Studies on the regulatory roles for Retinoic Acid (RA) during host-microbial interaction: implications for *S. aureus* and *C. albicans* infections

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DECLARATION

I hereby declare that the work presented in this thesis is the result of investigation carried out by me under the supervision of Prof. K. N. Balaji, at the Department of Microbiology and Cell Biology (MCB), Indian Institute of Science, Bangalore, India. This work is submitted for the award of the Ph. D. degree of the Indian Institute of Science and does not form the subject matter for any other degree, diploma, associate-ship or membership of any institute or university.

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ABBREVIATIONS

ATRA	All Trans Retinoic Acid
APL	Acute promyelocytic leukaemia
AURKA	Aurora Kinase A
BMP	Bone Morphogenesis Protein
CARD	Caspase activation and recruitment domain
CCL	Chemokine ligand
CCR	Chemokine Receptor
DAMPs	Damaged molecular patterns
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra-acetic acid
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK-3β	Glycogen synthase kinase-3β
h	hours
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
IL	Interleukin
kDa	kilo Dalton
MDP	Muramyl dipeptide
mTOR	Mechanistic target of rapamycin
NaCl	Sodium Chloride
Na_3VO_4	Sodium ortho vanadate
NaF	Sodium Fluoride
NCoR	nuclear receptor corepressor
NF-κB	Nuclear factor kappa B
NK	Natural Killer
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptor
NP-40	Non-iodet P40
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
ONPG	O-Nirophenol β-D-galactopyranoside
pg	picogram
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGN	Peptidogycane
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase

PP4c	protein phosphatase 4 catalytic subunit
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
SDS	Sodium dodecyl sulfate
SOCS	Suppressor of cytokine signaling
TAK1	Transforming growth factor- beta activated kinase 1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor

Synopsis

Studies on the regulatory roles for Retinoic Acid (RA) during host-microbial interaction : implications for *S. aureus* and *C. albicans*infections

Chronic inflammatory disorder is one of the leading causes of morbidity and mortality worldwide. It underlies the development of metabolic diseases and arises in concert to genetic polymorphism and risk factors including infectious diseases etc. Association between pathogenic infection and inflammation governs tissue homeostasis, which often relies on extensive crosstalk among signaling networks and host derived immune modulating factors. Sensing of pathogen associated molecular patterns (PAMPs) such as cell wall components and cytosolic constituents relies on the expression of plethora of pattern recognition receptors (PRRs) displayed on or expressed inside immune and non-immune cells. PRRs can be classified into various families based on structural and functional divergence. These include: Toll like Receptors (TLRs), C-type lectins receptors (CLRs), Nucleotide binding and oligomerisation domain (NOD) - like receptors (NLR) and Retinoic acid inducible gene (RIG) – like receptors (RLRs). Interestingly, recent insight into studies on inflammatory disorders have shown that, vitamins play a central role in governing fate of diseases by regulating cellular metabolism. Significantly, vitamin A deficiency is known to effectuate several aberrations in innate and adaptive immune responses. In this regard, our current study involves exploration of regulatory role of vitamin A metabolite – Retinoic Acid (RA) in modulating host-microbial interaction. Though known for its conventional role in cell growth, differentiation and organogenesis, RA can also modulate immune responses as well. For example, it can potentiate CD103+ dendritic cells to promote the generation of Foxp3+ regulatory T cells in addition to IL-10. Moreover, RA treatment also regulates inflammatory responses, by supressing TNFα production and nitric oxide synthesis. RA also plays crucial

role in humoral responses by regulating IgA antibodies production by B cells during mucosal immunity. However effectiveness of RA to subvert microbial infection or modulating host-pathogen interactions still remains to be explored.

In this context, first part of our current investigation involves delineating the effectors that are involved in ADA driven suppression of *S. aureus* induced septic arthritis. The disease progression is marked primarily by deregulated inflammation driven by macrophage infiltration into the synovial joints. However, details on the factors mediating such inflammatory dysfunction still remain obscure. In our current study we have utilized intra-articular mouse model of *Staphylococcus aureus* infection-induced septic arthritis. Here we have uncovered a critical role for Aurora kinase A driven activation of mTOR-WNT signaling axis, which acts as a key regulator of pro-inflammatory cytokines expression (*Ccl 2, Ccl 4, Ccl 5, Ccl 11* and*Ccl 12*) in joint synovium. This was found to be associated with perturbation of articular cartilage with degradation of bone in murine knee joints. Septic arthritis often becomes incurable due to emerging antibiotic resistance, thus limiting the treatment to invasive surgical procedures. Therefore, exploring the utilization of vitamins therapy during septic arthritis offers a potential interface for the development of efficacious adjuncts against pathogen-driven responses. Based on these premise, our current investigation employs utilization of a synthetic derivative of RA, FDA approved drug - Adapalene (ADA), which is a known biologically active and key metabolite of vitamin A. Interestingly, we found that prophylactic treatment of ADA, rescued the inflammatory tissue damage and helped in preservation of articular cartilage with bone architecture. Mechanistically, we have demonstrated that treatment with ADAcould inhibit AURKA-mTOR-WNT signaling axis with a concomitant activation of HIPPO pathway. Activation of MST1/2 regulated HIPPO signaling was found to be associated with M2 macrophages (*Retnla, Cxc3r1* and *Ucp1*) activation. The observed association of ADA regulated

polarization of M2 macrophages was found to govern anti-inflammatory effects in stalling *S. aureus* induced septic arthritis. Along with these lines, we have also seen that silencing of MST1/2 has significantly attenuated the ability of ADA to induce pro-resolving mediators like chemR23, which was corroborated witha significant decrease of synovial inflammation and septic arthritis development.

In the second part of our current work, we have explored the regulatory effects of ADA in supressing *C. albicans* induced delayed wound healing. Several studies over last decade, have indicated an exponential increase in invasive mycosis due toinfection with *C.albicans*in immunocompromised individuals. Morbidity and mortality rate associated with *C. albicans* infection is highest amongstfungal pathogens. *Candida* induced sepsis is often fatal which may be attributed largely to perturbed immune response of host rather than pathogen itself. Thus, therapies targeted at augmentation of host immune system might be an interesting strategy to combat *C. albicans*associated pathology.*C.albicans* which belongs to class of saccharomycetes, often reside as skin and gut commensal in host, with capability to elicit lethal systemic infection. Although oropharyngeal, vulvovaginal and invasive candidiasis represent the majority of *Candida* induced pathogenesis, *C. albicans* is also found to be prevalent and dynamic contributor of delayed wound healing. Defect in wound healing is associated with recruitment of proinflammatory mediators that perturbs tissue repair. In this regard, resolving inflammation could provide a therapeutic strategy to enhance wound contraction and restore tissue homeostasis. Interestingly, literature support high correlation of vitamin A deficiency to aberration in host defence against invasive candidiasis. Our observation with administration of ADA to *C. albicans* infected wounds showed significant rescue of delayed wound healing with suppression of chronic inflammatory response. Data was corroborated with skewed anti-inflammatory response, as proinflammatory genes (*Cxcl1, Tnfa* and *Il-6*) were suppressed and excessive activation of anti-inflammatory M2

macrophages genes (*Retnla, Cxc3r1* and *Ucp1*) was observed. BMP signaling which was established previously for its significant contribution to wound delay, was also found to be abrogated upon treatment with ADA. Mechanistically, we have elucidated crucial role of ADA in regulating dynamicity of actin filament assembly. Significantly, administration of ADA to *C. albicans* infected macrophages showed targeted inhibition of an actin-binding protein known as cofilin with significant increase in phagocytic ability of macrophages. It governed such effect through inhibition of *C.albicans* induced BMP signaling which was found to crosstalk with down-stream effectors of Rho/Rac GTPases. Thus, current work explores the beneficial effects of RA in modulating wound healing during *C. albicans* infection and tissue homeostasis.

In summary, through our current investigation we have uncovered the potential therapeutic effects of vitamin A metabolite, RAin contributing a suppressive effects upon hyper-inflammation with augmented tissue repair. ADA administration was found to mitigate inflammatory disorders with direct effect on host disease burden. On one side we have observed that RA potentiated HIPPO signaling governed anti-inflammatory effects of RA, which suppressed*S.aureus* induced septic arthritis. On other hand,a significant inhibition of BMP signalling was attributed to RA treatment which resulted in enhanced wound healing during *C. albicans*infection.

CHAPTER 1: INTRODUCTION

Retinoic acid mediated modulation of host immune signaling events during *S. aureus* and *C. albicans*infection

Various complex interactions between host and pathogens with constantly evolving antimicrobials significantly impact disease manifestation and response to anti-microbial therapy. Immune evasion strategies employed by Invading pathogens often involves impaired recognition of infected cells orprocured resistance to immune effectors mechanism.During severe microbial infection,uncontrolled or hyperactivated innate immune responses by host can lead to detrimental systemic response which include intravascular coagulation, hyper-inflammation, tissue injury and eventually death. In view of this perspective, for our current studywe have attempted to elucidate molecular pathways mediated by RA during infection of host with *S. aureus* or *C. albicans*.

Infection with *S.aureus* is responsible for vast majority of diseases in host ranging from impetigo or infected skin abrasions to more invasive infections such as folliculitis, endocarditis and sepsis. *S. aureus* survival within migratory phagocytic cells is often associated with its systemic dissemination, in addition to cytotoxic effects by its internalisation in non-phagocytic cells such as fibroblast and endothelial cells which ultimately contributes to enhanced burden of the said pathogen with severely affected host survival. Colonization of invading *S. aureus* inside host cells is an important risk factor, which is determined by expression of host effectors versus secretion of pathogenic virulence factors.

On the other hand, *C. albicans* which is a major fungal pathogen, often resides as a commensal with ability to invade both mucosal and deeper tissue surfaces, pertaining to superficial or life-threatening infection of host. *C. albicans* pathogenesis is stimulated by an array of coordinated and timely expression of several virulence factors in varying combination depending on host micro-environment and activated signaling pathways

effectors. Thus, dynamic interaction of *C. albicans* associated virulence factors with fitness attributes of host dictates its colonization in diverse host niches which ascertain its pathogenies.

To subvert microbial associated pathogenesis, several drugs or antimicrobial agents have been utilized to target deleterious infection to host, which often failed to make an impact in controlled clinical trials. In contrast to this, approaches that were aimed at modulating host immune responses were found to complement several deficiencies with ability to minimize the drawbacks of conventional therapy. In this regard, several clinical evidences correlate deficiency of vitamins with enhances percentage incidence, progression and augmentation of disease associated abnormalities. However, utilization of vitamins as effective adjuvants in prevention or reversal of chronic diseases still requires extensive investigation. Based on these premises our current investigation involves exploration of *S. aureus* and *C. albicans* infection induced perturbation of host signaling pathways. We have made an attempt to explore utilization of vitamin as host directed therapy to suppress exacerbated damage to host tissue along with restoration of immune system homeostasis. In this regard, for our current work we haveutilize anti-inflammatory effects of Vitamin A metabolite – Retinoic Acid (RA), as a therapy, to suppress infection induced chronic inflammatory damage to host with augmented anti-microbial defense and prevention of irreversible tissue damage.

1.1 RA family and their transcriptional regulation

RA is an active metabolic form of Vitamin A, which is obtained through diet either in the form of precursor β-carotene or directly as retinyl ester form. Retinoic acid has three metabolically active forms - All Trans Retinoic Acid (ATRA) that represent approximately 75% of total RA pool, followed by 21% of 9-cis-retinoic acidand 3% of 13-cis-retinoic acid(1). RA binds to retinoic acid receptors which belong to the large family of nuclear

receptors including steroids, thyroids hormones, retinoids and vitamin D3. Mechanistically, RA can exerts its effects through nuclear retinoid receptors namely Retinoic acid receptor (RAR) and Retinoid X receptor (RXR). Such nuclear receptors are ligand dependent regulators whose through homo or heterodimerization binding at the cognate promoter orchestrates the transcription of target genes. RAR receptor has three isoforms, RAR-α, β and γ, which can binds to both ATRA as well as 9-cis-RA. RXR also have three isoforms RXR- α, β and γ, whose cognate ligand is 9-cis-RA(2). In response to RA signaling, RAR/RXR occupy promoter which has a characteristic RA response element (RARE), that is typically composed oftwo direct repeats of a core hexameric motif, PuG(G/T)TCA. Majority of RARE is classified as 5 base pair spaced direct repeat (DR5), however RAR/RXR can also bind to direct repeat separated by 2 base pair (DR2) or 1 base pair (DR1) element(3). Following the binding of ligand regulated retinoid receptors at the promoter sequence, battery of chromatin remodellers, transcriptional coactivators and DNA modifiers are recruited which in turn direct the action of RNA polymerase II and general transcription factors to activate target genes.

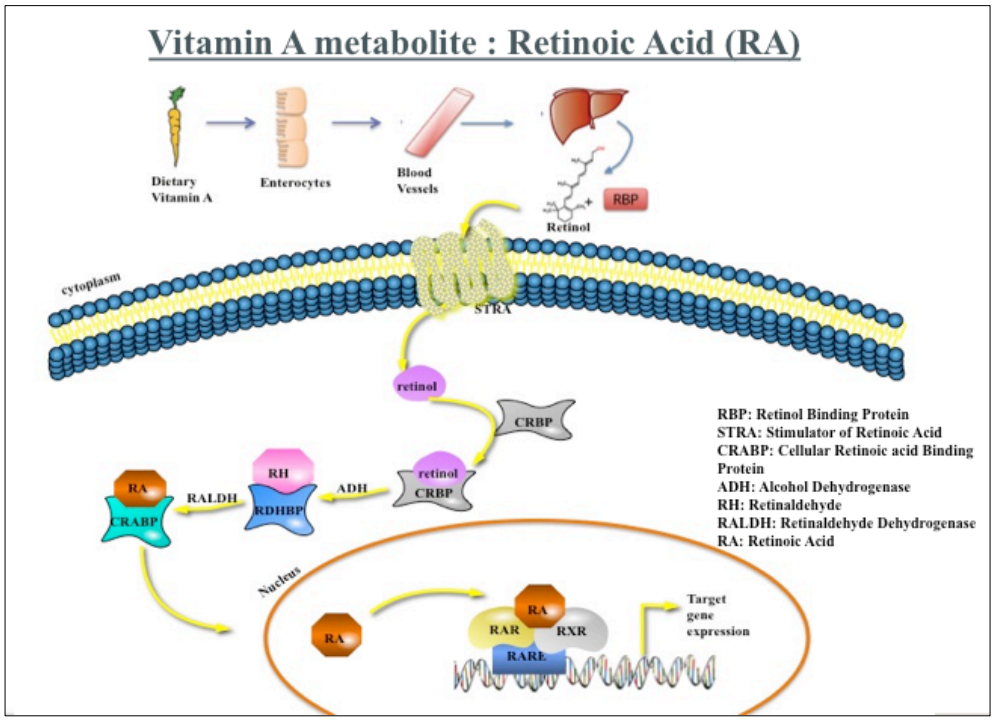

Figure 1. Vitamin A metabolism and signaling pathway - Vitamin A obtained through dietary precursors carotenoids enters in blood stream via enterocytes (small intestinal lining). This travels to distant location and enters liver that acts as storage reservoir. In extrahepatic tissue, Retinol binding protein (RBP) bound retinol enters cells via STRA-6 (Stimulated by retinoic acid 6) receptor. Free retinol derived from lipoprotein lipase and albumin bound RA, may enter by passive diffusion. Inside cells, retinol is esterified and stored as retinyl esters form. This is further metabolized to retinoic acid (RA) by sequential oxidation by ADH and RADH. RA enters inside nucleus, which on binding to RAR/RXR receptors leads to activation of target genes

1.2 Role of RA in infection and immunity

RA has ability to govern pleotropic effects, which includes the regulationof limb patterning, organogenesis and neuronal differentiation. In addition to regulating the developmental effects, accumulating evidences now point towards the immunological roles of RA.Itis known to direct the lineage fate of hematopoietic stem cells into dendritic cells (DCs), innate lymphoid cells (ILCs) and CD4+ T cells(4). Studies on murine macrophages showed that RA can act as a strong anti-inflammatory moleculewhichcan significantly inhibitTumor necrosis factor (TNF), nitric oxide (NO) and IL-12 production(5, 6). Interestingly, zymogen stimulated DC showed enhanced RA signaling, which was found topromote Treg responses with activation of Suppressor of cytokine signaling 3 (SOCS-3)(7). Moreover few reports also suggest thattreatment with RA have potential toinhibit TLR2 receptors expression and its associated functions(8). In line with these, treatment with RA was found to potentiate M2 macrophages phenotype with elevatedM2 marker Arginase-1 gene expression(9) .

Interestingly, RA is proven to be beneficial against hepatitis viral infection. Therapeutic administration of RA was found to reduces viral burden by supressing its replication and release, as observed both at*in vitro* as well as in patients suffering from chronic hepatitis C infection(10, 11). In addition, there are many reports that suggest intestinal immunity often being dictated by micronutrient status. In particularly, RA modulates the local cue to control innate lymphoid cells (ILCs) population which are known

as a potent mediators of barrier maintenance, tissue repair, and host defence. Utilising vitamin A–deficient (VAI) mice, the alteration inILC distribution was observed with respective change in cytokine signature. Herethe ILC3 derived IL-22 and IL-17 governed the significant increase in ILC2 as well as IL-5, and IL-4 level in the gut of VAI mice with was implicated to subvert severe pathological outcome(12).

1.3 Therapeutic role of RA

Retinoids are explored for its ability to prevent neo-plastic tumors from progressing to cancer. The most promising role of RA comes from acute promyelocytic leukaemia (APL) cure, which revolutionized cancer treatment and established RA as a therapeutic agent.Cytogenetically, APL is characterized by a reciprocal translocation between chromosomes 15 and 17, which results in the fusion between the promyelocytic leukemia *(PML)* gene and retinoic acid receptor α (*RAR*α) gene. It generates a PML–RARα fusion protein that causes a block in differentiation of promyelocytes which is characterized with the severe haemorrhagia that has been ascribed to disseminated intravascular coagulation (DIC). Mode of action of RA treatment follows – ATRA (as known RAR agonists) binding to the PML–RAR ligand binding domain, resultes in dissociation of the corepressor complex. This relieves the histone deacetylase activity (HDACs) dependent block of differentiation and, through association of coactivator complexes, triggers the transcriptional programmes that are now endowed by RA signaling(13). In addition to this, another landmark study by Crame*et al.,* showed new hope for treatment of Alzheimer's disease. For this study, administration of RXR agonist – Bexarotene was utilized, in mice expressing a mutant form of the β-amyloid precursor peptide (APP) gene. This resulted in rapid clearance of soluble β-amyloid peptide (Aβ) from the brain of mice with reduced neuritic plaque burden, improved cognition and reversed behavioural deficits(14).

Based on these observations, our current study elucidates regulatory effects of RA upon selected pathogen induced disease progression. Since administration with RA is known to mediate intricate balance of host immune system with subversion of massive tissue damage, our current study aims at exploring the immunomodulatory role of RA against *C. albicans* and *S. aureus*infection induced chronic inflammatory disorders.

S. aureus infection driven modulation of host innate immunity

As described, commensal microbes like fungi, bacteria or viruses have established themselves as symbionts, which are instrumental in shaping immune system homeostasis and regulation of diverse signaling pathways in host. Although C. albicans associated mortality rate is high in infected host yet there exist another commensal known as S. aureus which is equally potent disrupting host immune system homeostasis with mounting of an adverse pathogenic response.

S. aureus is a Gram-positive commensal bacterium with potential to cause both superficial and invasive infections like endocarditis, sepsis, bacteraemia and osteomyelitis. Typically, infection with *S.aureus* leads to characteristic abscess formation which is filled with damaged leukocytes and pus leads to necrotic tissue lesions. So far it has gained resistance to every antimicrobial therapy, which includes antibiotics as well as vaccines. Outburst to methicillin resistance has gained considerable interest due to associated high mortality and public health burden. Studies indicate huge clinical burden associated with methicillin associated *S. aureus* (MRSA)USA300 and USA 400 strains that are known to account for 60% to 75% of staphylococcus infected cases(15).

1.5 Development of infection induce septic arthritis

Septic arthritis is a severe purulent joint inflammatory disorder, most commonly caused by bacterial infection. *S. aureus* is a major clinical isolate and also the primary causal

agent that is accountable for 37% of the reported septic arthritis cases. Such infectious arthritis often renders host debilitated with permanent joint dysfunction that require invasive surgeries as final cure. Annually, incidences of septic arthritis amongst all age groups range from 4-10 cases per 100 000 persons in Western Europe (16). However, the situation is dire in non-industrialized nations where incidences are significantly higher ranging from 14 – 30 cases per 100 000 persons (17).

1.6*S. aureus* virulence factors and clinical manifestations

Clinical manifestation of *S. aureus* infection is governed by various virulence factors. Such pathogenic determinants promote tissue colonization, avoid immune system activation and secrete various toxins in host.

(i) Adherence factors:*S. aureus* invade and survive inside a variety of host cells including macrophages, fibroblasts, osteoblasts, endothelial and epithelial cells. Numerous adhesins are displayed on the surface of *S. aureus* which includestaphylococcal protein A (SpA), fibronectin-binding proteins A and B (FnbpA and FnbpB), collagen-binding protein, and clumping factor (Clf) A and B proteins (18–20).

The N terminal conserved region of SpA binds to IgG, which is an important mechanism to evade host immune signaling. SpA also acts as supraantigen to V_H regions of B cells receptor which in turn leads to clonal expansion of B cells and its apoptosis(21). Interestingly, intravenous challenge of mice with SpA deficient *S. aureus* Newman strain, resulted in significant reduction of bacterial load with decreased kidney abscesses formation within 18 days post infection as compared to wild type strain(22). In addition, another class of adhesins include FnbpA and FnbpB, which have the potency to bind to host cell fibronectin receptor integrin $\alpha_5\beta_1$. Studies utilising murine model showed that systemic infection of *S .aureus* fnbpA or fnbpB mutants can dramatically abolish lethal effects of *S .aureus* infection in contrast to wild type strain. This data was further corroborated with other pathological

parameters including reduced colonization of mutant bacterial strains with its reduced multiplication and abscess formation in the kidney (23).

(ii)Expression of exotoxins:*S. aureus* possess numerous exotoxins like *α*-hemolysin, *β*-hemolysin, and leukocidin. These form pore like cytotoxic beta barrel channel in host cell, which perturbs cellular osmolarity and ultimately ruptures it(24). Leukocidin enhances susceptibility of neutrophil towards cytolytic activity, by binding to C5aR and C5L2 on the surface of neutrophils(25). Another class of toxins is alpha-haemolysin (Hla) which interacts with host disintegrin and metalloprotease 10 (ADAM10), that ultimately generate cytolytic pores(26). Passive immunization with Hla antisera was found to significantly reduce the size dermonecrotic skin lesions in contrast to infection with wild type bacterial strain(27).

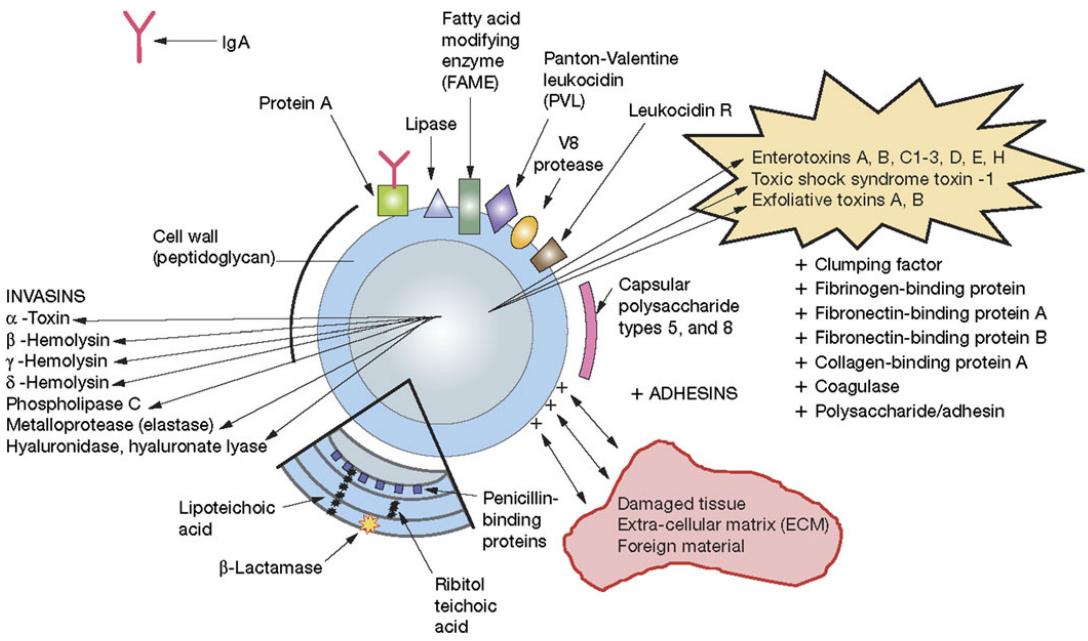

Figure 2. *S. aureus* associated virulence factors and subversion of host innate immunity–*S. aureus* virulence factors play pivotal role in mediating its proliferation and survival in host cells. Factors such as protein A binds to the Fc region of all human IgG subclasses thereby eliciting hampering of the host adaptive immunity. Secreted coagulase has an ability to clot plasma in the absence of calcium which along with staphylokinase can bind to plasminogen and activates the fibrinolytic enzymes. Hyaluronidase aids in the invasiveness of *S. aureus*

in tissue by breaking cell associated hyaluronate. Production of haemolysin by bacteria can severely effect host with lysis of RBCs and its associated cytotoxicity. (Adapted from (28))

1.7 Immune evasion of host by *S. aureus*

As described before,*S. aureus* infection often results in manifestation of acute or chronic persistence of diseases in host that are refractory to antibiotic treatment and results subsequently in high morbidity and mortality. With expression of enormous virulence factors and adaptation to harsh cellular microenvironment, bacteria can evade host defence mechanisms.

(i) Modulation of innate immune responses – *S. aureus* infection counteracts the anatomic barrier at first to initiate its colonization and infection in host cells. It breaches the epithelial defence by means of adhesins which include key factors like glycopolymer cell wall teichoic acid of *S. aureus*, that directly interacts with nasal epithelial surface through a type F scavenger receptor named SREC-1(29). Another important mediator is clumping factor B, which have potency to bind to host fibrinogen, cytokeratin and layer of squamous cells(30). Second line of innate defence constitutes of the complement system that is orchestrated by a cascade of proteolytic enzymes which act directly on invading bacteria. Complement evasion by *S. aureus* involves the interaction between staphylococcal binder of immunoglobins (Sbi) and complement component C3 via contact involving C3a anaphylatoxin domain and promoting its futile consumption (31).

S. aureus can also escape neutrophil mediated killing by subverting the effects of anti-microbial peptides, reactive oxygen, nitrogen species and proteolytic enzymes. Staphyloxanthin are carotenoid pigmented molecules that prevent neutrophilic killing by abolishing hydrogen peroxides and hydroxyl radical generation(32). Similarly expression of catalase (Kat A) and alkyl hydrogen peroxide reductase (AhpC) provide oxidative-stress resistance to *S aureus*, thereby aiding its survival in hostile environment(33).

(ii) Modulation of adaptive immune responses – In addition to the above mentioned observations, *S. aureus* has ability to manipulate both B and T cell responses as well. SpA secretion form majorly all clinicalisolates is a well-studied example to show that majority of infection can even occur in immune competent individuals with no defect in host phagocytic machinery. SpA is a sortase-anchored surface protein with high affinity for immunoglobulins IgA, IgD, IgG1-4, IgM and IgE(34). Immunosuppressive attribute of SpA is ascribed due to its interaction with Fcγ domain and with the Fab domains of antibodies. SpA crosslinking to V_H domain of IgM promotes B cell apoptotic cell death (21).

Superantigens secreted by *S. aureus* bind directly to MHCII and beta chain of T cell receptors to bypass conventional MHC restricted antigen processing and presentation. The most common staphylococcus associated superaantigens are toxic shock syndrome toxin 1 (TSST-1), enterotoxin B and enterotoxin C. Amongst these, TSST results in life-threatening conditions that involve multi-organ failure and death of host, which results from exacerbated release of cytokines (*Il-2, Ifng*and *Tnfa*) from highly activated superantigen stimulated T cells(35). In addition to this, persistent infection of *S. aureus* can also lead to T-cell anergy*in vivo,* thereby leading to its unresponsiveness towards invading pathogen (36).

1.8 Host directed therapies against *S. aureus* infection

Host directed therapies are promising strategies directed against invading bacteria to sustain spread of infection and provide protective benefits to the host. Bacterial evasion of host immune system involves exploitation of host factors and perturbation of host signaling pathways thus pressing the need to study of host- pathogen interactions as a promising avenue to identify potential novel targets. Plethora of host-directed strategies includes utilisation of monoclonal antibodies, vitamins, recombinant proteins and repurposed drugs.

(i) Interleukins therapy- *S. aureus* cutaneous infection toγδ T cell–deficient mice, had substantially larger skin lesions with higher bacterial counts and impaired neutrophil

recruitment compared with WT mice. With single dose of IL-17 significantly restored the impaired immune system of host with reduction in lesion size and reduced bacterial counts(37).

(ii) Utilization of antibodies- Altastaph was the first IgG based antibody that went upto phase II of clinical trial. Mechanistically, it was found to elicit opsonophagocytosis in mice infected with *S. aureus* with reduced bacterial counts obtained from kidney, liver and peritoneal lavage thereby conferring protective benefits under immunization(38). In addition to this, another antisera was developed from fibrinogen-binding proteins ClfA which was tested for its therapeutic potential. Treatment with veronate showed enhanced opsonization when rodents were intraperitoneally challenged with *S .aureus*. It however failed to reduce late onset of sepsis in phase III clinical trial(39, 40).

(iii) Protection by T cells and macrophages– Vaccination with*S. aureus* surface protein ClfA and TLR9 agonist CpG promotes Th1 immune response, which accelerates bacterial clearance in murine model of recurrent S aureus induced peritonitis(41). Another study suggests that, adoptive transfer of MRSA primed bone marrow derived macrophages can confer protective memory in naïve recipient mice. Data was further corroborated with, altered cytokine signature and increase in staphylocidal activity in vivo murine model(42).

However, due to the limited success with available antibiotics and constantly failing vaccines, a shift in passive immunization and modulation of host immune system is very much required.In light of the multitude of virulence factors utilised by *S. aureus* to cause disease, it may not be surprising that there have been numerous failed attempts to develop effective vaccines or immunotherapies when therapeutic regiments have been approached from a single-target angle. In this context, It is of paramount importance that novel therapies should be explored which could target the multifaceted makeup of the *S. aureus* pathogenic lifesty

C. albicans infection driven modulation of host innate immunity

In general, mounting evidences shows that fungal population lives in a dynamic relationship with host, wherein the commensal fungal community plays a significant role in development, homeostasis and function of immune system. In line with this it is widely considered that commensal have co-evolved with their respectivehost along with development of tolerance mechanism in order to establish its colonization. Of the various fungi that are known, *C. albicans* is well characterized commensal of gastrointestinal tract (43)and gastrourinary tract(44), with ability to cause severe aberration to host immune system as well. *C. albicans* is ubiquitously present in environment, which often leads to life threatening systemic infection with upto 40 % mortality rate in immunocompromised host(45). Ability of *Candida* to survive and infect diverse host is dependent onits virulence factors and fitness attributes. It is known that during the course of intestinal colonization,*C. albicans*can modulateEFG1 transcription factor which allows its yeast to hyphal transition and help in invasion to host tissue(46). Upon transition to pathogenic form,*C. albicans* significantlycontributors to oropharyngeal, vulvovaginal, and invasive candidiasis. Despite the availability of various pharmacological agents and therapies, manifestation of *C. albicans* pathology still remains high. This partial success in combating invading *Candida* could be attributed to acquired resistance to employed anti-fungal drugs. Thus in this context, we hypothesised to utilise host directed therapy to modulate immune system as a strategy employed to combat disease burden associated with *C. albicans.*

1.9Background:

Cell wall of*C. albicans* is composed of chitin, β-glucans and mannose. The polysaccharide structure of cell wall is recognised by PRRs of host innate immune system. Majorly, polymorphic *Candida* is recognised by TLRs and CLRs. TLR2, TLR4 and TLR9 can directly interact with phospholypomannan and O- linked mannose residue of *C.*

albicans(47, 48). In addition to this, interaction of β-glucan and mannan are associated with activation of CLR signaling associated surface receptors Dectin-1 and Dectin-2 respectively(49, 50). The dynamic interaction between host PRRs and *C. albicans*associated virulence factors often governs thepathogenesis. While responding to invading *C. albicans*, host activates diverse signaling pathways and employs various signaling mediators to counteract against it. Reports suggest that,host directed biphasic innate immune response help in discrimination between yeast and hyphae form of *C. albicans* during invasion of epithelial cells. Under this response, the activated MAPK/MKP1/c-Fos pathway is reported to be critical to transmit the danger signal to host which represent pathogenic switch of commensal microbe(51). In this regard, perturbation of host protective signals often leads to development of chronic diseases wherein several reports indicate a prevalent contribution of *C. albicans*to cutaneous infection and its association with greater then 46% of fungus positive wounds(52). The wound microbiome with *C. albicans*biofilm are hypothesized to disrupt highly coordinated and sequential wound healing process.

1.10Prognosis of wound and its associated microbiome

Skin being the largest organ of body, provides a protective shelter against harsh external environment and invading pathogens. Maintenance of skin tissue integrity is of paramount importance, which among many factors relies on extensive network of host innate and adaptive immune response that host showcases throughout its life time. Skin breach, which is often acute, is immediately addressed by circulating neutrophils and macrophages that, along with rapid angiogenesis blocks skin rupture. Wound healing is a dynamic process that includes four major phases. These include rapid vasoconstriction and platelets aggregation to maintain homeostasis within hours to tissue injury. This is followed by an inflammatory phase that consists of macrophages and neutrophils recruitment to the site of injury. Third phase lasts for weeks which includes fibroblast proliferation, collagen synthesis

and skin tissue re-epithelization. These phases end up with tissue healing which is governed by extra cellular matrix remodelling (53). Perturbation at any of these steps may hinder tissue repair with adverse scar formation and irreversible damage. Chronic wounds are characterised by poor angiogenesis with hyper-inflammatory milieu and stalled re-epithelization, thus resulting in aberrant wound contraction and scar formation. In this regard, several studies confirm that chronic wound microenvironment is associated with microbial persistence which makes such wounds refractory to antibiotics or any other medical intervention (54).

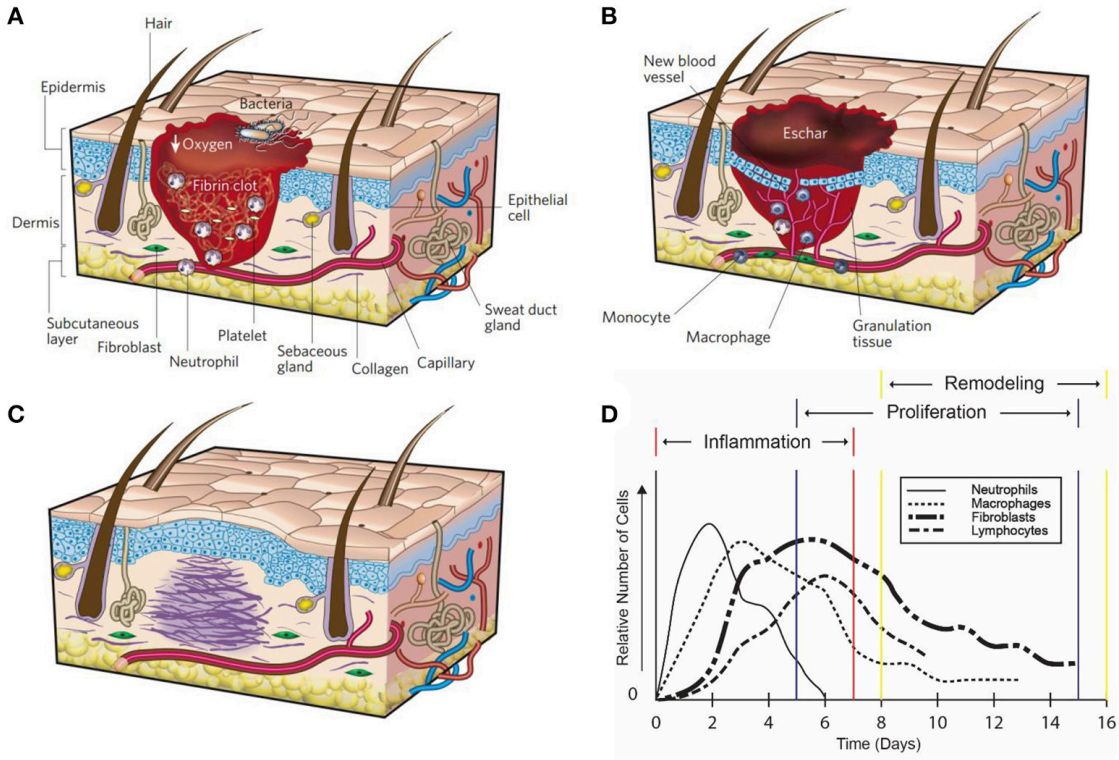

Figure 3. Phases of wound healing–wound healing is marked by three classical stages: **(a) inflammation –** this is the first stage which lasts for 48 h that is characterised by fibrin clot formation with ischemic microenvironment of wound bed. Such inflammation may be associated with presence of microbe with recruitment of neutrophils and platelets. **(b) Re-epithelization of matrix –** this stage lasts for upto 2 days after injury. As depicted a scabe has formed on the surface of wound with populated new blood vessels and migration of epithelial cells. **(c) Tissue remodelling –** This stage lasts for upto weeks after injury, marked by deposition of collagen and fibroblasts. This results in wound contraction and healing. (adapted from: (55))

1.11Role of *C. albicans* infection in wound pathogenesis

Interestingly, nutritional deprivation of host and microbial prevalence at wound microenvironment significantly abrogate highly coordinated physiological wound healing cascade(56). Clinical data primarily focuses on prevalence of diverse bacterial species in wound microbiome, however association of fungus with wound still require very extensive investigations.Only few reports hints towards the crucial contribution and interaction of fungus in shaping wound pathophysiology. Study by Chellan*et al.,* showed that *Candida* species (25% *C. parapsilosis*, 22.7% *C. tropicalis*, and 10.6% *C. albicans*) represented 75% of the total pathogenic strains isolated from wound microbiome of diabetic foot ulcers(57). Moreover fungal-positive wounds were found to be represented by 46% of the total clinical cases of chronic wounds (52). Interestingly, recent literatures also support the high prevalence of *C. albicans* in wound prognosis as well(58).

1.12Pathogenicity factors associated with *C. albicans*

C. albicans is a known commensal that resides on skin and gut mucosal surface of host. Such microbial interaction often breach host protection in immunocompromised individuals and result in disease pathogenesis. Various factors are associated with *C. albicans* virulence, as detailed below:

(i) Biofilm formation: Candidial biofilm is typically composed of both yeast and hyphal consortium that is typically interspersed by extracellular matrix. Maturation of such complex architecture of biofilms governs resistance to antibiotics and host immune signaling(59). Such mechanism also triggers a protective response against killing by neutrophils with compromised ROS production by leukocytes(60).

(ii) Transition from yeast to hyphal forms:*C. albicans* belong to the class of polymorphic fungus, which can grow either as yeast, or as elongated ellipsoid cells called pseudohyphae which have constriction at septa and can even show formation of hyphae as well. While yeast

and pseudohyphae are less pathogenic, transition to hyphal form imparts virulence to *Candida* with capacity to invade host cells. A range of environment cues govern the transitions of*C. albicans* morphologies, includingincrease in pH, CO2 and temperature, which predominantly allow *Candida* to form hyphal network (61). Interestingly, morphogenesis of *C. albicans* transition is under genetic control of Cph1 and Efg1 genes, and the double mutant of such genes are locked in non-filamentous form and found to be avirulent in mouse model(62).

(iii) Secretion of hydrolases: Interaction of host cells with *C. albicans* hyphae follows secretion of hydrolases which ultimately results in penetrance of *Candida* inside the cells. Secreted hydrolases comprises of three different classes: proteases, phospholipases and lipases. The family of secreted aspartic proteases (Saps) is comprised of ten members, Sap1–10. Immunomodulatory role of Sap1-3 was found in oral candidiasis model, which showed elicited damage to reconstituted epithelium in vitro (63) and also governs its systemic dissemination in host(64). The family of phospholipases contributes to pathogenicity by disrupting host cell membrane. Both *plb1* and *plb5* double mutants have been shown to be attenuated in virulence in a mouse model of systemic infection (65, 66) . The third family consists of secreted lipases with 10 members in total. For example, Lip8 *C. albicans* mutant are significantly less virulent in intravenous systemic murine infection model (67)

1.13Immune evasion mechanism employed by *C. albicans*

Candida employs multiple mechanisms to evade host immunity. These range from inhibition of phagosome maturation or phagocytosis, induced activation of stress responsive signaling mediators and expression of decoys against host.

(i) Dynamicity of *C. albicans* cell wall – Due to dynamic nature of fungal cell wall, *C. albicans* retains the ability to change its composition under environmental stress and

pathogenesis. For example, cell wall comprised of β-1,3-glucan polymers, are shielded by outer mannan layer, that prevents its interaction with host immune receptors - Dectin-1(68).

(ii) Prevention of phagosome maturation and phagocytosis – Internalization of *C. albicans*by phagocytosis results in rapid reactive oxygen species burst. Both Cap1 and Hog1 genes are established as a response regulators for*C. albicans* against oxidative stress of host cellular environment(69, 70) The key function of these genes are upregulation of anti-oxidant system, which involves the catalase, glutathione and thioredoxins along with NADPH production via pentose phosphate pathway. Catalase gene Cat1 detoxifies host driven H_20_2 thereby increasing fungus tolerance. More importantly, *C .albicans* employs the glutathione and thioredoxin systems to repair oxidatively damaged proteins.

(iii) Activation of stress responsive mediators- *C. albicans* inactivates a wide range of anti-microbial peptides to mount defence against host immune signaling. In this regard, studies revealed expression of polyamine efflux transporter Flu 1 to reduce toxicity of histatin 5, a known anti-fungus peptide(71). In addition to this, activation of high-osmolarity glycerol (HOG) pathway mediated resistance to antimicrobial peptides. Triggered mitogen-activated protein kinase (MAPK) Hog1 and β-defensins orchestrates response to cellular damage by reducing ROS production and ATP efflux (69)

1.14Immunotherapies against fungal burden

Utilisation of immunomodulatory therapy as an adjunct to antifungal agents has a potential to improve host immune system which can help in enhanced clearance of pathogens. In this regard various strategies have been employed in past few decades:

(i) Leukocytes therapy-Polymorphonuclear cells (PMNs), monocytes and macrophages are one of the most important cells of the innate immune system. Interestingly, utilization of granulocyte colony-stimulating factor (filgrastim and lenograstim) therapy directed against neutropenia is in phase IV randomised clinical trial. This therapy has proven to be beneficial

in patients with candidiasis withprofound decease observed for mortalities associated with invasive fungal diseases (IFD)(72). In addition to this, GM-CSF (trade name: Sargramostium) utilisation with voriconazole and amphotericin B, resulted in full recovery of patients treated for *Aspergillus* ventriculitis(73).

(ii) Monoclonal antibodies therapy- Mycograb a recombinant antibody against fungal heat shock protein 90 (HSP90), is studied for phase II clinical trials. With synergism to amphotericin B, Mycograb showed marked reduction in *Candida*-related mortality from 26% (controls) to 6% (Mycograb)(74)

(iii) IL-12 and enhanced Th1 mediated immunity- IL-12p40 is a potent inducer of Th1 responses(75). Interestingly, studies suggest that 1L12 -/- mice can display disseminated candidiasis following oral infection with *C. albicans*(76). Moreover, administration of recombinant 1L-12 with enolase which is a known *C. albicans* antigen, serves a protective benefit in mice with increase in its survival and decrease fungal burden in kidneys(77).

Thus, the identification and exploration of host directed therapies can provide new avenue to blunt *C. albicans* associated delay in wound healing. In this context, therapies directed against stalling chronic inflammation in addition to abrogation of *Candida*infection burden may prove to be beneficial to host.

1.15 Rationale of current study:

1.15.1 *S. aureus* activated AURKA-WNT signaling axis and its implication on development of septic arthritis

As described, host-pathogen interaction results in complex array of signaling events, where successful invasion by pathogen results from exploitation of host machinery to develop its own replicative niche. However, this can also proves detrimental to host which can result in various pathologies with sustained inflammation. Thus, it becomes imperative to explore such signaling events that play limiting role in determining outcome of host-pathogen crosstalk. Our previous work showed crucial role of NOD2 triggered WNT signaling in development of acute arthritis(78). In this study bacterial cell wall component muramyl dipeptide (MDP) was utilised as synthetic ligand to establish acutearthritis, with characteristic recruitment of proinflammatory cytokines and neutrophils in murine knee joints. With extension of this model, our current work aims at exploring septic arthritis which is a chronic inflammatory disorder that results from the colonization of infectious agents, majorly bacteria, in the joints. While pathogenic strains of *S. aureus* such as - Newman and LS1 have been demonstrated to cause arthritis with significant bone erosion (79), we lack an intricate understanding of the host-pathogen interactions and associated signaling networks in this process. Studies suggested that, protein A positive *S. aureus* Cowan1 is a septic arthritis isolate that strongly induced the production of serum IgM rheumatoid factor (80) and anchored arthritogenicity associated virulence factors such as the fibrinogen adhesin clumping factor A (ClfA), protein A and fibronectin-binding proteins (FnBP). These virulence factors potentiate the tissue invasion ability of Cowan 1 by recognizing and binding to host cartilage with high affinity. However, despite being a clinical arthritis isolate, Cowan1 has not been studied extensively in septic arthritis. Thus, to explore the role of *S. aureus* Cowan1 during infection induced arthritis; we utilized mice model for septic arthritis with the concurrent focus on exploring the role of pathogen-induced signaling pathways.

1.15.2 *C. albicans* impaired wound contraction and its effect on perturbation of Rho/Rac GTPase signaling

The complex interaction of host and microbe is often reflected by adverse fungal pathogenesis, wherein the defect in host immune effectors results in susceptibility to invasive diseases. Ubiquitously present fungus has co-evolved with host with ability to colonize every niche within in human body. For example, environmental opportunist like *Aspergillus fumigatus* or commensal like *C. albicans* can cause devastating host response when immune homeostasis is perturbed. In fact, *C.albicans* is known to be associated with self-limiting cutaneous lesions to manifestation of life threatening infections.

Of the various diseases like oropharyngeal, vulvovaginal and invasive candidiasis, the association of *C. albicans* with cutaneous wound infection still remains an unexplored field. Interestingly, a comprehensive molecular diagnostic report on clinical specimens showed high correlation between chronic wound and fungus microbiome (52). In line with this, our previous has demonstrated the crucial role of C. albicans in delayed wound healing(81). In this study, we have identified the indispensable role of Dectin-2 coupled Syk kinase in the destabilization of RNA-binding protein AUF, which in turn leads to activation of BMP signaling. Moreover, we had found the activated BMP pathway could orchestrate the recruitment of repressive EZH2 mark at the promoter of ECM remodelling genes. Although perturbation of these genes disrupt timely wound closure, we still lack understanding of effectors that mediates active phagocytosis of *C. albicans* and associated fungal burden at the site of infection. Such perturbation in wound contraction is often associated with hyperinflammation of the infected site that severely effect repair of wounded tissue. In this context we sought to identify mediators that could regulate invasion of *Candida* in host tissue upon infection.

1.15.3 Regulatory effects of RA in modulating host -microbial pathogenesis

Host armamentarium to combat chronic diseases is limited which often culminates to irreversible damage to tissue. Despite the availability of various treatment modalities ranging from expression of host derived anti-microbial peptide to utilisation of synthetic antibiotics, we still observe high prevalence of chronic diseases.Moreover treatment of chronic inflammatory disorders like wound healing and septic arthritis are often untreatable owing to development of refractoriness to antibiotics thereby limiting treatment to surgical intervention only. In this context we made an attempt to utilise vitamin A therapy to stall chronic inflammatory disorders. Vitamin A deficiency in the host is shown to effectuate several aberrations in innate and adaptive immune responses leading to increased susceptibility to *S. aureus* infection (82). Importantly, a direct role of RA in amelioration of collagen-induced inflammatory arthritis was documented, thereby establishing RA as a treatment modality in improving the clinical symptoms of autoimmune diseases (83). In line with this,administration of RA is also known to supress *C. albicans* infection induced secretion of proinflammatory genes (TNFα, IL6 and IL12) in human monocytes (84)Interestingly, literature indicatesstrong evidence of fungistatic efficacy of RA against *C. albicans*, suggesting potential usage of vitamin as adjuvant therapy for prevention of fungal diseases(85). However, there is a dearth of scientific exposition on the regulatory effects of RA against infectious microbial agents like *S. aureus*and *C. albicans*. To this end, synthetic derivative of RA – Adapalene (ADA) is utilized in the current study.As mentioned, ADA is a third generation RAR(β) selective agonist, approved by the U.S. Food and Drug Administration with a broad safety profile and it is chemically stable (FDA - 090962). It is an established comedolytic (86) and efficacious chemotherapeutic agent (87). Based on these premises, we have attempted to utilize ADA as a therapeutic, to prevent severe aberration of host tissue and preserve its architecture by supressing development of chronic inflammatory disorders upon infection with *S. aureus*and *C. albicans.*

1.16 Objectives of the current study:

As described, interaction between host and pathogen is dependent upon intricate balance between effective host defence and expression of virulence attributesby pathogens. Factors mediating any imbalance to such interactions often culminates to development of disease, which if unchecked leads to chronic irreversible damage to host. Lack of efficacious immunotherapeutics with increase in global disease burden, presses the need upon development of new effective treatment against invading pathogens. Several reports highlights the beneficial effects of vitamins supplementation in prevention or control of diseases progression. These observations prompted us to undertake the current study, wherein vitamin A metabolite was explored for its immunomodulatory role against selected pathogeninduced chronic inflammatory disorders.

In this context, we hypothesized the utilization of RA therapy to supress *S. aureus* induced chronic inflammation or septic arthritis development. The rise of *S. aureus* as a formidable pathogen is attributed to diverse virulence factors, altered antibiotics susceptibility and multiple signaling mechanism directed against host immune system.It is widely accepted that with availability of limited treatment modalities and increased antibiotics resistance,cure to septic arthritis is unarguably difficult.In this regard, we made an attempt to utilize anti-inflammatory ability of ADA, a synthetic retinoid derivative, to subdue septic arthritis development. Through our current work, we have found the differential effects of ADA in modulating host directed innate immune signaling, wherein AURKA regulated WNT pathway was inhibited with concomitant activation of HIPPO signaling. Mechanistically, we have observed that HIPPO pathway governed the anti-inflammatory effects of ADA on modulating the activation of M2 macrophages and lipid derived resolvin cognate receptor ChemR23. In addition to this, our work also revealed the implications of *in vivo* administration of ADA to intraarticularly *S. aureus* infected mice. We uncovered the

prophylactic effects of ADA in restoration of articular cartilage and bone architecture in septic arthritic mice. Quantification of bone morphometry also confirmed marked recue of bone porosity with preservation of tissue homeostasis and reduced damage **(Chapter 3)**.

High prevalence of fungus associated wounds, led us to explore *C.albicans* induced perturbation of wound healing with studies on associated signaling mediators governing the said *Candida* pathogenesis. For our current study we have utilized the mice model of dermal skin excision and studied the kinetics of wound closure with elucidation of skin regenerative differentiation markers associated with dermal contraction. Administration of synthetic RA derivative – ADA, was found as a promising therapy to subvert *C. albicans* induced wound healing with definitive role in modulating host innate immune signaling pathways. Our work highlighted the crosstalk of ADA regulated BMP signaling with effectors of cell cytoskeletal like LIMK and COFILIN. In this regard, the implications of membrane associated Rho/Rac GTPases was explored to study effects of RA on uptake of invading *C. albicans* inside macrophages **(Chapter 4).**

Thus, our study attributes the beneficial effects of administered RA, upon host derived signaling mediators in abrogating *C. albicans* induced delayed wound healing and *S. aureus* induced septic arthritis development.

CHAPTER 2: METHODS

2.1 Cells, mice and bacteria

BALB/c, *tlr2*-KO, *tlr4*$^{Lps\text{-}d}$ mice were purchased from Jackson Laboratory and maintained in the Central Animal Facility, Indian Institute of Sciences. Mice were intraperitoneally injected with 1.5ml of 8% Brewer thioglycolate. After 4 days, mice were sacrificed and peritoneal macrophages were isolated via gastric lavage from peritoneal cavity with ice – cold PBS. Isolated cells were cultured in DMEM (Gibco-Invitrogen/Thermo Fisher Scientific) containing 10% FBS (Gibco- Invitrogen/Thermo Fisher Scientific) for 12h and adherent cells were used as peritoneal macrophages. Murine RAW264.7 macrophage cell line was obtained from the National Centre for Cell Sciences, Pune, India.

*S. aureus C*owan 1(MTCC - 902) was obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India. For *in vitro* experiments, *S. aureus* were grown in Luria Broth at 37°C overnight and then diluted and grown for an additional 4 h until the OD600 reached 0.2, corresponding to approx. 8 x 10^7 colony-forming unit (CFU) ml. Bacteria were harvested by centrifugation, washed with phosphate-buffered saline (PBS) and resuspended in the cell culture DMEM complete medium without antibiotics. These *S. aureus* were used at Multiplicity of infection (MOI) of 10:1 (Macrophage: *S.aureus*) for in vitro peritoneal macrophages infection.

C. albicans (MTCC 4748) was obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India and was grown on Yeast Peptone agar plates. The HLC52 (mutant in the CAI4 background) *C. albicans* strains were generous gifts from Dr. S. Lata Panwar, Jawaharlal Nehru University, New Delhi, India. CEC 2684, mutant GFP *C. albicans* strain was a generous gift from Prof. KaustavSanyal, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR),Karnataka, India.These fungi were grown on yeast peptone dextrose agar plates and colonies were picked and subjected to liquid cultures at 30℃. Log-phase cultures were harvested, and the yeast form of C. albicans was enumerated for infection using Neubauer chamber. *In vitro* experiments were performed at MOI of 10:1 (Macrophage: *Candida*)

Ethics statement

All studies involving mice and virulent mycobacterial strains were carried out after the approval from the Institutional Ethics Committee for animal experimentation as well as from

Institutional Biosafety Committee. The animal care and use protocol adhered were approved by national guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2 Design of *in vivo* models

2.2.1 Intra-articular infection of mice with *S. aureus*

For *in vivo* experiments, mice were anesthetized with single dose of ketamine and xylazine cocktail. Hind limbs were shaved and left knee was intraarticularly infected with 1 x 10^8 CFU of live *S. aureus* dissolved in 10µl of 1XPBS. Right knee was injected with PBS as vehicle control. Mice were monitored for next 7 days for limping and restricted hind limb movement.

2.2.2 Mice dermal skin excisional wound model with local infection of C. albicans

Mice skin excisional wounds were created on the dorsal side of BALB/c mice using previously described protocols(88). Briefly, on day 0, mice were anesthetized with intraperitoneal administration of ketamine hydrochloride and xylazine (Aneket; Neon Laboratories, Mumbai, India). After depilation, the dorsal skin was picked up at the midline and punched through the skin with a sterile disposable biopsy punch (4 mm in diameter; Integra Miltex) tool, generating one wound on each side of the midline. Following this, wounds were locally treated with PBS (on the left wound) or infected with *C. albicans* (1 x 10^5) resuspended in PBS (on the right wound). Thus, day 0 represented the day of injury with subsequent observations that were carried out every 24 h. Kinetics of wound closure was followed for 9 days to analyse wound repair and skin regeneration. Each wound site was digitally photographed on indicated days, and change in wound areas were quantitated using ImageJ software (version 6.0). Each group was composed of a minimum of five mice. Changes in wound areas over time were expressed as the percentage of the initial wound areas.

2.3 Reagents and antibodies

Anti-β-actin (A3854) antibody was purchased from Sigma-Aldrich (USA). Anti-Ser-33/37/Thr-41 phospho-β-catenin (9561), anti-β-catenin (9562), anti-Ser-9 phospho-GSK-3β (9322), anti-Thr-288 phospho-Aurora Kinase A (3079), anti-Ser-2448 phospho mTOR (2971), anti-Thr-1079 phosphor-LATS1 (8654), anti-LATS-1 (3417), anti-Thr-183 Phopho-MST1/Thr-180MST2 (3681), anti-MST1 (14946), anti-MST2 (3952), p-Smad1/5/8 (9511)

antibodies were obtained from Cell Signaling Technology (USA). BMP2 (500-P195) was purchased from PeproTech Inc. Anti-CMKLR1/ChemR23 (ab64881) was purchased from Abcam, USA. HRP conjugated anti-rabbit IgG antibody (111-035-045) was purchased from Jackson Immuno Research (USA). Non-targeting small interfering RNA (siRNA) (D-001210-01-20), *Stk4*/*Mst1* (M-059385-01- 0005), *Stk3*/*Mst2* (M-040440-01-0005) and *Lats1* (M-063467-01-0005) siRNAs were obtained from Dharmacon as siGENOME SMART-pool reagents.

Plasmids pcDNA-EGF-Rac1-T17N (12982) and pcDNA3-EGF-RhaA -T19N (12967) were purchased from Addgene. We acknowledge the generous gift of plasmids from Dr. Kohei Miyazono, The Cancer Institute, Tokyo (BMPR1a CA) and Dr. Nick Morrell, Cambridge, UK (BMPR2 DN).

2.4 Treatment with pharmacological reagents

In all experiments involving pharmacological reagents, titrated concentration was used after assessing the viability of the macrophages using Trypan Blue assay. In all *in vitro* experiments peritoneal macrophages were pre-treated for 1 hour with inhibitors at following concentrations: Alisertib (1μM) (Cayman 1028486-01-2), Rapamycin (100nM) (Calbiochem, 553210), adapalene (10 μM) (Calbiochem, 114825) and DMH1 (20 mM) (D8946; Sigma-Aldrich).

2.5 RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from macrophages, pulverised knee samples and crushed wounds samples in liquid nitrogen, using TRI reagent (T9424; Sigma-Aldrich). 1.5 μg RNA was used for cDNA synthesis using first strand cDNA synthesis kit (M3682; Promega). Quantitative real time PCR was performed with SYBR green PCR mix (RR420A, TAKARA) in ABI machine. All the experiments were repeated in biological triplicate with technical duplicates taken in each experiment, to ensure the reproducibility of data. Mean Ct values were normalized to internal control *Gapdh*. Primers used for quantitative real time PCR are: *Gapdh*Fwd 5'-GAGCCAAACGGG TCATCATCT-3', Rvs5'-GAGGGGCCATCCACAGTCTT-3', *Cxcl1* Fwd 5'-TGTTGTGCG AAAAGAAGTGC-3', Rvs 5'-CGAGACGAGACCAGGAGAAA-3', *Ccl2* Fwd 5'-TAAAAACCTGGATCGGAACCAAA-3', Rvs 5'-GCATTAGCTTCAGATTTACGGGT-3', *Ccl12* Fwd5'-ATTTCCACACTTCTATGCCTCCT-3', Rvs 5'-

ATCCAGTATGGTCCTGAAGA TCA-3', *Ccl5* Fwd5'-CCCTCACCATCATCCTCACT-3', Rvs5'-CCTTCGAGTGACAAACACGA-3', *Il-1β*Fwd 5'-GAAATGCCACCTTTTGACAGTG-3', Rvs 5'-TGGATGCTCTCATCAGGACAG-3', *Retnla*Fwd 5'-CCCTCCACTGTA ACGAAGACTC-3', Rvs 5'-CACACCCAGTAGCAGTCATCC -3', *Ym-1* Fwd 5'- CATGAGCAAGACTT GCGTGA-3', Rvs5'GGTCCAAACTTCCATCCTCCA-3', *Mrc-1* Fwd 5'-TCTTTTACGAGAAGTTGGGGTCAG-3', Rvs 5'-ATCATTCCGTTCACCAGAGGG-3', *Il13ra2* Fwd5'-GGAAAGGAGGACAAAGAGGTC-3', Rvs 5'-GATTTAGTGTGCTGAAAGCTCTACTC-3', *Ccl22* Fwd5'-TGCCATCACGTTTAGTGAAGG-3', Rvs 5'-CGGCAGGATTTTGAGGTCCA-3', *Ucp-1* Fwd5'-GTGAAGGTCAGAATGCAAGC-3', Rvs5'-AGGGCCCCCTTCATGAGG TC-3', *Cx3cr1* Fwd5'-CAGCATCGACCGGTACCTT-3', Rvs 5'-GCTGCACTGTCCGGT TGTT-3', *Il10* Fwd 5'-GGACTTTAAGGGTTACTTGGGTTGCC-3', Rvs5'-CATTTTGATCATCATGTATGCTTCT – 3', *Tgfb*Fwd 3'- CCCTATATTTGGAGCCTGGA – 3' , Rvs 5'- GTTGGTTGTAGAGGGCAAGG – 3', *Il12*Fwd 5'-GACTTGAAGATGTACCAGACAG – 3', Rvs 5'- GAGATGAGATGTGATGGGAG – 3', *Tnfa*Fwd 5'- AGCCCACGTCGTAGCAAACCACCAA – 3', Rvs 5'-ACACCCATTCCCTTCACAGAGCAAT – 3', *Arg 1*Fwd 5'- GTGAAGAACCCACGGTCT GT – 3', Rvs – 5' CTGGTTGTCAGGGGAGTGTT- 3'.

2.6 Immunoblotting

Mice knee or biopsy from wounds were crushed in liquid nitrogen and total protein was isolated, in addition to protein isolation from treated macrophages by lysing in RIPA buffer constituted of 50 mM TRIS-HCl (pH 7.4), 1% NP-40, 0.25% Sodium deoxycholate, 150 mMNaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml of each aprotinin, leupeptin, pepstatin, 1 mM Na3VO4 and 1 mMNaF. Quantitative estimation of protein supernatant was performed through Bradford's method. An equal amount of protein from each cell lysate was resolved on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, IPVH00010) by the semidry transfer (Bio-Rad, 170-3940) method. Nonspecific binding was blocked with 5% nonfat dry milk powder in TBST [20 mM TRIS-HCl (pH 7.4), 137 mMNaCl, and 0.1% Tween 20] for 60 min. The blots were incubated overnight at 4 °C with primary antibody followed by incubation with anti-rabbit-HRP or anti-mouse-HRP secondary antibody in 5% BSA for 2 h. After washing in TBST, the immunoblots were developed with enhanced chemiluminescence detection system (Perkin Elmer,

NEL105001EA) as per the manufacturer's instructions. β-actin was used as loading control. For probing another protein in the same region of the PVDF membrane, the blots were stripped in the stripping buffer (62.5 mM TRIS-HCl (pH 6.8), 2% SDS and 0.7% β-mercaptoethanol) at 60 °C on a shaker, blocked with 5% nonfat dry milk powder and probed with antibodies as mentioned above.

2.7 Transient transfection

Transient transfection of $0.5x10^6$ RAW264.7 macrophages with 5μg of mentioned plasmid constructs was performed using Polyethylenimine (PEI, Sigma-Aldrich). Peritoneal macrophages were transiently transfected with 100nM targeted siRNA and miRNA mimic using low molecular weight polyethyleneimine (Sigma-Aldrich, 40872-7). Further 36 h post transfection cells were treated or infected as indicated for required time and processed for analysis.

2.8 Micro computed tomography

Limbs were scanned and reconstructed into a three-dimensional (3D) structure with XRADIA XRM 500 with a voxel size of 35 μm. The scanning was done at 100 kV and 455 mA, with a 0.2-mm aluminium filter. The exposure time was 109 ms. The X-ray projections were obtained at 0.7˚intervals with a scanning angular rotation of 180˚. This also resulted 35.26 of image pixel size. The projection images were reconstructed into three-dimensional images and analyzed using AVIZO software.

2.9 Histopathological examination

Tissue sections (5 microns) obtained through brief fixation in 4% formaldehyde, bone decalcification and paraffin embedding were stained with haematoxylin and eosin. Double blinded scoring was performed, including experienced pathologist to analyse severity of arthritis. Histological examination was performed based on parameters discussed in published report(89). Synovial inflammation was scored as 0 = normal; 1 = minimal infiltration of inflammatory cells in periarticular tissue; 2 = mild infiltration; 3 = moderate infiltration, with moderate edema; 4 = marked infiltration, with marked edema; and 5 = severe infiltration, with severe edema. Cartilage damage and bone erosion were scored as 0 = normal; 1 = minimal (minimal-to-mild loss of cartilage and bone); 2 = mild (mild loss of cartilage and bone); 3 = moderate (moderate loss of cartilage and bone); 4 = marked (marked loss of cartilage and bone); and 5 = severe (severe diffuse loss of cartilage and bone).

Tissues sections from dorsal wounds were obtained from punch biopsy tool (6mm diameter) and briefly fixed in 4% formaldehyde. These were embedded in paraffin and sectioned at 5 microns followed by staining with haematoxylin and eosin. Sectioned by examined and severity of tissue damage was observed by pathologist.

2.10 Immunohistochemistry (IHC)

Tissue sections (5microns) obtained from decalcified and paraffin-embedding blocks were subjected to deparaffinization and rehydration. For antigen retrieval, sections were kept at boiling temperature in 10mM sodium citrate buffer (pH 6) for 15 min. These were further treated with 2% H_2O_2 to block endogenous tissue peroxidase activity. After blocking for 1 h in 1% BSA, sections were incubated with primary antibodies (1: 100) made in 1% BSA and 1% Tween 20 solution, for overnight at 4^{o}C. After incubating with secondary anti-rabbit HRP conjugated antibody for 2 h, sections were stained with 0.1% diaminobenzidine (DAB, Sigma-Aldrich) in 1% H_2O_2 solution. Slides were washed with PBS to remove excess of DAB and counterstained with haematoxylin, dehydrated with ethanol and mounted on DPX.

2.11 Immunofluorescence (IF)

For in vitro CHEMR23 visualization experiment, peritoneal macrophages from BALB/c mice were pre-treated with Adapalene for 2 h followed by bacteria infection. Coverslips were incubated with primary CHEMR23 antibody overnight at 4 c, followed by 1 h incubation with DyLight 488–conjugated secondary Ab and nuclei staining with DAPI for 5 min. coverslip were mounted on glycerol for confocal imaging. For in vivo tissue imaging, infected Synovial tissue from mice was isolated, as previously described(90). Briefly, mice were positioned such that the knee joints were exposed by a midline skin incision. Transverse resection of the quadriceps at the middle followed by distally reversing the tissue exposed the synovium, patella, and patellar ligament. Synovial tissue was resected and immediately preserved in at -80c. Punch biopsy samples (6mm diameter), were obtained from PBS treated or C. albicans infected wounds from dorsal skin of BALB/c mice.

From isolated synovial tissue as well as wound biopsy samples, Cryosections of 10micron were made in optimum cutting temperature (OCT) medium in Leica CM 1510 S and stained with primary antibodies incubated overnight at 4c. Sections were incubated with DyLight 488–conjugated secondary Ab for 1 h, and nuclei were stained with DAPI for 5 min. A coverslip was mounted on the section with glycerol as the medium. Confocal images were

taken using a Zeiss LSM 710 Meta confocal laser scanning microscope using a Plan-Achromat 633/1.4 Oil DIC objective (both from Carl Zeiss), and images were analysed using ZEN 2009 software.

2.12 Statistical analysis

Levels of significance for comparison between samples were determined by the Student's t-test distribution and one-way ANOVA followed by Tukey's multiple-comparisons. The data in the graphs are expressed as the mean ± S.E for the values from at least 3 or more independent experiments and P values < 0.05 were defined as significant. GraphPad Prism 6.0 software (GraphPad Software) was used for all the statistical analysis.

CHAPTER 3: SELECTIVE ACTIVATION OF MST1/2 KINASES BY RETINOID AGONIST ADAPALENE ABROGATES AURKA-REGULATED SEPTIC ARTHRITIS

Selective activation of MST1/2 kinases by retinoid agonist Adapalene abrogates AURKA-regulated septic arthritis

3.1 Introduction

S. aureus although being a commensal can successfully established a mild to severe life threatening infection. Abundance of widely expressed virulence factors are the key aspects that govern the *S. aureus* induced pathogenicity and evasion of host immune responses. While it is widely known that expression of adhesins proteins promote the *S. aureus* invasion to host tissue, the secretion of cytolytic toxins can also cause severe damage to it. Based on these pretexts, *S. aureus* is a successful pathogen that evades host immune system along with the inhibition of complement activation, blockage of phagocytic uptake, and modification of normal B cell and T cell responses.Case fatality rate associated with MRSA is highest at 14% of all the identified bacterial and fungal pathogens(91).Thus, it requires immediate attention to subvert *S. aureus* induced pathogenesis and prevent fatal damage to host.

Antibiotic treatment of *S. aureus* infection is often inadequate, and this may be partially explained by deficiencies in arms of the immune system that typically work in concert with antibiotics to eradicate infection. The high prevalence of *S. aureus* infections in patients undergoing surgical procedure with less availability of efficacious of treatment has led to vigorous attempts to develop new host directed therapy (HDT) against pathogen. In this context, IL-17 was explored, which showed crucial role against systemic dissemination of lethal *S. aureus* infection. This study showed the absolute requirement of Nlrp3-driven IL-1β in activating IL-17 production by γδT cells, which mediated immune protection against *S. aureus* infection(92). Microbial sensing though PRRs are also believed to contribute significantly to *S. aureus* pathogenesis. Reports have showed that the intravenous challenge of *S. aureus* to TLR2-/- mice can significantly elicit IL-10 production with concomitant

upregulation of TNF-α and IL-6 blood serum level in contrast to WT mice. Thus TLR2 deficiency can contribute to dysregulated cytokines balance with impaired bacterial clearance and effect mice survival. Although membrane bound microbial sensors TLR2 and TLR4 are known to interact with *S. aureus*; it was demonstrated that macrophages knocked out for TLR2/4 still retain responsiveness to *S. aureus*infection, suggesting a role for an alternative intracellular receptors such as NOD2 in mitigating the host response(93). Work by Hruz et al., showed the critical role of NOD2 in cutaneous host defence against infection with *S. aureus*. It was found that NOD2 mediated bacterial clearance was associated with exacerbated IL-1β and IL-6 production which are key cytokines that were implicated in innate immune defence(94). *Staphylococcus* Infection mediated activation of type I IFNs, was found to mediate tissue homeostasis with beneficial effects to the host. Studies performed on murine skin cutaneous (s.c.) infection model, showed that exogenously provided IFN-β accelerated the clearance of *S. aureus* from infected skin lesions thereby promoting effective host defence against said pathogen (95)

The factors contributing to *S. aureus* pathogenesis include resistance to antimicrobial peptides with expression and secretion of diverse pathogenic factors like coagulase, lipases, protein A, fibrinogen and fibronectin binding proteins, TSST-1, haemolysins, pentone leucocidins. Infection with *Staphylococcus* is found to cause numerous mild to life threatening disease like meningitis, endocarditis, toxic shock syndrome, septic arthritis and osteomyelitis. Of these septic arthritis is of particular importance as progression to chronic stage of this disease is associated with irreversible joint damage which often renders the host with permanent joint dysfunction. Mechanistically progression of septic arthritis involves severe damage to articular cartilage with partial to complete loss of chondrocytes and meniscus fibrous tissues **(Fig 3).** With degradation of cartilage matrix, rapid burst of proinflammatory meditators occurs in the synovial fluid that ultimately results in the

development of chronic inflammatory stage. Such progression is often found to be associated with activation of proteases and collagenases which perturbs the endochondral ossification of bone that are known to adversely affect the subchondral bone morphology and disruption of its architecture.

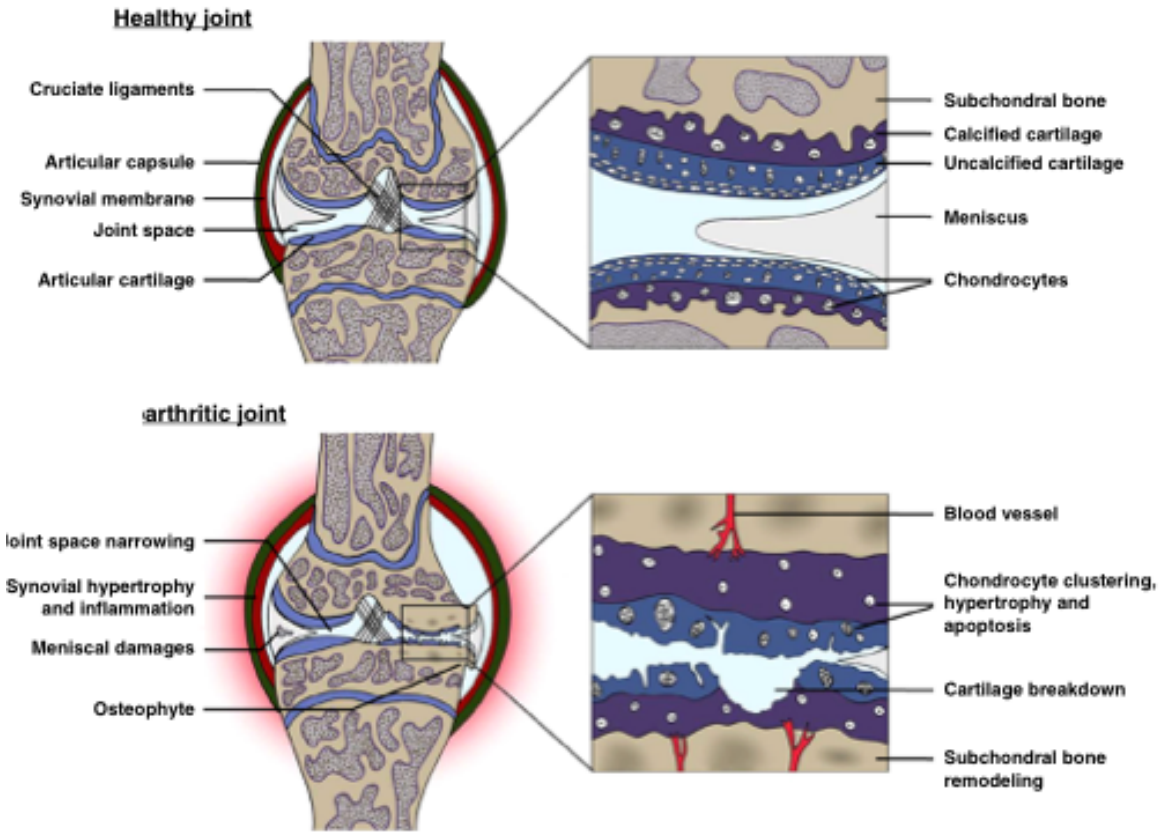

Figure 3. Damage of knee joint in septic arthritis–Septic arthritis is a chronic inflammatory disorder which is characterised by loss of articular cartilage with synovial hypertrophy and sclerosis of subchondrial bone. As the cartilage fibrillation precedes, the splitting of superficial chondrocytes layer occurs which perturbates the endochondral ossification process that involves intricate balance between continuous cell division of chondrocytes and remodelling of extracellular matrix. With hypertrophy of outer synovial layer, vascularization is also increased that recruits proinflammatory mediators and activation of matrix degrading enzymes which includes matrix metalloproteinases, disintegrins and thrombospondin. Thus activation of such cascade impedes the irreversible damage to host that culminates to permanent dysfunction of joints. Adapted from (96)

In the context of above-mentioned pathophysiological conditions of septic arthritis, clinically proven disease-modifying antirheumatic drugs (DMARDs) assumes important roles in mitigating host-*S. aureus* interactions as well as hyperinflammation leading to immune dysregulations. However, in many cases, treatment of septic arthritis among various sections of patients becomes ineffective owing to development of refractoriness not only to DMARDs as well as to various biologics and biosimilars.Current state for septic arthritis treatments involve combinatorial therapy involving co-treatment with antibiotics and corticosteroids, which supress bacterial growth and host hyperinflammatory immune response respectively(97). However, cure for septic arthritis often fails due to the emergence of antibiotic-resistant bacterial strains and unresponsiveness to corticosteroids when administered at late or chronic stage of infection. Despite various therapeutic interventions, mortality rate still remains as high as 8-20 % amongst septic arthritis cases(98, 99).

Interestingly, therapeutic intervention with vitamin A is associated with perturbation of *S. aureus* susceptibility of host (82). RA is established as treatment modality in improving the clinical symptoms of collagen induced inflammatory arthritis. Under given study, it was found that treatment with RA could elicit production of Foxp3+ regulatory T cells which in turn showed the perturbation of osteoclastogenesis activity and repressed IL-17 expression (100). In this context, our current work utilized the administration of RA to explore its regulatory effects on the host driven signaling pathways against *S. aureus* induced septic arthritis.

3.2 Results:

3.2.1 Establishment of arthritis by S. aureus infection

Despite reported studies, molecular pathways that govern host –*S. aureus* interactions as well as immune dysregulations arising from hyperinflammation requires extensive

investigations. In this context, we have utilised murine model of *S. aureus* induced septic arthritis, wherein BALB/c mice were given intra-articular infection of *S. aureus* in the left knee and the right knee received PBS which served as contralateral control. The mice were kept under observation, and on day 3 post infection, symptoms of clinical arthritis including observable limping and restricted leg movement were observed. Mice were sacrificed on day 7 and subjected to micro-CT analysis to evaluate changes in bone morphology and architecture. 3D reconstruction of mouse anterior knee joints **(Fig. 3.1A)** and distal femur transverse cross-section **(Fig. 3.1B)** revealed a visual decrease in bone porosity. Quantification of perturbed bone architecture upon infection, showed significant decrease in the percentage of bone volume over total bone volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number per mm area (Tb.N) with concomitant increase in trabecular spaces (Tb. Sp). **(Fig. 3.1C).** Immunohistochemistry of knee sections was performed to depict gross changes in tissue morphology of articular cartilage and bone. Upon assessment of H & E stained knee sections by pathologist, a significant erosion of bone was observed along with characterised recruitment of a sheet of inflammatory cells within the joint space. Significant level of accumulation of neutrophils were found between skeletal muscle bundles, indicating extension of inflammation from the joint into the soft tissue of the limb. In addition to neutrophils, the peripheral smear was also found to be associated with an Inflammatory sheets of cells with numerous plasmocytes as well as histiocytes that included monocytes or macrophages **(Fig. 3.1D).** The scoring of synovial and bone erosion revealed a significant increase in synovial inflammation in addition to increase in damage to bone and cartilage**(Fig. 3.1E).**Studies on various arthritic patients have reported an altered cytokine profiles in their synovial fluid(101).In order to evaluate mechanisms that regulate *S. aureus* infection triggered inflammation, expression of a panel of inflammatory mediators was analysed. As indicated, *In vitro* screening of various proinflammatory and anti-inflammatory

genes showed *S. aureus*-responsive expression **(Fig. 3.1F).** However,in contrast to this, *in vivo* tissue samples from *S. aureus* infected knee showed heightened expression of M1 macrophages genes as compared to M2 macrophages **(Fig. 3.1G).**Thus, these findings suggest that infection with *S. aureus*cowan 1, leads to differential expression of inflammatory mediators which in turn governs the increased bone porosity and bone erosion during development of septic arthritis in mice.

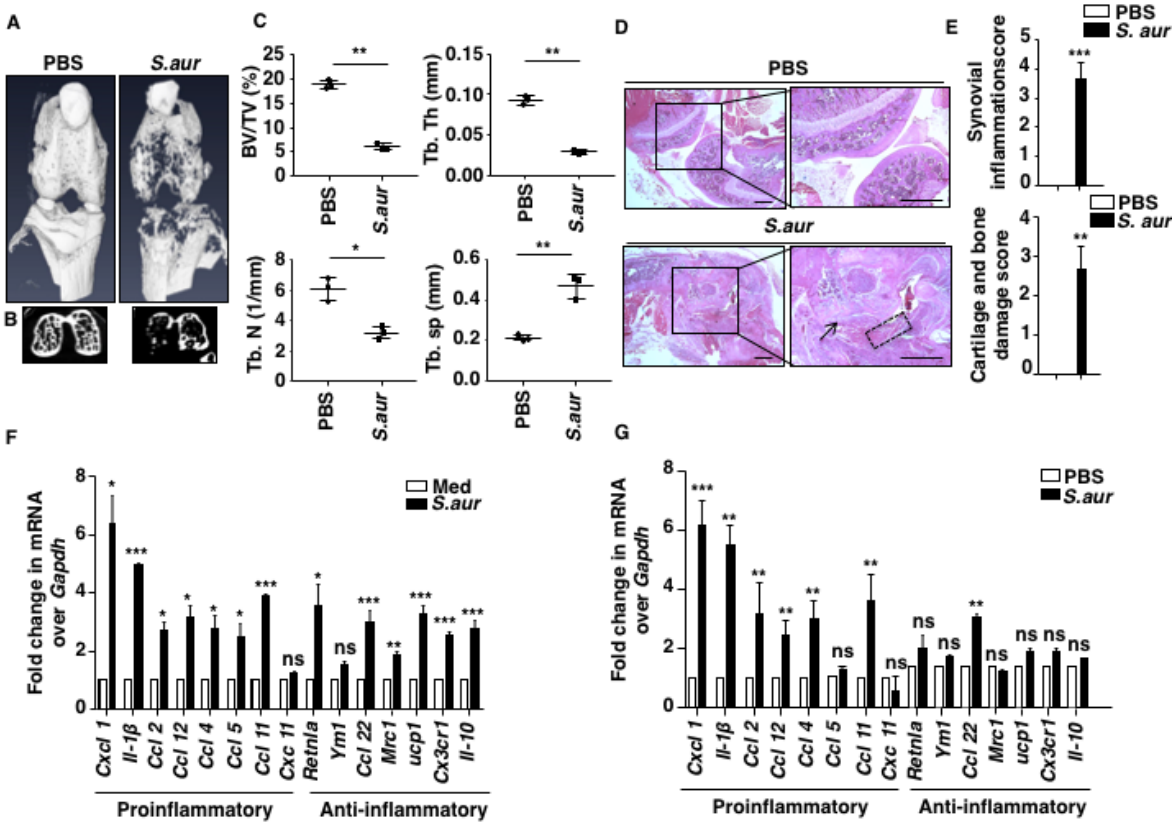

Fig 3.1 .*S. aureus* Cowan1 is a causative agent of septic arthritis.(A-E,G) Left knee of BALB/c mice were intraarticularly injected with 1 X 10^8 CFU of live *S. aureus* on Day 0. Contralateral knee received PBS and following this, the mice were observed for 7 days. **(A)** Representative micro CT images of day 7, showing anterior knee in 3D and (**B)** distal femur transverse cross-section of mice treated with PBS and *S. aureus.* (**C)** Hind limbs were dissected for bone morphometric analysis utilizing different indices; bone volume/total volume (BV/TV %), trabecular thickness Tb .Th (mm), trabecular number Tb N (per mm) and trabecular spacing Tb.sp (mm) score. **(D)** Representative H&E stained knee joint at 2X and 20X magnifications. Arrow indicates neutrophilic aggregates within hyaline cartilage and box represents sheet of inflammatory cells within joint space.**(E)**H &E sections were scored for severity of synovial inflammation, cartilage and bone damage. **(F)**

Peritoneal macrophages from BALB/c mice were infected with live *S. aureus* at MOI 1:5 for 12 hand quantitative real time PCR analysis was performed for indicated genes. **(G)** On day 7, intraarticularly injected knees of BALB/c were pulverized and quantitative real time PCR analysis was performed for indicated molecules**.**Data represent the mean ± SEM of three independent experiments. * $P< 0.05$, ** $P < 0.005$, *** $P< 0.001$(One-way ANOVA followed by Tukey's multiple-comparisons test). Bar, 50μm with original magnifications 2X and 5μm with original magnifications 20X.

3.2.2 AURKA responsive WNT signaling regulates *S. aureus* infection triggered septic arthritis

Mounting evidences validate the critical role of NOD2signalingduring *S. aureus* pathogenesis. In particularly, studies have revealed that NOD2 KO mice displayed higher susceptibility to *S. aureus* infection with reduction in median survival to almost half as compared to TLR2 KO mice(102). Additionally, previous reports from our lab and others have suggested an important role of NOD2-triggered WNT signaling in the development of acute arthritis(78, 103). While it is known that WNTsignaling axis process entails increased inhibitory phosphorylation of GSK-3β with concomitant decrease in pβ-CATENIN levels (hallmarks of active WNT signaling), molecular players orchestrating these events still remain unexplored. Interestingly both mTOR and AURKA transcripts levels were found to be elevated in osteoclasts and B cells of patients with rheumatoid arthritis(104, 105). In this perspective, we explored role for Aurora kinase A (AURKA) as well mTOR in regulating WNT signaling pathways during *S. aureus* infection triggered arthritis. Immunohistochemistry analysis of *S. aureus* infected mice knee sections demonstrated activated AURKA, mTOR and WNT signaling axis**(Fig. 3.2A).**In order to evaluate role for NOD2, macrophages were transfected with NOD2-specfic-siRNA followed by infection with *S. aureus*. As shown in **Fig. 3.2B**, RNA interference of NOD2 significantly diminished the ability of *S. aureus* to trigger AURKA, mTOR and WNT signaling axis**(Fig.3.2B).** In addition, *S. aureus* infection induced significant activation of AURKA, mTOR and WNT signalling events in macrophages derived from TLR2 null mice or from $tlr4^{Lps\text{-}d}$ (tlr4 mutant) compared to WT macrophages clearly implicating that both TLR2 and TLR4 are dispensable

for infection induced activation of said signaling pathway**(Fig. 3.2C).**To evaluate dependency of WNT signalling activation on AURKA or mTOR, macrophages were pretreated with Alisertib (an AURKA specific inhibitor) or Rapamycin (a mTOR specific inhibitor) followed by infection with by *S. aureus.* We observed that Alisertib significantly abrogated phosphorylation status of mTOR and WNT signaling proteins. On the other hand, though rapamycin abrogated WNT pathway activation, it failed to inhibit AURKA phosphorylation. This suggested that AURKA regulated the phosphorylation of mTOR, which subsequently activated WNT signaling**(Fig. 3.2D).**In order to bring relevance to *in vivo* significance of AURKA activation, mice that were infected with

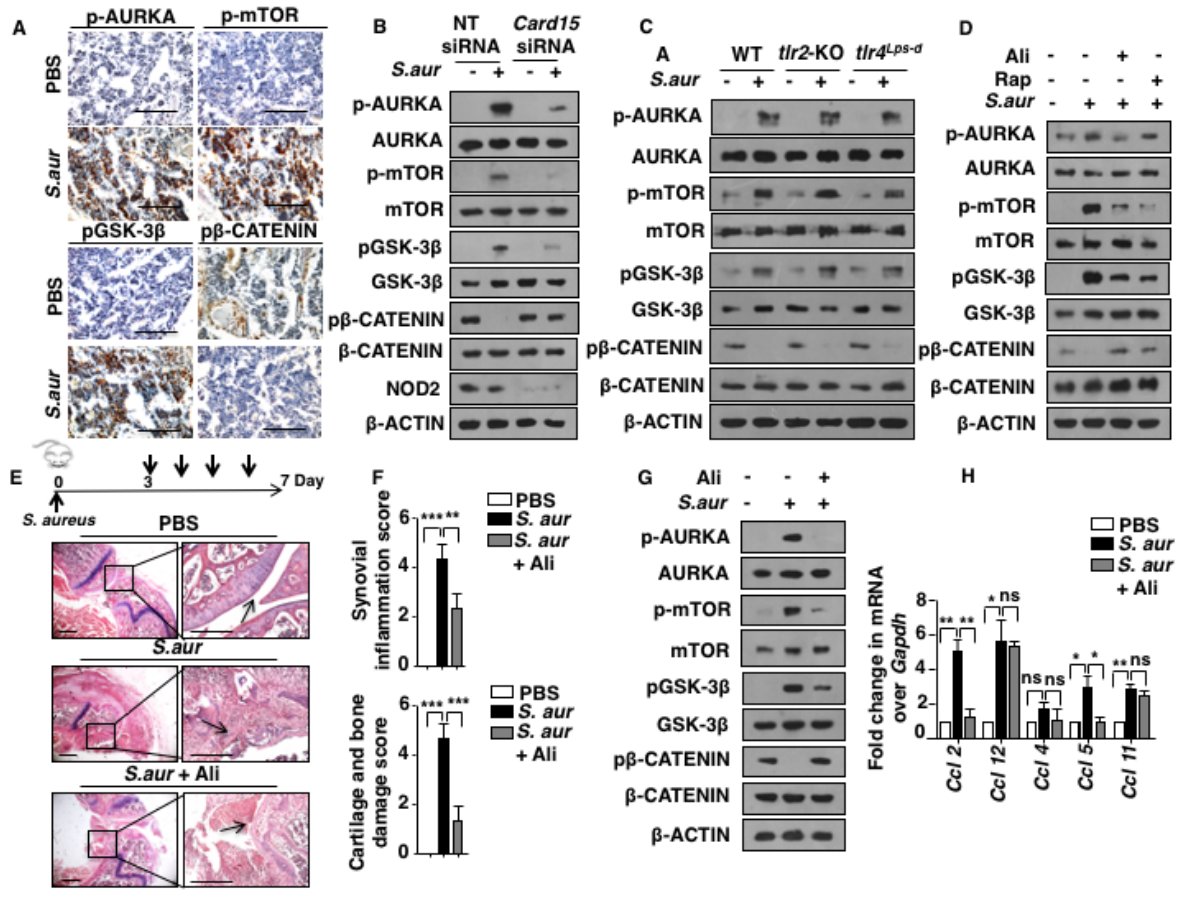

Fig 3.2 . *S. aureus* induces NOD2-dependent activation of the AURKA-mTOR-WNT signaling axis. (A) Left knee of BALB/c mice were intraarticularly injected with 1 X 10^8 CFU of live *S. aureus* at day 0.

Contralateral knee received PBS and kept for 7 days. Representative immunohistochemistry was performed on day 7 to check activation of indicated molecules. Peritoneal macrophages **(B)** from BALB/c mice transfected with NT or *Card15* siRNA, **(C)** from WT, *tlr2*-KO and *tlr4*$^{Lps\text{-}d}$ mice, were infected with live *S. aureus* at MOI 1:5 for two 2 h. Immunoblotting was performed for indicated molecules. **(D)** Peritoneal macrophages from BALB/c mice were pre-treated with alisertib and rapamycin for 1 h followed by infection with live *S. aureus* at MOI 1:5 for 2 h, to perform immunoblotting. **(E-F)** Post three days of infection, mice were treated daily with alisertib i.p. (3mg/Kg). After 7 days knees were **(E)**sectioned for H &Estaining and **(F)** scored for severity of synovial inflammation, cartilage and bone damage. Knees from alisertib treated mice were also pulverized to perform **(G)** immunoblotting and **(H)** quantitative real time PCR. Data represent the mean ± SEM of three independent experiments. * $P< 0.05$, ** $P < 0.005$, *** $P< 0.001$(One-way ANOVA followed by Tukey's multiple-comparisons test and all blots are representative of 3 independent experiments).NT-non-targeting. Bar, 50µm with original magnifications 2X and 5µm with original magnifications 20X.

S. aureus for 3 days were administrated daily with Alisertib (3mg/kg). Mice were sacrificed on day 7 and their hind limbs were sectioned and stained to observe histopathology and score for the severity of arthritis. As per evaluation by pathologist, the alisertib treatment showed only partial rescue of inflammatory damage to articular cartilage. However, inflammatory cells within the joint space focally involving periarticular soft tissue was still prevalent**(Fig. 3.2E).**Moreover upon assessment of synovial inflammation along with cartilage and bone destruction score, revealed that treatment with alisertib showed partial rescue of *S. aureus* induced damage to host **(Fig. 3.2F).** Immunoblotting of pulverised knee samples from treated mice showed the decreased activation mark of p-AURKA-mTOR and WNT signaling axis upon alisertib treatment**(Fig. 3.2G).** Moreover, inhibition of AURKA significantly reduced *S. aureus* induced expression of proinflammatory cytokines **(Fig. 3.2H).** Collectively, our findings confirmed that alisertib could potentiate the abrogation of WNT-induced cartilage damage, with partial reduction in inflammatory cells recruitment.

3.2.3 ADA rescues *S. aureus* induced cartilage damage

In the perspective of above mentioned results, we have explored the role for RA in curtailing pathogenesis of *S. aureus* triggered septic arthritis. Despite effects of RA on

various cellular platforms and pathways such as differentiation, inflammation, regulatory T cells etc is known, delineation of signaling intermediates playing significant roles during said therapeutic effects of retinoic acid requires extensive investigations. For our current work we have utilised Adapalene (ADA) as a synthetic derivative of RA. ADA was administered daily to mice as described starting from day 3, wherein day 0 represented *S. aureus* infection. At day 7 post infection, radiological examination of anterior knee **(Fig. 3.3A)** and distal femur transverse cross-section **(Fig. 3.3B)**, demonstrated visual changes in rescued bone damage upon ADA prophylaxis along with concomitant increase in bone porosity. Further, bone morphometric measurement confirmed the increased bone volume, with significant increase in trabecular bone thickness and number along with decreased trabecular spaces **(Fig. 3.3C)**.

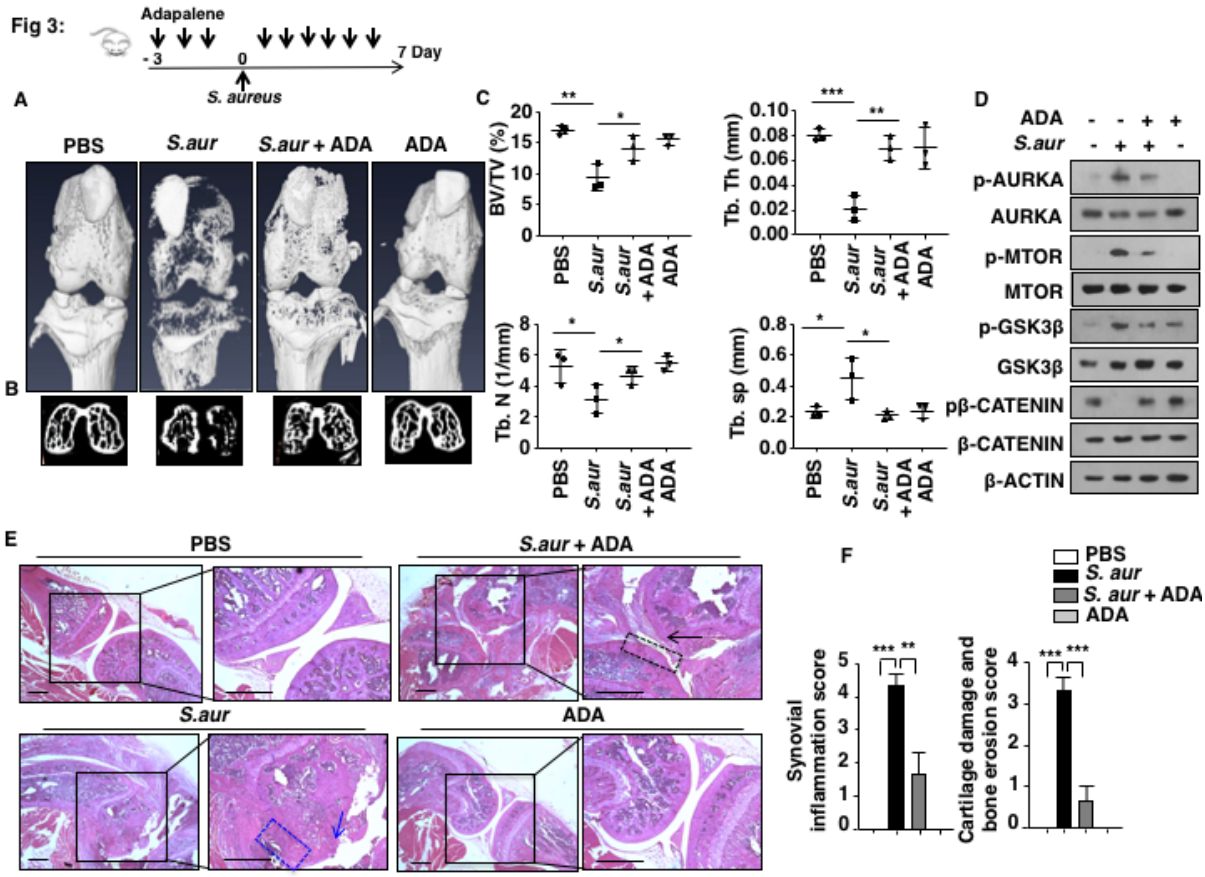

Fig 3.3. ADA negatively regulates *S. aureus*-induced septic arthritis. (A-F) Left knee of BALB/c mice were intraarticularly injected at day 0 with 1 X 10^8 CFU of live *S. aureus*. Contralateral knee received PBS. Mice

were treated daily with ADA i.p. (5mg/Kg) starting at – 3 day of bacterial infection and kept for 7 days. **(A)** Representative micro CT images taken at 7 day of infection, showing anterior knee and (**B)** distal femur cross-section followed by (**C)** bone morphometric analysis utilizing different indices; bone volume/total volume (BV/TV %), trabecular thickness Tb .Th (mm), trabecular number Tb N (per mm) and trabecular spacing Tb.sp (mm)score**. (D)** Representative immunoblotting from pulverised knee of ADA treated mice. **(E)**H& E stained knee section from BALB/c mice joints. Blue arrow indicates acute inflammation within joint space. Blue box represent extension of inflammation into and destroying articular cartilage. Black arrow indicates neutrophilic presence within the joint space and extending into the surface of the articular cartilage. Black box indicates intact articular cartilage with no sign of bone erosion. **(F)** H &E sections were scored for severity of synovial inflammation, cartilage and bone damage. Data represent the mean ± SEM of three independent experiments. * $P< 0.05$, ** $P < 0.005$, *** $P< 0.001$ (One-way ANOVA followed by Tukey's multiple-comparisons test and all blots are representative of 3 independent experiments). ADA-Adapalene. Bar, 50μm with original magnifications 2X and 5μm with original magnifications 20X.

Data was further corroborated with immunoblotting from pulverised knee samples from *S. aureus* infected mice that were treated with ADA, which showed significant decrease of AURKA regulated WNT signaling pathway **(Fig. 3.3D)**. In addition to this, analysis of immunohistochemistry of knee sections by pathologist, revealed that ADA treatment could significantly decrease recruitment of inflammatory cells along with preservation of articular cartilage and bone structure in *S. aureus* infected mice **(Fig. 3.3E)**. Significantly, double blinded scoring demonstrated a significant decrease in synovial inflammation as well as decrease in bone and cartilage damage score upon treatment with ADA **(Fig 3.3F)**. Overall, these results strongly suggest efficacy of ADA to downregulate the ability of *S. aureus* to elicit septic arthritis.

3.2.4 ADA differentially regulates WNT and HIPPOsignaling

After establishing the suppression of *S. aureus* induced AURKA-mTOR-WNT signaling axis by ADA, the possible mechanisms for ADA mediated suppression were explored. In this regard, it has been shown that Hippo signaling regulates susceptibility to bacterial sepsis(106). Interestingly, RARγ gain-of-function can regulate aberrant expression of an important effector of HIPPO signaling–YAP, thus regulating colorectal

tumorigenesis(107). Therefore, the regulatory effects of RA on HIPPOsignaling was assessed.Phosphorylation of MST1/2 and LATS1/2 are indicative of Hippo signaling activation. Significantly, immunoblotting of infected knee samples of mice showed augmented activation of Hippo signalling by ADA compared with *S. aureus* infection alone as evaluated by activatory phosphorylation of MST1, MST2 and LATS1. Interestingly, ADA strongly suppressed *S. aureus* induced WNT signalling as shown by inhibitory phosphorylation of GSK-3β or β-CATENIN. However, total levels of MST1, MST2 and LATS1 remained unchanged **(Fig. 3.4A).**

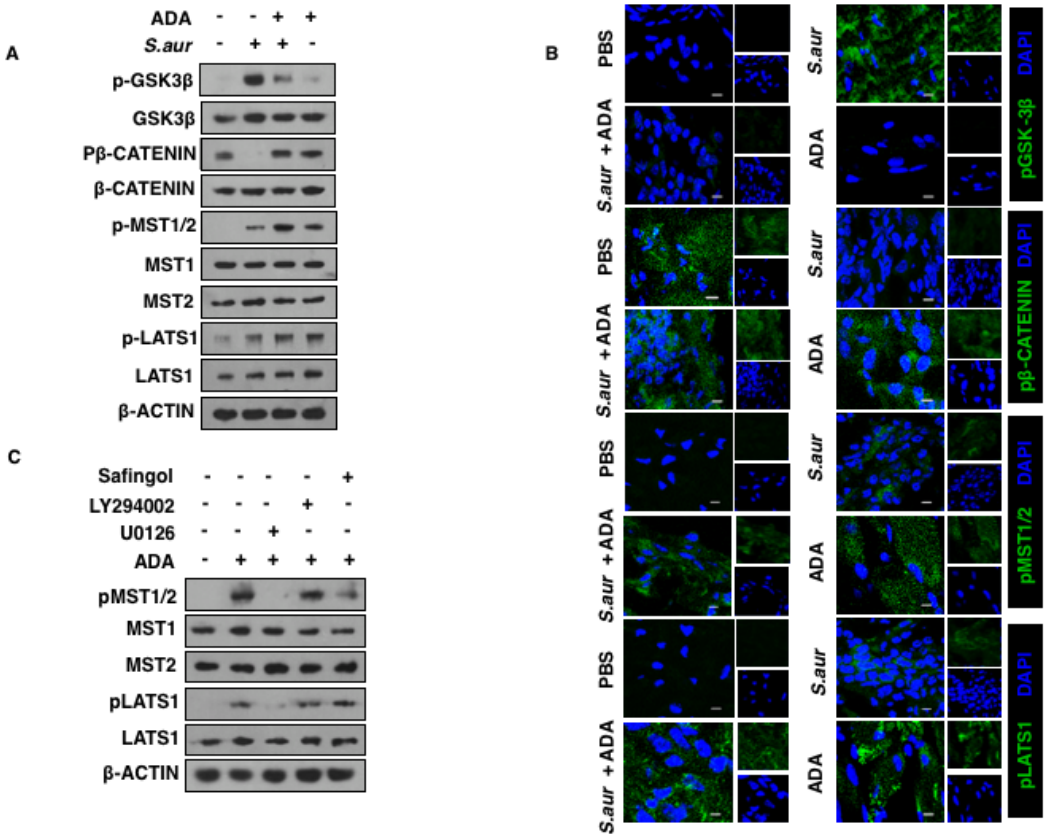

Fig 3.4.ADA treatment inhibits WNT and activates HIPPO signaling. (A-B) Left knee of BALB/c mice were intraarticularly injected at day 0 with 1 X 10^8 CFU of live *S. aureus*. Contralateral knee received PBS. Mice were treated daily with ADA i.p. (5mg/Kg) starting at – 3 day of bacterial infection and kept for 7 days. Representative **(A)** immunoblotting of pulverised knee samples and **(B)** immunofluorescence from knee synovium cryosections was performed to access activation of WNT and HIPPO signaling**(C)** Peritoneal macrophages from BLAB/c mice were pretreated with indicated inhibitors for 1 h followed by treatment with

ADA (5μM) for 1 h. Immunoblotting was performed to access activation of HIPPO signaling. (All blots are representative of 3 independent experiments).ADA-Adapalene. Bar, 5μm with original magnifications 40X

Importantly, synovial tissue of mice demonstrated inhibition of WNT signaling and enhancement of HIPPO signalingupon treatment of ADA **(Fig. 3.4B).** To understand the mechanism of activation of HIPPOsignaling by ADA, a screen for various kinases – MAPK(108), PI3K(109) and PKC(110) previously known to be activated by retinoic acid was performed. Upon treatment with specific inhibitor of MAPK/ERK (UO126), PI3K (LY29004) and pan PKC (Safingol), MAPK kinase inhibition showed abrogation of ADA-induced p-MST1/2 and p-LATS1 protein level **(Fig. 3.4C).** Thus, MAPK/ERK mediated the activation of HIPPOsignaling upon ADA treatment.

3.2.5 HIPPO signaling potentiated by ADA regulates pro-resolving mediators

To further delineate prophylactic role of ADA, its effect on inflammatory and anti-inflammatory mediators was further explored. As shown, ADA treatment on *S. aureus* infected macrophages showed a marked increase in expression of anti-inflammatory genes with a concomitant reduction in expression of proinflammatory **(Fig. 3.5A)**. Perturbations with siRNA mediated silencing of MST1/2 showed reduced level of anti-inflammatory mediators upon ADA treatment **(Fig. 3.5B).**

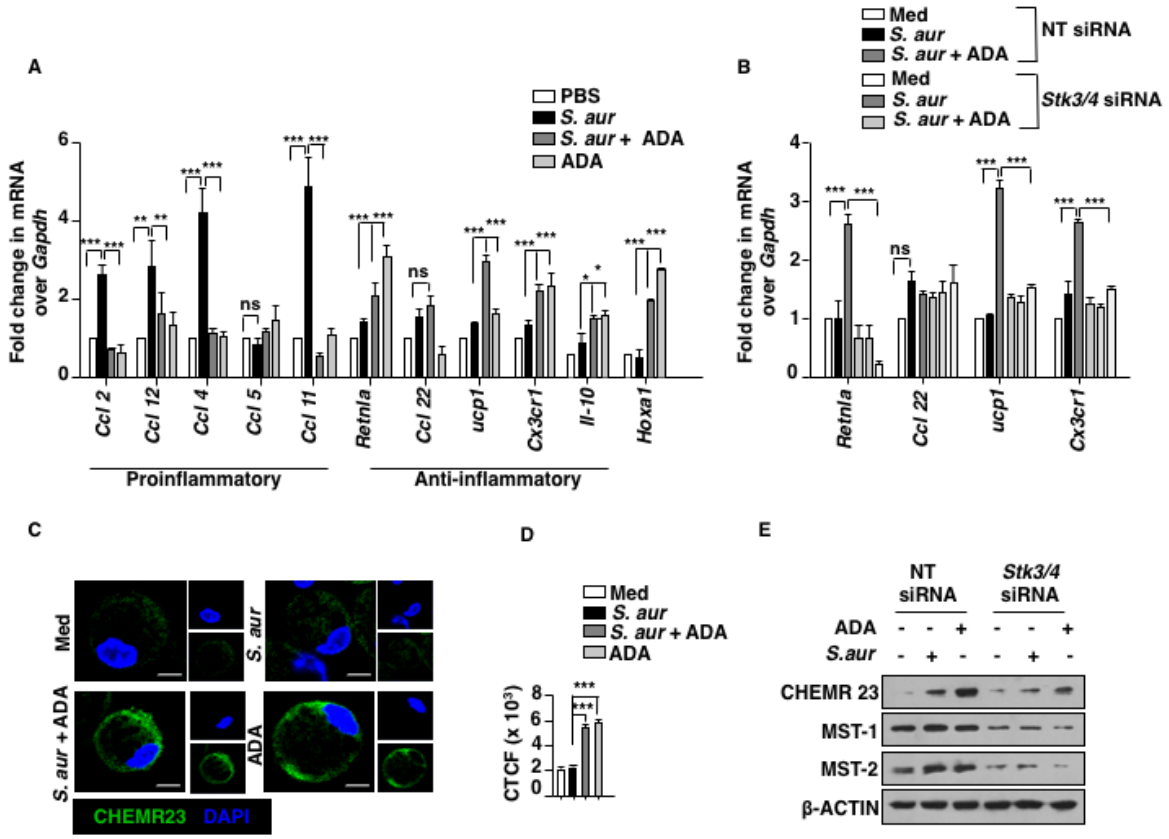

Fig 3.5. ADA-induced HIPPO signaling regulates M2 macrophages and CHEMR23 expression. (A) Left knee of BALB/c mice were intraarticularly injected at day 0 with 1 X 10^8 CFU of live *S. aureus*. Contralateral knee received PBS. Mice were treated daily with ADA i.p. (5mg/Kg) starting at – 3 day of bacterial infection. After 7 days knee were pulverized to perform quantitative real time PCR. **(B)** Peritoneal macrophages from BALB/c mice transfected with NT or *STK3/4* siRNA, were pretreated with ADA for 2 h followed by infection with live *S. aureus* for 10 h. Quantitative real time PCR was performed to analyse transcript level of M2 macrophages genes. **(C)** Representative immunofluorescence images of CHEMR23 receptor localization in peritoneal macrophages from BALB/c mice, pretreated with ADA (5µM) for 2 h followed by infection with live *S. aureus* (MOI 1:5)for 10 h. **(D)** Based on the IF images, CTCF were calculated (n = 100, each treatment) and plotted. **(E)**Peritoneal macrophages from BALB/c mice transfected with NT or *STK3/4* siRNA were treated with ADA (5µM) and live *S. aureus* infection for 12 h. Immunoblotting was performed to check CHEMR23 protein level. Data represent the mean ± SEM of three independent experiments. * $P< 0.05$, ** $P < 0.005$, *** $P< 0.001$ (One-way ANOVA followed by Tukey's multiple-comparisons test and all blots are representative of 3 independent experiments). Med-medium; NT-non-targeting. Bar, 5µm with original magnifications 63X

In line with above observations, we sought to explore effect of ADA on expression of *S. aureus* induced CHEMR23, a G protein– coupled receptor, which is known to resolve acute as well as chronic inflammation in presence of its ligand Resolvin-E1(RvE1)(111). For example, RvE1 blocks TNF-α induced NF-kB signaling and enhances phagocytosis of microbial particles and apoptotic neutrophils by human macrophages in a CHEMR23-dependent manner(112, 113). In this regard, we sought to explore the status of receptor of RvE1- CHEMR23, upon adapalene treatment.Immunofluorescence imaging demonstrated a significant cell membrane localization of CHEMR23 upon ADA treatment compared to *S. aureus* infection alone **(Fig. 3.5C, D).** Interestingly, silencing of MST1/2 showed abrogation of CHEMR23 protein expression **(Fig. 3.5E).** Thus, these results implicate pro-resolving role of ADA-induced HIPPO signaling in subduing septic arthritis development with M2 macrophages recruitment.

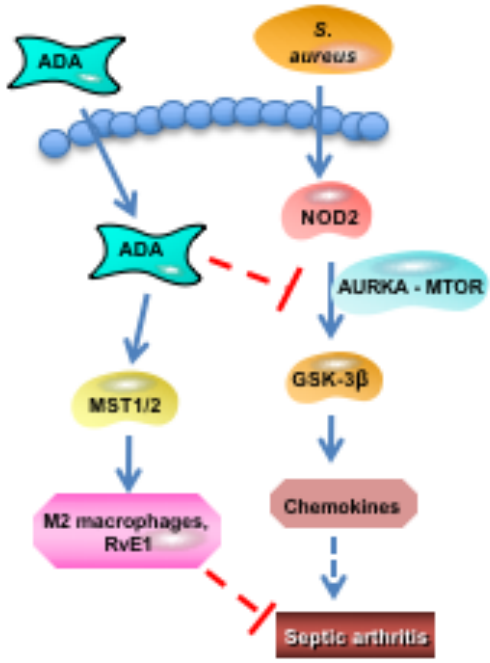

Fig 3.6. Model depicting regulatory effects of ADA in perturbation of *S. aureus* induced septic arthritis:*S. aureus* infection leads to NOD2 triggered activation of AURKA-mTOR-WNT signaling which results in enhanced recruitment proinflammatory chemokines leading to development of septic arthritis. ADA prophylaxis differentially mediates inhibition of WNT signalling while selectively enhancing activation of HIPPO pathway. MST1/2 Kinases acts as a signaling mediators of HIPPO pathway, which derives pro-resolvin action of adapalene by mounting activation of M2 macrophages and RvE1 cognate receptor CHEMR23, ultimately results in abrogation of septic arthritis.

In summary, through our current work we have elucidated a crucial role for the AURKA-WNT or HIPPO signaling axis in the regulation of septic arthritis. Moreover, we also observed a differential regulation of the pro- and anti-inflammatory mediators by ADA via the enhanced activation of HIPPO signaling. These findings strongly suggest ADA treatment as a prophylaxis to subdue *S. aureus*-induced cartilage damage **(Fig. 3.6)**

3.3 Discussion:

A broad spectrum of infectious agents are isolated from septic arthritic cases; of which *S. aureus* Cowan 1 has remained an enigma. Although a septic arthritic isolate, the implication of this particular strain in a murine septic arthritis model was never explored. The current study establishes for the first time, Cowan 1 as the causative agent for septic arthritis and uncovers novel signaling mechanisms that govern the disease progression.

One of the major pathogenic outcomes of septic arthritis is significant increase risk for Rheumatoid arthritis (RA). Importantly, patients undergoing joint replacement surgery or suffering immunodeficiencies due to lack of various immunological factors demonstrate enhanced vulnerability to septic arthritis. As described, *S. aureus* is a causative agent for septic arthritis in majority of RA patients, results in prolonged immune activation at joints with a chronic induction of Th2 or M2 macrophage cytokines/chemokines resulting a highly skewed immune effector functions. Thus, we explored role for unidentified regulatory circuits regulating host immune responses to *S. aureus* infection during septic arthritis. In the current study we have utilised intra-articular injection of *S. aureus* tofacilitate bacterial

colonization to knee, which ensured progressive loss of cartilage. In this study, infection with *S. aureus* Cowan1 showed significant increase in bone porosity and reduced bone volume, which was further validated through histopathological examination of knee sections.Upon delineation of regulatory circuits regulating host immune responses, pattern recognition receptor, NOD2, significantly regulated the *S. aureus* infection triggered signaling pathways. In this context, cell-cycle regulator AURKA(114), also acts as an immunomodulator, known to promote inflammation and contributes to pathogenesis of human gastric neoplasia(115). Notably, targeted inhibition of AURKA by VX680 could inhibit rheumatoid arthritis development, which is an autoimmune disorder by inducing B cell apoptosis (105). Congruent to these observations, our work showed proinflammatory role for AURKA by inducing WNT signaling pathway. As detailed in results, Aurora kinase A (AURKA) activated by *S. aureus* infection act as a key nodal kinase in regulating septic arthritis. In agreement to these observations, we utilized aphase III clinical trial AURKA inhibitor – alisertib, which was shown to have far fewer side effects than earlier established inhibitor like VX680. Utilizing alisertib intraperitoneal administration, we have observed a partial rescue in septic arthritis development with preserved cartilage and bone morphology.

As mentioned, septic arthritis is a rapidly progressing disease that often becomes difficult to cure and eventually reaches a chronic stage requiring surgical interventions. In view of these, we explored the role for retinoic acid in limiting *S. aureus* induced septic arthritis. Upon treatment with ADA, which is a FDA approved therapeutic, we have observed strong suppression of*S. aureus* triggered joint inflammation with preservation of articular cartilage layer with reduced changes in bone porosity. Interestingly, we have observed the differential effects of ADA,which while potentiating *S. aureus* triggered HIPPO signalling, showed significant abrogation of AURKA-responsive WNT signalling, thereby regulating the progression of septic arthritis development.

As detailed in introduction, in case of host-microbial interactions, resolution of ensuing inflammation often involves recruitment of lipid-derived mediators such as lipoxins and resolvins to local inflammatory milieu. These mediators serve a protective role by exhibiting dual activity of skewing anti-inflammatory processes and promoting pro-resolution activities. *In vivo* studies have revealed that late onset of resolvins could reduce excess leukocyte infiltration at the site of injury, with increased macrophage phagocytic activity and induction of anti-microbial defensins to restore homeostasis (111, 116). Of the various classes of resolvins, RvE1 is well established to play anti-inflammatory role. Mice osteolysis model revealed that Resolvin E1 negatively regulates osteoclastogenesis and enhance bone regeneration under TNF-a induced inflammatory conditions(117). Exudate isolated from *S. aureus* infected murine dorsal air pouch, showed recruitment of pro-resolving mediators which enhanced nonphlogistic activity of host derived macrophages leading to bacterial clearance(118). In line with these findings, we have demonstrated that HIPPO signaling potentiated by ADA regulated the level of membrane bound CHEMR23 in macrophages. However the direct effects of RvE1 treatment is not explored in current work.

In summary, we have elucidated a novel mechanism of AURKA-WNT signaling mediated septic arthritis development. Moreover, it was observed that ADA treatment differentially regulated the activation of HIPPO pathway and, in contrast, inhibited WNT signaling, which further leads to the recruitment of resolvins and M2 macrophages. Thus, we propose that adapalene could act as a promising candidate to treat septic arthritis.

CHAPTER 4: EXPLORATION OF REGULATORY EFFECTS OF RA DURING *C. ALBICANS* INDUCED DELAYED WOUND HEALING AND PATHOGENESIS

Exploration of regulatory effects of RA during *C. albicans* induced delayed wound healing and pathogenesis

4.1 introduction

C. albicans belongs to the class of Saccharomycetes that resides as a commensal on skin and gut mucosa, which often leads to superficial, cutaneous and sometimes life threating systemic infection in immunocompromised individuals. Several virulence factors including secretion of hydrolases together with its ability to form biofilm and transition effortlessly from yeast to hyphae are associated with *Candida* pathogenicity. Fungal infection often gets complicated with chronic inflammatory response which increases mortality rate amongst higher risk groups. Mortality rate pertains to 40% in immunocompromised individuals or those who areundergoing immunosuppressive therapy(119).

Sentinels cells such as macrophages, dendritic cells or natural killer cells employ pattern recognition receptors (PRRs) including C-type lectins such as dectin-1, dectin-2, mannose receptor (MR), Mincle, Galectin-3, TLRs (including TLR2 and TLR4) to recognise *C. albicans* cell wall associated molecular patterns. Such association culminates to activation of various host driven signalling cascades that help in protection against pathogen insult. For example, PRR driven inflammasome activation can regulate fungal infection in host, as mice defective in NLRP3/ASC caspase-1 activity were found to be susceptible to disseminated candidiasis with concomitant increase in kidney fungal burden(120). Moreover, Th17 are predominant cell type that secrete numerous cytokines including IL-17A, IL-17F and IL-22 which are proven to limit *Candida* growth and confer protective response against fungal infection(121). In corroboration to such studies, IL-17RC$^{-/-}$ mice are shown to be susceptible to oropharyngeal candidiasis (122)

Interestingly, *C. albicans* often evades host immune response by employing multiple avenues to blunt defences like blocking recognition and inhibiting the release of anti-

microbial effectors. *C. albicans* has the ability to perturb host microenvironment by detoxifying extracellular Reactive oxygen species (ROS)that are produced in macrophages through the secreted superoxide dismutases (SOD1-6) and catalase enzymes(123). Additionally, anti-reactive nitrogen species (anti-RNS) defences are synthesised intracellularly in the form of three flavohemoglobin enzymes (*Yhb1, Yhb4, Yhb5*) wherein, deletion of *YHB1* renders cells hypersensitive to NO stress *in vitro*(124)

Although oropharyngeal, vulvovaginal and invasive candidiasis represent the majority of candidial pathogenesis, *C. albicans* is also found to be one of the prevalent and dynamic causes of delayed wound healing among other potential pathogens. Chronic non-healing wounds are strong risk factors associated with high morbidity and mortality amongst diabetic, obese and elderly population. The chronic wounds are marked by characteristic recruitment of metalloproteinases such as MMP9, MMP8 etc. which, in association with elastase activity and recruited proinflammatory cytokines like *Il-1b, Il-6* and *Tnf-α* hinders wound healing and tissue repair. Restoration of damaged tissue homeostasis and augmentation of wound repair remain elusive targets for health-care, as unaddressed wounds increase susceptibility to colonization of pathogenic agent that may lead to lethal systemic infection.

Significantly, constant genetic drift and prevailing antibiotic resistance has profoundly impacted the pathogenicity of *C. albicans* infection, which shifted the focus on utilization of host immune response as an adjunct therapy. Such therapeutics aid in acceleration of maximum fungus killing with minimized inflammatory tissue damage. In this regard, the introduction of vitamins supplementation may provide beneficial to patients with underlying disorders and diseases. Interestingly, therapeutic effects of RA has long been studied during fungal infection, which could supress exacerbated inflammation and promote tolerance against pathogen insult(84). RA in concert with TLR2,effectuated resistance to disseminated *Candida* infection(125) and can also potentiate expansion of $Foxp3^{+}$ and IL-

10^{+} CD4 T cells(48). In this context, our current investigation involves unravelling the mechanistic details outlining the *C. albicans* induced modulation of host immune signaling with perturbation of wound healing. We have also explored administration of RA as a therapyto effectuate skin defects and as an immunomodulator against infection of *C. albicans.*

4.2 Results

4.2.1 Adapalene treatment modulates *C. albicans* induced delayed wound closure

Mice skin excisional wounds were createdusing previously described protocol(88), to explore the effects of RA administration on wound contraction. Full dermal thickness wounds were made symmetrically by punch biopsy on dorsal side of BALB/c mice. The wound on the right side was locally infected with *C. albicans*at 1 x 10^{5}CFU, whereas the wound on the left side was given PBS treatment to serve as contralateral control (**Fig 4.1A)**. Kinetics of wound closure was followed for 9 days to analyze wound repair and skin regeneration. Measurement of wound closure area at indicated days revealed that PBS treated wounds underwent progressive healing with complete re-epithelialization by day 9. Infection of wounds with virulent strain of wild type *C. albicans* (SC5314),disrupted wound contraction, with the most prominent difference observed on day 3 and this pattern continued for next 9 days (**Fig 4.1B)**. Infection induced delayed wound healing was comparable between wild type *C. albicans* which is known to form hyphae as well as yeast locked mutant *C. albicans* strain (HLC52) (**Fig 4.1C)**.

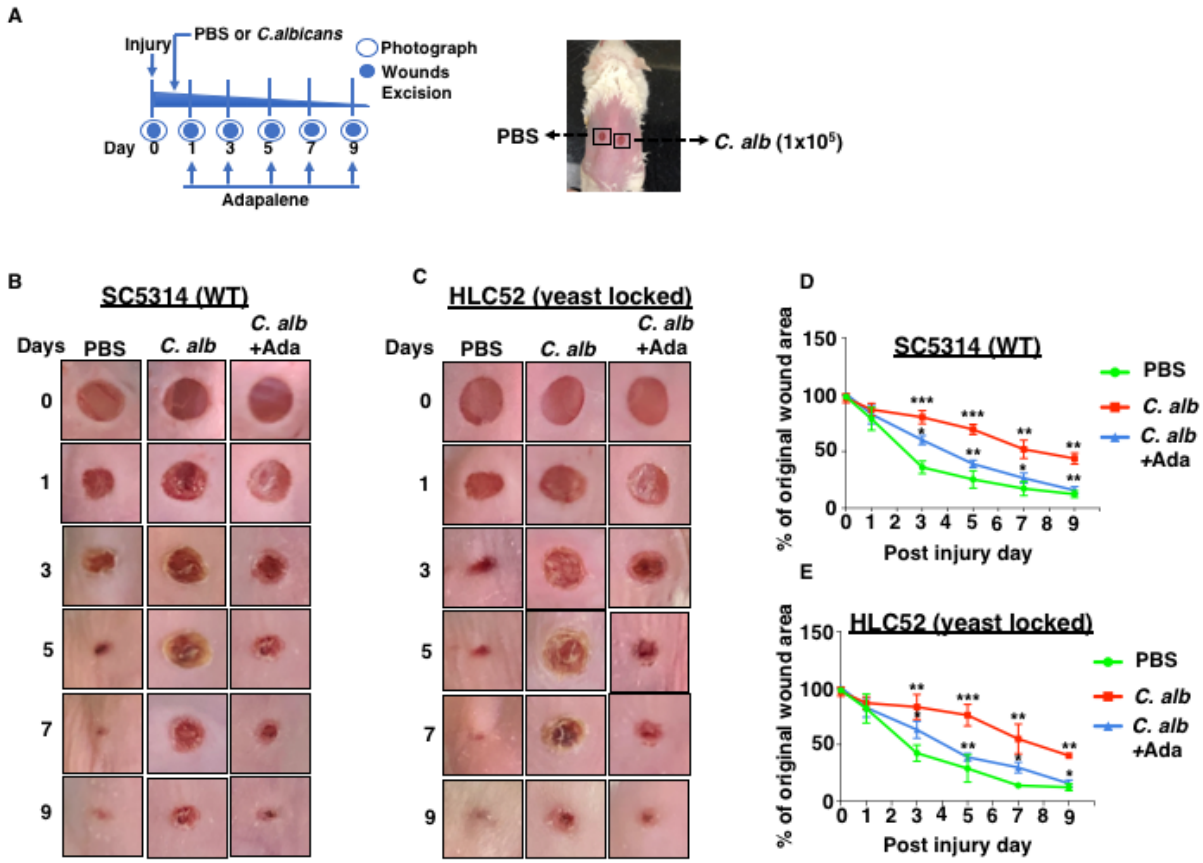

Fig: 4.1 Administration of RA augments *C. albicans* induced delayed wound contraction. (A) Schematic of wound healing model. Full dermal thickness wounds created symmetrically on dorsal side of shaved BALB/c mice. The wound on the right side was infected with *C. albicans* ($1x10^5$), whereas the wound on the left side was given PBS treatment. Adapalene was intraperitoneally administered daily after 12 h of *C. albicans* infection and wounds were photographed daily until day 9. **(B)** Representative photographs of dorsal side from PBS treated, wild type *C. albicans* (SC5314) infected mice, **(C)**yeast locked mutant *C. albicans* (HLC52) infected and adapalene treated mice, depicting the wound closure on indicated days. **(D and E)** Photographs to quantitatively measure wounds closure of PBS treated and *C. albicans* infected mice, were analyzed using Image J software. Data represent the mean ± SEM of three independent experiments. * $P< 0.05$, ** $P < 0.005$, *** $P< 0.001$(One-way ANOVA followed by Tukey's multiple-comparisons test and all are representative of 3 independent experiments)

To explore regulatory effects of RA on tissue repair, mice wounds were given intraperitoneal administration of ADA, a synthetic derivative of RA which is also an FDA approved drug. Daily treatment of mice with ADA starting at 12 hours post infection, markedly accelerated the rate of wound healing (**Fig 4.1Band C**). Quantitative assessment of wound closure in the ADA treated mice revealed upto 60% decrease in wound area on day 5 in contrast to *C. albicans* infected wounds which decreased only upto 20% of initial wound

area and continued until end of experiment (**Fig 4.1D)**. We have observed similar result for ADA treatment to yeast locked mutant*C. albicans* strain HLC52 infected wounds, wherein upto 70% decrease in wound area was observed on day 5in contrast to 15 % decrease of initial wound area in non-ADA treated wounds. (**Fig 4.1E)**. Thus, administration of RA significantly restored and augmented cutaneous wound healing.

4.2.2 *C. albicans* infectionimpairs wound healing and modulates inflammatory transcripts profile

In order to elucidate the molecular mechanism governing RA mediated wound closure, we shifted our focus primarily on day 3rd which showed significant difference in wound contraction.The histology of regenerated skin was assessed by hematoxylin-eosin staining of wound biopsy sections at day 3from PBS treated and *C. albicans* infected samples (**Fig 4.2A)**. As evaluated by pathologist, section of normal unwounded skin revealed well preserved epidermis followed by dermis layer, with characteristic hair follicles and sebaceous glands. Upon assessment of PBS treated wounds, the development of mild skin ulceration was observed withthe association of edema in the deeper tissues and an infiltration of acute and chronic inflammatory cells in the serous, subcutis and the region between skeletal muscle fibers. In contrast to PBS, *C. albicans* infected wound sections showed extensive central ulcers covered by fibrinous neutrophilic exudate, within which *C. albicans* hyphal forms were seen. The observed inflammation was seen to be extending into deeper tissues and markedly damaging serous and subcutis tissues.

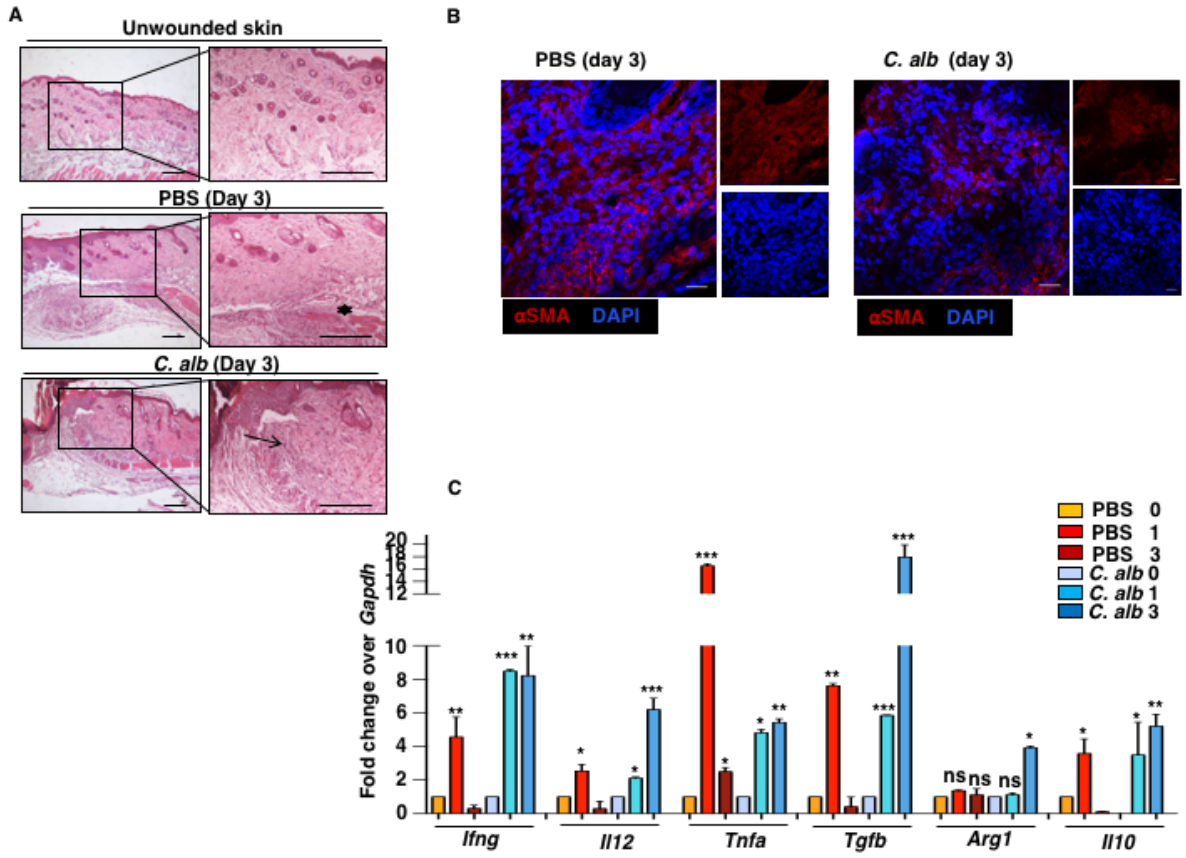

Fig: 4.2)*C. albicans* infection imparts healing defects to wounded skin. (A) Hematoxylin and eosin stained of skin section treated with PBS or infected with *C. albicans.* **(B)** Immunofluorescence imaging of wound section stained with a-smooth muscle actin. **(C)** wound biopsy samples were crushed to isolate RNA and real time quantitative RT- PCR analysis was performed for indicated genes. Data represent the mean ± SEM of three independent experiments. * $P< 0.05$, ** $P < 0.005$, *** $P< 0.001$(One-way ANOVA followed by Tukey's multiple-comparisons test and all are representative of 3 independent experiments). Bar, 50µm with original magnifications 2X and 5µm with original magnifications 20X.

To further analyze dermal tissue, we determined fibroblast proliferation and differentiation as a hallmark of scar-less tissue repair. Microscopic assessment of cutaneous wound closure showed high myofibroblast activity with enhanced immunofluorescence for alpha smooth muscle actin in PBS wound section in contrast to sections from *C. albicans* infection (**Fig 4.2B)**. As described in literature, both temporal and spatial expression of inflammatory cytokines are long been speculated to regulate wound repair(126). Wound microenvironment is often dictated by proinflammatory cytokines that persist at the site of injury for initial few hours to days. In this context, we have found that *C. albicans* strongly

elicited expression and secretion of both proinflammatory cytokines – *Ifng, Il-12* and *Tnfα* as well as anti-inflammatory cytokines *Tgfb, Arg1* and *Il-10*, within 24h of *C. albicans infection* (**Fig 4.2C)**, and this effect persisted upto day 3. Thus, *C. albicans* infection perturbs homeostasis of wound microenvironment that deregulates both pro and anti-inflammatory arms of immune response.

4.2.3 RA augments phagocytic uptake of *C. albicans* without effecting its survival

To delineate the effects of RA treatment on *C. albicans* survival, in vitro CFU assays were performed. Peritoneal macrophages from BALB/c mice was pretreated with adapalene followed by infection with *C. albicans* at 5:1 MOI for 90min to ensure phagocytic uptake of fungus. Following this, the rate of phagocytosis was enumerated at 8 h and 18 h post infection. CFU plating revealed no observable difference between infected and adapalene treated cells, suggesting that adapalene treatment does not alter *C. albicans* burden of macrophages (**Fig 4.3A)**. To further explore the immunomodulatory effects of RA, expression levels of inflammatory genes were analyzed. Interestingly, adapalene treatment significantly reduced proinflammatory genes and skewed the response towards expression of anti-inflammatory transcripts which are often marker for M2 macrophages activation (**Fig 4.3B)**.

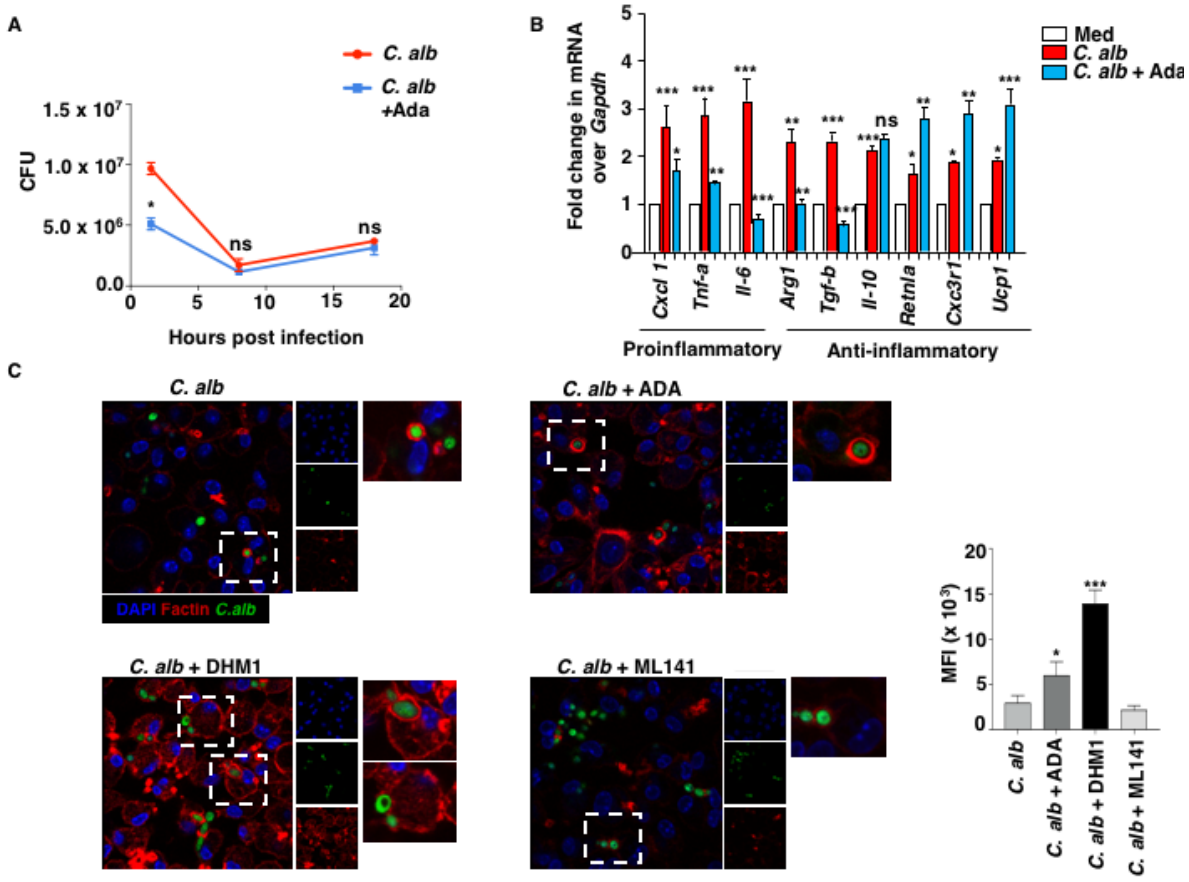

Fig: 4.3) Treatment with adapalene skews M2 macrophages with increased phagocytic uptake of C. *albicans.* (A-C) Peritoneal macrophages from BALB/c mice pretreated with adapalene followed by infection with*C. albicans* at MOI 5:1. **(A)** CFU was enumerated at indicated time to assess fungal burden. **(B)** RNA was isolated to analyze indicated transcript level by quantitative real time RT-PCR. **(C)** Peritoneal macrophages pretreated adapalene, BMP inhibitor (DHM1) and CDC42 inhibitor (ML141) were infected *C. albicans* at MOI 5:1 for 20min. Immunofluorescence analysis of stained phalloidin to observe F-actin assembly and phagocytic uptake of *C. albicans.* Data represent the mean ± SEM of three independent experiments. * $P< 0.05$, ** $P < 0.005$, *** $P< 0.001$(One-way ANOVA followed by Tukey's multiple-comparisons test and all are representative of 3 independent experiments). Bar, 10μm

M2 macrophages are frontiers of immunity that govern tissue homeostasis. In general, M2 phenotype switching provides defense against pathogen and impedes its survival by secreting chemotactic factors, clearing apoptotic cells and increasing angiogenesis with significant mounting of phagocytic response(127). In this regard, immunofluorescence imaging of *in-vitro* infected peritoneal macrophages with GFP-tagged *C. albicans*, revealed formation of phagophores through assembled F-actin network (**Fig 4.3C**). The association of *Candida* with macrophages was increased upon adapalene treatment with concomitant

increase in actin assembly. Since, from our previous work we have observed that BMP signaling could effectuate *C. albicans* infection during delayed wound healing(81), inhibitor of BMP signaling-DHM1 was utilized. Data showed the crucial role of BMP signaling,whose inhibition by DHM1 significantly enhanced phagophore formation around invading *C. albicans*. Here ML141 was utilized as a potent and selective inhibitor of Rho family GTPases cdc42, to block assembly of F-actin, which showed perturbed *C. albicans*-phagophores formation. Thus, our data showed that both RA treatment and inhibition of BMP signaling can regulate phagocytic uptake of *C. albicans* by macrophages.

4.2.4 Crosstalk of BMP signaling with downstream effectors of Rho/Rac GTPases

Uptake of *C. albicans*by macrophages leads to the relay of diverse signaling pathways which regulates systemic dissemination of said *C. albicans* in host. Coordinated action of Rho/Rac GTPases are known to regulate cellular phagocytosis by recruiting actin binding protein – COFILIN (128). LIMK is a potent negative regulator of COFILIN, which regulates actin reorganization by reversing COFILIN induced actin depolymerization(129). Immunoblotting of *in vivo* wound samples showed induced p-COFILIN inhibitory level augmented on day 5 and this pattern sustained until day 9, indicating enhanced activation of F-actin assembly. On the contrary, *C. albicans* infection mounted inhibition of COFILIN activity from day 0 to day 3, which might be required to entrap fungus burden inside cells. However, post 3 days of infection, activation of COFILIN was induced that perturbed actin assembly and possible phagocytic rescue. Confirming our previous work, BMP signaling was found to be elevated in *C. albicans* infection induced deregulated wound repair(81), with concomitant activation of MMP9, which is known for its crucial role in remodeling extracellular matrix (**Fig 4.4A)**.

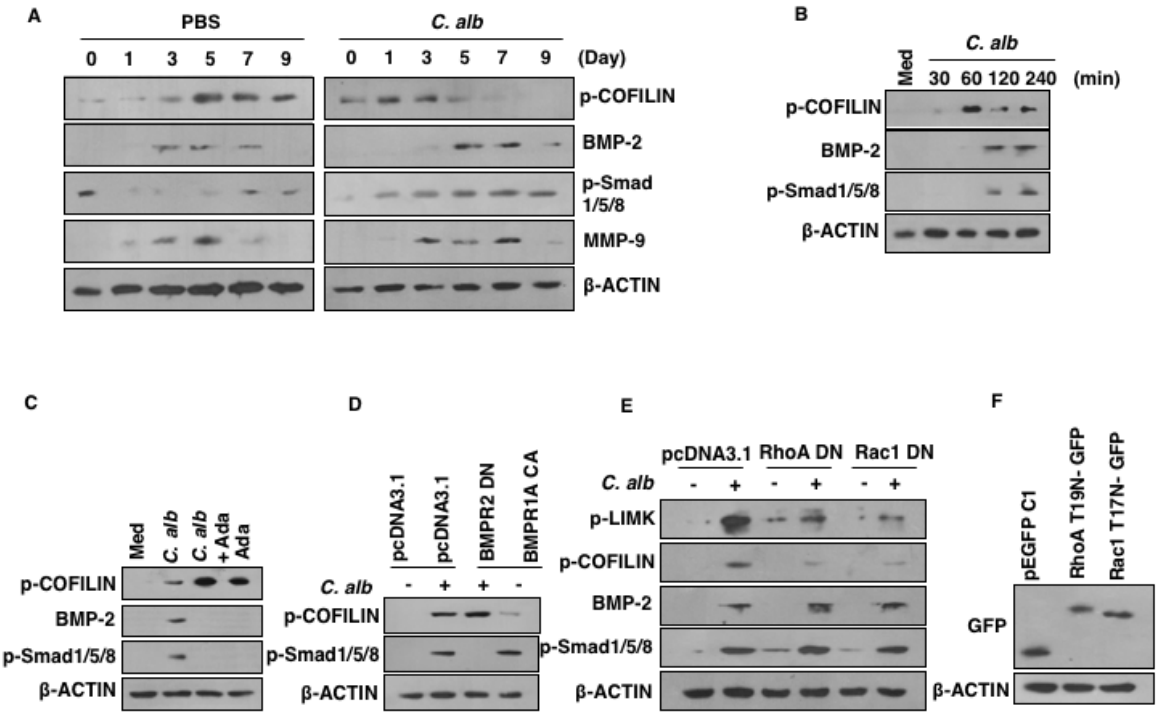

Fig: 4.4 Coordinated action of Rho/Rac GTPases in mediating regulatory effects of RA on F-actin polymerization. (A) Immunoblotting analysis of wound sections from PBS treated and *C. albicans* infected mice to analyze protein expression of indicated molecules during time course of wound healing. **(B-C)** Peritoneal macrophages from BALB/c mice were infected with *C. albicans* at 5:1 MOI **(B)** time kinetics was performed and **(C)** pretreated with adapalene to assess activation of BMP signaling and COFILIN. **(D-F)** RAW264.7 cells overexpressing indicated constructs were infected with *C. albicans* at MOI 5:1 for 4 h and immunoblotting was performed to assess activation of BMP signaling and Rho/Rac signaling downstream effectors LIMK and COFILIN. Data representative of 3 independent experiments.

Since both adapalene and inhibition of BMP signaling were regulating *C. albicans* phagocytosis, we wanted to elucidate functional cross talk between RA and BMP in regulating Rho/Rac effectors. Time kinetics showed that inhibitory phosphorylation of COFILIN started at 60min post *C. albicans* infectionand remain sustained until 4 h along with activation of BMP signaling, whose level was induced at 120min after infection with *C. albicans*. (**Fig 4.4B)**. Administration of adapalene to *C. albicans* infected peritoneal macrophages inhibited BMP signaling, but enhanced level of p-COFILIN (**Fig 4.4C)**. Thus, adapalene governed differential effect upon inhibiting BMP and activating Rho/Rac-LIMK signaling axis. Overexpression of constitutively active BMP receptor abolishes inhibitory p-COFILIN level, whereas BMPR dominant negative construct expression further enhanced it

(**Fig 4.4D)**, indicating that BMP signaling significantly inhibits Rho/Rac-LIMK signaling axis. To explore if Rho/Rac can regulate BMP signaling on its own, we have utilized overexpression of dominant negative RhoA and Rac1 constructs. Data showed that although LIMK and p-COFILIN are modulated byoverexpression of dominant negative Rho/Rac kinases, it did not perturb BMP signaling axis (**Fig 4.4E)**. Immunoblotting of GFP tagged RhoAT19N dominant negative and Rac1T17N dominant negative constructs was performed as a control to validate their overexpression (**Fig 4.4F)**.

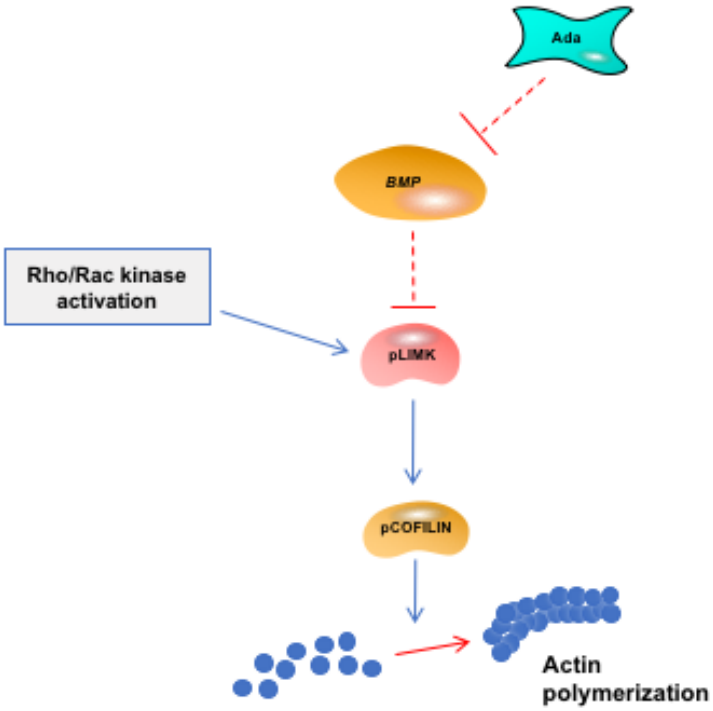

Fig: 4.5 Model depicting adapalene regulated interaction of BMP and Rho/Rac effectors. RA rescues *C. albicans* infected delayed wound healing by inhibiting COFILIN activity on F-actin assembly. Administration of adapalene inhibits BMP signaling which leads to activation of LIMK kinase, independent of Rho A and Rac1 kinases. Activated LIMK blocks actin binding function of COFILIN to increase F-actin polymerization, thereby enhancing phagocytosis of *C. albicans*.

Thus, current model indicates that COFILIN, which is a known downstream effector of Rho/Rac GTPases, is under negative regulation of BMP signaling axis. This suppressive effect is rescued upon administration of adapalene that further enhanced F-actin phagophore

assembly and these results suggest that, RA driven inhibition of BMP signaling protects mice from *C. albicans* induced delayed wound healing (**Fig 4. 5).**

4.3 Discussion:

The current study uncovered the therapeutic role for RA in abrogating *C. albicans* induced delayed would healing. Colonization with *C. albicans* perturbed homeostatic balance of wound microenvironment that severely damaged tissue repair. Assessment of *C. albicans* infected wound sections showed the development of central ulcers in deep injured tissue which were found to be associated with fibrinous neutrophilic exudate.

Various studies impede the compelling need for relative abundance of effectors which are known to govern wound contraction phenotype. Differentiation of fibroblast to myofibroblast is known to play a crucial role in remodeling disrupted extracellular matrix(130). With our observation of immunofluorescence imaged *C. albicans* infected mice wound sections, we have confirmed reduced fibroblast differentiation with low expression of smooth muscle actin. Contrary to fungal infection, PBS treatment to sterile wounds showed higher contractile activity of myofibroblast cells, thereby suggesting that wound contraction is severely affected due to *C. albicans* infection.

Inflammation plays a pivotal role during repair of injured tissue by supplying necessary growth factors and cytokines signals(131). In this regard, analysis of recruited cytokines from wound sections revealed perturbed homeostatic balance of both pro and anti-inflammatory cytokines. Resolution of inflammation is instrumental for successful wound repair. Our observation with administration of adapalene, showed skewed anti-inflammatory cytokines with activation of M2 macrophages genes. Such effect was corroborated with augmented wound contraction in adapalene treated mice thereby resulting in rescuing *C. albicans* infection induced wound healing delay.

To delineate the molecular mechanism governing adapalene regulated responses, we enumerated survival of *C. albicans* under adapalene treatment.Since we did not observe any marked effect of adapalene treatment on *C. albicans* CFU, we hypothesized that adapalene treatment might be regulating enhanced association of *C. albicans* with activated M2 macrophages. Immunofluorescence analysis demonstrated that adapalene treatment increased actin phagophore formation of macrophages which resulted in engulfment of invading C. *albicans.* As described, membrane phagophore formation utilizes F actin assembly network that is under coordinated action of Rho A and Rac 1 GTPases. Since both adapalene and inhibition of BMP signaling were regulating F actin polymerization, we hypothesized a possible crosstalk of adapalene and BMP with Rho/Rac signaling axis. Our results defined the crucial immunomodulatory role of RA in abrogating BMP signaling axis which was found to be instrumental for coordinating assembly of actin binding proteins. Taken together we have found that RA mediated inhibition of BMP signaling positively regulated LIMK kinase activity, which inturninhibited action of COFILIN thereby enhancing *C. albicans* – phagophore formation. RA mediated enhanced phagocytosis of invading *C. albicans* resulted in rescuing of delayed wound healing and helped in restoration of damaged cutaneous tissue.

PERSPECTIVE

In regard of identifying new avenues of host directed therapies, our current study established the unidentified role of retinoic acid therapy against *S. aureus* and *C. albicans* induced chronic inflammatory disorders. Mechanistically, the present work highlight the critical role of AURKA in regulating mTOR dependent WNT signaling activation, which dictates the development of *S. aureus* infection induced septic arthritis, with marked changes and degradation observed for associated joint tissues morphologies. In this context,we have utilised ADA prophylaxis which showed significant inhibition of AURKA-WNT signaling pathway with concomitant activation of HIPPO signaling. Our work highlights the implication of HIPPO activated anti-inflammatory mediators which ultimately governed the rescue of *S. aureus* induced damage to articular cartilage and bone architecture (chapter 3). In addition to this, we also found beneficial role of ADA against *C. albicans* induced delayed wound healing. Here we have investigated the novel mechanism of functional crosstalk between deregulated BMP signaling and effectors of Rho/Rac GTPases which in turn regulates cortical actin network and phagocytic uptake of *C. albicans*. We underscored the regulatory role of ADA which mediated the p-LIMK regulated inhibition of COFILIN that increased the macrophages and *C. albicans* interaction and in turnresulted in enhanced wound healing with restoration of damaged tissue (chapter 4). Together, our study provide the novel insight into therapeutic role of retinoic acid which helped in maintaining intricate balance between host tissue homeostasis and pathogen induced hyper-inflammatory response.

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- **Preeti Yadav**, Bharat Bhatt and Kithiganahalli Narayanaswamy Balaji. "Selective activation of MST1/2 kinases by retinoid agonist Adapalene abrogates AURKA-regulated septic arthritis" (under circulation).

- **Preeti Yadav**, Ankita Ghoshwal and Kithiganahalli Narayanaswamy Balaji. "Adapalene targets Cofilin to regulate*C. albicans* infection induced delayed wound healing" (Manuscript in preparation).