall % of CD 4 cells are elevated but the % of regulatory T cells has been found to be decreased. This alteration indicates that the development of auto-immunity which eventually destroys host's own cells. This imbalance of Tregs might lead to the development of persisting polyarthralgia.

In contrary, NKT cell population is found to be increased in patients suffering from persisting polyarthralgia. In consequence of the viral infection, NKT cells are expanded in these patient populations to induce inflammatory cytokines [232]. It is known that NKT

cells involves with Siglecs which are accountable for inflammation in diseases like systemic sclerosis and Systemic Lupus Erythematosus [233, 234]. Rush of these cells at the small joints provides the idea of inflammation procedure due to chikungunya infection.

Here comes the Physiological correlation study. Correlation of Siglecs with standard immune markers has been studied. This shows interesting results. Any new biomarker needs to be established by correlating it with some standard immune mediators or established biomarkers. Same has been done in this study.

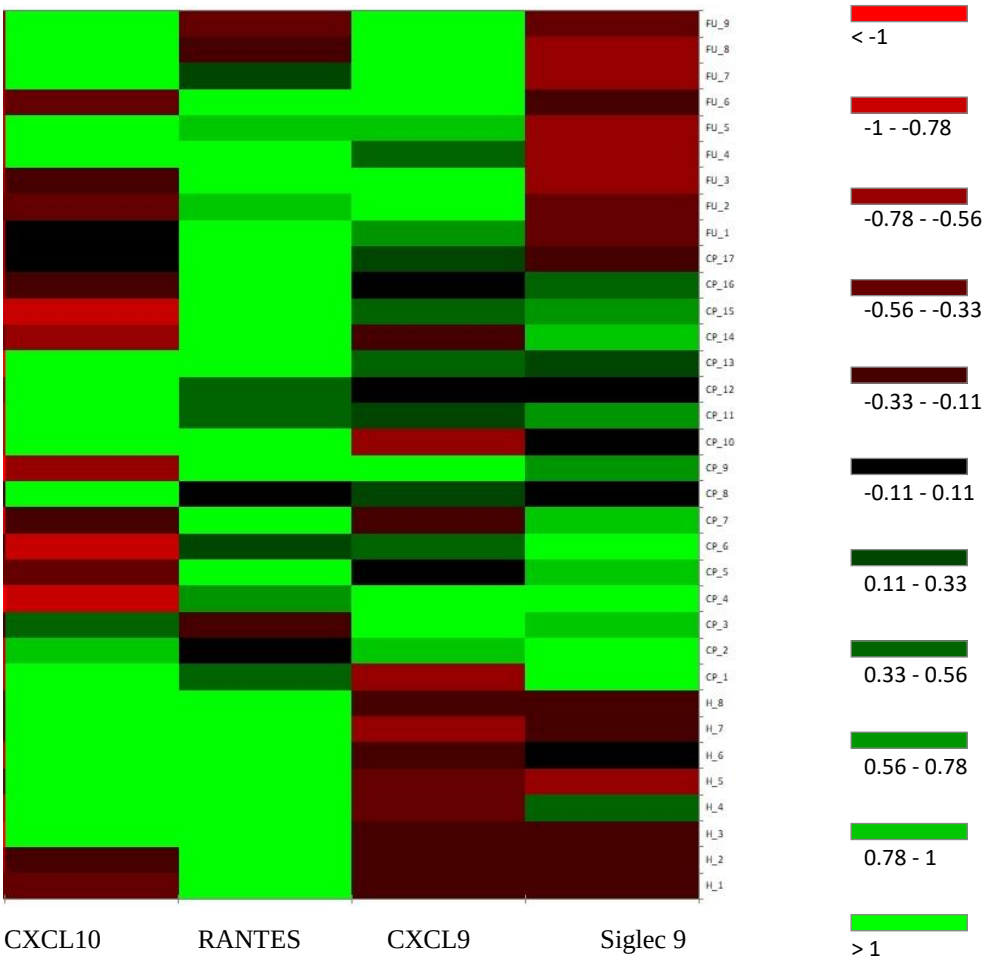


Figure 13: *Heat Map of the expression of Siglec 9 along with other chemokines found in some Healthy controls (H); Chikungunya patients (CP) and Follow up cases (FU) in this study.*

This heat map is generated by XLSTAT software, which is an extension of Microsoft Excel. Color scale: Red to green through black. Red is minimum value, Green is maximum value whereas the black is median value.

This heat map shows that in the prognosis of Chikungunya related persisitent polyarthralgia is the best marker among CXCL 9, CXCL 10, RANTES and Siglec 9. Because, only this marker shows decrease in follow up and the values are similar to healthy individuals. Though other markers are significant when grouped with all patient details, bu if individual patient and controls are studied, then it shows the Siglec 9 becomes better prognostic biomarker for chikungunya induced polyarthralgia.

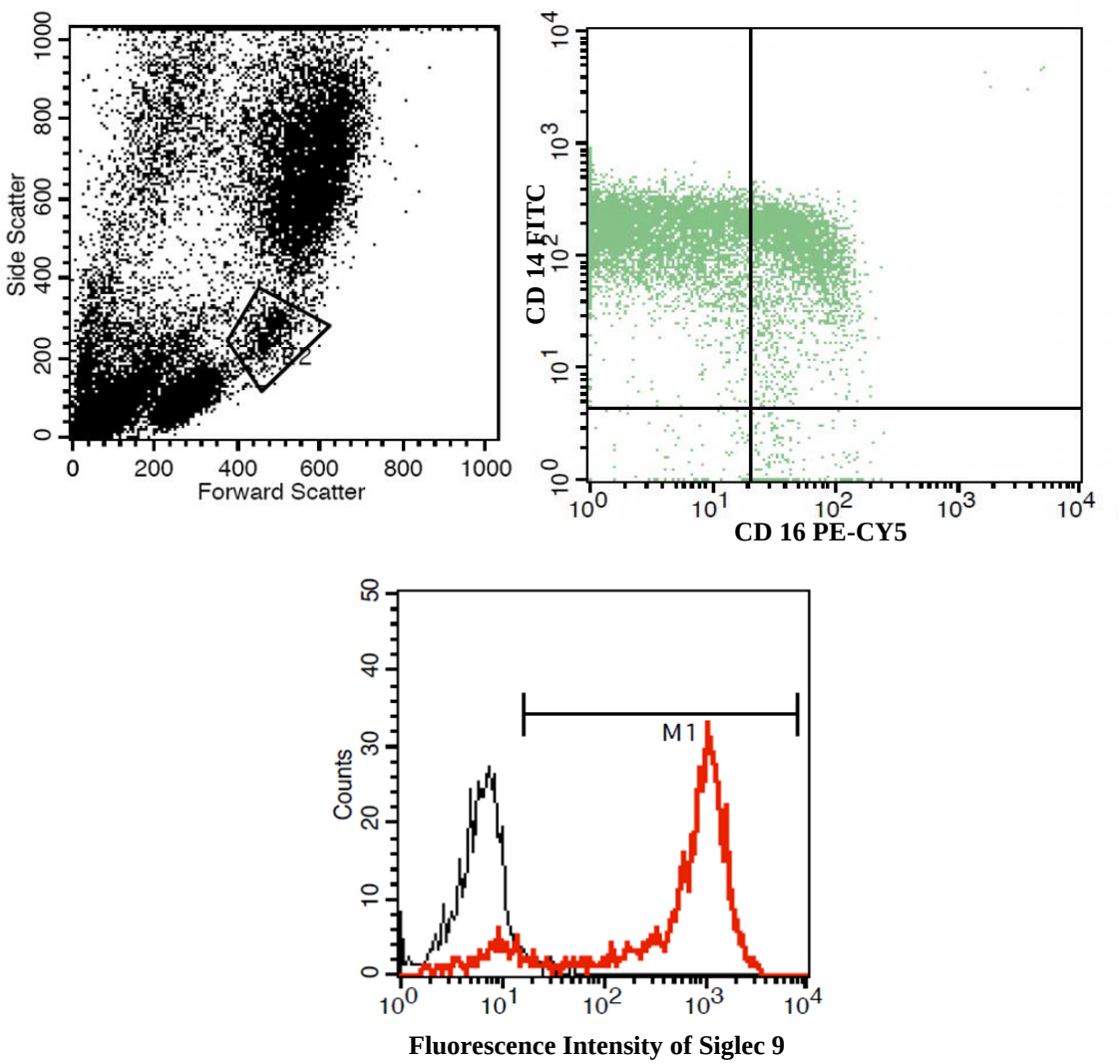


Figure 14: *Study of Siglec 9 expression on the surface of monocytes/macrophages*

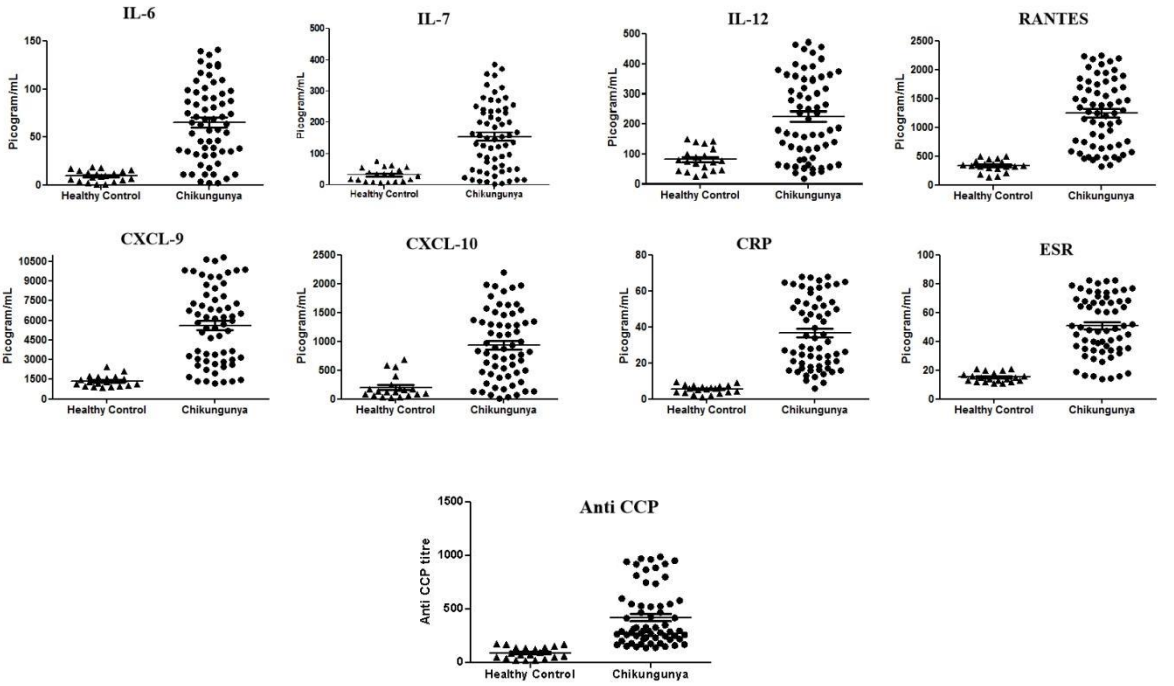
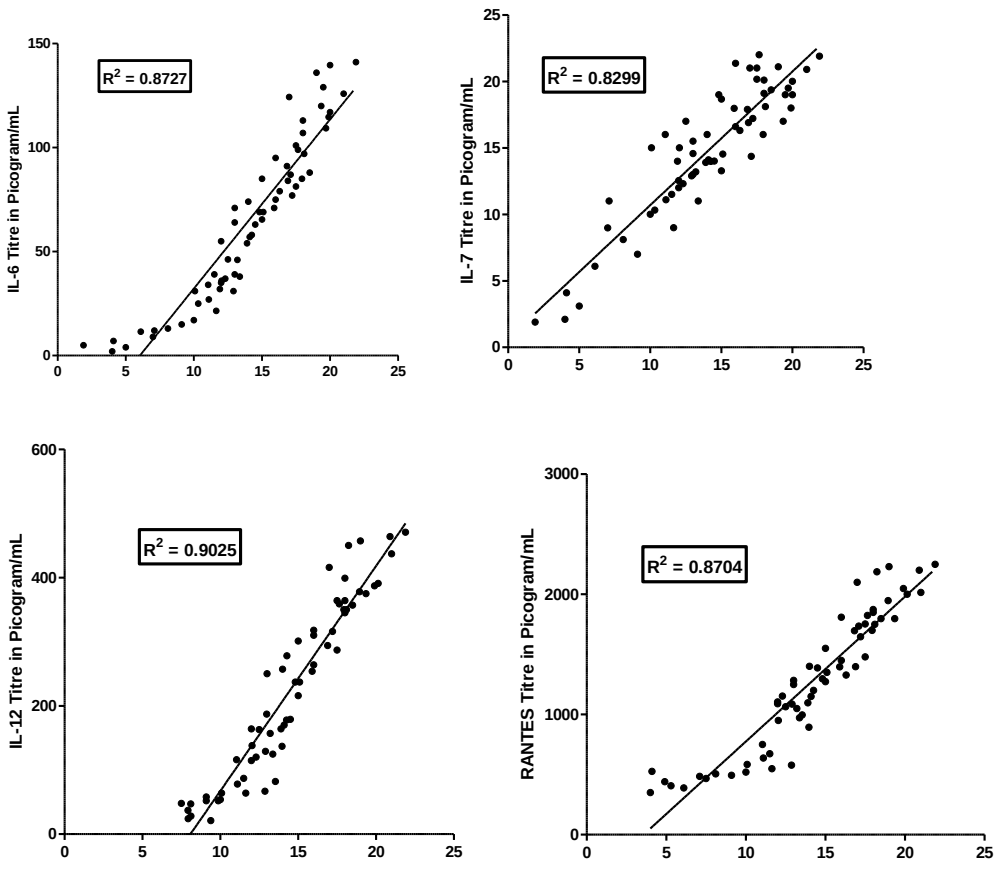


Figure 15: *Status of different Inflammatory parameters in Chikungunya patients (n=65) and Healthy controls (n=25) P<0.0001****



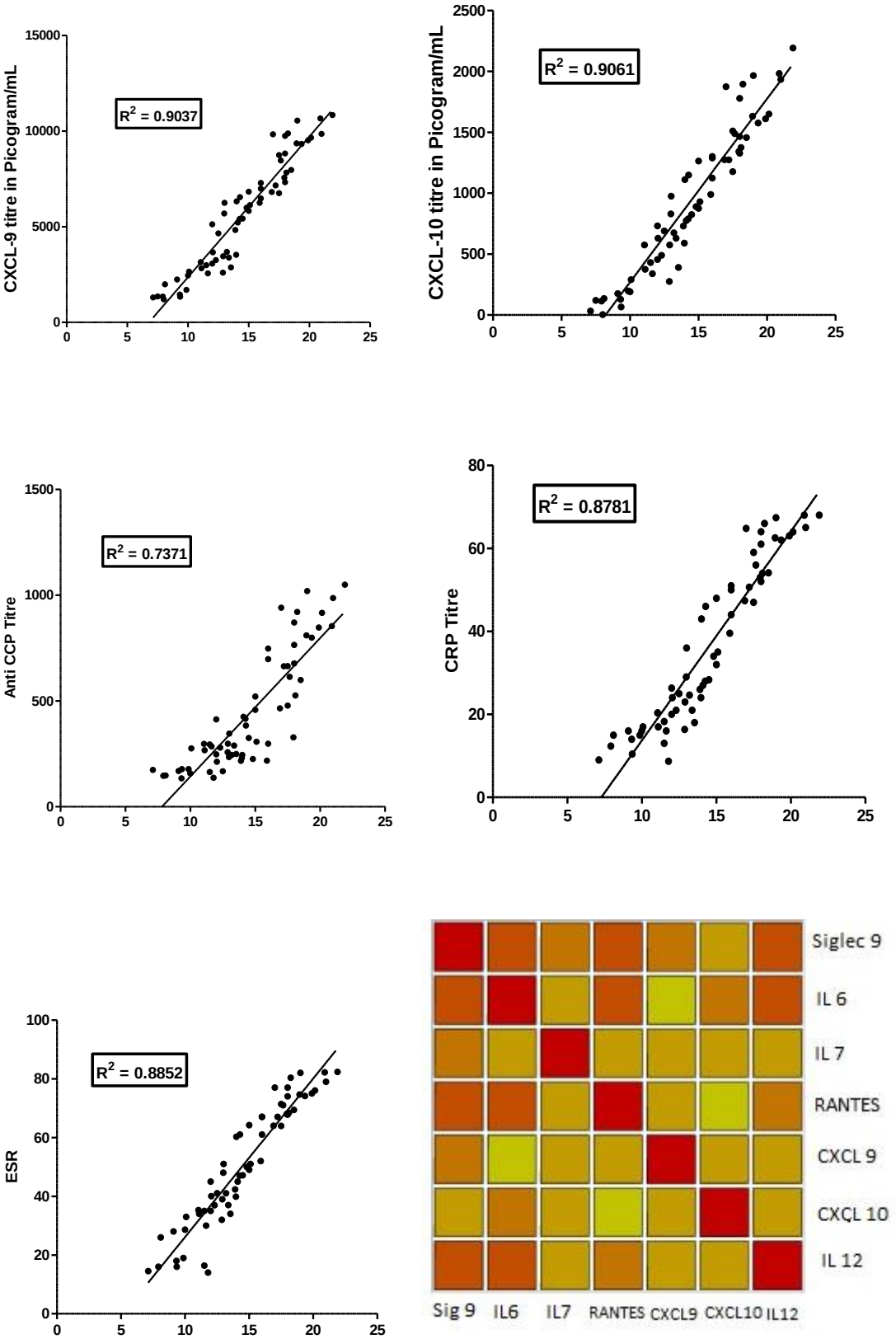


Figure 16: *Correlation of Siglec 9 MFI (Normalised to 10^{-1} level) with other inflammatory parameters.*

It implies that Siglec 9 has average to good correlation with several inflammatory markrers whih makes it a good prognostic biomarker for chikungunya induced polyarthralgia.

Standard biochemical parameters of oxidative stress were measured to get the glimpse of internal REDOX imbalance scenario in the patient as this imbalance marks tissue damage, inflammation and persistence of the disease activity [149]. Malondialdehyde or MDA level in plasma, which depicts lipid peroxidation, was found to be significantly (P>0.0001) 1.6 fold high in ChikWPP group when compared to healthy controls. Nitric Oxide or NO, which is a crucial player of REDOX signaling was 1.75 fold increased in ChikWPP patients significantly (P>0.0001) than controls [154]. Thiols are traditional markers of oxidative stress and found to be decreased in Chikungunya patients earlier [38]. Sulfhydryl group containing Thiols are important players of enzyme activity and can potentially decrease the damage caused by oxidative stress. In this study, it has been found that there is 1.34 fold decreased Thiol content of the patient plasma. This significant (P>0.0001) change describes that there is higher oxidative damage in those persisting polyarthralgia patients after Chikungunya infection. Similarly Carbonylation of protein marks protein damage and there is significantly (P>0.0001) high levels of protein carbonyls present in patient plasma which when compared to controls was found to be 3.2 fold higher. So it is evident that, there is higher level of oxidative damage in patients suffering from post Chikungunya persisting polyarthralgia and the oxidative damage is well represented by the plasma levels of several standard biochemical markers.

To combat increased oxidative damage, human body is well equipped with anti-oxidant mechanisms which sustain proper working of the body systems [39]. In diseased condition, there is imbalance among these mechanisms and the manifestation affects the life style of

the patient [128]. Plasma anti-oxidant potential was investigated by evaluating Superoxide dismutase enzyme activity and DPPH radical scavenging activity in this study. Both the parameters were found to be significantly (P>0.0001) low in patient plasma when considered along with healthy individuals. SOD level was 1.03 fold low whereas DPPH radical scavenging activity was found to be 1.17 fold lower in patients than controls. SOD activity was found to be increased in patients who had no sign of polyarthralgia indicates significant role of it in the management of oxidative damage. This poor anti-oxidant potential of patient plasma explains the inability of the patient system to combat with such higher oxidative damage. MDA/SOD ratio is an established marker to indicate REDOX imbalance in diseases [37]. This ratio is markedly changed in ChikWPP group. When compared to Healthy controls, a 1.6 fold higher value is observed.

Intracellular ROS was determined in CD 14^{+} monocytes and the expression of Siglec-9 was also measured in same cells by both Flow Cytometry and semi-qPCR. In this study, Dichlorofluroscein fluorescence intensity was measured for determining the level of intracellular ROS. Higher is the amount of free radicals higher will be the fluorescence in FL1 channel. Siglec-9 is reported early to be increased with response to oxidative stress and have a protective role which eventually controls the mammalian lifespan [40]. There is 2.41 fold increased intracellular ROS in monocytes which is statistically significant when undergone t-test (P>0.0001). Siglec-9 surface expression was determined by Median Fluorescence intensity and there was significantly increased surface expression of Siglec-9 observed on CD 14^{+} monocytes. Siglec-9 ELISA depicted significantly higher plasma levels of Siglec-9 protein.

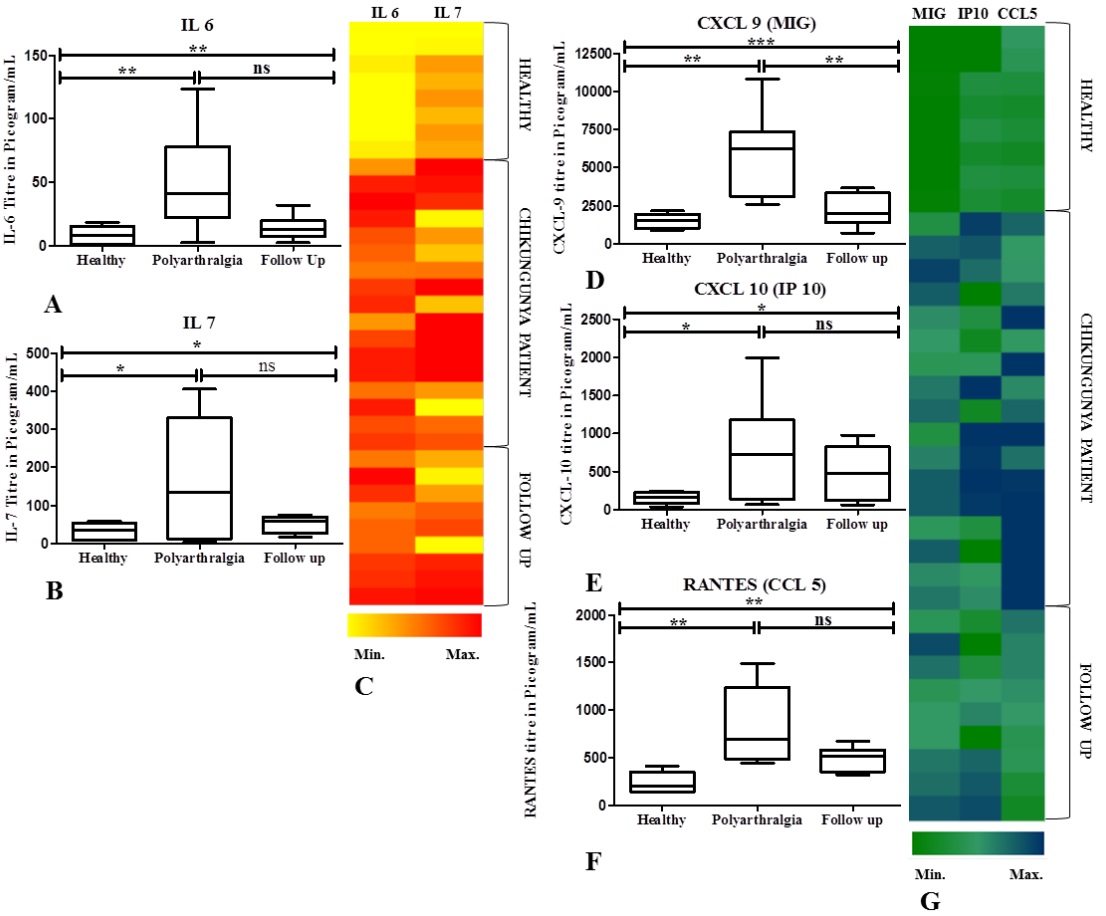


Fig 17: *Status of different Cytokines and Chemokines*

A,B,D,E,F are Box-n-Whiskers plot of IL-6, IL-7, CXCL 9, CXCL 10 and RANTES respectively in three study groups namely Healthy (Healthy Controls), Polyarthralgia (CHIK patients with Polyarthralgia) and Follow UP. Statistically significant P value of ANOVA and t-test analysis is shown by *****($P<0.05$ = *, $P<0.05$ = **, $P<0.005$ = ***). C & G are Heat maps of those Cytokines and Chemokines after normalizing titers in $\log_{10}$ scale in all the study subjects generated through Microsoft Excel 7 Software.

Cytokines are array of secreted proteins playing pivotal role in cellular crosstalk [42]. Pain is the response of nervous system after interaction with inflammatory signals [41]. Cytokines are involved in such communications and therefore provides glimpses of the

internal inflammatory manifestations [42]. Pro-inflammatory, inflammatory and anti-inflammatory cytokines are classified with respect to their roles in inflammation. Now, inflammation can be developed through many ways. It may happen immediately after interaction in between host and pathogen or may develop later due to the perturbing host immunity. Whatever may be the cause, inflammation is characterized by the levels of cytokines in peripheral blood in a regular fashion. A panel of cytokines were studied involving all the study subjects and it was observed that IL-6 and IFN-γ are significantly ($P<0.05$) elevated among the pro-inflammatory cytokines and correlates well with intracellular ROS. CXCL-9, which is a chemokines and reported to be increased in Chikungunya earlier [43] correlates well with ROS levels. Anti-inflammatory cytokine IL-10 is found to be significantly decreased in ChikWPP group similar to some previous study [44]. TGFβ1 levels are increased in the same group when compared with Healthy controls. Eventually, these levels are found to be corroborated with previous studies reporting cytokine levels in Chikungunya [41-44].

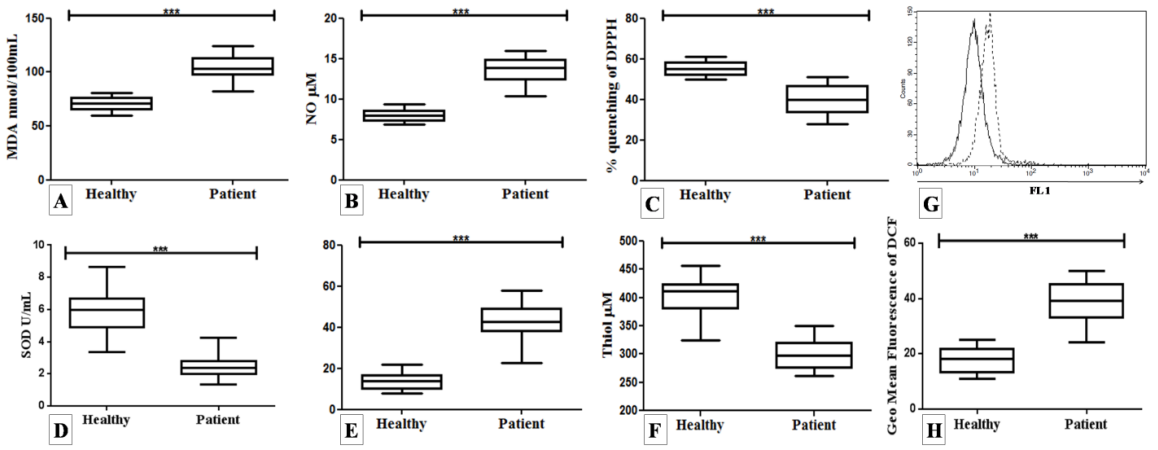


Figure 18:*Status of various oxidative stress markers in CHIK patients and healthy controls*

Figures A, B, C, D, E, F describes significant changes in the levels of MDA, NO, % quenching of DPPH, SOD, MDA/SOD and Thiol respectively among healthy donors and CHIK patients suffering from polyarthralgia. t-test was performed among two groups and $P<0.05$ was taken as significant. 18G is the representative histogram of DCF intensity in Healthy controls (solid line) and CHIK patients (dotted line). Geo Mean Fluorescence intensity of DCF in all the study subjects is analyzed statistically and represented as graph in 18H. $P<0.05$ is taken as significant after doing t-test. (n=35)

ROS is known to be the mediator of inflammation in various diseases [45] which has driven the investigators to study the same in Chikungunya induced persisting polyarthralgia. To the best of the knowledge of the authors, there is no such report till date. Chikungunya is an arboviral infection causing acute febrile illness followed by rash and debilitating joint pain. Acute viral infection causes upheavals of the immune system and results in oxidative damage [46]. But in chronic diseases there becomes a balance between damage caused by oxidative stress and its rejuvenation by the anti-oxidant system present in host system [39]. It depends on the health of the patient immune system and possibly the

potent cause behind the suffering of a group of individuals with previous Chikungunya infection to develop persisting polyarthralgia months after the initial acute febrile illness [161,166].

It was observed that younger individuals resolved their acute joint pain within 10 days of their infection in most of the cases. This observation becomes crucial, as this indicates the 'health' of the immune system and natural correlation with ageing. As some previous studies has also depicted similar observations [47,167]. It is well known that our immune system becomes weaker with the progress of time and degenerative changes may come in middle or late thirties [48]. These observations make it stronger to investigate several host factors like intracellular ROS, biochemical markers of oxidative damage along with a relatively new marker of ageing in mammals; Siglec9.

Reactive oxygen species are continuously formed within cells but their levels must be at minimal to proper working of the cellular entity [192]. In any pathological condition, these are the earliest markers of REDOX imbalance of the host. When cells produce excessive ROS, it not only destroys itself but eventually affects the cellular milieu badly [49]. It behaves like a 'double edged sword' which is essential for pathogen killing but simultaneously destroys own cells [50]. Higher level of intracellular Reactive Oxygen Species are evident in viral infection but persistence of the same after 10 days of infection indicates pathological conditions in which the host continuously produces those free radicals. After resolution of joint pain, ChikWoPP patients also shows higher ROS than controls but that possibly neutralized by anti-oxidation mechanism of the person as evident from the level of such parameters. ChikWPP patients show even higher free radicals but effective neutralization is hampered in those cases leading to destruction of joint tissue.

Studied Oxidative Parameters	Correlation with ROS in ChikWoPP	Correlation with ROS in ChikWPP
MDA nmol/100ml	r = 0.826; P < 0.05	r = 0.952; P < 0.05
SOD U/mL	r = -0.887; P<0.05	r = -0.552; P < 0.05
MDA/SOD	r = 0.882; P < 0.05	r = 0.702; P < 0.05
NO μM	r = 0.904; P < 0.05	r = 0.568; P < 0.05
Protein Carbonyls nmol/mg of protein	r = 0.881; P < 0.05	r = 0.478; P < 0.05
% quenching of DPPH	r = -0.814; P < 0.05	r = -0.441; P < 0.05
Thiol μM	r = -0.892; P < 0.05	r = -0.818; P < 0.05
Siglec-9 pg/mL	r = 0.858; P < 0.05	r = 0.732; P < 0.05
Siglec-9 MFI	r = 0.824; P < 0.05	r = 0.403; P < 0.05
Studied Cytokine titers		
IL-6 pg/mL	r = 0.878; P<0.05	r = 0.634; P<0.05
IFN-γ pg/mL	r = 0.913; P<0.05	r = 0.617; P<0.05
CXCL-9 pg/mL	r = 0.892; P <0.05	r = 0.787; P <0.05
IL-10 pg/mL	r = -0.881; P<0.05	r = 0.328; P = 0.112
TGF-β1 pg/mL	r = 0.724; P = 0.167	r = 0.631; P = 0.052

Table 5: *Correlation between Intracellular ROS and all studied parameters*

An oxidative marker like nitric oxide is an essential part of cellular REDOX signaling and any alteration from nominal healthy level indicates the cataclysms of the immune system [50]. Similar to intracellular ROS, NO levels are also higher than ChikWoPP and healthy controls. MDA is well known to determine the amount of lipid peroxidation and carbonylated proteins are of the same for protein damage [45]. Any alteration of Thiol levels point towards enzymatic dysfunction and as a whole these parameters indicate the degree of oxidative cellular damage in any individual [52]. All these parameters were found to be significantly altered in Chikungunya patients specifically speaks about the grade of oxidative imbalance of the host. In ChikWoPP group higher oxidation level is present along with sufficient amount of anti-oxidation markers. But the same levels in ChikWPP group clearly shows imbalance.

Anti-oxidant potential of peripheral blood plasma is generally studied oxidative damage markers to enquire about the quality of the redox balance [53]. In this study, Superoxide dismutase activity and DPPH radical scavenging activity was studied for the same purpose [54]. Chikungunya patients suffering from persisting polyarthralgia were found to have decreased levels of those activities [201]. It may possibly happen that the host immune system is not capable enough to overcome the oxidative stress or the amount is not enough to combat with the same. ChikWoPP group shows higher DPPH radical scavenging activity, which possibly neutralizes generated ROS [193]. In a previous study it was reported that early treatment with high dose of anti-oxidant relieved the patient from all pathological manifestation of Chikungunya [194]. So these observations indicate about possible clinical interventions in Chikungunya. MDA/SOD ratio is used to indicate oxidative imbalance in patients. Results from this study indicate significantly higher MDA/SOD ratio gives clear insight about the patient REDOX profile.

Siglecs are Sialic acid binding Immunoglobulin like lectins expressed on the surface of hematopoietic cells [52]. Siglec-9 is known to express on monocytes and reported to control the mammalian life span. In recent years there are several studies involving the status and possible role of Siglecs in infection and inflammation due to their potential role to control intracellular function as well as cell signaling. Siglec-9 surface expression indicates the cellular response to inflammation [40]. In two such previous works, Siglec-9 is reported to suppress arthritis and increased expression of the same is observed in lung airway inflammation [45]. As the Siglec-9 molecule is well related with inflammation and ageing, the investigators of this study was also curious to know its status in Chikungunya induced joint inflammation. Increased expression of Siglec-9 was similar to previous reports and establishes its possible role in inflammation [45]. Plasma Siglec-9 titers was

also found to be increased significantly which can be easily determined in patients suffering from pain and inflammation.

Cytokines and chemokines are well established markers of inflammation and some previous works reported higher levels of pro-inflammatory cytokines in Chikungunya from different parts of the world [54,199]. The investigators studied those established cytokines in a new clinical setup involving patients from different populations from previous studies and correlated these established markers with intracellular ROS in this study. IL-6 is reported as biomarker of Chikungunya, and in this study also it becomes a key marker establishing again those previous reports [42,200]. Interestingly, IL-6 titers in Chikungunya patients correlate well with intracellular ROS. This indicates that due to higher production of pro-inflammatory cytokines, inflammatory pathways are on after initial viral infection and continuously produces reactive oxygen species which is dangerous for the host. IFN-γ is a general marker of inflammation and there are many reports available about it [53]. This well-known marker also correlates well with ROS.

IL-10 levels are increased in ChikWoPP group than healthy controls but significantly lower in persisting polyarthralgia group. Previous report shows decreased regulatory T cells in Chikungunya patients with polyarthralgia and as these cells are producers of IL-10 so their low levels are obvious resulting in the imbalance of the inflammatory scenario. TGFβ1 is reported to have increased in joint inflammation and similar results are observed in ChikWPP group of this study [48,198]. Previous work also reported about increased TGFβ in Chikungunya model of mice and samples from humans [48]. But it has no significant correlation with intracellular ROS as evident from this study.

Interestingly, higher value of correlation coefficient (Pearson r) is found when correlation studies were done among Intracellular ROS and all other studied parameters in ChikWoPP group.

	Adjusted OR	**95% CI**
IL-6	1.69	0.54 to 5.28
IL-7	1.19	0.38 to 3.69
CXCL 9	1.69	0.54 to 5.28
CXCL 10	2.42	0.76 to 7.69
RANTES	2.64	0.78 to 8.88
Siglec 9	110.25	17.29 to 702.68

Table 6: *Prognostic ODDs RATIO (adjusted) of all the studied immune mediators.*

It means that higher generation of ROS resembles with other oxidative parameters, pro-inflammatory cytokines and also there is parity between high ROS and anti-inflammatory markers. In ChikWPP group, there is significant correlation, but Pearson r value is lower indicating weaker correlation. This clearly indicates that those patients suffering from persisting polyarthralgia are not capable enough to manage the generated ROS successfully which eventually leads to debilitating joint pain.

	IL-6	IL-7	CXCL 9	CXCL 10	RANTES	Siglec 9
Fischer's Exact test P Value	0.5559	1.000	0.5559	0.2380	0.2167	<0.0001
Chi-square test P value	0.3763	0.7681	0.3763	0.1404	0.1228	<0.0001

Table 7: *Association study between joint inflammation > 6 locations and all the studied immune mediators by determining Fischer's Exact Test and Chi-square test P value. P<0.05 was taken as significant.*

In conclusion it can be said that elevated levels of intracellular ROS and other oxidative damage parameters are possible cause as well as indicators of persisting polyarthralgia. Despite of poor plasma anti-oxidant potential; cells try to attenuate such an excessive amount of oxidative damage by over-expressing Siglec-9 [197]. Cytokines can be easily enumerated in peripheral blood and provides an insight about the REDOX status of the host system. Being one of the chief causes of joint inflammation altered levels of cytokines switches on oxidative damage which eventually damages host tissue causing severe pain and persisting inflammation. The age and overall 'health' of the immune system is possible other important factors in the development of post Chikungunya persisting polyarthralgia. This study describes the levels of different oxidative damage markers Chikungunya patients along with cytokines which provides insight about the disease manifestation and indicates future therapeutic targets.

To determine the free radical scavenging potential of *T. cordifolia* leaf extract, peripheral blood mononuclear cells isolated from the enrolled subjects were briefly incubated with the plant formulation. PBMC of 10^6 order was treated with ethanolic extract of *T. cordifolia* leaves in concentrations of 0.5 and 1 g/mL for 1 h at room temperature [129]. The analysis was done, and the presence of ROS was documented. P>0.05 was taken significantly in all cases. T-test was done to determine statistical significance among two groups of data and one-way ANOVA was done for the same within three groups.

In this present study, the prevalence rate of CHIK infection was calculated comprising the positive cases among the screened populations and found to be 16.82%. Patients who suffered from polyarthralgia 3 months post-infection were studied and mentioned as 'CHIK Patient' in this work. Persisting polyarthralgia associated with CHIK infection after 3 months was the key clinical determinant of this study. It shows joint involvement in a patient after 3 months of CHIK infection. An arboviral infection like CHIK elicits intense immune reaction within the host.

Several immune pathways are switched on and off to combat the consequences of viral infection. DCFDA assay revealed 2.11-fold increased intracellular Reactive oxygen species in PBMC from CHIK patients when compared to healthy individuals.

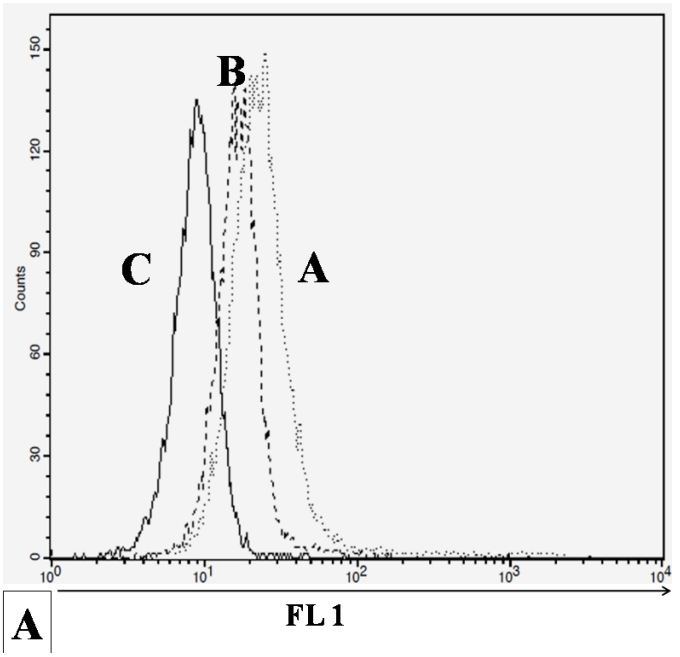


Fig 19: *Effect of Gulancha extracts on PBMC of CHIK patients and Healthy controls in Ex vivo condition.*

A is a representative histogram plot of DCF intensity after treatment with Tinospora cordifolia leaf extract in various concentrations (A = 0µg/mL, B = 0.5µg/mL, C = 1µg/mL) in a CHIK patient.

This intracellular assay directly enumerates ROS within PBMC. More than a twofold increase of intracellular ROS gives an insight into the CHIKpathophysiology [130]. Interestingly, this extract decreased intracellular reactive oxygen species in both healthy controls and CHIK patients significantly.

In healthy individuals, treatment with 0.5 g/mL Tinospora extract decreased ROS by 1.56-fold whereas, with an increased dose of 1 g/mL, there was no significant change in ROS level. Interestingly, in PBMC isolated from CHIK patients suffering from persisting polyarthralgia, intracellular ROS decreased from 37.96 ± 1.51 to 24.48 ± 0.67 after

treatment with 0.5 g/mL plant extract whereas ROS decreased from 37.96 ± 1.51 to 14.75 ± 0.66 after treatment with 1 lg/mL extract.

Free radicals are established markers of joint destruction, as those chemicals destroy the cartilage and bony tissue and degrade the synovial fluid [196]. Chikungunya is a viral infection and elicits a high number of free radicals which eventually degrades synovial joints in some patients to cause persisting polyarthralgia [133]. Taken together, CHIK infection develops intracellular ROS within the host, which might cause tissue damage leading to severe and persisting joint pain among some patients.

Conclusion and indication for future research

Enhanced CD 56^{dim} population ($P<0.0001$) is observed in Chikungunya patients having severe polyarthralgia.

These findings report for the first time 3 fold decreased expression of CD 328($p<0.0001$) in CHIK patients having Severe Joint Pain.

Siglec 7+ CD 56dim NK cell population produces little intracellular Interferon gamma but have high degranulation activity.

NKT cells are increased but Regulator T cells are decreased in patients suffering from persisting polyarthralgia due to chikungunya infection.

High titres of pro-inflammatory cytokine like IL-6 and Chemokines like CXCL 9 & CXCL 10 bear good correlation with high expression of Siglec 9 on Monocytes.

Altered expressions of Siglec7/9 have important pathophysiological roles in Chikungunya infection

Tinospora cordifolia leaf extract can scavenge the intracellular ROS developed in peripheral blood cellsof chikungunya patient and that is useful to ameliorate the oxidative damage. This finally provides insights towards further study.

As Siglecs can be used as prognostic markers so, the therapeutic effectes of Tinospora cordifolia can be physiologically measured by the Siglec expression.

This study strongly suggests that Tinospora cordifolia leaf extract can be used as a therapeutic agent for patients suffering with persisting polyarthralgia as well as Siglecs can be used as a prognostic biomarker of disease severity in case of Chikungunya induced persisting polyarthralgia.

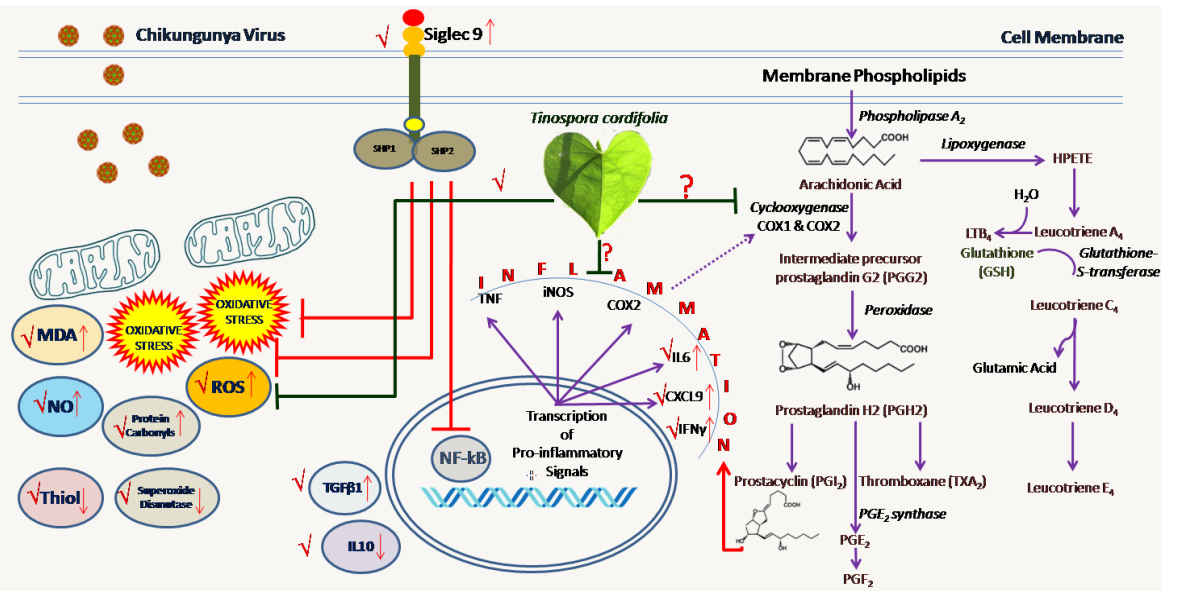


Fig 20: *Pictorial representation of future work that can be done from this thesis work in future*

ॐ पूर्णमदः पूर्णमिदं पूर्णात्पूर्णमुदच्यते।
पूर्णस्य पूर्णमादाय पूर्णमेवावशिष्यते॥
ॐ शान्तिः शान्तिः शान्तिः॥

Om Puurnnam-Adah Puurnnam-Idam Puurnnaat-Puurnnam-Udacyate |
Puurnnasya Puurnnam-Aadaaya Puurnnam-Eva-Avashissyate ||
Om Shaantih Shaantih Shaantih ||

Shukla YajurVeda, Isa Upanishad, Shanti Mantra

Om, That (Outer World) is Purna (Full with Divine Consciousness); This (Inner World) is also Purna (Full with Divine Consciousness); From Purna is manifested Purna (From the Fullness of Divine Consciousness the World is manifested); Taking Purna from Purna, Purna indeed remains (Because Divine Consciousness is Non-Dual and Infinite),
Om, Peace, Peace, Peace.

পরব্রহ্ম পূর্ণ, নামরূপ ব্রহ্মও পূর্ণ; পূর্ণ থেকে পূর্ণ উদ্গত হন; পূর্ণের পূর্ণত্ব বিদ্যা সহায়ে গ্রহণ করলে পূর্ণই (পরব্রহ্মই) অবশিষ্ট থাকেন। ওঁ আধ্যাত্মিক, আধিদৈবিক ও আধিভৌতিক – এই ত্রিবিধ বিঘ্নের বিনাশ হোক।

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Altered Profile of Regulatory T Cells and NKT Cells As Characteristic of Chikungunya-Associated Polyarthralgia

Nilotpal Banerjee, Bibhuti Saha & Sumi Mukhopadhyay

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ঠাকুরমার ঝুলিতে ডেঙ্গি, চিকুনগুনিয়া

দু’টো রোগই আজকের নয়। বাঙালির রূপকথার গল্পেও কিন্তু তাদের প্রবল প্রতাপ। **নীলোৎপল বন্দ্যোপাধ্যায়**

গত কয়েক বছর ধরে কলকাতা তথা সারা দেশেই প্রবল মাথাব্যথা হয়ে উঠেছে ডেঙ্গি। এ কি কোনও নতুন রোগ? আগে তো নাম শুনিনি, এত ঘটাপটা দেখিনি? তবে পঞ্চাশের দশকে, বিশেষত যারা দমদম, বেলগাছিয়া, বরানগরে থাকতেন তারা হয়তো মনে করতে পারবেন এক জ্বরের কথা, যে জ্বরের সঙ্গে ছিল গাঁটে গাঁটে অসহ্য যন্ত্রণা।

রবীন্দ্রগানের প্রখ্যাত শিল্পী প্রসাদ সেনের কাছে শুনেছিলাম তাঁর কলেজবেলার কথা। ১৯৪১ সাল, সদ্য শান্তিনিকেতনে এসেছেন তিনি, সঙ্গী বন্ধু অশোকতরু বন্দ্যোপাধ্যায়। দু’জনেই তরুণ, সুস্বাস্থ্যের অধিকারী। এমন সময় শান্তিনিকেতনে হল ‘ডেঙু’। এই উচ্চারণই বলেছিলেন। হোস্টেলসুদ্ধু সবাই ‘ডেঙুজ্বর’-এ কাবু। প্রায় হামাগুড়ি দিয়ে হাঁটতেন।

১৯৫২-তে আফ্রিকার মোজাম্বিক ও তানজানিয়ার সীমান্তে মাকোন্ডে উপত্যকায় শনাক্ত হল নতুন ভাইরাস। স্থানীয় জনজাতির ভাষায় নাম ‘চিকুনগুনিয়া’। মূল শব্দ ‘কুনগুনিয়ালা’, অর্থ ‘যা বেঁকে যায়’। তা থেকেই ভাইরাসটির নাম হয়েছে চিকুনগুনিয়া। কারণ, এই জ্বর হলে রোগী সোজা হয়ে হাঁটতে পারে না। হাঁটতে হয় খুঁড়িয়ে, লেংচে। তা হলে শান্তিনিকেতনে ওঁদের যে ‘ডেঙু’ হয়েছিল, তা কি সত্যি ডেঙ্গি, না চিকুনগুনিয়া? এই ভাইরাসেরও বহু নাম— চিকেন গুনিয়া, চিকেন গিনি।

বাংলায় ‘আরবোভাইরাস’ অর্থাৎ মশাবাহিত ভাইরাসদের মধ্যে তিনটের প্রকোপ বেশি— ডেঙ্গি, চিকুনগুনিয়া আর জাপানি এনসেফালাইটিস (জে ই)। জে ই মূলত আক্রমণ করে শিশুদের। মস্তিষ্কের সংক্রমণ বলে বাচ্চারা অজ্ঞান হয়ে যায়। মেরুদণ্ডের রস নিয়ে পরীক্ষা করতে হয়। মৃত্যুহারও বেশি। তবে গত ক’বছরে যে হারে ডেঙ্গিতে মানুষ মারা যাচ্ছে তাতে মনে পড়ে যায় রবীন্দ্রনাথের ছোটবেলার কথা। তখন কলকাতায় এত লোক মরছিল যে ঠাকুর পরিবারের সবাই পানিহাটিতে আশ্রয় নেন।

তখন ডিএনএ-আরএনএ আবিষ্কার হয়নি। তাই জানা যায়নি, ডেঙ্গির কোন কোন সেরোটাইপ তখন কলকাতায় সার্কুলেট করছিল। এখন জানা গিয়েছে, কলকাতায় ডেঙ্গির ২ আর ৪ সেরোটাইপে মানুষ আক্রান্ত হচ্ছেন বেশি, সেগুলোরই মৃত্যুহার বেশি। চার দিকে ডেঙ্গি নিয়ে হইচই, তার পাশে আলোচনাতে প্রায় আসেই না চিকুনগুনিয়া। যেহেতু (সৌভাগ্যক্রমে) চিকুনগুনিয়ায় মৃত্যু হয় না বললেই চলে, তাই মিডিয়ার উৎসাহ নেই। তবে একেবারেই হয় না বললে ভুল হবে। এগুলো আরএনএ ভাইরাস, যে কোনও দিন রূপ বদলে ফিরে আসতে পারে প্রাণঘাতী হয়ে। ডেঙ্গির পাশাপাশি চিকুনগুনিয়া নিয়েও মানুষের সচেতনতা জরুরি।

দুঃখের কথা, চিকিৎসকরাও তেমন সচেতন নন। অনেক ক্ষেত্রেই দেখা যায়, ‘আরবোভাইরাল’ জ্বরের লক্ষণ দেখলেই ডাক্তাররা ডেঙ্গির এনএস১ বা আইজিএম এলাইজা করতে দিচ্ছেন। রিপোর্ট ভাল হলে রোগীকে ছেড়ে দিচ্ছেন। পরে যখন রোগী গাঁটের প্রচণ্ড যন্ত্রণায় ভুগছেন, তখন পাড়ার ওষুধের দোকান থেকে ব্যথা কমানোর ওষুধ কিনে খেয়ে শরীরের সাড়ে সর্বনাশ করছেন। চিকিৎসক যদি ডেঙ্গির পাশাপাশি চিকুনগুনিয়ারও পরীক্ষা করালে হয়তো এই বিপদ কিছুটা হলেও এড়ানো যেত। সেখানেও সমস্যা। চিকুনগুনিয়া আইজিএম এলাইজা পশ্চিমবঙ্গে হাতে-গোনা বেসরকারি ল্যাব-এ হয়। সরকারি জায়গাতে খুব কমই হয় এই পরীক্ষা। যেহেতু মৃত্যুহার কম, তাই নজরও কম!

কিন্তু কথা হল, চিকুনগুনিয়াও কম ভয়ানক নয়। ডেঙ্গিতে ভুগলে মোটামুটি ১৫টা কাজের দিন নষ্ট। কিন্তু চিকুনগুনিয়া হলে? রোগীদের আকুতি শুনেছি, “কবে অফিস যেতে পারব? আমার যে প্রাইভেট অফিস, চাকরি চলে যাবে!” শুধু ২০০৬ সালেই চিকুনগুনিয়ার জন্য অর্থনীতির হিসেবে ক্ষতি হয়েছে ভারতীয় মুদ্রায় ৩৯ কোটি ১০ লক্ষ। ‘ডিসেবিলিটি এডজাস্টেড লাইফ ইয়ারস’ বা ‘ডালি’ একটা বিশেষ সূচক, যা দিয়ে বোঝা যায় কোনও রোগের কারণে এক জন মানুষের কতটা সময় নষ্ট হল। সেই হিসেবে শুধু ২০০৬ সালেই চিকুনগুনিয়ার জন্য ভারতে ২৫,৫৮৮ টি ‘ডালি’ নষ্ট হয়েছে। মানবসম্পদের কী অপূরণীয় ক্ষতি! এখন শনাক্তকরণের হার বেড়েছে, ন্যাশনাল ইন্সটিটিউট অব ভাইরোলজি-তে সঠিক এলাইজা কিট তৈরি হয়েছে। তবু চিকুনগুনিয়া নিয়ে সেই সচেতনতা কোথায়?

আফ্রিকান নামটা খটোমটো, তবে এক্ষুনি ‘হাড়মুড়্‌মুড়ী ব্যারাম’-এর নাম করলে অনেকেই বলবেন, এ রোগের কথা শুনেছি তো, ‘ঠাকুরমার ঝুলি’-তে! দক্ষিণারঞ্জন মিত্র মজুমদারের ‘ঠাকুরমার ঝুলি’-তে ছিল ‘সোনার কাটি রূপার কাটি’ গল্প। রাজপুত্র, মন্ত্রীপুত্র, কোটালপুত্র, সদাগরপুত্র দেশ ছেড়ে বেরিয়ে, তেপান্তরের মাঠ পেরিয়ে পৌঁছন বনে। বনের ভূপ্রকৃতি আর গাছপালার বর্ণনা শুনলে মনে হয়, এ বাংলারই বন, ‘কেবল পাথর কাঁকর আর বড় বড় বট পাকুড় তাল শিমুলের গাছ!’ খাবারের খোঁজ করতে করতে সামনেই হরিণের মাথা দেখা, এবং তা কাটতে গিয়ে রাজপুত্র ছাড়া বাকিরা সবাই তাদের ঘোড়াসহ রাক্ষসীর পেটে। রাক্ষসী রাজপুত্রকেও খেতে চায়। রাজপুত্র আর এক রাজার রাজত্বে পালিয়ে এসে, এক আমগাছের ভিতর লুকিয়ে থাকে, আর রাক্ষসী আমগাছের নীচে পরমাসুন্দরী সেজে কাঁদতে থাকে। সে দেশের রাজা বনে এসে কান্না শুনে সেই সুন্দরীকে বিয়ে করলেন। এই বার শুরু রাক্ষসীর খেলা। সেই রাজপুত্রকে খাওয়ার জন্য বা তাকে মারার জন্য গল্পে কিসের অবতারণা হয়, সেটাই আসলে দেখার। দক্ষিণারঞ্জন সারা বাংলা খুঁজে, দিদিমা-ঠাকুরমাদের মুখের গল্প সংগ্রহ করে, অবিকল সেই মুখের ভাষাতেই রচনা করেছিলেন ‘ঠাকুরমার ঝুলি’।

এ বার ঠাকুরমাদের মুখের ভাষায় সেই হাড়মুড়্‌মুড়ী ব্যারামের ‘ইটিওলজি’ দেখা যাক। রাক্ষসী ‘সাত বাসি পান্তা, চৌদ্দ বাসি তেঁতুলের অম্বল খাইয়া অসুখ বানাইয়া বসিল।’ কী অসুখ? গা-গরম। সাবক্লিনিক্যাল ফিভার। মানে ওই একটু জ্বর আর কী। এ রকম বাসি খাবার খেয়ে পেটের রোগ হওয়াই স্বাভাবিক। টাইফয়েডে তা-ই হয়। আর তেঁতুলের জলের সঙ্গে জ্বরের একটা যোগাযোগ বাংলার মানুষের মনে চিরকালই রয়েছে। পশ্চিম বাংলার মানুষেরা খুব পুরনো তেঁতুলের টক খেতে ভালবাসেন। কথামৃতে পাই: ‘জ্বর বিকারের রোগীর ঘরে তেঁতুল আর জলের জালা’। রানি যে রোগী সাজতে চাইছেন তার প্রথম লক্ষণ, হালকা জ্বর। আর? ‘রাক্ষসী বিছানার নীচে শোলাকাটি পাতিল। পাতিয়া সেই বিছানায় শুইয়া রঙ্গীমুখ ভঙ্গী করিয়া...’ এখানে একটু থামা দরকার। ঠাকুরমা কিন্তু বলছেন না, ‘রঙ্গী মুখভঙ্গী করিয়া’। বলছেন ‘রঙ্গীমুখ ভঙ্গী করিয়া’। মানে রাঙা মুখে নানা বিকৃতি করে, উঃআঃ করে। এ বার আসুন চিকুনগুনিয়ায়। জ্বরের সঙ্গে রোগীর (বিশেষত মেয়েদের) নাক আর গালের দু’পাশ লাল হয়ে যায়। হেমারেজিক ডেঙ্গির ক্ষেত্রেও কিন্তু জ্বরের সঙ্গে সঙ্গে মুখ লাল হয় না। হয় জ্বরের কিছু দিন বাদে, যখন ‘হেমারেজিক ম্যানিফেস্টেশন’ শুরু হয়। চিকুনগুনিয়ায় মুখ লাল হয়ে মুখে র‍্যাশ দেখা যায়, শরীরের অন্য জায়গার মতোই। তবে লাল হওয়াটা মুখেই সবচেয়ে বেশি দেখা যায়।

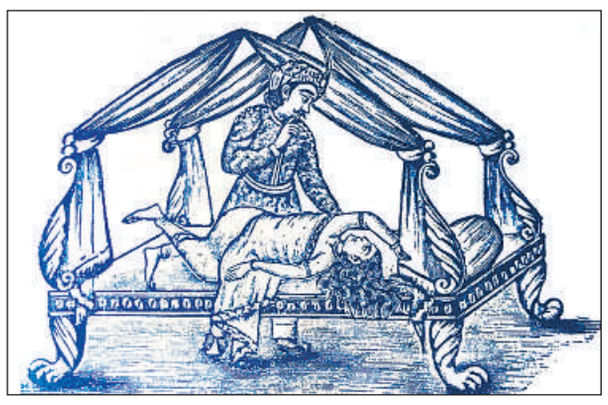
আরও পড়ি। ‘একবার ফিরে এ-পাশ, একবার ফিরে ও-পাশ’। রাক্ষসী রানির শরীরের চাপে শোলাকাঠি ভাঙে আর রাজা ভাবেন, রানির হাড়গুলো মুড়মুড় করছে। ভাইরাল জ্বরে রোগীর অস্বস্তির কী সুন্দর উপস্থাপন: ‘রাজা আসিয়া দেখেন, রাণী খান না, দান না, শুক্‌ন ঘরে জল ঢালিয়া চাঁচর চুলে আঁচড় কাটিয়া, রাণী শুইয়া আছেন।’ চিকুনগুনিয়াতেও রোগী সারা শরীরে অস্বস্তি অনুভব করেন। চিকিৎসা পরিভাষায় যাকে ‘সাইন’ ও ‘সিম্পটম’ বলা হয়, রাক্ষসী রানি দুয়েরই অবতারণা করেছেন সফল ভাবে। শেষে ডায়াগনোসিসও নিজেই করে ‘কোঁকাইয়া কোঁকাইয়া’ বলছেন ‘আমার হাড়মুড়্‌মুড়ীর ব্যারাম হইয়াছে’ (কারণ ওকে তো রাজপুত্রকে মারতে হবে)। শুনে ‘রাজা বলিলেন— হায় কি হইবে! রাজ্যের রাজা যখন রোগের নাম শুনে বলেন ‘কী হবে’, তখন এটা নিশ্চিত যে এর ওষুধ জানা নেই। আর রোগটাও সে রাজ্যে খুব সাধারণ, কারণ রাজা বলছেন না, বদ্যি ডেকে পরীক্ষা করাই তোমার কী রোগ হয়েছে। মানে, এ রোগের লক্ষণ ঠাকুমা-দিদিমাদের ভালই জানা ছিল। ‘কত ওষুধ, কত চিকিৎসা; রাণীর কি যে-সে অসুখ? অসুখ সারিল না!’ রাক্ষসী তো আসলে রোগের অভিনয় করছে। দু’টো ব্যাপার স্পষ্ট: এ রোগের কোনও চিকিৎসা ছিল না, আর এ রোগ বহু দিন ভোগায়। ‘পারসিস্টিং পলিআরথ্রালজিয়া’র সঙ্গে মিলে যাচ্ছে? মানে— ছ’মাস থেকে এক বছর গাঁটে গাঁটে যন্ত্রণা।

তখনকার দিনে আরও বহু রোগ জানা ছিল সবার। তার কিছু না কিছু চিকিৎসাও জানা ছিল। কিন্তু যে রোগ বাংলায় প্রায়ই হয় অথচ ওষুধ নেই, সেটাই রানির অভিনয়ের জন্য আদর্শ রোগ। এ বার মোক্ষম কথা বলল রাক্ষসী: ‘ওষুধে তো কিছু হইবে না, বনের সেই আমগাছ কাটিয়া তাহার তক্তার ধোঁয়া ঘরে দিলে তবে আমার ব্যারাম সারিবে।’ একেবারে অ্যাসিড টেস্ট। মানে এ ব্যাপারটা জানা, ওষুধে এ রোগ সারে না, কিন্তু ঘরে ধোঁয়া দিলে প্রকোপ কমে। তা হলে কি মশা থেকে এ রোগ হয়, সে ধারণা ছিল? নিশ্চয়ই ছিল, তাই রাজপুত্রকে মারার ফন্দিতে রাক্ষসী এমন রোগের রোগী সাজলেন যার কোনও চিকিৎসা নেই, অথচ এটা জানা যে ঘরে ধোঁয়া দিলে তার প্রকোপ কমে!

এই গল্পেই পরে দেখি ওই রাক্ষসীর মা, বুড়ি ‘আয়ীমা’-কে। সকাল হতেই সে ‘কাজে’ বেরিয়ে যায়, ফিরে রাজকন্যাকে বলে পায়ে প্রদীপের তেল মালিশ করে দিতে। বুড়ি মানুষ, বাতের ব্যথা থাকাই স্বাভাবিক। আমরা যাকে বলি গেঁটে বাত। কিন্তু যে বুড়ো মানুষের বাত, সে কি সারা দিন ‘হাঁপাইয়া হুঁপাইয়া’ খাবারের সন্ধান করতে পারে?

বয়স্ক বাতের রোগী মোটেই দৌড়ঝাঁপ করতে পারেন না। বরং চিকুনগুনিয়া রোগীদের দেখেছি, সারা দিন কাজের পর ঘরে ফিরলে তাঁরা ব্যথাটা বেশি টের পান। ক্লান্ত অস্থিসন্ধি প্রদাহ জানান দেয়। সারা দিন কাজের তোড়ে ব্যথা টের পান না। স্টেরয়েড দিয়ে ব্যথা কমানোর কথা মনে পড়ে যাচ্ছে? একটু পুরনো চিকুনগুনিয়া রোগীদের এই কথাই আমরা বলতে শুনি। বরং বাত রোগীরা ‘মর্নিং স্টিফনেস’-এর কথা বলেন। ঘুম থেকে উঠে গাঁটে ব্যথা থাকলে কিন্তু রাক্ষসী বুড়ি আয়ীমা কাজে বেরোতে পারত না। তাই সন্ধ্যায় ফিরে এসে বলে ‘হ্যাঁ লো হ্যাঁ নাঁত্‌নি, পাঁ-টা তোঁ কঁট্ কঁট্‌ই কচ্ছে। একটু টিপিয়া দিঁবি।’ তর্কের খাতিরে কেউ অস্টিওআরথ্রাইটিস-এর কথা তুলতে পারেন। এটুকুই বলার, সেই রোগীরা দৌড়ঝাঁপ করতে পারেন না। অতএব বোঝাই যাচ্ছে, বুড়ির কিছু কাল আগে হাড়মুড়্‌মুড়ীর ব্যারাম হয়েছিল। সেই ব্যথায় তিনি এখনও ভুগছেন। আর তার মেয়েটি মায়ের রোগেরই অভিনয় করছে। এই গল্পে আমরা ‘অ্যাকিউট’ ও ‘ক্রনিক’, দু’রকম চিকুনগুনিয়ারই বর্ণনা পাচ্ছি।

‘শিবের গীত’-এর মাহাত্ম্য বোঝা গেল? বাংলায় প্রাচীনকাল থেকেই চিকুনগুনিয়ার প্রকোপ ছিল। হালে আফ্রিকায় ভাইরাসটিকে ডেঙ্গির থেকে জেনেটিক্যালি আলাদা করা গেছে। আগে পশ্চিমের দেশগুলোতে ডেঙ্গিকেই ‘বোন-ব্রেকিং ফিভার’ বলা হত। সেই রোগীরা ডেঙ্গির নয়, চিকুনগুনিয়ার শিকার ছিলেন। এটা পরিষ্কার যে মশাবাহিত রোগ বাংলায় নতুন নয়। আগে আধুনিক প্রযুক্তি ছিল না বলে রোগের ঠিক শনাক্তকরণ সম্ভব ছিল না। মশাবাহিত প্রতিটি রোগ সম্পর্কেই জনসচেতনতা দরকার। আমরা ক্রান্তীয় অঞ্চলের মানুষ, ‘রেতে মশা দিনে মাছি’ নিয়েই আমাদের বাস করতে হবে। দরকার শুধু ঠিক জ্ঞান, আর ঠিক রোগের ঠিক চিকিৎসা। সঙ্গে মশারির নিত্য ব্যবহার।



অসুখ: ‘হাড়মুড়্‌মুড়ীর ব্যারাম’, মানে চিকুনগুনিয়ার ‘অ্যাকিউট’ অবস্থা। উপরে, ‘পাঁ-টা কঁট্‌কঁট্ কচ্ছে’। চিকুনগুনিয়ার ‘ক্রনিক’ অস্থিসন্ধি যন্ত্রণা

বাইজির নাচে আবিরে ফুটে উঠত পদ্মফুল

সেই সঙ্গে রুপোর পিচকিরিতে সুগন্ধি রং। ঠাকুরদালানে গহরজান, মালকাজান। কলকাতার বনেদি বাড়ির দোল। **বিভূতিসুন্দর ভট্টাচার্য**

তিনশো বছর আগে এক বসন্তদিনে কয়েক জন ইংরেজ যুবক গঙ্গার তীরে ঘুরতে বেরিয়েছিলেন। কলকাতা তখন গণ্ডগ্রাম। সে দিন ছিল দোল। গঙ্গার তীর থেকে গ্রামের ভিতরে হাঁটতে হাঁটতে তাঁদের কানে এল গানের এক অদ্ভুত সুর। সেই সুরের টানে তাঁরা পৌঁছলেন এক দিঘির পাড়ে। বিশাল দিঘির দু’দিকে দু’টি মঞ্চ। একটিতে রাখা গোবিন্দজি, অন্যটিতে রাধিকার বিগ্রহ। মাঝখানে দোল খেলা চলছিল। দিঘির উত্তর পাড়ে রাধাবাজারে স্তূপ করে রাখা আবির। পথঘাট, দিঘির জল লাল।

গোপিনীদের নাচগান আর চারপাশের পরিবেশ সেই সাহেব যুবকদের প্ররোচিত করেছিল। এই গোপিনীরা যে আসলে পুরুষ, ভাবতে পারেননি তারা। প্রাচীন গ্রিসের ‘স্যাটারনালিয়া’র সঙ্গে মিল খুঁজে পেয়ে তারা ভেবেছিল এ বুঝি ‘কামোৎসব’। ভিড়ের মধ্যে ঢোকার চেষ্টা করতেই, নেটিভরা তাদের বাধা দিলেন। বাধা না মানায় সাহেবদের ভাগ্যে চড়থাপ্পড়ও জুটেছিল!

এ তো গল্প। পরে ঔপনিবেশিক প্রভাবে যে নব্য বাবুসমাজ গড়ে উঠেছিল, হোলি উৎসব তাতে প্রাধান্য পেয়েছিল। এই উদ্‌যাপনও ছিল বিচিত্র। রুপোর রেকাবি থেকে আতর মেশানো আবির উড়িয়ে, রুপোর পিচকারির সুগন্ধী রঙিন জল ছিটিয়ে, গেলাসে রঙিন পানীয় হাতে, হোলির ঠুমরি বা দাদরার তালে ইয়ারদের নিয়ে মাতাল হয়ে ওঠার আনন্দটা কেমন, বাবুরাই জানতেন! বাবু কালচারের হোলির বিবরণ দিতে গিয়ে কবি ঈশ্বর গুপ্ত লিখেছিলেন ‘ক্রমেতে হোলির খেলা, নবীনা নাগরী মেলা, ছুটে মুটে যায় এক ঠাঁই। উড়ায় আবির যত, কুড়ায় লোকেতে কত, জুড়ায় দেখিলে মন তায়। ঢালিয়া গোলাপ জল, অঙ্গ করে সুশীতল, মাঝে মাঝে হয় কোলাহল।’

‘কলিকাতার ইতিবৃত্ত’ বইতে প্রাণকৃষ্ণ দত্ত লিখছেন, ‘সে সময়ে কোনও ব্যক্তির বেদাগ বস্ত্র থাকিত না, দলে দলে মিছিল বাহির হইতেছে, পিচ্‌কারি ও আবিরে পথঘাট ঘরবাড়ি লালে লাল হইয়া যাইতেছে।’ চিৎপুর অঞ্চলের দোল সম্পর্কে লেখা: ‘মিছিলওয়ালারা সুশ্রাব্য ও অশ্রাব্য গীতিতে পাড়া মাতাইয়া এবং নরনারী যাহাকে সম্মুখে পাইত, তাহাকে আবির ও পিচকারিতে ব্যতিব্যস্ত করিয়া চলিয়া যাইত। এমন অশ্রাব্য গীত এবং কুৎসিত সং প্রকাশ্যে পথে বাহির করিতেন যে, এখনকার লোকে তাহা কল্পনা করিতে পারে না। কর্তারা কিন্তু তাহা লইয়া আমোদ করিতেন। গৃহিণীও বালক-বালিকাদের সহিত শ্রবণ করিতেন।’

প্রাচীন ভারতে বসন্তে উদ্‌যাপিত হত মদনোৎসব ও কামমহোৎসব। কামসূত্রে, ‘রত্নাবলী’ বা ‘মালতী মাধব’ নাটকে এর উল্লেখ আছে। একাদশ শতকে আল বিরুনির ভারত বিবরণেও এর উল্লেখ রয়েছে। উৎসবে পুজো হত মদন ও রতির। তবে তা হত চৈত্র মাসে, ফাল্গুনে নয়। গবেষকদের মতে, সাবেক কলকাতার দোল উৎসব প্রাচীন মদনোৎসবের আদলে গড়া। সে কালেও দোলে চটুলতা ছিল। প্রমাণ মেলে ইতিহাসের পাতায়।

দোল উপলক্ষে বাবুদের বাড়িতে পুজো হত, তৈরি হত মিষ্টি, শরবতও। কিছু কিছু পুরনো পরিবারে আজও এই সব রীতি দেখা যায়। শোভাবাজার রাজপরিবারের বড় তরফে গৃহদেবতা রাধাগোবিন্দজিউয়ের দোল হয় দোলের পরের দিন প্রতিপদে। দোলের দিন সন্ধ্যায় হয় চাঁচর। পর দিন বিশেষ পুজো। দোলের আগের দিন হয় নারায়ণের চাঁচর। আগে সন্ধেবেলা ঠাকুরদালানে বসত গানের আসর। গানের শেষে উড়ত ফাগ।

মুক্তারামবাবু স্ট্রিটে রামচাঁদ শীলের পরিবারে গৃহদেবতা দামোদর জিউয়ের দোল উৎসবে গান গাইতেন গহরজান, মালকাজান। এখানকার দোলে গান রচনা করেছেন গিরিশ ঘোষও। রাজা রাজেন্দ্র মল্লিকের মার্বেল প্যালেসেও দোল হয়ে আসছে অতীতের ধারা বজায় রেখেই।

ব্যতিক্রমী ছিল জোড়াসাঁকোর ঠাকুর পরিবার। গুণেন্দ্রনাথ ঠাকুরের উদ্যোগে গোলাপজলের পিচকিরি, কাচের গড়গড়া, ফুল দিয়ে বৈঠকখানা সাজানো হত। প্রায় আধ হাত উঁচু আবিরের ফরাসের উপর বিছিয়ে দেওয়া হত পাতলা কাপড়, তাতে ফুটে উঠত রক্তিমাভা। গানের আসর বসত, তানপুরা হাতে আসতেন অক্ষয় চৌধুরী এবং শ্যামসুন্দর। গুণেন্দ্রনাথের সামনেই থাকত গোলাপজলের পিচকিরি। কাচের গড়গড়ায় টান দিলে দেখা যেত তার মধ্যে গোলাপ পাপড়ির ওঠানামা।

কলকাতার বেশ কিছু বনেদি পরিবারের নাচঘরে বা বৈঠকখানায় বিছিয়ে দেওয়া হত পুরু আবির। উপরে পাতা হত পাতলা কাপড়। নর্তকীর নাচ শেষে যখন কাপড়টি তুলে নিলে দেখা যেত, নর্তকীর পায়ের চাপে আবিরের উপরে ফুটে উঠেছে পদ্মফুল, আরও কত নকশা।

নজর টানত হোলির গানও। রেকর্ড কোম্পানি থেকে প্রকাশিত হত নতুন রেকর্ড। পঞ্চাশের দশকের শেষ পর্যন্ত বজায় ছিল এই ট্রাডিশন। শিল্পী-তালিকায় কে না ছিলেন— গহরজান, মৈজুদ্দিন খান, জোহরাবাই থেকে শুরু করে আখতারিবাই, বড়ে গুলাম আলি খান, কমলা ঝরিয়া, কৃষ্ণচন্দ্র দে, যূথিকা রায়।

দোলসন্ধ্যা: শোভাবাজার রাজবাড়িতে সুসজ্জিত রাধাগোবিন্দজিউ

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REVIEW ARTICLE

Viral glycoproteins: biological role and application in diagnosis

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Abstract The viruses that infect humans cause a huge global disease burden and produce immense challenge towards healthcare system. Glycoproteins are one of the major components of human pathogenic viruses. They have been demonstrated to have important role(s) in infection and immunity. Concomitantly high titres of antibodies against these antigenic viral glycoproteins have paved the way for development of novel diagnostics. Availability of appropriate biomarkers is necessary for advance diagnosis of infectious diseases especially in case of outbreaks. As human mobilization has increased manifold nowadays, dissemination of infectious agents became quicker that paves the need of rapid diagnostic system. In case of viral infection it is an emergency as virus spreads and mutates very fast. This review encircles the vast arena of viral glycoproteins, their importance in health and disease and their diagnostic applications.

Keywords Viral glycoprotein · Immunodiagnostics · Biomarker · Viral pathogenesis

Introduction

Being an obligate intracellular parasite [32], virus is the most deadly microbe to be dealt with. Globally it accounts for extremely high morbidity and mortality throughout the age groups of people [3, 62]. Thousands of new viral strains are discovered till date affecting people producing a huge global burden of viral infections resulting immense challenge towards healthcare system [9, 20, 48]. With the capability of fast mutation, viruses affect the host cells with new and newer mechanisms. So to detect them at the earliest, there is an extreme need of dynamic diagnostic system.

Glycans are major components of the outermost surface of viruses. Thus, majority of the interactions of viral pathogens with their hosts are influenced by the pattern of glycans and glycan-binding receptors that each expresses. [5, 95, 98] Glycans are most complex biomolecules due to extensive branching of carbohydrates, and a variety of glycoproteins have been identified in human viral pathogens. These pathogenic glycans either virus encoded or host derived usually elicit high humoral responses in human body [34]. These virus specific high levels of glycan specific antibodies have been exploited to develop novel diagnostic assays.

Viral diagnostic tests can be broadly classified into three categories in general. Those are direct detection, indirect examination (virus isolation), and by serology. In case of direct detection, the clinical sample is examined directly to identify any presence of virus particles, virus antigen or viral nucleic acids. In case of indirect examination, the sample has to be added into cell culture, eggs or animals to grow the virus in vitro. This is known as virus isolation. Serology always constitutes the bulk of the work of any virology laboratory, especially in overpopulated third world countries. Serological diagnosis is generally made by detecting titres of antibody in infection [8]. Generally, the majority of common viral infections are diagnosed by serology [86]. Viruses can be directly detected through electron microscopy. It can also be enumerated by molecular biological techniques like PCR/RTPCR by detecting viral genomes. These techniques are extremely

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useful but are technically demanding, costly and require skilled personnel. On the other hand, indirect detection by virus isolation is dependent on cell culture techniques. The major problem of cell culture is it takes a long time (up to 4 weeks). Also, the sensitivity is poor and depends on many factors, such as the specimen condition and the condition of the cell line. Cell cultures are also very susceptible to microbial contamination and toxins present in the specimen. Also, many viruses do not grow at all in cell culture e.g. Hepatitis B and C, viruses causing diarrhea, parvovirus etc. Serology is the mainstream of viral diagnosis [8, 44]. With increase in the growth of sophisticated immunoassay techniques, effective viral immunodiagnostic assays are now available in the market [13, 91, 96].

The detection of structural glycoproteins of viruses or early glycoprotein antigen formation in the host due to viral infection or the quantification of titres of antibodies against viral antigenic glycoprotein is an emerging discipline in viral immunodiagnostics [47]. The detection of these structural glycoproteins of viruses is done by lectins or monoclonal antibodies acting as probe or by measuring the titres of host antibodies against antigenic glycoprotein.

There are several good review works on viral glycoproteins. Namely, the work of Kazuya I.P.J. Hidari and Takashi Suzuki on Glycan receptor in influenza Virus [43]. Yuan et al. [116] worked on receptor glycoprotein interaction in *Zaire Ebola Virus* (*ZEV*). This review attempts to conglomerate the importance of glycoprotein in widely studied viral infection and their application in diagnosis.

Viral glycoproteins

A fully assembled infectious virus is known as virion. The simplest virions consist of two basic components, namely nucleic acid (single- or double-stranded RNA or DNA) and a capsid, which is a protein coat, functions as a shell to protect the viral genome from nucleases. This capsid comes into play during infection to attach the virion to specific receptors exposed on the prospective host cell. Capsid proteins are coded by the viral genome. Due to its limited size, the genome codes for only a few structural proteins (besides non-structural regulatory proteins involved in virus replication). Capsids are formed as single or double protein shells and consist of only one or a few structural protein species. Therefore, multiple protein copies must self assemble to form the continuous three-dimensional capsid structure [35]. The structural viral proteins are extremely important to the virus, so as to facilitate the transfer of the viral nucleic acid from one host cell to another. The proteins determine the antigenicity of the virus. Host's primary immune response is directed against the antigenic determinants of these proteins rather glycoprotein in major cases.

There are enveloped Viruses and these envelopes are made up of either lipid or glycoprotein. Viral envelopes mainly consist of Envelope proteins (E), Membrane proteins (M) and Spike proteins (S) [24]. Lipid envelopes are derived from the host cell. Whereas the envelope glycoproteins are virus encoded. However, there are sugars attached to the viral glycoproteins which often reflect the host cell that harboured the virus. The surface glycoproteins of an enveloped virus attach the virion to a target host cell by properly interacting with a cellular receptor [22]. Structural biological analysis of viral envelope glycoproteins reveals that viruses have wide range of folds to facilitate their attachment with proper host receptors. Bowden et al. [10] stated that Arenaviridae group of viruses have α/β fold, whereas Filoviridae possess 'Chalice' of GP1. Similarly, Paramyxoviridae shows six bladed β propeller and large trimeric haemaglutinin is shown by Orthomyxoviridae. Glycosylated GP120 trimer is observed in the Lentiviruses of Retroviridae. Viruses exhibit 'Semaphorins' which are family of cell surface signalling glycoproteins [19]. These semaphorins binds with cell surface receptors to initiate important physiological processes. These observations are made by recent study of viral glycoproteins by employing Macromolecular crystallography [10]. The M and S proteins of the virus are usually rich in N glycosylated proteins, which have been demonstrated as important virulent factor of viruses [98]. Thus, E, M and S viral glycoproteins are involved in viral host binding and subsequent virus-host membrane fusion to establish the pathogenesis of the virus. Two envelope glycoproteins, namely E1 and E2 develop the viral spike of the virions of Flaviviridae family [61] are involved in the engagement with host receptor and conformational change required for membrane fusion (Fig. 1c). Studies show that E2 can express independently but E1 is dependent upon E2 in case of HCV. SARS Coronavirus possess a spike(S) glycoprotein [70], which itself performs the membrane fusion for the entry of the virion and its fusion with host cell [115]. In case of Chikungunya virus, attachment is facilitated by the E2 glycoprotein [89] and fusion mainly by the E1 glycoprotein, thus both the processes are mutually exclusive, whereas in Dengue virus, it is carried by the same E protein (http://www.uniprot.org/uniprot/Q8JUX5). Interestingly, the dengue virus apart from synthesizing the basic capsid, membrane and envelope proteins also produces seven non-structural secretory glycoproteins NS1,2A, 2B, 3, 4A,4B,5 [46]. These proteins are not integrated in the virus but secreted in the host. Studies have found heterogeneity in the E glycoprotein of Dengue virus [56]. Five different glycans are present in this glycoconjugate including Mannose, GalNac and GlcNac,

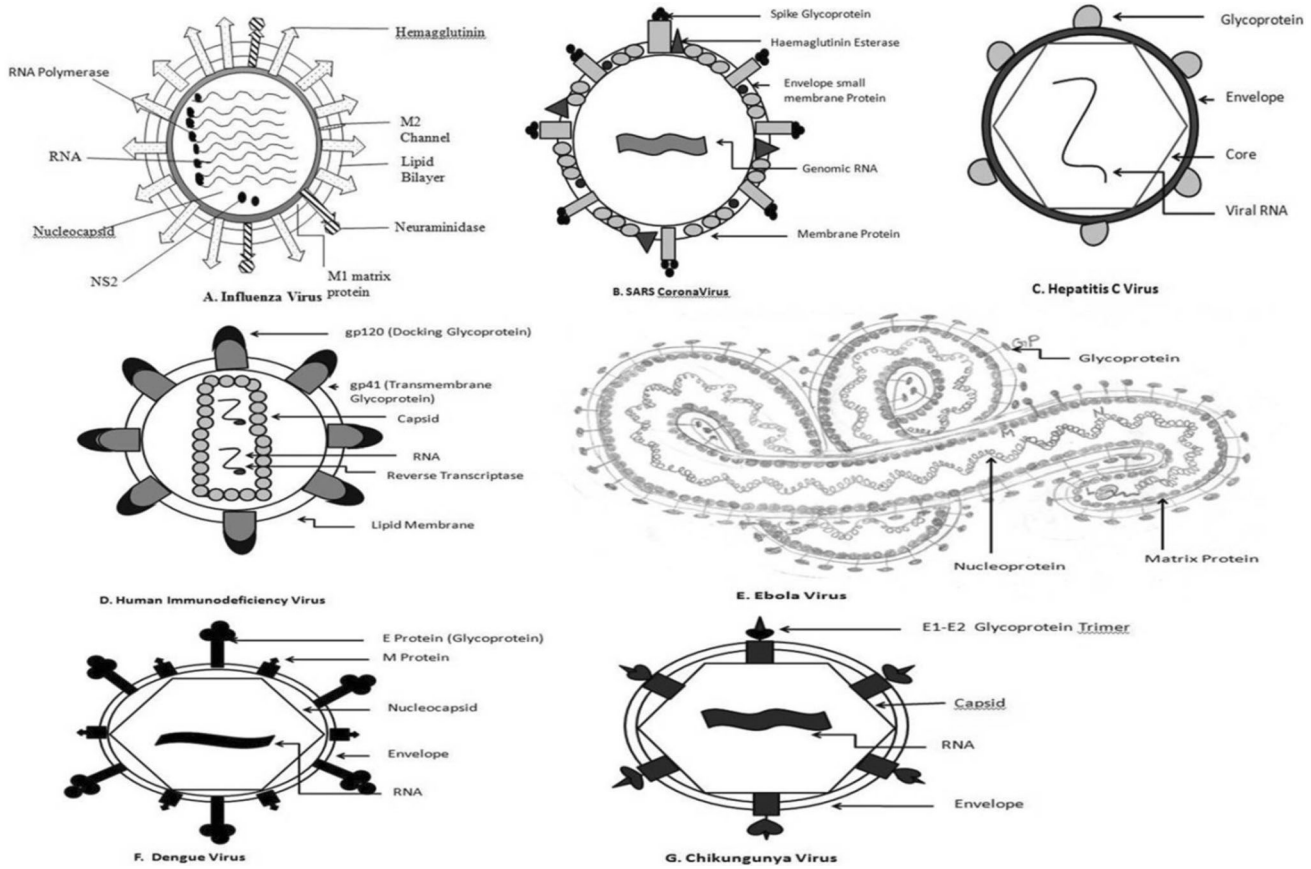

Fig. 1 Distribution of Glycoproteins on the surfaces of different viruses **a** influenza virus, **b** SARS Coronavirus, **c** Hepatitis C virus, **d** human immunodeficiency virus, **e** Ebola virus, **f** Dengue virus and **g** Chikungunya virus

Fucose and Sialic Acid. B cell and T-cell epitopes are predicted in a study by analysing this E glycoprotein [46]. The Dengue Viral envelope is more ordered than the inner viral core, as the envelope is composed of 90 glycoprotein E dimer icosahedral scaffold [58]. Computational studies are there to develop vaccines against Dengue virus [4, 97].

There are three glycoproteins present in HIV [1]; namely gp 120, gp 160 and gp 41 [38]. All these are encoded by the ENV gene [76]. The HIV envelope glycoprotein gp120 contains nine disulphide bridges and is highly glycosylated, carrying on average 24 N-linked glycans (Fig. 1d) [73]. Experiments proved that the glycan part of the gp 41 protein has important role in the efficient intracellular transport of another glycoprotein gp 160. Those gp 160 proteins lack gp41 are arrested in golgi complex after their biosynthesis [27]. Zaire Ebola Virus is the member of Filoviridae group, and the Glycoproteins (GP) have found to be major pathogenic determinants [24–26, 63, 75, 99]. In the Ebola virion GP gene is the 4th gene among total seven genes in the linear gene order. This synthesizes several proteins. Among them two are predominant. Those are sGP and Δ-peptide (delta peptide). These two proteins are produced due to a furin cleavage of a precursor pre-sGP protein. The GP is actually a Spike protein which is composed of two subunits joined by disulphide linkage Gp1-Gp2 [92].

The Chikungunya Virus on the other hand are known to produce 4 Non structural glycoproteins (nsP1-4) [91] these nsPs have been demonstrated to have important role in keeping the replicase complex of the virus intact in the host as well as to circumvent important host immune responses. Chikungunya Virus has two envelope proteins, namely E1 and E2 [11]. Thus, viral glycoproteins have diverse structure and function. Taken together, glycoproteins are important components of the virus structure and each have unique role to establish pathogenesis. [17].

Viral glycoproteins have a definite role in their pathogenesis. The primary goal of viral infection is to identify a receptor on the host cell surface and binding with it. Subsequently this will pave the way of viral entry into the host cell. In most cases, the first attachment site of the virus is a glycan, either a glycoprotein or a glycolipid. So, glycoproteins play a crucial role in viral pathogenesis. The study of glycoproteins in viral infection is most important to know the disease process as well as to develop antiviral treatments. Glycoprotein–receptor interactions also play

important roles in pathogen pattern recognition and in the regulatory signals that control the activities of cells of the immune system. The most important cause behind viral infection is that it has evolved to present its own sugars and receptors in a manner that mimics or interferes with host glycan-based immune functions. Glycomic studies are ongoing in several viruses. Several advanced technologies are there to decipher structural and functional aspects of glycans like Glycan microarray [40], Mass Spectrometry and Nano LC. Glycan array represents the actual in vivo interaction in silico. The arrayed multivalent demonstration of polysaccharides mimics the cell surface display. There are two types of carbohydrate microarray. Those are polysaccharide and oligosaccharide microarray [53]. Natural polysaccharides are randomly immobilized on solid matrices exploiting hydrophobic physical absorption or charge-based interaction. Polysaccharide microarrays are useful for comparative antigenicity analyses. Being hydrophilic in nature, oligosaccharides need chemical derivatization before arraying. Through oligosaccharide microarray we can study structure–activity relationships [52]. Microarrays were developed on maleimide-functionalized surfaces using seven thiol-containing synthetic high-mannose oligosaccharides for the identification of human immunodeficiency virus (HIV) vaccine candidate antigens [2]. The binding profile reveals that several proteins which interact with gp120 of HIV, like the receptor of the innate immune system known as DC-SIGN (CD 209). In case of Influenza glycans, there are protocols for fluorescent labeling of virus, coupling of virus to a glycan microarray, analysis of a glycan microarray slide experiment, and data interpretation. Studies have shown that there are α2, 3-linked sialic acid motif (SA2, 3Gal) in avian, equine, and canine species. Whereas α2, 6-linked sialic acid motif (SA2, 6Gal) is present in humans. SAα2, 3Gal and SAα2,6Gal are present in swine, these are causing corresponding host tropism. Zhao et al. [117] showed that, association mining results of glycan microarray [2] data with 211 influenza viruses from five host groups: humans, swine, canine, migratory waterfowl, and terrestrial birds [72]. The study suggest that besides Neu5Acα2-6Galβ, human-origin viruses could bind glycans with Neu5Acα2-8Neu5Acα2-8Neu5Ac and Neu5Gcα2-6Galβ1-4GlcNAc substructures; Galβ and GlcNAcβ terminal substructures, without sialic acid branches these were linked with the binding of human, swine, and avian origin viruses. Sulfated Neu5Acα2-3 substructures were associated with the binding of human- and swine-origin viruses. Finally, through three-dimensional structure characterization, it has been revealed that the role of glycan chain shapes is more important than that of torsion angles [117].

Though characterization of Glycoproteins is tough but, through Mass Spectrometry, it is now easier to identify structural details of complex glycoproteins. Mass spectrometry derived glycoproteomics [118] helps us to precisely identify viral and cellular proteins that are functionally, structurally, and dynamically altered during virus infection, but enables us to identify important proteins having active role in the infection pathway. Additionally, isolation and purification techniques along with quantitative strategies in conjunction with MS significantly improve its sensitivity to detect low-abundant proteins. With time, more virus and host genomes are being sequenced and MS-based glycoproteomics is becoming a very important tool for virology. A work by Barrientos et al. [7] revealed that post translational modification of secretory glycoprotein of Zaire Ebola Virus can be characterized by Mass spectrometry. MALDI-TOF MS (Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry) also enables to identify regions susceptible to limited proteolysis in sGP of ZEV.

Another work by Anastassia et al. [54] shows that 0.1 microgram of viral glycoprotein can be purified by Nano-Liquid Chromatography. After Nano-LC the sample is analysed through mass spectrometry. One more work showed that they purified Heat shock protein 90 by NanoLC-MS of Respiratory syncitial virus which have important role in virus particle assembly [54, 80]. So it is evident that using these modern techniques, the biological roles of glycoproteins can be studied more conveniently.

Viral glycoproteins and their biological role

Virus is a nucleic acid surrounded by proteins. This infective particle is called a virion. In most cases this virion is covered with a fascinating coat composed of glycoproteins through which the virus communicates with its host. The co-evolution of host and virus leads the way of making the glycoprotein coat so fascinating [23]. It is evident that the infectivity of a virus rather of its nucleic acid is fully dependent on its glycoproteins. Enveloped viruses generally encode membrane proteins and these special proteins are necessary to mediate the specific binding against host cell ligands. This also directs initial events of membrane fusion and viral internalization. These fascinating envelope proteins are generally glycosylated [15]. The process of glycosylation takes place in the endoplasmic reticulum (ER)-Golgi complex secretory pathway. The host cell encoded glycosyl-transferase enzyme catalyses the glycosylation. This glycosylation is necessary to make the virion host compatible, which is needed by the virus for its pathogenesis. So glycans present at the envelop proteins acts as immunological barriers to resist evasion by the host immune system [22].

Several Viruses exhibit different glycoproteins on their surface (Table 1). Hepatitis C Virus has two envelope glycoproteins namely E1 and E2 [59, 98]. These two proteins play an important role in viral infectivity and can be used as candidate subunit vaccine [98]. On the other hand, in case of Ebola virus there are glycoproteins GP1 and GP2 which causes cell attachment and cell fusion and therefore the main target of the host antibodies. The Ebola virus has an RNA editing mechanism to regulate these GP 1 and 2 genes which when expressed at high level, disrupts normal cell physiology [24]. In case of HIV-1, the gp-120 protein initiates viral entry to the CD4 cells [16]. Recent studies proved that several types of glycans in HIV-1 produce different levels in infectivity. Those viruses have more oligomannose and less structural complexity, infects more efficiently. This ultimately proves that mature oligosaccharide structure of the envelope glycans play a pivotal role in the infection process of HIV-1. Due to *N*-linked glycosylation of gp-160 protein in the endoplasmic reticulum, and further folding and cleaving in the golgi complex; the gp-120 protein of the envelope is produced [71].

There are two popular glycoproteins present on the surface of Influenza virus namely, Haemaglutinin (HA) and Neuraminidase (NA) [84, 88]. These are the key molecules for the viral infection which binds with Sialic acid. Initially, HA binds with sialic acid during the initiation of infection. After viral replication NA degrades its substrate sialic acid to accelerate release of new viruses [30].

So it is evident from the examples of viruses of diverse family that glycoproteins are the key molecules for a virus to establish an infection within the host and to survive further within the host system.

In the study of viral pathogenesis, a special type of glycoprotein, called Semaphorin have been established [6]. Semaphorins are family of cell surface signaling glycoproteins which binds to the family of plexin glycoprotein cell surface receptors. Semaphorins also activate repulsive guidance pathways having active part in axon guidance, immune regulation and activation, and vascular development [57, 93]. Semaphorins have eight known classes. Among them two are found in invertebrates, five in vertebrates, and the eighth class in viruses which are known as 'viral semaphorins' [19]. The ectodomains of cellular semaphorins contain C-terminal domain elaborations like PSI (plexin, semaphorin and integrin) domains, immunoglobulin (Ig)-like domains, thrombospondin domains and PDZ-domain-binding sites which occassionally attach to the cell-surface. Whereas the N-terminal having a plexin-binding sema-domain, is conserved in all cases of virus host cell attachment. The sema-domain is the

Table 1 Status of glycoproteins in some well studied viruses and their disease burden

Name of the Virus	Glycoproteins identified	Specific role	Disease burden
Influenza virus	Haemaglutinin and Neuraminidase [18, 43]	Fusion with host cell membrane Sialic Acid and attachment [43]	3–5 million cases Worldwide [78, 105]
SARS-CoV	Spike(S) glycoprotein [25, 115]	Membrane fusion [115]	8422 within the duration of 1st November 2002 to 7th August 2003 occurring worldwide [113, 114]
Hepatitis C virus	E1 and E2 [55, 98]	Binding to Host receptor and Conformational change necessary for membrane fusion [98]	130 to 150 million people globally [103, 106]
Human immunodeficiency virus 1	gp120, gp160, gp41 [16]	Intracellular transport [16]	35 million globally up to 2013 [83, 104, 108, 112]
Zaire Ebola virus	Spike Protein Gp1-Gp2 [64]	Primary Host cell activation [64]	up to 28th June 2015 total 27,550 cases [107, 110, 111]
Dengue virus	E (dimer) [64]	Host cell fusion and attachment [64]	WHO reported recently that there are 390 million dengue infections per year globally [109]. Presently Dengue is endemic in 112 countries [109].
Chikungunya virus	E1 and E2 [41, 51]	Host cell binding	According to WHO, this disease occurs mainly in Africa, Asia and Indian Sub-continent [102]. But recently in the 2005 outbreak around the Indian Ocean, there were imported cases in Europe and USA through travellers. In 2005 there were 1.9 million reported cases around Indian Ocean [102]. On 21st October 2014 France has reported 4 local Chikungunya infections and in late 2014 there was an outbreak in the pacific islands [102]. In India, NVBDCP reports that up to 29th June 2015 there were 10,317 total suspected Chikungunya cases.

only component found in viruses. Crystallographic studies by Bowden et al. [10] have revealed that human Sema3A and mouse Sema4D semadomains comprises of structurally conserved homodimer of seven-bladed β-propellers [6, 50, 65, 69, 77]. The immune-regulatory semaphorins like Sema3A, 4A, 4D, and 7A helps in B cell mediated immunity (Sema4D), T cell activation as well as differentiation (Sema4A, Sema3A, and Sema4D), and inflammation (Sema7A) [93]. These semaphorins provide a molecular basis for how viruses can optimize their own proteins to override normal physiological interactions.

A work by Shirato H as 'Norovirus and histo-blood group antigens' in the journal Jpn J Infect Dis. (2011;64(2):95–103) describes that NoroVirus (NoV) causes viral gastroenteritis and interestingly bind to histo-blood group antigens (HBGAs), like ABH antigens and Lewis antigens. It has been shown epidemiologically that persons with different ABH phenotypes are infected with NoV strains in a genotype-dependant fashion. An in vitro binding assay using NoV virus-like particles (VLPs) showed a uniform recognition pattern for type 1 and 2 core structures of histo blood group antigens. NoV VLPs bind more tightly to type 1 carbohydrates than to type 2. Type 1 carbohydrates are found to be expressed at the surface of the small intestine and targeted by NoV. This property speaks about NoV tissue specificity.

So it is evident that glycoproteins perform a major and active role in viral pathogenesis and disease progression.

Glycoproteins provide tissue tropism to the virus. Some virues used to infect the respiratory system whereas some affects the liver. The cause is the type of glycoprotein with which the virus binds to accelerate its invasion.

In a study by Raska et al. [81], it has been proved that there are differential glycosylation in viruses like HIV1 depending upon the cells which produce the virus. *N*-glycosylation of reconbinant gp120 of HIV1 is varied and affected the recognition by serum antibodies. Glycosylation of gp120 protein of HIV1 affects its recognition by neutralizing and non neutralizing monoclonal antibodies. This study also says that this glycosylation is cell specific.

Another study by Lin et al. [64] stated that there are C-type lectins expressed on the Dendritic Cell surface known as DC-SIGN(Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) or CD 209 and DC-SIGNR which binds to HIV1 and transmit to T cells through the viral envelope Env glycoprotein. But interestingly other highly glycosylated Viruses failed to interact with DC-SIGNR [64]. Lin et al. showed that DC-SIGN (R) or CD 209 selectively binds with HIV1 Env and Zaire Ebola Virus glycoproteins containing more high-mannose. By modulating N-glycans on Env or glycoprotein during virus production in different primary cells or in the presence of the mannosidase I inhibitor deoxymannojirimycin affected DC-SIGN(R) infectivity enhancement. They also predict that viruses containing glycoproteins with a high amount of high-mannose N-glycans effectively interact with DC-SIGN(R), but those viruses having only complex N-glycans cannot effectively react with DC-SIGN(R). So it is evident that virus-producing cell type is a crucial factor in depicting both N-glycan status and virus interactions with DC-SIGN(R), which establishes virus tropism and infection within the human body [64].

Liu et al. [66] described in their study that sialic acid present on cell surface is essential for Human EnteroVirus D 68 (EV-D68) entry. Crystallographic studies showed that EV-D68 with sialylated glycan receptor analogues binds on the viral surface. Sialic acid receptor induces a cascade of conformational changes within the virus to secrete a fatty-acid-like molecule which regulates the stability of the virus. So, it is evident that binding of virus to a sialic acid receptor and to immunoglobulin-like receptors facilitates viral entry in enteroviruses.

Application of viral glycoproteins in diagnostics

Glycan based viral immunodiagnostics usually have high sensitivity and specificity. Glycoprotein based IgM serology was developed for the diagnosis of recent primary rubella virus infections and significant sensitivity and specificity was obtained. Similarly, Glycoprotein based serology tests to detect antibodies to herpes simplex virus glycoproteins G-1 and G-2, which evoke a type-specific antibody response have also been developed. These tests are used to confirm a diagnosis of genital herpes, and also to establish diagnosis of HSV infection in patients with atypical complaints, to identify asymptomatic carriers, and identify persons at risk for acquiring HSV. Glycan based immunodiagnostics have also been developed for the rapid identification of different strains of Influenza Virus. A novel peptide based ELISA which has sensitivity and specificity of 96.55 and 74.4 % respectively is very promising. On the other hand, the main diagnostic challenge related to SARS is to diagnose it differently with atypical pneumonia [68, 79]. The key diagnostic tools are immunofluorescent staining, ELISA and RT-PCR [67]. But all these techniques are extremely sophisticated and of little use in case of epidemics; especially in the developing world. SARS Coronavirus possess a spike(S) glycoprotein, which itself performs the membrane fusion for the entry of the virion and its fusion with host cell (Fig. 1b) [115]. IgG based diagnostics against this S protein has been developed [114]. Indirect ELISA test has been developed by using recombinant SARS 'S' protein and the N (nucleoprotein) protein. The sensitivity of SARS 'S' and 'N' proteins are

100 and 96.7 % respectively whereas the specificity of SARS 'S' and 'N' are 98.55 and 98.4 % [114].

Similarly, as acute Hepatitis C infection is asymptomatic, so it is difficult to diagnose early. Generally patients come to the clinic with damaged liver. Initially patients are screened by anti-HCV antibody test and further confirmed by testing Viral RNA. There are two glycoproteins E1 and E2 in HCV. Standardised PCR system is available in HCV diagnostics but a Core antigen test is also in the market. In 1995 Tanaka et al. [21] suggested that the HCV core proteins can be used as antigens for the chronic stage. Studies for developing Algorithms confirms 99.05 % sensitivity in case of RT-PCR whereas 98.10 % for the Core Antigen Test [82]. Another novel glycoproteomic serum biomarker has been identified which can diagnose HCV along with progressive liver cirrhosis is Wisteria floribunda agglutinin positive Mac-2-binding protein (WFA+-M2BP) [29]. The diagnostic threshold for cut-off index values of this protein is 1.435 and 4.615 in HCV negative and HCV positive patients showing progressive liver cirrhosis [39].

In case of HIV1, ELISA and PCR are two Gold standard tests. Type specific conformational epitopes of the gp160 and gp 41 glycoproteins of the HIV envelope are used for the recognition of 'early HIV antibodies' [14]. Enzyme Immuno assay (EIA) and RT-PCR are used in these assays. These tests are very specific but they fail to diagnose early infection [14]. The most challenging part in HIV diagnosis is to diagnose the acute stage and differentiate "Window Phase" patients from the serologically positive patients [87]. Popular Serological tests fail to diagnose all patients of HIV as they exploit the GAG proteins which literally decrease after disease progression [60, 74]. There are three glycoproteins present in HIV; namely gp 120, gp 160 and gp 41. All these are encoded by the ENV gene [76]. The HIV envelope glycoprotein gp120 contains nine disulphide bridges and is highly glycosylated, carrying on average 24 N-linked glycans (Fig. 1d) [73]. Experiments proved that the glycan part of the gp 41 protein has important role in the efficient intracellular transport of another glycoprotein gp 160. Those gp 160 proteins lack gp41 are arrested in golgi complex after their biosynthesis [27]. As stated above, there are limitations of the popular GAG antigen based serological tests which cannot diagnose HIV patients of different clinical stage. But antibodies against precursor gp160 ENV protein and final ENV proteins gp 120 and gp 41 can detect all clinical stages of HIV [74]. The Sensitivity of current available diagnostic system is 38 % at $<$7 days, 97 % at 7–41 days and 95 % at 42–93 days [74] The Specificity is almost 95 % [90].

In case of Zaire Ebola Virus, initially there was controversy about the role of glycoproteins in the pathogenesis of EBV. But later on, scientific researches proved that the primary host cell activation by the EBV is mediated by GP1-2 [100]. An antigen capture ELISA has been developed in Zaire EBOV using mAbs [85]. It has been reported that these tests have both high sensitivity and specificity [85].

Similarly, Dengue (DENV) NS1 is a highly conserved glycoprotein, expressed as both membrane-associated and secreted forms [33, 36, 94]. Secreted NS1 has been detected ranging from 2–0.04 μg/mL in the serum of dengue-infected patients during the early stages of the disease. A high NS1 level has been demonstrated to circulate as early as 1 day after onset of symptoms up to early convalescences thus provides an alternative to virus culture or PCR for early dengue diagnosis when IgM or IgG antibodies are not present yet in dengue infected patients [42, 49]. Circulating dengue NS1 in sera can be detected either using ELISA assay or lateral flow based RDTs [31]. Thus, glycan based Viral Immunodiagnostics or Glyco-Immunodiagnostics are helpful in early diagnosis of patients with viral infection [28].

Current diagnosis scenario in Chikungunya is IgM and IgG based ELISA and Nucleic Acid detection by RT-PCR [12, 51]. But there is no Antigen based ELISA. This makes the condition crucial as the primary health care providers in the Virus affected countries do not have RT-PCR facilities. It is not recommended to maintain RT-PCR facilities in Primary Health care centre by policy. It is extremely costly and demands expertise. It is not possible to provide such facility in the densely populated tropical countries. As Chikungunya causes short duration fever, often the patients are not diagnosed properly. The joint pain generally persists for some days but can be present for a year [102]. There is a study which shows even multi organ failure in Chikungunya infected patients [45]. CHIKV has two envelope glycoproteins, namely E1 and E2 (Fig. 1g) [11]. Recombinant CHIKV E1 and E2 glycoprotein based ELISA showed a sensitivity of 77.5 and 90 % respectively whereas the specificity for both cases was 100 % [11, 54] highlighting the potential for these two glycoproteins in the diagnosis.

Conclusion

Viral glycoproteins are integral parts of enveloped viruses and they actively take part in their pathogenesis. Exploting glycoproteins, viruses enter into their host and combat with host immune system. Recent advances in technology deciphers different role of glycoproteins which are dependent on their structures.

Different viruses have different mode of pathogenesis and glycoproteins directly takes part in the host binding and entry. During maturation from host cell viruses have

host glycoproteins on their surface to avoid the immunity of the host. So, to detect viruses and to decide for developing vaccines [37], glycoproteins always play a key role.

Antibody production is a prominent feature of the immune response in patients with viral infection, and particular isotypes correlate with resistance or susceptibility to infection [101]. A substantial proportion of the antibodies detected in patients with acute or chronic infections is directed against viral glycan epitopes. As levels of anti glycan antibody are high and specific for each viral infection this permits diagnostic discrimination between the different viral infections. Taken together, viral glycoproteins have important functions in pathogenesis and can be exploited to develop viral diagnostics.

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ORIGINAL ARTICLE

Oxidative damage markers and inflammatory cytokines are altered in patients suffering with post-chikungunya persisting polyarthralgia

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ABSTRACT

Redox homoeostasis is necessary for the maintenance of living systems. Chikungunya viral infection manifests into joint inflammation and debilitating polyarthralgia affecting the life style of the patient badly. The disease pathophysiology is poorly understood and there is a lack of targeted therapeutics. The pathogenic role of free radicals in arthritis is well established. This study aims for the first time to evaluate the status of several standard oxidative stress markers and their correlation in chikungunya patients suffering with polyarthralgia. Expression of Siglec-9 on monocytes; which can modulate oxidative stress is studied along with intracellular reactive oxygen species (ROS), cellular lipid and protein damage markers in chikungunya patients with/without persisting polyarthralgia along with healthy controls. Furthermore, plasma NO level, antioxidant status was investigated along with some inflammatory cytokines namely IL-6, IFN-γ, CXCL-9, IL-10 and TGFβ1. Interestingly, all oxidative damage markers are altered significantly in groups but their alteration levels vary in patients with/without persisting polyarthralgia. Siglec-9 expression level is increased in patients revealing cellular response to manage oxidative stress with respect to controls. Correlation studies reveal that intracellular ROS correlates well with most of the studied parameters but the correlation coefficient (Pearson r) differs with disease manifestation demonstrating strong role of these factors in a pro-oxidant milieu. The presence of free radicals increases the availability of neoantigens continuously, which possibly further cascades oxidative damage and development of persisting polyarthralgia.

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Chikungunya; cytokines; intracellular ROS; oxidative damage; persisting polyarthralgia; Siglec-9

Introduction

Chikungunya is an arboviral infection characterised by acute febrile illness along with moderate to severe joint pain affecting the life style of the individuals [1]. The productivity loss in terms of income due to chikungunya associated joint pain in a tropical country like India was estimated INR 391 million in the year of 2006 [2]. Similarly, in Latin America, it was estimated as USD 73.6 million and in Europe as 35,000 euros per capita elucidating a huge economic burden of the disease manifestation [3,4]. The pathogenesis of persisting polyarthralgia post-chikungunya infection which continues till several months or years is not well understood but becomes very crucial for better patient management and development of appropriate therapeutics. Possible underlying mediators include immunological, biochemical, genetic and environmental factors which collectively develop joint inflammation and pain [5]. Existing literatures suggest an upheaval of immune system due to chikungunya but there is no such study elucidating oxidative stress; which plays a crucial role in development of arthralgia [6,7]. Former studies endorse production of higher amount of soluble immune complexes, proinflammatory cytokines, chemokines and autoantibodies in chikungunya patients and all these altered levels indicate development of oxidative stress [8–12]. Increased frequency of CD8 T cell has also been reported in chikungunya earlier and this may be due to the oxidative induction of chemokines [13,14]. Destruction of cartilage has been previously reported in chikungunya and the degree of oxidative stress in peripheral blood could represent the level of such damage in patients easily [15]. Host has the machinery to combat oxidative damage. Siglec-9 is known to manage oxidative stress and its expression represents the same in different inflammatory conditions [16]. This study aims to evaluate the amount of reactive oxygen species (ROS), oxidative stress markers, antioxidant potential and cytokines in peripheral blood to get the glimpse of oxidative stress generated at pathologic milieu and as a whole in the host system.

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Materials and methods

Study population

This study was done at Carmichael Hospital for Tropical Diseases, School of Tropical Medicine, Kolkata, India. Patients were enrolled during the period of April 2015 to December 2017 with prior approval from Institutional Human Ethics Committee. Peripheral blood was collected from only informed and written consented patients following the Helsinki guidelines. Detailed history was taken by filling a predetermined questionnaire from every patient. Chikungunya infection status was confirmed by a WHO recommended MAC-ELISA kit provided by Indian Council for Medical Research-National Institute of Virology, Pune, Maharashtra and/or by Real Time PCR with genesig® Chikungunya standard Kit obtained from Primerdesign® Ltd. UK.

Patients were grouped as chikungunya patients without persisting polyarthralgia or ChikWoPP and chikungunya patients with persisting polyarthralgia or ChikWPP. Persisting polyarthralgia was determined by clinical signs and symptoms presented from 10 to 30 days of infection. Total 15 patients were available for follow-up who were not suffering with any joint pain after 10 days of infection and 25 patients were came to hospital for follow-up suffering with joint pain and inflammation. Healthy donors ($n = 25$) include age and sex matched non-smoking, non-alcoholic fellow researchers, scientists, clinicians of the institute without any history of fever and/or joint pain in last 3 months.

Study of standard markers of oxidative damage

Estimation of malondialdehyde, NO, thiol and protein carbonyls in plasma

Malondialdehyde (MDA) was estimated in plasma of all ($n = 65$) study subjects by following Pasha and Sadasivudu's method [17]. MDA reacts with thiobarbituric acid (TBA) to produce a coloured compound, which was measured spectrophotometrically at 530 nm. TBA detects only free MDA in peroxidizing lipid system. In this study, TBA was obtained from Sigma-Aldrich®.

Nitric oxide or NO present in the plasma sample was measured by using Griess reagent (modified) obtained from Sigma-Aldrich®. The method followed in this study was earlier described by Griess [18]. Samples ($n = 65$) used for determination of NO level were collected in EDTA coated vial as heparin produces precipitate with Griess reagent [18].

Thiol content in the plasma samples of both patients ($n = 40$) and healthy controls ($n = 25$) were determined using Ellman's reagent or 5,5′-dithiobis (2-nitrobenzoic acid) obtained from Sigma-Aldrich® following the description elsewhere [19].

Protein carbonylation status was estimated by derivatization with 2,4-dinitrophenylhydrazine as described previously [20].

Determination of antioxidant status by DPPH and superoxide dismutase assay

DPPH or 2,2-diphenyl-1-picrylhydrazyl was obtained from Sigma-Aldrich® and the assay was performed following the method described by Janaszewaska and Bartosz earlier [21].

Superoxide dismutase enzymatic activity with respect to inhibition of auto oxidation of pyrogallol in the plasma was determined by following the method of Marklund as described by P. Jyothi et al. [22].

Study of intracellular ROS in peripheral blood mononuclear cells (PBMC)

Within 1 hour of collection of peripheral blood, PBMC or peripheral Blood Mononuclear cells were layered by Lymphoprep™ obtained from Axis-Shield, Oslo, Norway and washed with phosphate buffered saline or PBS of physiological pH. Intracellular ROS was determined using 2′,7′-dichlorofluorescin diacetate or DCFDA obtained from Sigma Aldrich, Missouri, USA by Flow cytometry as described earlier [23]. Total 10,000 gated events were acquired through BD FACSCalibur™ and analysed using BD CellQuestPro™ software. Cells treated with H_2O_2 were considered as positive control.

Status of Siglec-9 expression on CD 14^+ cells

Siglec-9 expression on monocytes were determined by both flow cytometry and semiquantitative PCR.

Whole blood was incubated with FITC conjugated CD14 (M5E2) and PE conjugated CD329 (K8) (Siglec-9) and treated with BD Pharm Lyse. Further acquisition was done using a FACSCalibur Flow Cytometer and the data was analysed using BD Cell Quest Pro software.

$CD14^+$ cells were sorted from whole blood using Mojosort Magnetic cell separation kit obtained from BioLegend, USA. Further, mRNA was extracted using a Qiagen RNeasy mini kit followed by SYBR Green based semiquantitative PCR in an Applied Biosystems 7500 Fast platform. Gene expression level was determined with respect to β-actin expression following ΔΔCT method.

Siglec-9 and cytokine, chemokine ELISA

Sandwich ELISA was performed by using standard kits obtained from RayBiotech, USA for each of Siglec-9,

IL-6, TGFβ1, IL-10, IFN-γ and CXCL-9 according to manufacturer's protocol.

Statistical analysis

GraphPad Prism software version 5.0 (Graph Pad Software Inc, La Jolla, CA) was used for all statistical analysis. $p < .05$ was taken significant in all cases. *t*-test was done to determine statistical significance among two groups of data and one-way ANOVA was done for the same within three groups. All data are represented as mean ± SEM.

Results

This study was involved with 65 individuals comprising 25 chikungunya patients suffering from persisting polyarthralgia post-10 days of infection, 15 patients without any joint involvement after 10 days along with 25 healthy controls of similar sex and age group (Table 1).

Table 1. Study population.

Parameters	Healthy controls ($N = 25$)	Chikungunya patients without persisting polyarthralgia ($N = 15$)	Chikungunya patients with persisting polyarthralgia ($N = 25$)
Male:Female	12:13	6:9	10:15
Age in years [Median with range]	47 [23–75]	39 [20–64]	55 [35–76]

As evident in a viral infection, there is no gender bias but persisting polyarthralgia was found in older patients similar to previous studies [24,25].

Altered biochemical parameters of oxidative damage is observed in patients

Standard biochemical parameters of oxidative stress were measured to get the glimpse of internal Redox imbalance scenario in the patient, as these marks tissue damage, inflammation and persistence of the disease activity. Malondialdehyde or MDA level in plasma, which depicts lipid peroxidation, was found to be significantly ($p < .0001$) 1.58-fold high in ChikWPP group when compared to healthy controls (Figure 1(A)). Nitric oxide or NO, which is a crucial player of Redox signalling was 1.82-fold increased in ChikWPP patients significantly ($p < .0001$) than controls (Figure 1(D)). Sulphhydryl group containing thiols are important players of enzyme activity and can potentially decrease the damage caused by oxidative stress [26]. In this study, it has been found that there is 1.34-fold decreased thiol content in the ChikWPP patient plasma (Figure 1(G)). This significant ($p < .0001$) change describes that there is higher oxidative damage in those persisting polyarthralgia patients after chikungunya infection. Patients without any joint pain have higher levels of thiols than ChikWPP. Similarly, carbonylation of protein marks protein damage and there is significantly ($p < .0001$) high levels of protein carbonyls present in patient plasma

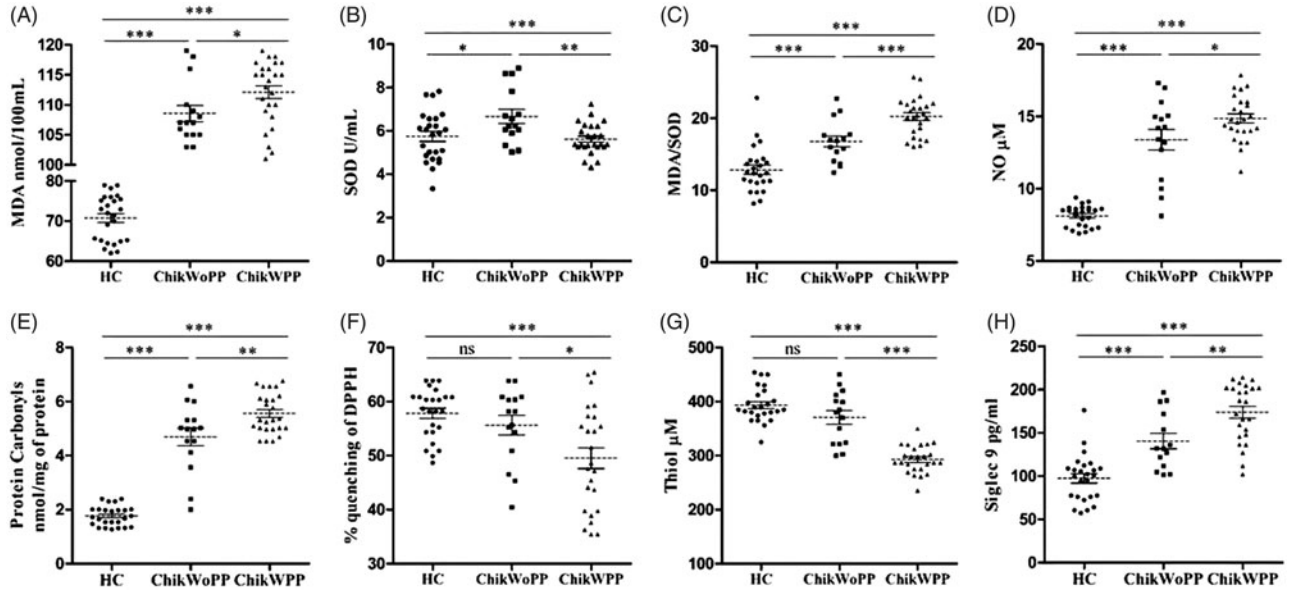

Figure 1. Study of oxidative damage markers in all study subjects. (A) represents scatter plot of MDA in HC, chikungunya patients without persisting polyarthralgia (ChikWoPP) and chikungunya patients with persisting polyarthralgia (ChikWPP). Horizontal bars represent Mean ± SEM. $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$. Similarly (B) represents SOD, (C) MDA/SOD, (D) NO, (E) Protein Carbonyls, (F) % quenching of DPPH, (G) Thiol and (H) Siglec-9 titres respectively.

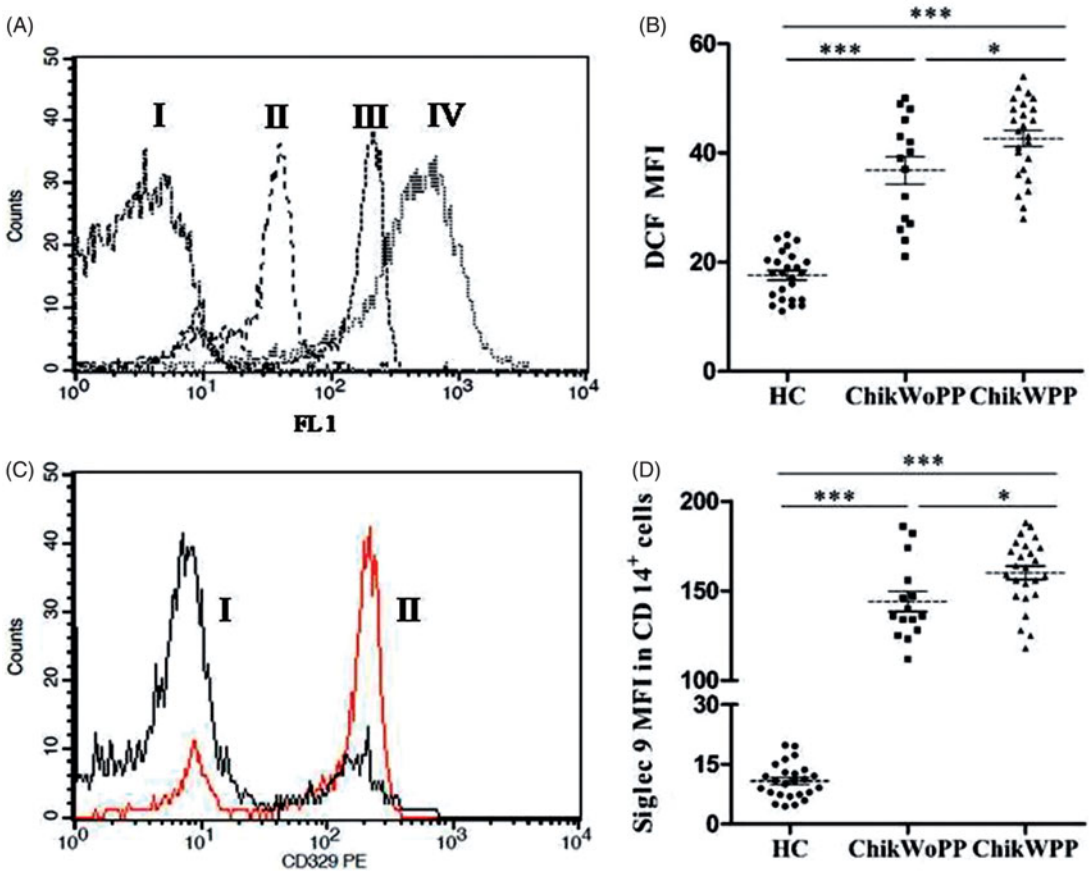

Figure 2. Intracellular ROS and Siglec-9 expression in study subjects. (A) is a representative histogram overlay of Dichlorofluoroscein or DCF Fluorescence Intensity vs. cell counts in peripheral blood mononuclear cells. AI is without any presence of DCF, AII is Healthy Control, AIII is patient, AIV is positive control. (B) is the scatter plot of DCF Median Fluorescence Intensity in all study subjects. (C) representative histogram plot of Siglec 9 (CD 329) Fluorescence in Healthy control(I) and patient(II). (D) is the scatter plot of Median Fluorescence Intensity of Siglec 9 in all study groups. Horizontal bars represent Mean ± SEM. $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$.

which when compared to controls was found to be 3.15-fold higher (Figure 1(E)). So it is evident that, there is higher level of oxidative damage in patients suffering from post-chikungunya persisting polyarthralgia and the oxidative damage is well represented by the plasma levels of several standard biochemical markers.

Patient plasma has poor antioxidant potential

To combat increased oxidative damage, human body is well equipped with antioxidant mechanisms which sustain proper working of the body systems [26]. In diseased condition, there is imbalance among these mechanisms and the manifestation affects the life style of the patient. Plasma antioxidant potential was investigated by evaluating superoxide dismutase enzyme activity and DPPH radical scavenging activity in this study. Both the parameters were found to be significantly ($p < .0001$) low in patient plasma when considered along with healthy individuals. SOD level was 1.02-fold low, whereas % radical scavenging activity was found to be 1.16-fold lower in patients than controls (Figure 1(B,F)). SOD activity was found to be increased in patients who had no sign of polyarthralgia indicates significant role of it in the management of oxidative damage. This poor antioxidant potential of ChikWPP patient plasma explains the inability of the system to combat with such higher oxidative damage. MDA/SOD ratio is an established marker to indicate Redox imbalance in diseases [22]. This ratio is markedly changed in ChikWPP group. When compared to healthy controls; a 1.57-fold higher value is observed (Figure 1(C)).

High intracellular ROS is observed in patients along with higher expression of Siglec-9

In this study, dichlorofluoroscein fluorescence intensity was measured for determining the level of intracellular ROS. Higher is the amount of free radicals higher will be the fluorescence in FL1 channel. Siglec-9 is reported early to be increased with response to oxidative stress and have a protective role which eventually controls

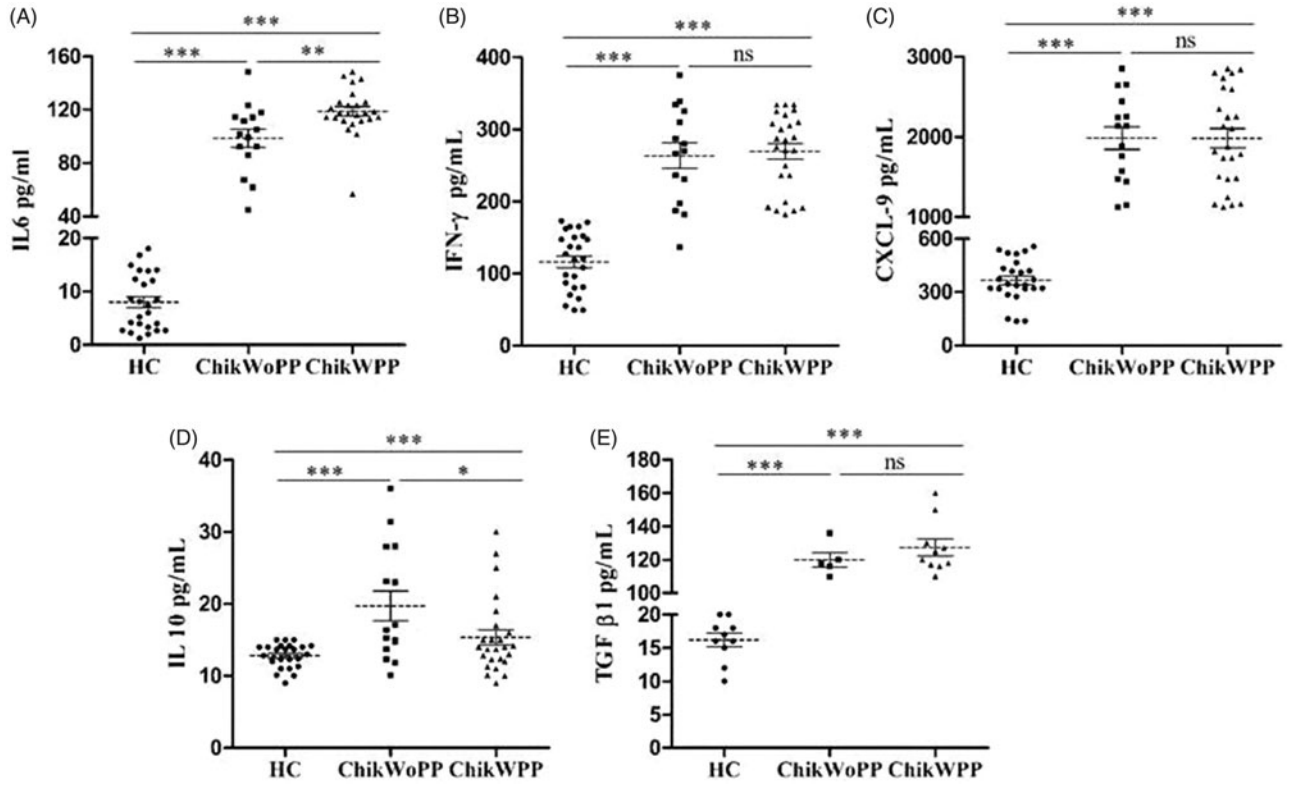

Figure 3. Cytokine pattern in different study groups. (A–E) represents scatter plot of IL-6, IFN-γ, CXCL-9, IL-10 and TGFβ1 titres in Healthy controls and ChikWoPP and ChikWPP patients. Horizontal bars represent Mean ± SEM. $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$.

Table 2. Correlation between intracellular ROS and all studied parameters.

	Correlation with ROS in ChikWoPP	Correlation with ROS in ChikWPP
Studied oxidative parameters		
MDA nmol/100 mL	$r = 0.825$; $p < .05$	$r = 0.951$; $p < .05$
SOD U/mL	$r = -0.888$; $p < .05$	$r = -0.553$; $p < .05$
MDA/SOD	$r = 0.881$; $p < .05$	$r = 0.703$; $p < .05$
NO μM	$r = 0.905$; $p < .05$	$r = 0.569$; $p < .05$
Protein Carbonyls nmol/mg of protein	$r = 0.880$; $p < .05$	$r = 0.479$; $p < .05$
% quenching of DPPH	$r = -0.815$; $p < .05$	$r = -0.440$; $p < .05$
Thiol μM	$r = -0.893$; $p < .05$	$r = -0.817$; $p < .05$
Siglec-9 pg/mL	$r = 0.859$; $p < .05$	$r = 0.731$; $p < .05$
Siglec-9 MFI	$r = 0.823$; $p < .05$	$r = 0.404$; $p < .05$
Studied Cytokine titres		
IL-6 pg/mL	$r = 0.877$; $p < .05$	$r = 0.635$; $p < .05$
IFN-γ pg/mL	$r = 0.912$; $p < .05$	$r = 0.616$; $p < .05$
CXCL-9 pg/mL	$r = 0.891$; $p < .05$	$r = 0.789$; $p < .05$
IL-10 pg/mL	$r = -0.882$; $p < .05$	$r = 0.327$; $p = .111$
TGF-β1 pg/mL	$r = 0.725$; $p = .166$	$r = 0.630$; $p = .051$

the mammalian lifespan [16]. There is 2.42-fold increased intracellular ROS which is statistically significant when undergone *t-test* ($p < .0001$) (Figure 2(B)). Siglec-9 surface expression was determined by Median Fluorescence intensity and there was significantly increased surface expression of Siglec-9 observed on CD 14^{+} cells (Figure 2(D)). Gene expression studied also revealed similar results (data not shown). Siglec-9 ELISA depicted significantly higher plasma levels of Siglec-9 protein (Figure 1(H)).

Inflammatory cytokines are altered in patients

Cytokines are array of secreted proteins playing pivotal role in cellular cross talk [9]. Pain is the response of nervous system after interaction with inflammatory signals [10]. Cytokines are involved in such communications and therefore provides glimpses of the internal inflammatory manifestations [9]. Proinflammatory, inflammatory and anti-inflammatory cytokines are classified with respect to their roles in inflammation. A panel of

cytokines were studied involving all the study subjects and it was observed that IL-6 and IFN-γ are significantly ($p < .05$) elevated (Figure 3(A,B)) among the proinflammatory cytokines and correlates well (Table 2) with intracellular ROS. CXCL-9, which is a chemokine and reported to be increased in chikungunya earlier [8] correlates well with ROS levels (Table 2) (Figure 3(C)). Anti-inflammatory cytokine IL-10 is found to be significantly decreased ($p < .05$) (Figure 3(D)) in ChikWPP group similar to some previous study [14]. TGFβ1 levels are increased ($p < .05$) in the patients when compared with healthy controls (Figure 3(E)). Eventually, these levels are found to be corroborated with previous studies reporting cytokine levels in chikungunya [9,10,27].

Discussion

Redox homoeostasis is essential for normal functioning of living systems. Daily interaction of biological systems with environment causes generation of free radicals; which damages the building blocks of life like proteins and lipids heavily. Cells travel to great lengths to combat these free radicals which become necessary for proper working of any type of tissues within the body [28]. Antioxidants are known to protect human system from infections. This seems to be a paradox but possibly explains that those antioxidants are not sufficient enough to neutralise the immense amount of free radicals generated by cell organelles against those pathogens. So, any kind of Redox imbalance becomes amplified in the host system as initial oxidative stress leads to the production of cell debris which eventually acts as neoantigens and triggers further immune upheavals, finally more oxidative stress and damage to the host tissue [23].

Cartilage tissues present at skeletal joints are vulnerable to destruction by oxidative burst which is triggered by immune system through proinflammatory cytokines, soluble immune complexes and oxygen species as evidenced from previous works [29]. Persisting cryoglobulinaemia is observed in both arthralgia causing viral infections like hepatitis C and Chikungunya and have strong roles in development of inflammation [30]. Cartilage destruction is caused due to proteolysis by metalloproteinase enzymes secreted from macrophages, whose production and function are tightly regulated by cytokines and reactive oxygen species present in the surrounding milieu [31]. Thus, the same was evaluated in patients with or without persisting polyarthralgia post-chikungunya infection. Intracellular level of ROS was higher in patient vis-à-vis healthy controls, but its level is significantly higher in ChikWPP patients. Higher level of intracellular oxygen species makes increased level of oxidative damage as evidenced by the levels of lipid bilayer and protein destruction. MDA is a secondary product of lipid peroxidation, whereas protein carbonyls are recognised marker of protein oxidation and have been shown to be more vulnerable for cross-linking and cleavage that undergo rapid proteolysis [31]. Protein-bound sulphhydryl group or thiols have important role in antioxidant defence also studied and found to be decreased than healthy controls in ChikWPP group. Interestingly, ChikWoPP group shows higher thiols than persisting polyarthralgia patients. Higher MDA, protein carbonyls and lower thiols clearly indicate elevated oxidative damage in ChikWPP patients. Free radicals are known as mediators of joint tissue damage in arthritis, in combination with proinflammatory cytokines. The function of ROS and, especially of O_2^- ion in the destruction of cartilage and bone is established [31]. Cartilage is the only protein known to be fragmented by the superoxide anion, and SOD inhibits this destruction strongly [31]. ROS degrade synovial fluid and depolymerise hyaluronic acid, which leads to loss of the thickness in the joint, inactivation of antiproteinase enzymes and initiation of bone resorption [31]. O_2^- ion is produced by osteoclast cells during this bone resorption, and this occurs at the osteoclast––bone interface. Experimentally, it has been established that too much production of ROS leads to accelerated damage of joint cartilage and osteoclast activation; leading to damage of cartilage matrix [31]. SOD levels are significantly lower in ChikWPP patients representing higher degree of damage at the synovial joints of the patient. MDA/SOD ratio indicates higher oxidative damage and found to be elevated in persisting polyarthralgia patients. MDA level represents destruction of lipid bilayer whereas SOD directly indicates level of cartilage damage. Significantly higher MDA/SOD ratio translates the severe damage at joints in the patients of ChikWPP group. The oxidative modifications of proteins and lipids are recognised for their ability to increase the antigenicity of the same and are of particular significance in autoimmune diseases having an inflammatory arm [31].

A strong involvement of ROS signalling and NF-κB pathway has been suggested earlier [31]. Siglec-9 is known to be upregulated by NF-κB pathway and recognised to be increased in inflammatory disease conditions [31]. It has been described that Siglec-9 receptors modulates ROS and decreases ageing effects of the Redox imbalance. It seems very interesting that generation of excess ROS modulates NF-κB pathway which eventually upregulates the expression of Siglec-9 and

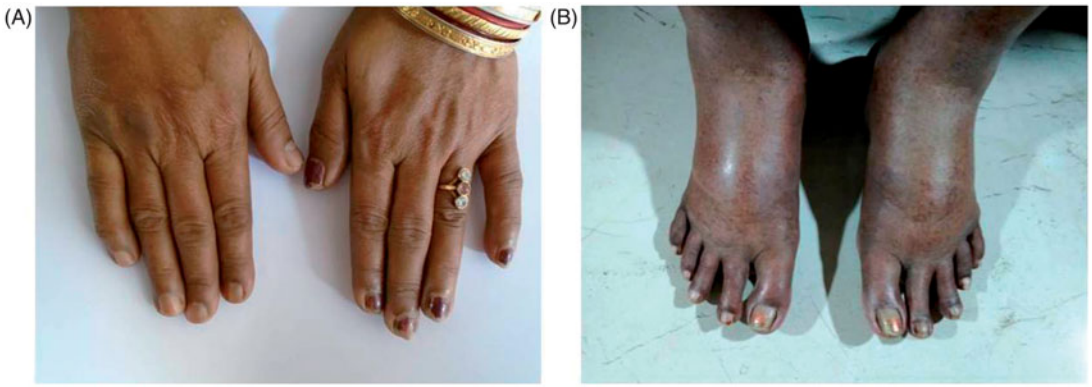

Figure 4. (A–B) are two photographs from two different female patients showing joint inflammation in hand and foot at 15th and 12th day of Chikungunya infection respectively.

both ROS as well as Siglec-9 are found to be increased in ChikWPP patients. So, elevated Siglec-9 expression indirectly indicates huge ROS generation within the cells. Such observation after resolution of initial febrile illness represents the oxidative imbalance present within the patients suffering from persisting polyarthralgia. Free radical scavenging activity of the plasma provides an insight about the antioxidant potential of the same. ChikWoPP group has similar DPPH scavenging activity when compared with healthy controls but the same is significantly decreased in ChikWPP. A negative correlation with ROS further indicates failure of the antioxidation mechanism of the body to attenuate those free radicals. Similar to intracellular oxygen species, NO level in plasma is high in patients with joint pain.

Cytokines and chemokines are well-established markers of inflammation and some previous works reported higher levels of proinflammatory cytokines in chikungunya from different parts of the world [9,10]. IL-6 is reported as biomarker of chikungunya, and in this study also, it becomes a key marker establishing again those previous reports [9]. Interestingly, IL-6 titres in chikungunya patients correlate well with intracellular ROS. This indicates that due to higher production of proinflammatory cytokines, inflammatory pathways are switches on after initial viral infection and continuously produces reactive oxygen species which is dangerous for the host. IFN-γ is a general marker of inflammation and there are many reports available about it [32]. This well-known marker also correlates well with ROS.

IL-10 levels are increased in ChikWoPP group than healthy controls but significantly lower in persisting polyarthralgia group. Previous report shows decreased regulatory T cells in chikungunya patients with polyarthralgia and as these cells are producers of IL-10 so their low levels are obvious resulting in the imbalance of the inflammatory scenario. TGFβ1 is reported to have increased in joint inflammation and similar results are observed in ChikWPP group of this study [14,32]. Previous work also reported about increased TGFβ in chikungunya model of mice and samples from humans [27].

Interestingly, higher value of correlation coefficient (Pearson *r*) is found when correlation studies were done among intracellular ROS and all other studied parameters in ChikWoPP group. It means that higher generation of ROS resembles with other oxidative parameters, proinflammatory cytokines and also there is parity between high ROS and anti-inflammatory markers indicating less damage. In ChikWPP group, anti-inflammatory markers are not present enough to manage the generated ROS successfully which eventually leads to debilitating joint pain.

Following chikungunya infection, free radicals are generated within the host to combat the viral infection and leads to the generation of auto antibodies, cytokines, immune complexes, cytotoxic T lymphocytes. As the virus migrates towards synovial joints, immune complexes trigger excessive ROS within the chondrocytes and osteoblasts and induce osteoclastogenesis. Infiltrating CD 8 T cells further destroys cells and all these available free radicals further generates neoantigens and triggers an inflammatory cycle "Immune complex-ROS-neoantigen-Immune complex". Those patients suffering with polyarthralgia possibly are more prone to produce higher amount of immune complexes (Figure 4). Literatures suggest IgG1 and IgG2 present in those soluble immune complexes mediate inflammatory signals to the cells they attach [33]. Different surrogate markers of oxidative damage provide insight about the pathophysiology of the disease manifestation.

Taken together, this study elaborates for the first time that, patients suffering from persisting polyarthralgia post-chikungunya infection generates higher amount of intracellular ROS along with altered oxidative

damage parameters and Siglec-9 expression on monocytes. ROS levels correlate differently with all parameters and cytokines in chikungunya patients having different disease manifestation. It is evident from this study that, oxidative damage markers provide insight about the disease progression and an important player in differential disease manifestation of chikungunya.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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4 Altered Profile of Regulatory T Cells and NKT Cells As Characteristic of Chikungunya-Associated Polyarthralgia

Nilotpal Banerjee, Bibhuti Saha, and Sumi Mukhopadhyay

Abstract

Chikungunya is an arboviral infection having huge disease burden throughout the tropics, including India. The chief manifestation of the febrile illness is the development of debilitating joint pain, which often leads to arthritis. Till date, very little is known about the disease pathophysiology, which is very important toward better patient management and development of therapeutics. This study aims to characterize different subpopulations of T cell in the peripheral blood of chikungunya patients which may give some idea about the immune homeostasis during acute infection. At a Medical research institute devoted to tropical diseases, 25 informed consented chikungunya IgM^+ patients of different age groups were enrolled during April 2015 to March 2017. After clinical examination, lymphocyte count of peripheral blood was noted followed by three-color flow cytometry. Interestingly, there is no significant ($P = 0.0583$) change in $CD3^+$ T cell population, but there is statistically significant increase in $CD3^+$ $CD56^+$ ($P = 0.0003$) population among chikungunya patients with/without polyarthralgia and healthy controls. $CD3^+$ $CD4^+$ $CD25^+$ (T_{regs}) are decreased significantly ($P < 0.0001$) in chikungunya patients with polyarthralgia. CD4:CD8 is also altered significantly in chikungunya patients. From this study, it is concluded that an imbalance in T_{reg} and NKT cell population is a characteristic of Chikungunya-associated polyarthralgia having possible immunopathological roles.

Keywords

Chikungunya · Polyarthralgia · Regulatory T cell · NKT cell

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4.1 Introduction

Chikungunya (CHIK) is a single-stranded RNA virus of genus *Alphavirus* of family *Togaviridae*. This is an arbovirus transmitted by *Aedes* mosquito [2]. CHIK infection in humans is mostly characterized by short-duration fever and rash along with varied levels of joint pain. Most of the clinical symptoms of CHIK viral infection last for maximum 7–10 days on an average, but the joint pain can persist for 1 year causing extreme distress to the patient along with effective man-hour loss toward the economy [4]. Currently, CHIK viral infection is not only the issue of tropical countries only. In recent years, CHIK has a huge global burden affecting millions of people [11, 12]. The interplay between virus and host is believed to be possible cause of joint inflammation due to chikungunya. Recent studies in mouse model have indicated the involvement of CHIK virus-specific $CD4^+$ but not $CD8^+$ T cells as essentials for the development of joint swelling. Further, the role of Tregs has also been demonstrated in CHIK virus pathogenesis in mice model [8]. Taken together, T cell immunology plays a significant role in CHIK-associated arthritis.

T lymphocytes are innate immune cells having pivotal role in cell-mediated immunity in viral infections. CD3 is the signature marker of T lymphocytes, and estimation of $CD3^+$ population in peripheral blood is a routine method in viral infections like HIV [1]. $CD3^+$ cells are usually further characterized by immunophenotyping with anti-human CD4 and CD8 monoclonal antibodies. NKT cells are $CD3^+$ $CD56^+$ cell population which functionally differs from other $CD3^+$ cells having both the properties of T lymphocytes and natural killer cells. $CD3^+$ $CD4^+$ $CD25^+$ cells are popularly known as regulatory T cells which play crucial role in inflammatory and autoimmune diseases. Regulatory T cells are also known as suppressor T cells and are known to maintain the self-nonself balance of the immune system by cascading signaling pathways to suppress other immune cells. T_{reg} balance is necessary for the homeostasis of the overall immune system, and any alteration in their frequency leads to destruction of normal healthy cells. The CD4:CD8 ratio in peripheral blood represents the tendency of the immune system toward development of inflammation [9]. These parameters are very important to determine the status of the disease manifestation at any given point of time. NKT cells are known to regulate immune response along with secretion of inflammatory cytokines upon activation by Ig-like lectins expressed by macrophages. So, the status of both NKT cells and T_{regs} is important to know the pathophysiology of joint inflammation and pain due to chikungunya infection. To the best of our knowledge, there is no such report regarding the status of those cells in chikungunya infection involving human subjects. In this background, this study aims to identify NKT cells and regulatory T cells in peripheral blood of chikungunya patients suffering from varied levels of joint pain along with healthy individuals by immunophenotyping which might give an explanation for Chikungunya-associated polyarthralgia.

4.2 Materials, Methods, and Subjects

Ethical permission for this study was given by the institutional ethical committee. This study has been done from April 2015 to March 2017, and 25 informed, written-consented patients along with 25 age- and sex-matched healthy donors were enrolled. After clinical evaluation of the patients, initial screening for the presence of IgM antibodies for arboviral infection was done by using WHO-recommended MAC ELISA kits provided by NIV, Pune, ICMR, Government of India. Dengue and chikungunya equivocal cases were repeated to confirm their status. After confirming disease status, peripheral blood was taken, and lymphocyte count was done by Sysmex KX-21 N™ Automated Hematology Analyzer. Furthermore, 100 μL of whole peripheral blood was taken in a BD Falcon™ 5 ml round-bottom tube (obtained from BD Biosciences, India Pvt. Ltd.), and surface was stained with 5 μL of PE/Cy5-conjugated anti-human CD3 mAb (Clone HIT3a) and FITC-conjugated anti-human CD56 mAb (HCD56) obtained from Biolegend®, San Diego, USA. For the immunophenotyping of T_{regs}, whole peripheral blood was mixed with PE/Cy5-conjugated anti-human CD3 mAb, FITC-conjugated anti-human CD4 (clone OKT4) or CD8 (clone SK1), and PE-conjugated anti-human CD25 (clone BC96) mAbs. After 30 min incubation with mAbs, the samples were treated with 2 mL of 1X BD Pharm Lyse™ buffer. The samples were processed as directed in the technical data sheet and undergone flow cytometric analysis through a BD FACSCalibur™ machine. The data generated through flow cytometer were further analyzed by BD CellQuest™ Pro Analysis software. Data was generated in duplicate, and further statistical analysis was done by using Graph Pad Prism 5 software.

4.3 Results

After initial clinical study, CHIK patients were divided into two groups, namely, chikungunya patients with and without polyarthralgia. According to a previous study by Gerardin P et al. (2009), subjects having joint pain involving more than six locations were treated as CHIK patients with polyarthralgia [7]. A total of 80% of CHIK patients were found to have polyarthralgia. Rest of the patients have joint pain in less than six locations. Total lymphocyte count in peripheral blood was found to be overall statistically significant but not significant among groups. This pathological parameter is patient specific but not disease specific. CD4 and CD8 ratio has been found to be increased significantly. NKT cells were found to have increased 2.53-fold in CHIK patients with polyarthralgia than healthy controls. Regulatory T cells are 8.73-fold decreased in CHIK patients suffering with polyarthralgia and 1.29-fold decreased in CHIK patients without polyarthralgia (Table 4.1, Fig. 4.1). This is found to be significant after statistical analysis.

Table 4.1 Different study parameters of the subjects

	Healthy controls[a] ($N = 25$)	CHIK patients without polyarthralgia[a] ($N = 05$)	CHIK patients with polyarthralgia[a] ($N = 20$)	P value* (one-way ANOVA)
Lymphocyte count	30 ± 10.58	32 ± 7.58	34.21 ± 4.68	0.0024
% of $CD3^+$ cells (among lymphocytes)	58.55 ± 1.23	62.15 ± 1.06	62.32 ± 1.13	0.0583
CD 4: CD8	1.51 ± 0.15	1.8 ± 0.10	2.1 ± 0.23	0.0206
% of $CD3^+$ $CD56^+$ cells (among lymphocytes)	4.37 ± 1.05	6.9 ± 3.62	11.07 ± 2.60	0.0003
% of $CD3^+$ $CD4^+$ $CD25^+$ cells (among lymphocytes)	23.75 ± 2.86	18.31 ± 4.61	2.72 ± 5.31	<0.0001

[a]Mean ± S.E.M
*$P < 0.05$ is significant

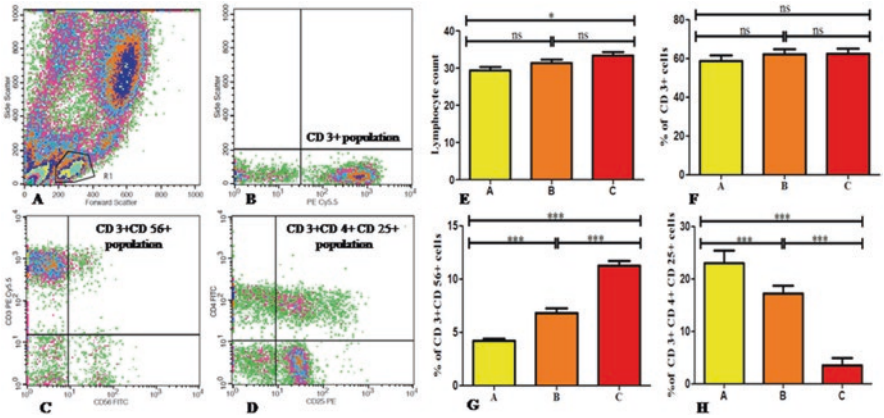

Fig. 4.1 (**a**–**d**) are representative intensity plots. (**a**) is the Forward vs. Side Scatter of white blood cells. (**b**) describes CD3 positive population. (**c**) shows $CD3^+$ $CD56^+$ cells. (**d**) represents $CD3^+$ $CD4^+$ $CD25^+$ regulatory T cells. (**e**–**h**) are graphical representation of study parameters among three groups. (**a**) is healthy control, (**b**) is chikungunya patients without polyarthralgia, (**c**) is chikungunya patients with polyarthralgia within the graphs. One-way ANOVA was done among three groups. Between two groups, *t-test* was done for determination of significance. $P < 0.05$ is statistically significant. Nonsignificance is represented as "ns." (**e**) is the total lymphocyte count among all the study groups. (**f**, **g**, **h**) are percentage of $CD3^+$ cells, NKT cells, and regulatory T cells among all the study groups, respectively

4.4 Discussion

Lymphocyte count of patients in comparison with healthy individuals is not sufficient enough to comment on their immunological role in the pathophysiology of CHIK infection. Rather there is clonal expansion of different subpopulations of lymphocytes observed during CHIK infection which is not reflected by routine pathological test. Interestingly, $CD3^+$ T lymphocytes were also not significantly changed in CHIK patients. Frequency of overall T cell population is not sufficient to comment on the cell-mediated immunity, which happens during viral infection like CHIK. Rather it becomes necessary to know the status of different subpopulations of T cells by immunophenotyping.

CD4 and CD25 positive regulatory T cells were found to be significantly decreased in study group, namely, chikungunya patients having arthralgia in more than six locations, whereas in CHIK patients who do not have developed polyarthralgia, they are not decreased significantly. So, it is clear that decrease of T_{regs} or suppressor T cells is observed in patients with polyarthralgia. CD4:CD8 ratio is also changed significantly. Interestingly, overall percentage of CD4 cells is increased, but that of regulatory T cells is decreased [Table 4.1]. This change indicated autoimmunity, which can destroy the host's own cells. The imbalance of Tregs which happens in some patients might lead to polyarthralgia. As from studies involving other diseases, it is clear that regulatory T cells have protective role against self-cell destruction [6]. Any deviation from the normal level of those cells possibly leads to destruction of host cells, which might be the cause of joint inflammation and pain in a significantly large subpopulation of persons suffering from CHIK infection.

On the other hand, NKT cells are found to be significantly increased in those patients having polyarthralgia. In response of the infection, NKT cells are expanded in some patients to produce inflammatory cytokines [10]. From previous studies, it is known that NKT cells involve with Ig-like lectins, which are responsible for inflammation in diseases like systemic sclerosis and systemic lupus erythematosus [3, 5]. Surge of such cells gives an idea about the inflammation process during CHIK infection. Those patients, who have an increased NKT cell frequency, suffer with more joint inflammation.

So from this study, it is clear that though there is no significant change in $CD3^+$ population among CHIK patients, there is expansion of subpopulations, which is not reflected by overall percentage of T lymphocytes. Detailed study revealed altered percentage of Tregs along with NKT cells, which possibly leads to development of polyarthralgia. This information becomes very helpful for further study toward development of therapeutics and more detailed study of the CHIK disease pathophysiology.

4.5 Conclusion

NKT cells are increased in CHIK patients and might have an important role in the development of joint inflammation. Regulatory T cells are decreased in CHIK patients with polyarthralgia. Alteration of normal level of those cells is a potential cause of self-cell destruction, and possibly this is the cause behind debilitating joint pain. For the first time, this study reports about NKT cells, T_{regs}, and CD4:CD8 data, which are the characteristics of chikungunya-associated polyarthralgia.

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SHORT COMMUNICATION

Intracellular ROS generated in chikungunya patients with persisting polyarthralgia can be reduced by *Tinospora cordifolia* leaf extract

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Abstract Chikungunya (CHIK) is an arboviral infection having huge global burden affecting the life style of the patient badly due to debilitating polyarthralgia. This study aims to evaluate intracellular reactive oxygen species (ROS) in peripheral blood of patients suffering with persisting polyarthralgia post CHIK infection and the potential of *Tinospora cordifolia* leaf extract in scavenging those free radicals in peripheral blood mononuclear cells (PBMC) of the patient. Peripheral blood was collected from written informed consented patients and intracellular ROS was measured in PBMC of patients suffering with persisting polyarthralgia 3 months post CHIK infection followed by the study of free radical scavenging by *T. cordifolia* leaf extract in those cells through flow cytometry. Control population comprising healthy donors were also included in the study. As compared to healthy subjects, twofold higher Intracellular ROS (17.89 ± 1.007 vs. 37.96 ± 1.510, $P < 0.0001$) was found in patient PBMC. Ex-vivo treatment of those PBMC with ethanolic extract of *T. cordifolia* leaf (1 µg/mL) decreased intracellular ROS significantly by twofold ($P < 0.0001$). This study reports that CHIK infection produces high level of intracellular ROS in the patients suffering with persisting polyarthralgia, which was significantly scavenged by ex vivo treatment with *T. cordifolia* leaf extract.

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Keywords Chikungunya · Polyarthralgia · ROS · *Tinospora cordifolia* · Flow cytometry

Chikungunya (CHIK) is an arthropod borne viral infection of humans characterized by febrile illness followed by moderate to severe joint pain affecting the life style of the individuals [2]. CHIK infection is caused by a single stranded RNA virus from genus *Alphavirus* of family *Togaviridae*. This virus is present in human–mosquito reservoir for long time as evident from ancient history of fevers with acute joint pain, but finally discovered in Southern Tanzania and also named in their Makonde language which means "that which bends up" [18]. A significant proportion of chikungunya patients suffer from recurrent polyarthralgia within a year of the infection. The productivity loss in terms of income due to CHIK associated joint pain in a tropical country like India, was estimated INR 391 million in the year of 2006 [9]. Similarly, in Latin America, it was estimated as USD 73.6 million [3]. In Europe it is estimated as 35,000 Euro per capita [8]. Small scale CHIK outbreak is also reported in China in recent years [10]. So it is evident that not only tropical countries, CHIK have affected Europe and American continents in the last decade heavily, challenging the present healthcare system globally [4]. Currently, the disease pathophysiology is poorly understood and there is lack of CHIK specific therapeutics.

Generation of Reactive Oxygen Species or ROS is reported to be the key player of all kind of disease manifestation. Free radical homeostasis is necessary to maintain the life processes in a healthy individual. Immune system runs on the movement and interactions of millions of immune cells throughout the body and generates ROS in regular fashion [12]. Evolutionary changes have provided better physiological mechanisms to manage these free

radicals within the immune system. There are some immunoglobulin-like lectins which are reported to be alter-expressed to counter the generation of excessive ROS and eventually, their expression correlates with the life span of the organism [16].

Management of ROS becomes crucial to minimize the suffering of the patient. *Tinospora cordifolia* is an herbaceous vine of *Menispermaceae* family indigenous to Indian subcontinent [17]. *Tinospora* is mentioned in the ancient texts of 'Ayurveda', the Indian system of medicine as 'Guduchi'. Gulancha is the Bengali name of the climber. In some south Indian languages, this plant is referred as 'Amrita balli' or 'the climber which gives immortality' signifying its medicinal potential [17]. The word 'Amrita' etymologically related to the Greek word 'Ambrosia' having the same meaning. *Tinospora* is mentioned as a reservoir of alkaloids, lactones, glycosides with immunomodulatory potential by Dr. R. N. Chopra from Calcutta School of Tropical Medicine in 1958 [5]. Recently, ministry of AYUSH, Government of India mentioned *Tinospora* extract for the treatment of fevers with joint pain or 'Sandhijwara' (http://www.ccras.nic.in/sites/default/files/viewpdf/Chikunguniya/22092016_MANAGEMENT%20OF%20CHIKUNGUNYA%20THROUGH%20AYURVEDA%20AND%20SIDDHAA%20TECHNICAL%20REPORT.pdf). The etiology and possible management of fever with joint pain was indicated in folklores and fairy tales of India (http://www.anandabazar.com/supplementary/rabibashoriyo/in-bengali-fairy-tales-there-was-strong-indication-about-dengue-and-chikungunya-1.761749). The anti-oxidant activity of *T. cordifolia* leaf extract has recently been demonstrated [15]. However, till date, there is no scientific study elucidating the potential of scavenging intracellular ROS by *T. cordifolia* leaf extract on living cells in ex vivo condition.

This study aims to evaluate intracellular ROS in patients suffering with persisting polyarthralgia post CHIK infection and the potential of *T. cordifolia* leaf extract in scavenging those free radicals in peripheral blood mononuclear cells of the patient.

Experimentally, patients were enrolled along with healthy controls. To know the status of intracellular ROS and the free radical scavenging potential of *T. cordifolia* leaf extract, a simple flow cytometry based technique was followed using 2′,7′-Dichlorofluorescin diacetate or DCFDA as probe. At the first instance, intracellular ROS was measured just after collecting peripheral blood from the study subjects and documented. Further, similar cells were isolated from blood and undergone brief incubation with the plant formulation and intracellular ROS was measured by using the same DCFDA assay. Thus, intracellular ROS is measured directly in the patient blood cells before and after treatment with the plant formulation. This provides a clear idea about the amount of ROS generated in the patient suffering with persistent polyarthralgia along with the effect of *T. cordifolia* leaf extract in lowering the amount of ROS in the patient.

Total 550 individuals were screened in this study during the period of April 2015 to December 2017. Among them 230 and 92 cases were diagnosed as dengue and chikungunya respectively. Rest of the screened patients was not diagnosed with any of the arboviral infection. After initial clinical evaluation, CHIK diagnosis was confirmed by MAC ELISA performed by a WHO recommended kit provided by ICMR-NIV Pune and/or Real Time PCR by genesig® chikungunya standard kit obtained from Primerdesign®Ltd UK. Further, among these 92 confirmed chikungunya cases, only 23 patients were suffering with persisting polyarthralgia. Thus the present study was initiated with 21 such patients (2 subjects, one each of pediatric and geriatric group has been excluded) along with age and sex matched 20 healthy controls. Adhering to the Helsinki protocol, this study was approved by Institutional ethical committee (IEC) and peripheral blood was collected in EDTA coated vials from informed and written consented individuals. Peripheral Blood Mononuclear cells(PBMC) were obtained by layering cells from whole blood using Lymphoprep™ obtained from Axis-Shield, Oslo, Norway.

Tinospora cordifolia leaves were collected from Nursery of West Bengal State Medicinal Plants Board, Department of Health & Family Welfare, Government of West Bengal (Memo No. 8861). The leaves were air dried, powdered and crude ethanolic extract was prepared as described previously [1]. The final extract was dissolved in propylene glycol and stored at 4 °C.

Intracellular ROS was measured using DCFDA as a probe, in PBMC of the study subjects. DCFDA or 2′,7′-Dichlorofluorescin diacetate is a non-fluorescent probe which is naturally permeable to cell. When de-esterified due to oxidation within a cell, 2′,7′-Dichlorofluorescin diacetate turns into highly fluorescent 2′,7′-Dichlorofluorescin [6]. To determine the optimal concentration of DCFDA, PBMC of 10^6 order was mixed with various concentrations of DCFDA (0.5–2.5 μM) at 37 °C for 30 min in dark condition. A concentration of 1 μM was found to be optimal and used throughout this study.

The intracellular ROS measurement assay was performed within 2 h of collection of the peripheral blood sample. PBMC of 10^6 order collected from study subjects were suspended in Phosphate buffered saline of physiological pH and treated with 1 μM 2′,7′-Dichlorofluorescin diacetate obtained from Sigma-Aldrich® in polystyrene tubes suitable for flow cytometry (obtained from BD Biosciences®) maintaining dark condition at room temperature for 30 min. Hydrogen peroxide was used as positive control. After incubation, samples were acquired through a BD

FACSCalibur™ flow Cytometer (Becton–Dickinson, San Jose, CA, USA) in FL 1 channel. Geo Mean fluorescence was taken for determining the presence of intracellular ROS in this study. Thus the level of intracellular ROS was evaluated in all study subjects by this experiment.

To determine the free radical scavenging potential of *T. cordifolia* leaf extract, peripheral blood mononuclear cells isolated from the enrolled subjects, were briefly incubated with the plant formulation. PBMC of 10^6 order was treated with ethanolic extract of *T. cordifolia* leaves in concentrations of 0.5 and 1 µg/mL for 1 h at room temperature. After incubation, intracellular ROS was again measured by the same DCFDA assay adding 1 µM DCFDA to each tube followed by 30 min incubation and flow cytometry. Analysis was done and presence of ROS was documented. Thus, the intracellular ROS level before and after treatment with plant formulation helped in determining the free radical scavenging potential of *T. cordifolia* leaf extract.

GraphPad Prism software version 5.0 (Graph Pad Software Inc., La Jolla, CA, USA) was used for all statistical analyses. $P < 0.05$ was taken significant in all cases. *t* test was done to determine statistical significance among two groups of data and One way ANOVA was done for the same within three groups. All data are represented as Mean ± SEM.

In this present study, prevalence rate of CHIK infection was calculated comprising the positive cases (n = 92) among screened population (n = 550) and found to be 16.72%. Patients who suffered from polyarthralgia 3 months post infection were studied and mentioned as 'CHIK Patient' in this work. All CHIK patients were presented with fever; rash and polyarthralgia (Fig. 1). Persisting polyarthralgia associated with CHIK infection after 3 months was the key clinical determinant of this study. Figure 1 clearly shows joint involvement in a patient after 3 months of CHIK infection.

Arboviral infection like CHIK elicits intense immune reaction within the host. Lymphocytes and monocytes are first line of defense within the host comprising the innate immune system. Several immune pathways are switched on and off to combat the consequences of the viral infection. These abrupt immune reactions develop huge free radicals like reactive oxygen species within the patients' blood and other tissues where generally the virus travels during the fever. Joint inflammation and pain is reported [11] to be caused by the damage of synovial joint tissue which leads to acute and chronic arthropathy.

DCFDA assay revealed 2.12 fold increased intracellular Reactive oxygen species in PBMC from CHIK patients, when compared to healthy individuals (Table 1). The increased value of ROS is found to be significant ($P < 0.0001$) after statistical analysis (Fig. 1). This intracellular assay directly enumerates ROS within PBMC. More than twofold increase of intracellular ROS gives an insight about the CHIK pathophysiology. It becomes evident that ROS might play a crucial role in the development

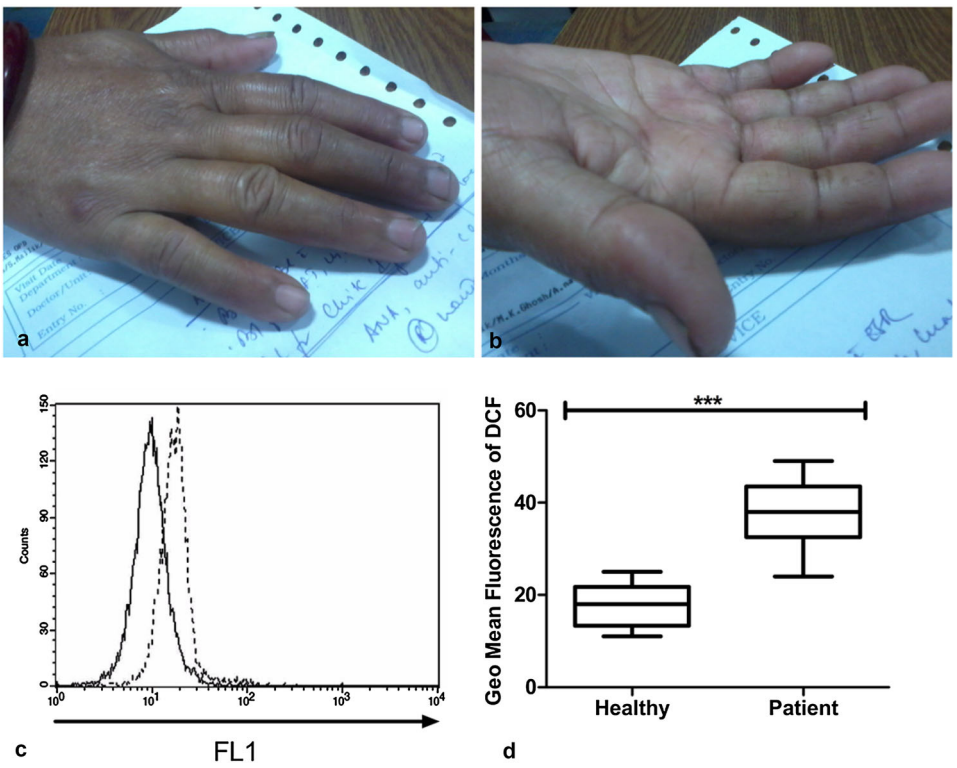

Fig. 1 Joint inflammation and intracellular ROS post 3 months chikungunya infection: **a**, **b** Shows inflamed joints in the right hand of a female patient. **c** is a representative histogram plot of Dichlorofluorescin or DCF intensity in Healthy controls (solid line) and CHIK patients (dotted line). GMF intensity of DCF in all the study subjects is analyzed statistically and represented as **d**. Statistical significance was obtained by *t test* and $P < 0.05$ was taken as significant

Table 1 Basic and oxidative parameters studied in all the study subjects

Parameters	Healthy controls (n = 20)	Chikungunya patients with persisting polyarthralgia (n = 21)
Male:female	1:1	1:1.1
Age in years	27	36
[Median with IQR]	[21.5–34.5]	[30.5–44.5]
Intracellular ROS[a]	17.89 ± 1.007	37.96 ± 1.510
[GMF of DCF intensity]		

[a]Mean ± SEM

of joint tissue damage as reported in previous studies [6]. Scavenging of ROS is very important from the beginning of the disease progression and this might control further damage which otherwise can eventually develop into debilitating joint pain.

Tinospora cordifolia is reported to have anti-oxidant property but not earlier evaluated in CHIK or similar diseases [14, 15]. Lymphocytes are important players of immune system having crucial role in disease pathophysiology of CHIK. Excessive generation of ROS damages those cells, as well as the surrounding milieu where those lymphocytes reside. Interestingly, this extract decreased intracellular reactive oxygen species in both healthy controls and CHIK patients significantly (Fig. 2). Two concentrations of *T. cordifolia* leaf extract were taken to know the appropriate dose in healthy controls and patients; as the minimal dose required for healthy control may not be suitable for patient. In healthy individuals, treatment with 0.5 μg/mL *Tinospora* extract decreased ROS by 1.56 fold whereas with an increased dose of 1 μg/mL, there was no

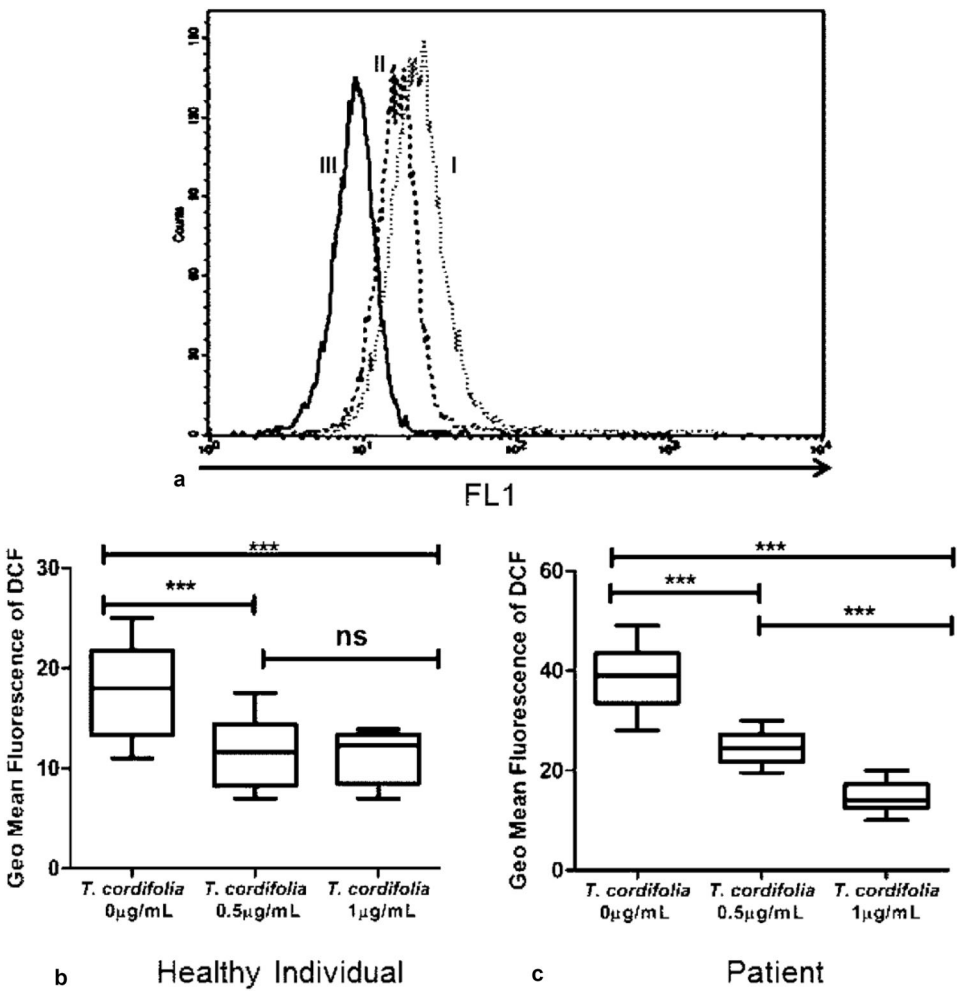

Fig. 2 Scavenging of Intracellular ROS by *T. cordifolia* leaf extract: **a** is a representative histogram plot of DCF intensity after treatment of Peripheral Blood Mononuclear Cells or PBMC with *T. cordifolia* leaf extract in various concentrations (I = 0 μg/mL, II = 0.5 μg/mL, III = 1 μg/mL) in a CHIK patient. **b**, **c** describes the effect of *T. cordifolia* treatment in Healthy and CHIK Patients respectively. Statistical significance was obtained by *t test* and $P < 0.05$ was taken as significant

significant change in ROS level. Interestingly, in PBMC isolated from CHIK patients suffering with persisting polyarthralgia, intracellular ROS decreased from 37.96 ± 1.51 to 24.48 ± 0.67 after treatment with 0.5 µg/mL plant extract whereas ROS decreased from 37.96 ± 1.51 to 14.75 ± 0.66 after treatment with 1 µg/mL extract. Both the changes of Geometric Mean Fluorescence of DCFDA were found to be statistically significant having P value < 0.0001. So, it was determined that a dose of 1 µg/mL extract of *T. cordifolia* leaf extract is required to decrease the intracellular ROS in CHIK patients suffering with persisting polyarthralgia comparable with the basal level of intracellular ROS in healthy individuals.

Tinospora cordifolia leaf extract was found to scavenge intracellular ROS in PBMC of CHIK patients suffering with persisting polyarthralgia. Treatment with 0.5 and 1 µg/mL of *Tinospora* leaf extract also decreased ROS in healthy individuals (Fig. 2). Earlier studies and use in folk medicine reports that *T. cordifolia* extract is non-toxic for humans and rich in alkaloids which might be the possible cause behind scavenging of intracellular ROS (http://www.ccras.nic.in/sites/default/files/viewpdf/Chikunguniya/22092016_MANAGEMENT%20OF%20CHIKUNGUNYA%20THROUGH%20AYURVEDA%20AND%20SIDDHAA%20TECHNICAL%20REPORT.pdf) [5, 11, 14, 17].

Free radicals are established mediators of joint destruction, as these radicals destroy cartilage and bone tissue and degrade synovial fluid. As a result, bone thickness decreases leading to joint inflammation and pain [7]. Chikungunya is a viral infection and elicits high amount of free radicals which eventually degrades synovial joints in some patients to cause persisting polyarthralgia. Though this is a multifactorial process, still ROS is one of the key players in development of joint pain. *T. cordifolia* leaf extract contains several potential anti-oxidant molecules as described earlier [13] and can easily ameliorate excess ROS without any further toxic effect. Plant formulations are popular and easy to use. Thus using those formulations are helpful in such multifaceted manifestation of a viral infection like chikungunya.

Taken together, CHIK infection develops intracellular ROS within the host, which might cause tissue damage leading to severe and persisting joint pain among some patients. Scavenging such high amount of ROS possibly is helpful to decrease the oxidative damage. *T. cordifolia* leaf extract is found to scavenge intracellular ROS in CHIK peripheral blood mononuclear cells and provide insights for further study.

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