Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence

A Thesis

Submitted in Partial

Fulfilment of Requirements for the Degree of

DOCTOR OF PHILOSOPHY

BY

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October, 2024

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STATEMENT

I do hereby declare that the matter embodied in this thesis entitled: **"Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence"**, is the result of investigations carried out during my PhD in School of Agro and Rural Technology, IIT Guwahati and KaviKrishna Laboratory, Guwahati, India under the guidance of Dr. Sanjukta Patra from SART, IIT Guwahati and Dr. Bikul Das from KaviKrishna Laboratory, Guwahati, India. This work has not been submitted elsewhere for a degree. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work in this thesis entitled: **"Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence"**, done by Ms Lekhika Pathak (Roll No. 196154102), a registered student of School of Agro and Rural Technology, IIT Guwahati for the award of degree of Doctor of Philosophy is an authentic record. This work has not been submitted elsewhere for a degree. Lekhika carried out the research work under my supervision and Dr. Bikul Das of KaviKrishna Laboratory, Guwahati, India as an external and part time student.

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CERTIFICATION OF INSTITUTIONAL ETHICS COMMITTEE

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Dear Lekhika,

The Institutional Ethics committee of KaviKrishna Laboratory meticulously reviewed your application on ethical approval for PhD project titled "**Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence**".

After comprehensive consideration and evaluation of the proposal's scientific significance, ethical implications, and methodology, the committee is pleased to convey its ethical approval for the research study. This approval is granted under the IEC protocol 2 (IEC/KKL/GBP/07/2018) for the project titled "Tumor stemness defense study using invitro and invivo model of oral squamous cell carcinoma" and renewed IEC protocol 5 (IEC/KKL/GBP/02/2019) for the project titled "Study of in-depth genetic heterogeneity with respect to resistome and compensatory adaptation of MDR Mtb clinical strains inside BM-mesenchymal stem cells circulating in the North East Region" provided to the PI of the project, Dr. Bikul Das, KaviKrishna Laboratory. It is suggested to conduct the research work for PhD under the supervision of project PI. Furthermore, any updates or modifications to the study protocol must be duly communicated and reviewed by the committee for continued ethical compliance.

Your Sincerely,

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Dear Lekhika,

The Institutional biosafety committee of KaviKrishna Laboratory has reviewed your PhD project titled "**Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence**" and suggested to conduct the research work for PhD under the supervision of project PI, Dr. Bikul Das, KaviKrishna Laboratory as per the below IBSC protocol and the recommendations.

IBSC protocol 2 (IBSC/KKL/GBP/07/2018): "Tumor stemness defense study using invitro and invivo model of oral squamous cell carcinoma"

IBSC protocol 5 (IBSC/KKL/GBP/02/2019): "Study of in-depth genetic heterogeneity with respect to resistome and compensatory adaptation of MDR Mtb clinical strains inside BM-mesenchymal stem cells circulating in the North East Region"

Recommendation: Approved. All experiments involving TB patient samples should be performed under BSC-3 hood following RNTCP protocol for sputum processing and culture. All experiments involving cancer patient samples should be performed under BSL-2 facility to be set up in Guwahati Biotech Park, IIT-G campus. All the waste should be autoclaved before disposing.

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CERTIFICATION OF INSTITUTIONAL COMMITTEE FOR STEM CELL RESEARCH APPROVAL

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To,

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Senior Research Fellow, KaviKrishna Laboratory,

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Dear Lekhika,

The Institutional Committee for Stem Cell Research (ICSCR) of KaviKrishna Laboratory has reviewed your PhD project titled "**Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence**" and approved under the below ICSCR protocol number with following recommendation:

ICSCR protocol 2 (ICSCR/KKL/GBP/07/2018): "Tumor stemness defense study using invitro and invivo model of oral squamous cell carcinoma"

ICSCR protocol 5 (ICSCR/KKL/GBP/02/2019): "Study of in-depth genetic heterogeneity with respect to resistome and compensatory adaptation of MDR Mtb clinical strains inside BM-mesenchymal stem cells circulating in the North East Region"

Recommendation: In accordance with ICMR guidelines, ICSCR, KaviKrishna approved the ICSCR protocol 2 and 5 on 30/07/2018, to be conducted at BSC-2 and BSC-3 cabinet of KaviKrishna Laboratory in coordination with KaviKrishna Telemedicine Care (KTC) under my guidance as project PI. Therefore, you are suggested to conduct the research work for PhD at KaviKrishna Laboratory following the ICSCR guidelines.

Your Sincerely,

Dr. Bikul Das

Principal Investigator, KaviKrishna Laboratory

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Acknowledgment

I would like to take the privilege to express my sincere thanks and gratitude to my Coordinating Supervisor, Dr. Sanjukta Patra, Professor, School of Agro and Rural Technology, whose guidance, motivation and valuable suggestions helped me greatly during my tenure of Ph.D. program until thesis preparation. I would also be thankful to her PhD students for their help whenever needed. I am very much thankful to the doctoral committee members of my PhD; Dr. Karuna Kalita, Dr. Bithiah J Jagannathan, Dr Siddharth Sinha and Thesis reviewers; Prof Dean Felsher and Dr. Dhruv Kumar for their incredible support, sincere review of the work and valuable suggestions.

I would like to take this opportunity to express my deepest sense of gratitude to my Local Supervisor, Dr. Bikul Das, Director and Senior Scientist of KaviKrishna Laboratory, IIT Guwahati Research Park, IIT Guwahati, for his guidance with constant encouragement, invaluable suggestions, and constructive criticisms throughout the period of PhD work experiments, manuscripts and thesis preparation. I am really grateful to him for showing believe in me to work on his concept of stem cell altruism in the field of infectious diseases and cancer. The idea of stem cell altruism is inspired by the Vedic Philosophic theory of avatarvada or “Vedic Jiva Upakar Vada”, which was mentioned in a poetry book titled “Sonali Nokhor Jui”, written by Krishna Ram Das, a philosopher poet of Sualkuchi, Assam. Several session of “satavata tarka” or debate between Krishna Ram Das, traditional healer Jagat Ghora, Vaishnav pandit Dayal Krishna Borah, Geeta pandit Hem bhai and Dr. Bikul Das led the emergence of stem cell altruism idea. Therefore, I would be always indebted for their contribution in my research journey. I would like to thank KaviKrishna, Sualkuchi for providing funding for my PhD research work described in this thesis. I am grateful towards the executive committee members specially Sailen Chandra Choudhury, Kabindra Das and Mallika Maral of KaviKrishna, Sualkuchi for agreeing to sponsor my PhD via providing facility to perform experiments at KaviKrishna Laboratory.

I delivered oral presentation on stem cell altruism work in 8th international translational cancer research conference at Banaras Hindu University, India and received Dr. B. Nagarajan memorial lecture award. Also, the underlying vedic philosophic theory

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of the stem cell altruism idea was appreciated in the conference by scientists like Prof Sen Pathak and Oommen V Ommen from USA. I am glad to receive suggestions and appreciation on my PhD work presented in international conference of society for immunotherapy of cancer held in Boston, USA from eminent scientists, researchers and entrepreneurs, which helped me to upgrade the experimental techniques in the study. I am thankful to the scientists and researchers of our collaborator lab in USA; Thoreau Lab for Global Health, UMass, Lowell, MA, USA namely Joel therein, Reza B Mokhtari and Ibrahim Akeel. A special thanks to Marcie Black from Thoreau lab for the brainstorming discussions, valuable suggestions and appreciation. I was fortunate to interact with many eminent personalities including academicians, scientists, social workers and writers such as Prof Kapil Kapoor, Hem Bhai, Sankar Das, Rakesh Bhatnagar, Rajendra Prasad, Rituraj Kalita, Purabi Bora, Asok Mukhopadhyai, Rubul Mout, Binoy Bordoloi etc which gave huge inspiration and courage in my research journey.

The research team members Partha Jyoti Saikia, Gokul Das, Shirsajit Mitra from KaviKrishna Lab and Tutumoni Baishya, Rupam Das, Chayanika Das, Nirala Medhi from KaviKrishna telemedicine care (KTC), Sualkuchi have been my strong support throughout the PhD journey. Different brainstorming sessions with them and other members Sukanya Gayan, Seema Bhuyan, Nihar Ranjan Das, Bidisha Pal, Debduti Dutta, Sandhya Sorra and Joyeeta Talukdar also helped me to craft my scientific thinking. The bachelor and master students who have done internship under my supervision during the 5 years of my PhD journey have incredibly motivated me to work more. Specially Riya Kanodia, Siddhant Biswas, Upasha Sarma, Sonali Das and Moana Gregori are exceptional. I have worked with many volunteers at KTC whose sincere patient follow up helped to complete the clinical study of my thesis work. I was always amazed by their kind words, specially of Nihar, Uddipana, Dimpi, Deepjyoti, Puja, Hima, Barsha, Nina and the list goes on. I also got motivation from the cancer survivors; Damayanti baideu, Bhula daa, Khanindra and weavers; Chitra baideu, lakhi baideu, Malati baideu as well as other team members Sailen Baishya, Bhargav Oja, Dhruba Kakati, Montu Medhi who are engaged in cancer patient rehabilitation project via muga silk culture revival at KaviKrishna Living Muga Museum. I have received immense love

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and support from all of them which made a hard PhD life a happy journey. Their expectations will always encourage me in doing good work.

Finally, I would like to thank my family Maa (mother); Parinita Pathak, Deuta (father); Sarat Chandra Pathak, Vaiti (younger brother); Luhit Ranjan Pathak and Vonti (younger sister); Lipika Pathak for always understanding my busy research life. I learnt working hard in life from Maa and Deuta though I can't match up to their hard work. My sad and failure days were compensated by fun filled conversations with vaiti, vonti and my few friends including Akram and Udisee. With their love and blessings from Krishna (God), my research journey has reached this stage.

Yours sincerely,

Date: 22.10.2024

Lekhika Pathak

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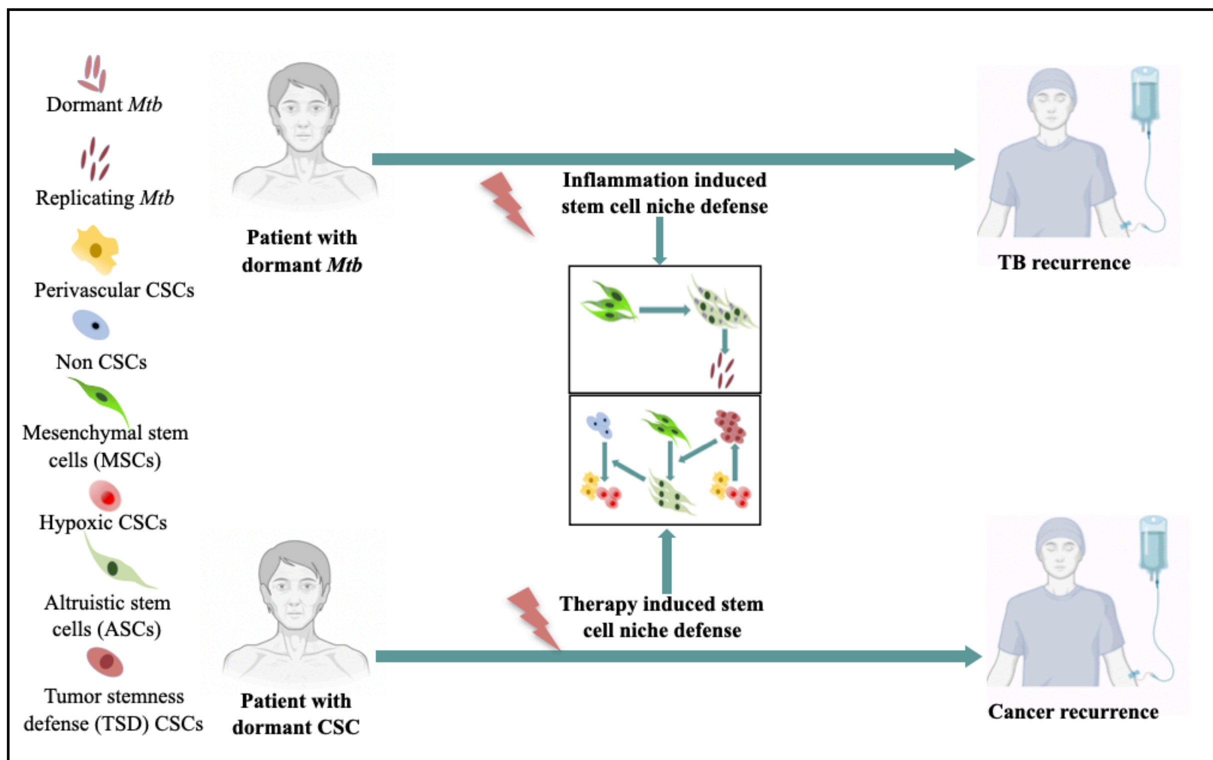
Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence

Abstract

Adult stem cells, residing within protective niches, can be threatened by invading pathogens and cancer cells. To counter these threats, stem cells have evolved defense mechanisms, including "trained immunity" and the adoption of a transient, p53-deficient "altruistic stem cell" (ASC) phenotype that prioritizes community survival over self-preservation. However, we propose that this ASC-based niche defense can be exploited by pathogens and cancer cells, leading to disease reactivation. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), and cancer both exhibit dormancy and reactivation patterns, posing significant therapeutic challenges. Our research demonstrates that CD45-/CD271+ mesenchymal stem cells (MSCs) mount a defense against both *Mtb* and head and neck squamous cell carcinoma (HNSCC)-derived cancer stem cells (CSCs). We observed that crosstalk between MSCs and *Mtb* or HNSCC-derived CSCs triggers the reprogramming of MSCs into ASCs. This, in turn, facilitates *Mtb* reactivation and promotes the pro-tumorigenic activity of CSCs, respectively. Furthermore, utilizing a rural telemedicine network, we found evidence linking ASC niche defense to TB and HNSCC recurrence in clinical subjects. These findings suggest that the ASC-based stem cell niche defense mechanism can be hijacked, contributing to disease relapse. This highlights a novel therapeutic target for preventing reactivation in both infectious diseases and cancer.



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Chapter 1: Introduction and Literature of Review

Abstract:

The transition from dormancy to reactivation is a hallmark of many infectious diseases and cancers, posing significant challenges to disease management and patient outcomes. Tuberculosis (TB) and head and neck squamous cell carcinoma (HNSCC) are two such diseases that disproportionately impact public health in India, particularly in the Northeast region. Reactivation or recurrence of these conditions often leads to increased mortality, underscoring the urgent need for novel therapeutic approaches. Current treatment strategies struggle to effectively address these challenges, particularly within the context of limited healthcare resources in India. Therefore, a deeper understanding of the shared mechanisms driving disease reactivation is crucial for developing more effective interventions. The dormancy to reactivation is a common feature of many infectious diseases as well as cancer. TB and HNSCC are 2 fatal diseases that have hugely affected the rural public health of India, especially North East India. Reactivation or recurrence of the TB and cancer leads to mortality for most of the patients. The current therapeutics and management strategies are not able to manage these two diseases especially in India. Therefore, there is a need to develop a novel approach to study a shared mechanism of reactivation. One such potential mechanism is the stem cell niche defense, a strategy employed by stem cells to protect their microenvironment (niche) from threats like pathogens or cancer cells. This defense mechanism involves both cytoprotective actions, shielding the niche from harm, and altruistic behaviours, where stem cells sacrifice themselves or alter their properties to safeguard the niche. This introduction delves into the biology of TB and a prevalent epithelial cancer, HNSCC, emphasising their impact on public health in rural India. We also explore the emerging understanding of stem cells as protective havens for both dormant *Mycobacterium tuberculosis* (*Mtb*) and cancer stem cells (CSCs). One such potential mechanism is the stem cell niche defense, a cytoprotective and altruistic defense mechanism exerted by stem cells to defend their niches against pathogens as well as cancer cells. We discuss how the stem cell niche defense mechanism may provide a novel insight into understanding the reactivation or recurrence of these two disease types.

1.1 Reactivation and recurrence of diseases

Reactivation is the mechanism whereby a latent or dormant microorganism that has infected a host cell switches to a lytic stage, undergoing productive replication and allowing the organism to spread. Latent infections have the potential to be reactivated into a lytic form. Thus the pathogen exploits this ability to move back and forth from latent to lytic infections to transmit from infected individuals to uninfected individuals (Traylen CM *et al.*, 2011, Forte E *et al.*, 2020, Grinde B *et al.*, 2013). The pathogens have another pattern of replication, where they are not eliminated from the host completely during primary infection. This is called "persistent replication", one example is hepatitis B virus (Traylen CM *et al.*, 2011, Forte E *et al.*, 2020). This latent and persistent replication is also associated with the Tuberculosis (TB) disease. 10% of latent TB subjects have the potential of reactivation in their lifetime (Lin and Flynn, 2010) and 10% of persistent TB subjects also have the potential of recurrence (Azhar, 2012, Global TB report by WHO, 2022). There are many other diseases which recur after successful treatment such as malaria (Mahittikorn *et al.*, 2021), cholera (Elimian *et al.*, 2020), pertussis (Koufakis *et al.*, 2017), influenza (Memoli *et al.*, 2020), pneumococcal disease (King *et al.*, 2003), and gonorrhea (Hosenfeld *et al.*, 2009) (Table 1). Importantly, successful vaccine based prevention and efficient treatment strategies are available for most of these diseases. Whereas despite decades of having an active control program, TB remains major killer worldwide. The causative agent of TB is *Mycobacterium tuberculosis (Mtb).* Remaining dormant inside the host and reactivate in a favourable condition may be the key strategy of the *Mtb* to maintain its transmission cycle in the community (Pathak L *et al.,* 2021). Another fatal disease with the reactivation and recurrence potential is cancer. Cancer recurrence occurs, when patients remain clinically asymptomatic for a long period after successful treatment of the primary tumor and then the cancer comes back (Mahvi DA *et al.*, 2018). Almost, all the cancer have more than 50% recurrence rate (Spiegelberg D *et al.*, 2022, Oriol A *et al.*, 2010, Denaro N *et al.*, 2016). India has the highest number of head and neck squamous cell carcinoma (HNSCC) with more than 30% recurrence probability. Therefore, there is a need of novel insight in the reactivation or recurrence of these 2 deadly diseases, TB and cancer, specially the HNSCC.

Table 1: The list of major diseases associated with reactivation or recurrence

SL no	Diseases associated with reactivation/ recurrence	Associated disease condition
1	Herpes simplex virus infection	Based on sites of infection, it causes encephalitis, keratitis, mucocutaneous disease
2	Human papilloma virus infection	Warts and precancerous condition. Can cause cervial, anal, penile and oropharyngeal cancer
3	Parvovirus infection	Can cause anemia and anemia crisis
4	Malaria	*P. vivax* and *P. ovale* have dormant liver stage parasites that reactivates. Symptoms include fever, anemia, jaundice, muscle ache
5	Cholera	Acute watery diarrhoea
6	Pertussis	Paroxysmal cough, often recur with subsequent respiratory infections for many months
7	Influenza	Severe malaise, dry cough. Can reactivate other dormant bacterial infection such as tubercle bacilli
8	Pneumococcal disease	Repeated asthma exacerbations, fever, abnormal auscultation
9	Gonorrhea	Irregular menstruation, Heavy bleeding, pain or burning while urinating
10	Cancer	All types of cancers show recurrence, Persistent and worsening pain, difficulty breathing, loss of appetite
11	Tubercuslosis	Chest pain and shortness breath. COPD or Asthma patients are potential to get reinfected
12	Hepatitis B	Serious illnesses such as cirrhosis and liver cancer

There are many diseases associated with reactivation and recurrence. Most of these diseases are infectious. These diseases become more fatal during recurrence after successful completion of initial therapy.

1.2 Tuberculosis statistics:

Every year, 10.6 million people are infected with TB. 1.6 million people died from TB in the year 2021. 8 countries contribute half of the TB cases in the world. India is in the top position in contributing TB followed by Indonesia, Pakistan, Nigeria, China, Bangladesh, Philippines, and Democratic Republic of the Congo (Varshney K *et al.*, 2023, Global TB report 2022). India contributes 27% of total TB cases in the world (Figure 1). India has the highest burden of TB with 2, 135,670 TB cases in the year 2021 and two deaths occurring every three minutes from TB. In India TB report, 2022, Central TB Division, reported that there is 19% increase in TB cases than previous year (India TB report, 2022, Global TB report 2022). Multi-drug resistance (MDR) TB is a big problem that hinders the progress of tuberculosis eradication program. Globally, the estimated proportion of new TB cases with MDR/RR-TB was 3.6% in 2021 and for India, it was 2.5%. Most importantly 18% of MDR cases of the world and 13% MDR cases of India are from previously treated TB cases (Sharma N *et al.*, 2020, Shivekar SS *et al.*, 2020, Global TB report 2022).

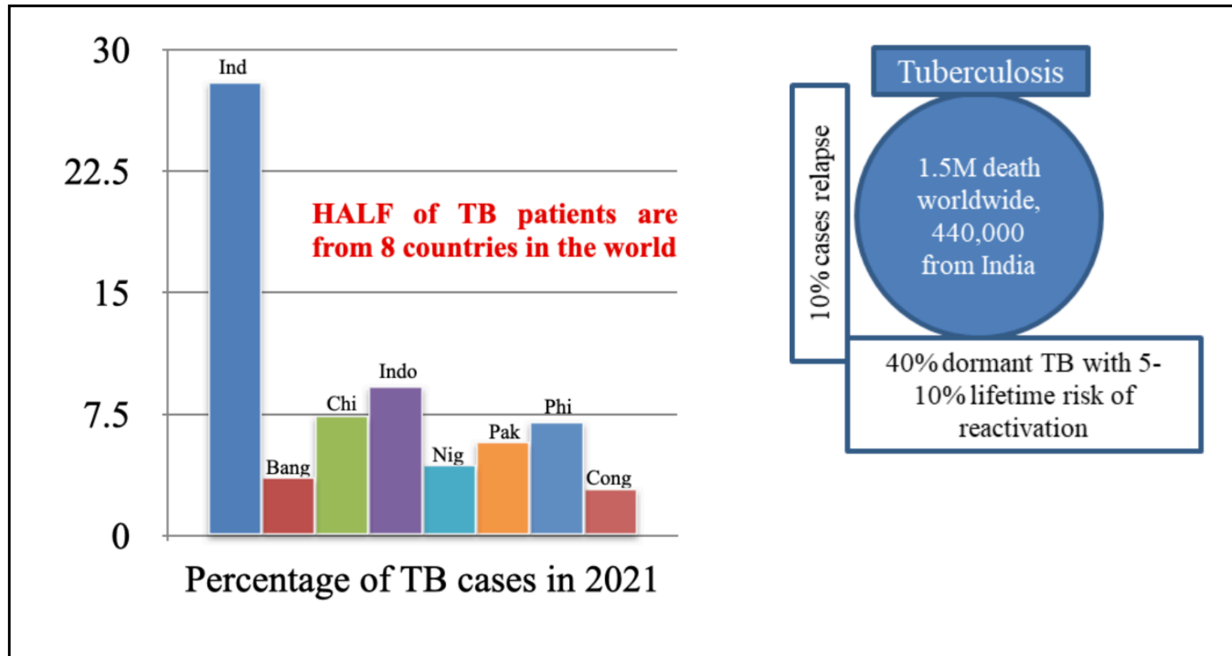

Figure 1: Representation of active and dormant TB incidences. *8 countries contribute most of the TB cases in the world. India contributes 27% of total TB cases worldwide. 33% of total population in the world and 40% of Indian population has dormant TB infection.*

Latent TB infection (LTBI) is another big problem that hinders the TB eradication program. 33% people worldwide have LTBI, whereas 40% Indian population has dormant TB (India TB report, 2022, Saha *et al.*, 2020). 10% of dormant TB subjects have the potential to reactivate the disease in their lifetime (Vishwakarma D *et al.*, 2023, Lin and Flynn, 2010). Some of the *Mtb* escape the anti TB drug remains dormant inside the host. Such successfully treated TB cases come back as recurrence under favourable condition. Recurrence rate is high (almost 10%) as per almost all the studies from India (Azhar GS, 2012), suggesting that *Mtb* remains dormant in the host escaping the therapy.

1.3 Pathogenesis of Tuberculosis

The causative agent; *Mtb* transmit from the infected host to the community via aerosol. First, *Mtb* enters, initiate primary infection in the lung of human host and generate active TB lesions including caseating granuloma formation. The most of the tubercle bacilli are ingested and destroyed by alveolar macrophages. The infected host develops robust lifelong immunity against primary TB due to vigorous cell-mediated immune response which leads to eventual calcification of the granuloma (Medlar EM, 1948, 1955; Hunter, 2018). This T cell mediated macrophage activation and killing *Mtb* occurs approximately 3 weeks after exposure. In this stage, the person has no symptoms and thus, the pathogen is not contagious. Slowly, pulmonary tissues are filled with heterogenous types of sterile, non-progressive, but highly organised granulomas that include active cavities (Hunter, 2018; Pathak L and Das B, 2021). In these highly organised granulomas, the dormant bacilli remain in a standoff with the immune cells. Due to immune system weakness, the bacilli disseminate from the granuloma and cause reactivation of the disease (Bucşan AN *et al.*, 2019, Behr MA *et al.*, 2018). The active granulomas expand into nearby bronchioles, and then enter into the sputum and the infected person spreads *Mtb* via aerosol into the community by vigorous coughing. This active TB disease is characterised by symptoms of persistent cough, fever, chest pain, weight loss, night sweat, loss of appetite, fatigue etc. TB infections mainly affect the lungs, called pulmonary TB (PTB). When the TB infection occurs in extra pulmonary sites such as bones, abdomens and lymph nodes, it is known as extra pulmonary TB (EPTB) (Fang *et al.*, 2022). In the context of TB transmission, PTB subjects are considered as potent transmitter as 80% of total TB cases in India are PTB as per central TB division, India. Several other studies from

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different region of world also suggest the higher prevalence of PTB than EPTB (Rolo *et al.*, 2023, Pang *et al.*, 2019). Currently, the active TB diseases are diagnosed by sputum smear microscopy, molecular diagnostic and other bacteriological evaluation by culturing the bacilli in Löwenstein–Jensen medium and 7H9 medium (Lewinsohn DM *et al.*, 2017, Pai M *et al.*, 2023). The TB treatment strategies (Figure 2) are based on whether the TB subjects are MDR, drug sensitive or extensively-drug resistant (XDR) (Decroo T *et al.*, 2020).

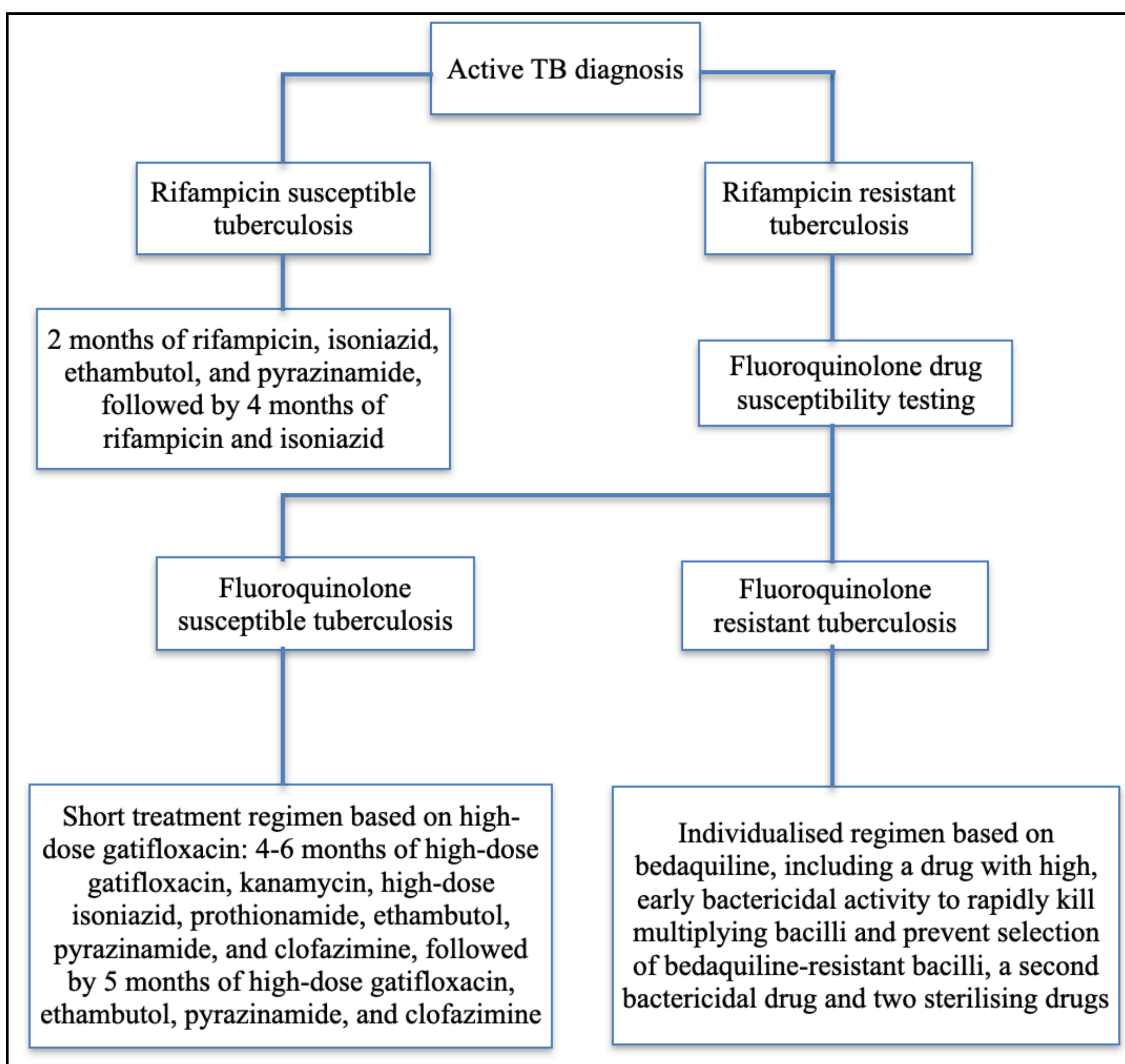

Figure 2: The different treatment strategies for Tuberculosis patients. *The active TB patients are treated based on their diagnosis on rifampicin status. Rifampicin susceptible TB patients are treated with first line anti TB drugs and rifampicin resistant TB patients are treated with second line anti TB drugs.*

Isoniazid and Rifampicin are the key drugs to treat TB disease. When the *Mtb* pathogen becomes resistant to these 2 drugs, it is called MDR TB. MDR mutation is occurred in RNA polymerase B (rpoB) region of the *Mtb* strain (Yue J *et al.*, 2003, Ma X *et al.*, 2006) and in some cases to compensate the low fitness of MDR, mutation occurs also in rpoC region (Li QJ *et al.*, 2016, Comas *et al.*, 2011). RIF-resistance is caused by mutations generally located in the short 81-bps region between codons 507 to 533 of the *rpoB* gene, also known as the rifampicin resistance determining region (RRDR) (Yue J *et al.*, 2003, Ma X *et al.*, 2006). The most common rpoB mutation is in 516, 526, 531 (Yue J *et al.*, 2003) and rpoC mutation is in 483, 491 (Li QJ *et al.*, 2016). The increased MDR TB cases indicate that current TB diagnostic strategies should also include rpoC mutation detection approach to identify potential TB subjects for future MDR.

1.4 Tuberculosis dormancy and reactivation

Mycobacterium tuberculosis, the causative organism of TB maintains disease transmission by remaining dormant inside the infected host followed by reactivation and then transmit via aerosol to the community (Pathak and Das, 2020). Primary *Mtb* infection generates active TB lesions in the lung by forming "caseating granuloma" (Medlar EM, 1948, 1955; Hunter, 2018). After primary infection, the infected immunocompetent adult develops life-long immunity against primary TB that leads calcification of the granuloma (North and Jung, 2004; Gengenbacher and Kaufmann, 2012). Then, active TB lesions reappear in the apical part of the lungs after 10–30 years of dormancy, which is termed as post-primary tuberculosis of the lungs (PPTBL) (Figure 3) (Cheeseman EA, 1952; Dubos R, 1987; Korzeniewska-Kosela *et al.*, 1994). Strikingly, one quarter of the world population is already infected with dormant tuberculosis (d*Mtb*). According to recent statistics, 40% Indian population has dormant TB (Saha *et al.*, 2020), 10% out of this population has lifetime risk of reactivation of the disease (Lin and Flynn, 2010). Also, in 10% successfully treated TB patients, the disease recur (Lin and Flynn, 2010), which indicates the drug resistance potential of *Mtb*. Epidemiological studies suggest that 90–95% of new *Mtb* infections may become dormant (Gideon and Flynn, 2011). Moreover, escaping from the anti-TB drugs due to treatment failure is not only causing persistent *Mtb* infection, but also contributing to the evolution of MDR *Mtb*. The MDR strain may spread around the world to cause many new active TB cases in future (Lillebaek *et al.*, 2002; Peddireddy, Doddam and

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Ahmed, 2017). Therefore, it is necessary to identify the dormant TB subjects with reactivation potential to prevent the future spread of active TB.

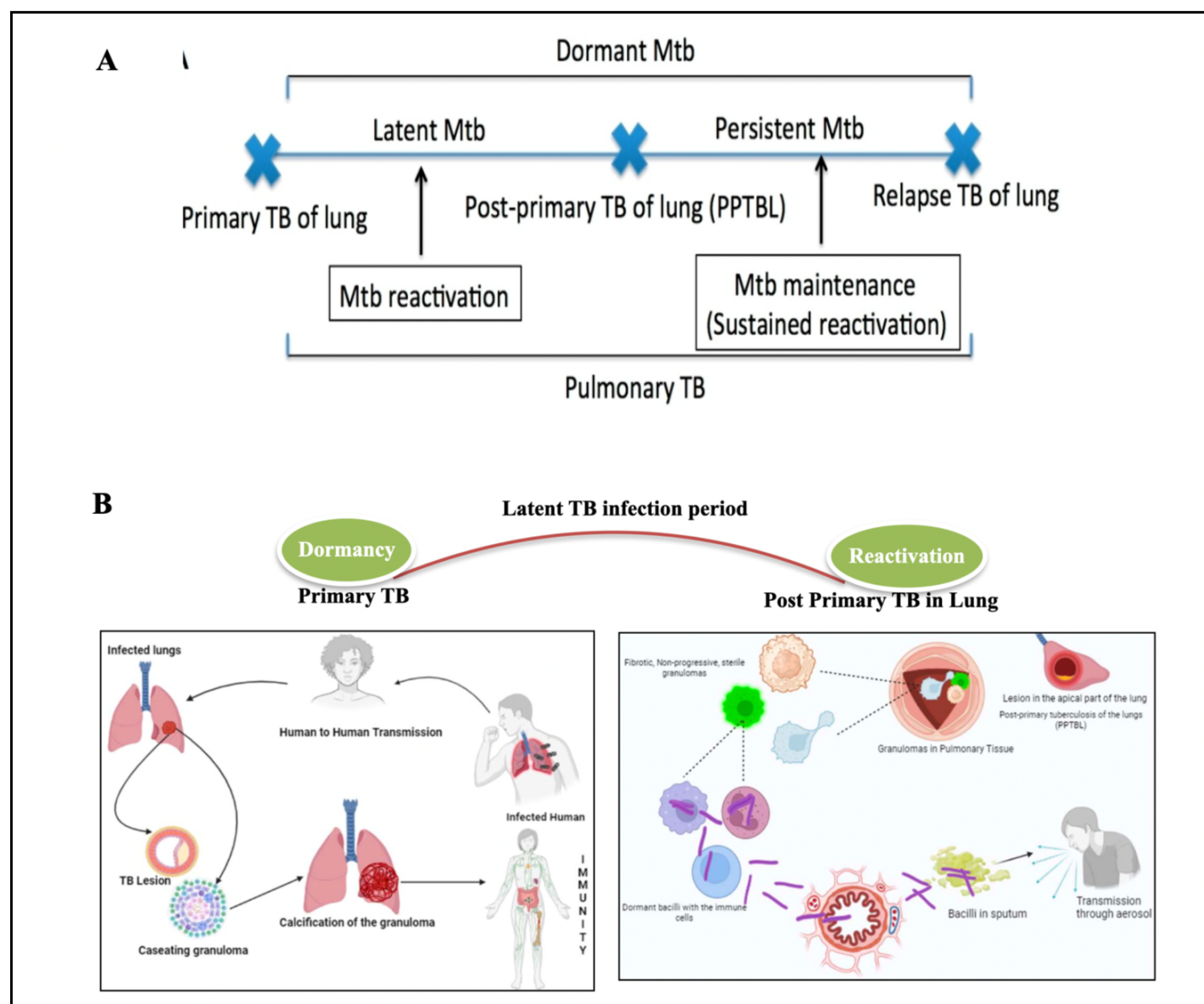

***Figure 3**: **The transmission cycle of pulmonary TB in the community.** A. The schematic is showing different clinical terminologies associated with TB dormancy to reactivation and persistent TB to TB recurrence or relapse. B. Figure is showing how Mtb cause primary infection and then cause active PPTBL via latent TB infection period.*

The source of endogenous dormant *Mtb* in an adult and how does it reactivate the disease is not yet clearly known. *Mtb* re-programs macrophages to remain in a relatively non-replicative dormant state (Eisner *et al.*, 2010; Zimmermann *et al.*, 2017). However, the viability of intracellular *Mtb* is poor (Biketov *et al.*, 2000). Also, so far, there is no evidence of direct isolation of viable *Mtb* from macrophages of LTBI subject (Gomez and McKinney, 2004; Das B

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et al., 2013). Importantly, dormant *Mtb* are highly immunogenic intracellular to macrophages as the bacteria fail to arrest phagosome maturation. Next, most *Mtb* are processed for efficient antigen presentation to stimulate adaptive immunity (Mariotti *et al.*, 2013). Thus, the pathogen in LTBI subjects will be killed by macrophage due to specific T cells mediated interferon gamma response with vigorous T cell mediated immunity.

Table 2**: **Proposed endogenous sites for dormant Mtb in latent TB subjects

SL no	Host cell type	Recovery of Mtb-DNA	Recovery of viable Mtb	Reference
1	Host cell free, sterile granuloma	Yes	No	Medlar EM, 1948, Balasubramanian V, 1994, Loomis HP 1830, Bloom BR 1999
2	Alveolar Macrophages	Yes	No	Gomez JE,2004, Hernandez-Pando R et al 2000, Biketov S et al 2000
3	Alveolar epithelial cells	Yes	No	Hernandez-Pando R et al 2000
4	Host cell free adipose tissue	Yes	No	Hunter RL, 2007, Balasubramanian V, 1994, Neyrolles O 2006
5	Adipocytes	Yes	No	Hunter RL, 2007, Balasubramanian V, 1994, Neyrolles O 2006
6	Macrophages	Yes	No	Balasubramanian V, 1994, Neyrolles O 2006, Gomez JE,2004
7	Fibroblast	Yes	No	Balasubramanian V, 1994, Neyrolles O 2006, Gomez JE,2004
8	CD271+ BM-MSCs	Yes	Yes	Das B et al 2013, Garhyan J et al 2015, Beamer et al 2014
9	CD34+ HSCs	Yes	Yes	Das B et al 2013, Charrier S et al 2002

The data from the table suggests that the viable Mtb could be recovered only from CD271+BM-MSCs and CD34+HSCs of the latent TB subjects.

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Therefore, it is highly unlikely that latent *Mtb* can persist intracellular to macrophages (Flynn *et al.*, 1993). Other potential sites for d*Mtb* were also proposed, however viable *Mtb* was not recovered from those sites (Table 2). For PPTBL development, the *Mtb* need to be remained in dormant state intracellular to an immunosuppressed host cell type (Balasubramanian *et al.*, 1994). Such strategic approach may lead the *Mtb* pathogen to migrate to the apical part of the lungs during inflammation to initiate PPTBL (Pathak and Das, 2020). Therefore, this stem cell based hypothesis of TB reactivation need to be investigated to confirm the mechanism of TB reactivation and develop strategy to prevent TB reactivation.

1.5 Role of Bone marrow (BM) stem cell in TB dormancy and reactivation

The BM stem cell niche is immunopriviledged (Fujisaki *et al.*, 2011; Tormin *et al.*, 2011). In the BM niche, hematopoietic stem cell (HSC), mesenchymal stem cell (MSC) and endothelial stem cells reside in their quiescent state (Aria F, 2008) with potential to self-renew (Aria F, 2008; Jones and Wagers, 2008). These BM-stem cells expand in the hypoxia/oxidative stress microenvironment (Danet *et al.*, 2003; Grayson *et al.*, 2007) and during infection, migrate to the area of inflammation (Crosby and Waters, 2010; Spaeth, Kidd and Marini, 2012). BM-stem cells also exhibit age-specific mobilization to specific tissues (Whetton and Graham, 1999; Mozid *et al.*, 2013). Stem cell's self-renewal property was shown to be utilized by other bacteria such as *Mycobacterium leprae* (*ML*) (Masaki *et al.*, 2013) and *Wolbachia* (Frydman *et al.*, 2006) for effective transmission. Intracellular *ML* reprograms the infected Schwann cells into stem cell like cells by up-regulating the epithelial mesenchymal transition (EMT) markers such as Twist-1, Snail2 and stem cell marker Sox2 (Masaki *et al.*, 2013). The reprogrammed stem cell like Schwann cells were found to migrate and efficiently transfer the intracellular *ML* to other tissue cells (Masaki *et al.*, 2013). *Wolbachia* were also found abundantly in somatic stem cell niche of long term infected host and migrate to the germline through the somatic stem cell niche for effective transmission (Frydman *et al.*, 2006). Thus, it can be presumed that BM stem cells' migratory and self-renewal abilities could be utilized by dormant *Mtb* for initiation and transmission of active TB (Pathak and Das, 2021). Dr. Das and his team identified human BM stem cell niche as protective site for *Mtb* dormancy (Das B *et al.*, 2013). The viable *Mtb* were recovered from CD271+BM-MSCs of PTB subjects successfully treated with anti-TB drugs (Das *et al.*, 2013). Tornack et al. reproduced this finding in LTBI subjects. They recovered *Mtb*-DNA

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from CD271+BM-MSCs of LTBI subjects (Tornack *et al.*, 2017). Studies reported CD271+BM-MSCs' migration from BM niche into the circulation (Whetton and Graham, 1999; Spaeth, Kidd and Marini, 2012) during tissue damage and inflammation (Iso *et al.*, 2012; Gójska-Grymajło *et al.*, 2018). Moreover, CD271+ BM-MSCs are significantly more mobilised in adults versus children (Iso *et al.*, 2012). Interestingly, this is the age group of PPTBL development. Importantly, the *Mtb*-DNA harboring CD271+BM-MSCs of post-PPTBL subjects were shown to exhibit high expression of hypoxia inducible factor 1 alpha (HIF1α) and low expression of CD146 (Garhyan J *et al.*, 2015). This result indicates that these *Mtb* infected BM-MSCs may reside in the hypoxic niche of BM and thus, the *Mtb* remain unreachable by anti-TB therapy (Beamer *et al.*, 2014; Garhyan *et al.*, 2015; Wang *et al.*, 2020). Importantly, hypoxia is known to induce dormancy in *Mtb* (Wayne and Sohaskey, 2001; Kumar *et al.*, 2007; Rustad *et al.*, 2009). Researchers found that *Mtb* intracellular to BM-stem cells of LTBI subjects express hypoxia induced dormancy genes *DosR*, *hspX* and *c-lat* (Tornack *et al.*, 2017).

In a streptomycin dependent mouse model of *Mtb* dormancy, our lab has recovered dormant *Mtb* intracellular to CD271+BM-MSCs even after 6 months of primary TB infection. The invivo transplantation assay confirmed the re-infection potential of these recovered d*Mtb* intracellular to CD271+BM-MSCs (Das B *et al.*, 2013) (Figure 4). Other researchers found the expression of dormancy related genes in the *Mtb* obtained from CD45-Sca1+BM-MSCs recovered from *Mtb* infected mice (Fatima *et al.*, 2020). In a cornel model of dormancy, d*Mtb* was recovered from ABCG2 expressing CD271+BM-MSCs despite prolonged anti-TB drug therapy (Das B *et al.*, 2013; Beamer *et al.*, 2014; Garhyan J *et al.*, 2015). ABCG2 is a drug efflux pump and thus, it can be presumed that the MSCs expressing drug efflux pump (Suzuki *et al.*, 2003; Haimeur *et al.*, 2004) may help intracellular *Mtb* to escape anti TB drug.

HSCs reside in the BM niche in their quiescent state with self renewing potential (Suzuki *et al.*, 2003; Haimeur *et al.*, 2004; Aria F, 2008; Fujisaki *et al.*, 2011). Our lab recovered d*Mtb* from CD34+HSCs of some of the previously treated PPTBL subjects (Das B *et al.*, 2013). This finding was confirmed by Tornack et al. who recovered dormant *Mtb* in HSCs of LTBI human peripheral blood (Tornack *et al.*, 2017). These human CD34+HSCs and mouse CD150+HSCs having non-replicating d*Mtb* were administered intratracheally to recipient immune-deficient mice. Indeed, the mice formed active TB lesions in the lungs (Tornack J *et al.*, 2017). These findings indicate the potential of reinfection capability of dormant *Mtb*.

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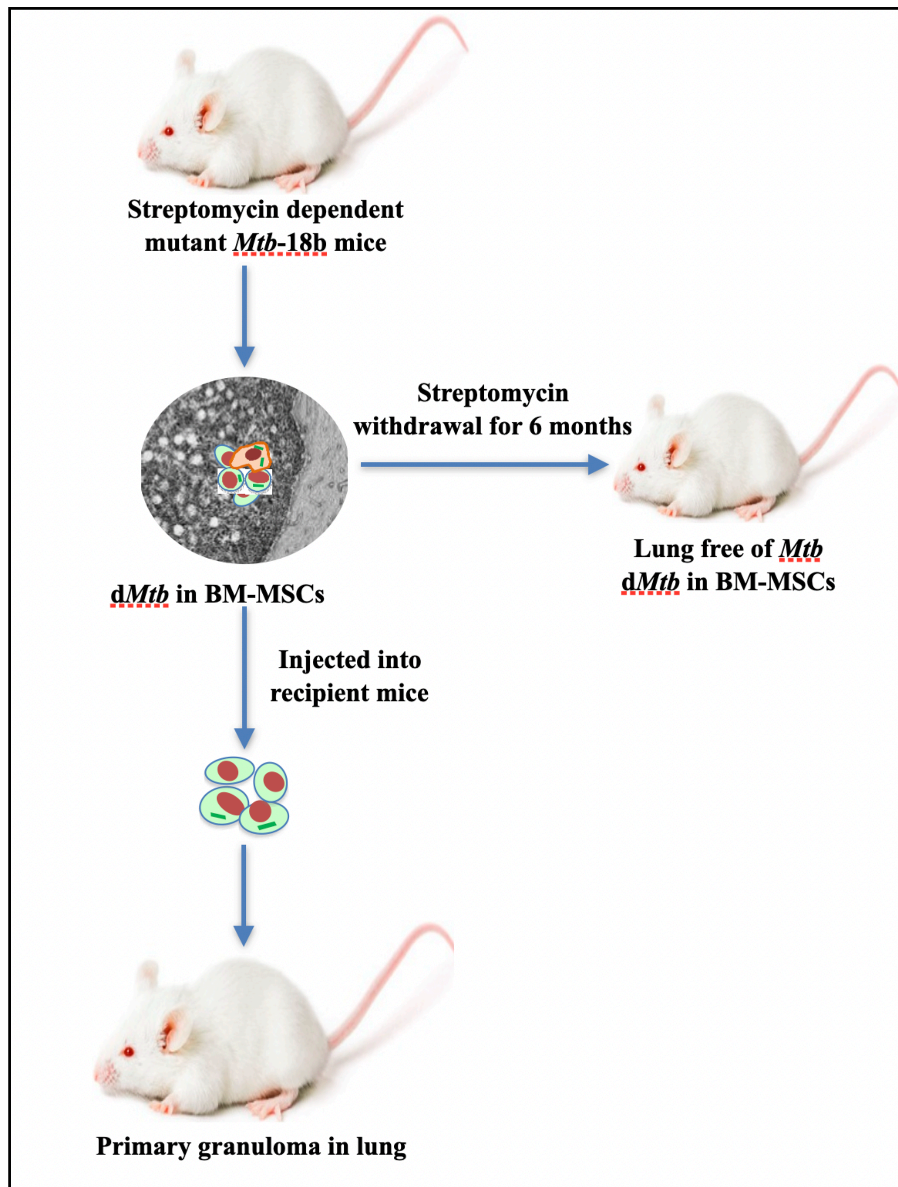

Figure 4: A mouse model of stem cell mediated TB dormancy. *In a streptomycin dependent m18b Mtb model, dormant Mtb was found intracellular to CD271+BM-MSCs even after 6 months of primary TB infection. These Mtb intracellular to CD271+BM-MSCs formed granuloma when injected to recipient mice.*

1.6 Stem Cell Niche defense mechanism and its role in TB dormancy and reactivation

The stemness state of BM- stem cells is characterized by the undifferentiated and self-renewal potential, which may be necessary for *Mtb* dormancy. It was reported that the CD271+BM-MSCs differentiate to mesenchymal stromal cells and lose the expression of stemness markers; CD271, CD133, ABCG2 and HIF-2α when grown in vitro in high serum

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media or with adipogenic agents (Das B *et al.*, 2013; Pal and Das, 2017; Tornack J *et al.*, 2017). It also significantly reduces the viability of intracellular d*Mtb* (Das B *et al.*, 2013). These findings confirm that the maintenance of the stemness state of stem cells is necessary for *Mtb* dormancy. Studies showed *Mtb* infected adipose tissue derived MSCs activate the autocrine pathway of PGE2 (Jain *et al.*, 2020). This pathway was also reported to be involved in the stemness of MSCs by enhancing the niche independent stemness of immunosuppressive MSCs (Pal and Das, 2017).

BM stem cell may modulate the stem cell niche to defend against pathogens (Pal and Das, 2017). HSCs and endothelial progenitor cells were reported to modulate BM niche to prevent pathogen infection (Nombela-Arrieta and Isringhausen, 2016). Our previous findings show that only a small fraction of BM-MSCs harbor the *Mtb* after intravenous injection of *Mtb* to mice (Das B *et al.*, 2013) which suggests that the stem cells may resist pathogen's invasion. In vitro studies found that autophagy and phagocytosis mechanism of MSCs can kill intracellular *Mtb* (Khan *et al.*, 2017). *Mtb* infected MSCs secrete nitric oxide (Khan *et al.*, 2017) that also kills the intracellular *Mtb* (Bogdan, 2015). Another study showed that rapamycin has the potential to reduce the dormant *Mtb* load inside MSCs by inducing autophagy (Fatima *et al.*, 2020). These findings indicate a potential stem cell mediated defense against *Mtb* invasion.

However, the findings of d*Mtb* inside BM-MSCs for prolonged period suggest that *Mtb* may exploit the stem cells' niche defense mechanism to remain dormant inside BM-MSCs escaping the anti-TB drug therapy. In this context, the present lab previously reported a stem cell niche defense mechanism in embryonic stem cells (ESCs) (Das B *et al.*, 2012), where during oxidative stress, a sub-population of ESCs reprogram to "enhanced stemness" phenotype by activating a HIF-2α stemness pathway. This "enhanced stemness" phenotype is p53 deficient. The low p53 state permits these cells to gain fitness and thus maintain a state of self-renewal and self-sufficiency in stress microenvironment (Das B *et al.*, 2012) (Figure 5A). However, instead of becoming the dominant subpopulation, these cells sacrifice their self-fitness to enhance the fitness of neighboring cells under stress by secreting glutathione. The culture supernatant containing the antioxidant glutathione of these reprogramed ES cells defended HSCs and MSCs from oxidative stress induced differentiation/apoptosis. Thus, the reprogrammed cells are termed as altruistic stem cell (ASCs) (Das B 2014, Pal B and Das B 2017, Pathak L and Das B 2021). This idea of stem cell altruism is inspired by the Indian Vedic altruism theory of Jiva Upakarvada

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(JUV) (Pathak L *et al.,* 2021, Mitra S *et al.,* 2023). JUV has extended the metaphysical concept of Avatar Kosha to Prakriti mata or mother Earth; Prithvi according to which biosphere may also have their own altruistic defense mechanism of Avatar Kosha during environmental stress. Inspired by this theory, it was proposed that like ordinary humans undergo immense transformation of an Avatar to protect the earth from atrocities, a few cells in the animal body may also transform into avatar or altruistic cells to protect the body from infectious pathogens as well as cancer cells (Das KR, 1992, Das B 2000, Das B, 2019). This philosophical insight led to the development of the idea of stem cell niche defense (Figure 14, page no 52, Das B 2000). Eventually, the ASC reprogramming was demonstrated in ESCs (Das B *et al*., 2012) and MSCs (Pathak L *et al*., 2021). The ASCs exhibit niche modulatory or altruistic stemness in the microenvironment of hypoxia/oxidative stress, serve as ASC niche defense mechanism (Figure 5B) (Pal and Das, 2017). It can be speculated that *Mtb* pathogen may exploit this stem cell niche defense mechanism (Pathak and Das, 2021) to enhance their fitness in the stress microenvironment of lung for TB reactivation. The d*Mtb* harboring BM-MSCs may migrate to lung following tissue injury or inflammation (Figure 1B, Pathak L and Das B, 2021). However, how the d*Mtb* reprograms the BM-MSC in lung niche for reactivation of the disease needs to be investigated.

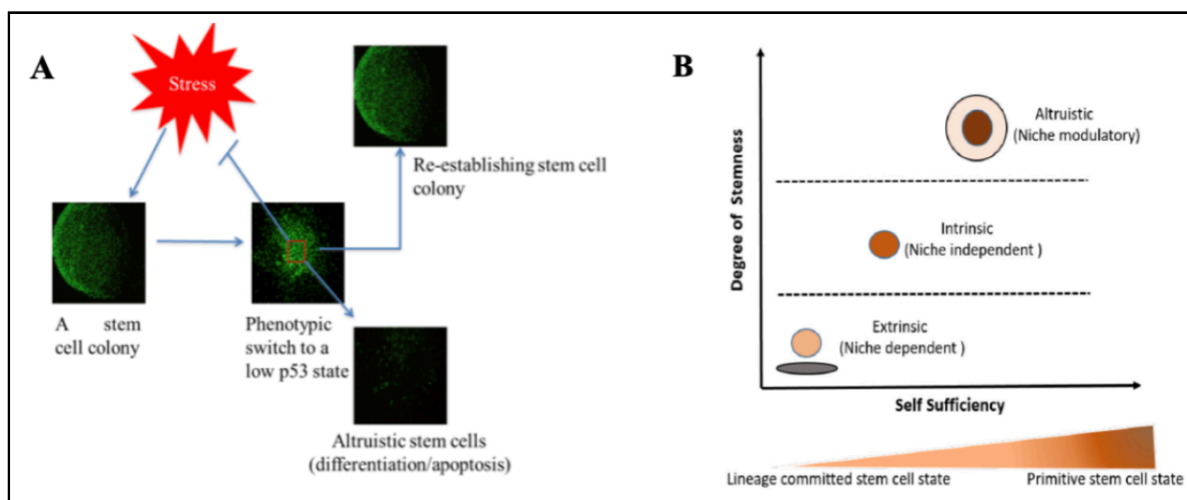

Figure 5: The characterisation of Altruistic stem cell (ASC) based stem cell niche defense mechanism. *A. The ASC reprogramming in embryonic stem cell population during stress (Das B et al 2012). B. Degree of stemness in adult stem cell niche, which could be exploited by Mtb for dormancy and reactivation.*

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1.7 Cancer statistics with special emphasis on Head and Neck Squamous Cell Carcinoma (HNSCC)

Cancer is a leading cause of death worldwide with a record of 10 million deaths, one in six deaths in 2020. Globally, there were an estimated 20 million new cases of cancer in 2020. The cancer burden will increase by approximately 60% over the next two decades (Chauhan R et al, 2022, Leemans CR *et al.*, 2011). In almost all cancer types, 30-50% cases show recurrence (Baker, 2016; Nishio *et al.*, 2017; Li, Young and Medeiros, 2018). HNSCC is the sixth most frequent cancer worldwide, accounting for 890,000 new cases and 450,000 deaths in 2018. Asia has 57.5% of global HNSCC cancer, where India contributes 30% (Leemans CR *et al.*, 2011). HNSCC incidence continues to rise and is expected to climb by 30% (1.08 million new cases per year) by 2030. India has the highest oral cavity cancer patients in the world with 1,19,992 new cases and 72,616 deaths due to oral cancers in 2018. 35% cancers in India are oral cancer (Chauhan R *et al.*, 2022, Leemans CR *et al.*, 2011) (Figure 6).

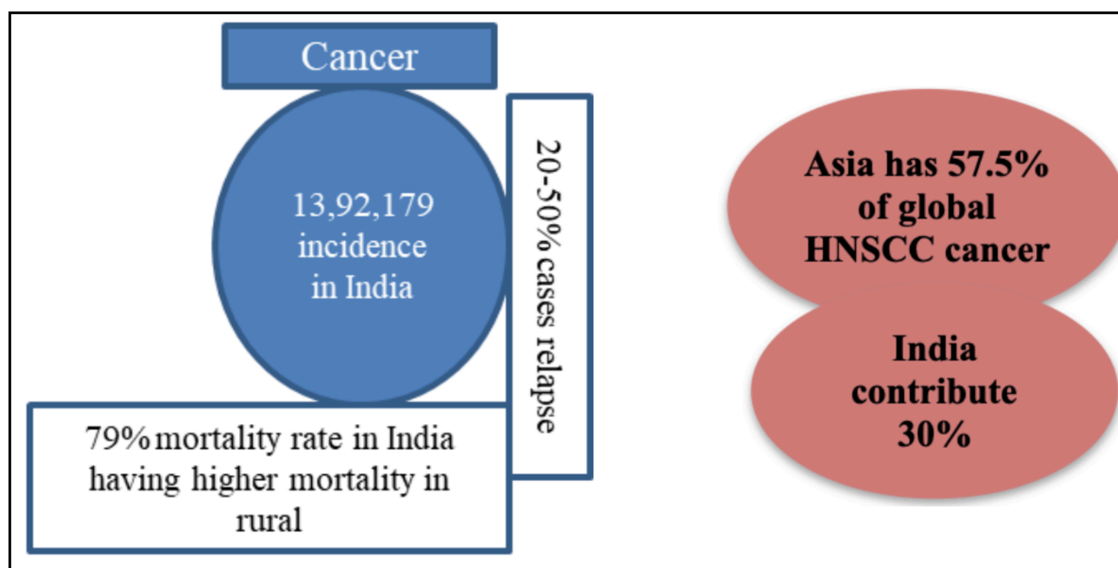

Figure 6: Worldwide cancer incidences with India's highest contribution in HNSCC incidences. *Cancer is one of the leading cause of death in India as well as in the world. India is the highest contributor in HNSCC development in the world.*

Most of the HNSCC cancer cases are diagnosed at stage III or stage IV, and 50% of these

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cases develop locoregional or distant recurrence despite chemo-radiation. Also, approximately

35% of patients need changes in treatment plan due to cisplatin induced toxicity (de Roest RH *et al.*, 2022). The overall survival period of more than 50% HNSCC cases is less than 3 years. Poor survival and distant recurrence indicate the therapy failure and lack of proper treatment management of advanced HNSCC cases. Therefore, the CSC niche defense may provide a new insight to the therapy failure and recurrence of HNSCCs.

1.8 Head and Neck Squamous Cell Carcinoma (HNSCC) progression and recurrence

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. Cancer development is characterised by 6 hallmarks as proposed by Hanahan and Weinberg; sustaining proliferative signalling, resistance to cell death, evading growth suppressor, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2011). HNSCCs are the most ubiquitous malignancies of the head and neck. The high prevalence of HNSCC in Southeast Asia and Australia is linked to consumption of specific carcinogen-containing products (Johnson DE *et al.*, 2020). Tobacco use, betel quid, and areca nut intake, as well as exposure to environmental contaminants and excessive alcohol consumption, are all risk factors for the development of HPV-negative HNSCC. Carcinogens undergo metabolic activation, which leads in the creation of reactive metabolites and DNA damage by mutations (Johnson DE *et al*, 2020). Changes in critical tumour suppressor genes or signalling pathways are linked to the initiation, progression, and poor prognosis of HPV-negative HNSCC (Johnson DE *et al*, 2020, Saikia PJ *et al*, 2023). HNSCC originates in the mucosal epithelium lining the oral cavity, pharynx, larynx, and sinonasal tract. HNSCC follows an ordered series of steps of epithelial cell hyperplasia, dysplasia (mild, moderate and severe), carcinoma in situ and, ultimately, invasive carcinoma (Solomon B *et al*, 2018). Normal adult stem or progenitor cells are potential candidates for the cell of origin, giving rise to HNSCC cancer stem cells (CSCs) with self-renewal and pluripotency after oncogenic transformation (Johnson DE *et al*, 2020). The PI3K-AKT-mTOR pathway is the most often altered oncogenic pathway in HNSCC among signalling pathways that commonly drive cancer formation (Psyrri A *et al*, 2013). In HNSCC, STAT3 signalling is hyperactivated, which correlates with a poor prognosis. STAT3 signalling promotes the expression of genes that promote cellular proliferation and survival, as well as genes that encode growth factors and immunosuppressive cytokines (such as VEGF, IL-6, IL-10, and TGF) (Xu Q

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et al., 2020). The RAS-MAPK pathway, which contributes to the growth and survival of HNSCC cancer cells, is infrequently altered in HNSCC tumours (Ngan HL *et al.*, 2020). Mutations in the genes that code for the protein tyrosine phosphatase receptors (PTPRs), PTPRT and PTPRD are common in HNSCC (Du Y *et al*, 2015). The up regulation of mesenchymal epithelial transition (MET) is associated with HNSCC progression by stimulating invasiveness, motility and proliferation. MET establishes crosstalk with epidermal growth factor receptor (EGFR) which further contribute in chemoresistance of HNSCC (Raj S *et al.*, 2022).

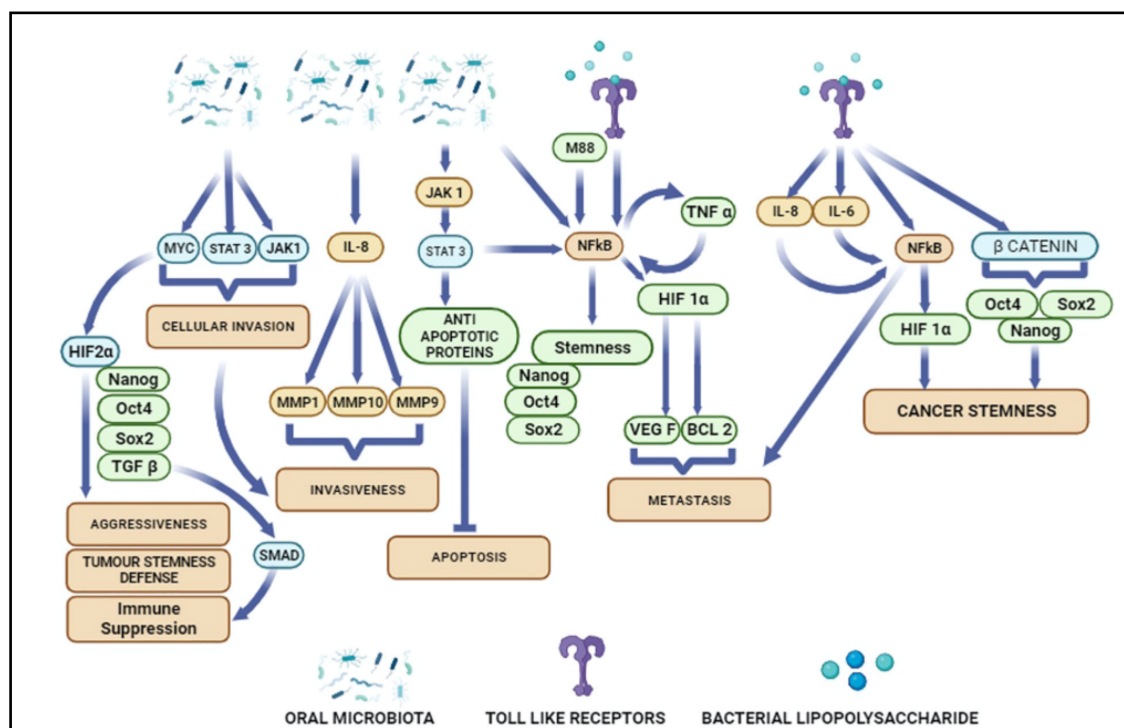

Figure 7: Different pathways involved in the HNSCC development and progression. *These stemness pathways are also involved in HNSCC recurrence. The pathways including NF-kB, and β-catenin can be induced by different factors including oral microbiota and endotoxin such as lipopolysaccharides by activating inflammatory receptors such as TLRs.*

Toll-like receptors (TLRs) and nuclear factor-kB (NF-kB) are two key inflammatory mediators that contribute in HNSCC initiation, progression and recurrence (Yeh D-W *et al.*, 2016; Han S *et al.*, 2026). Activation of TLRs stimulate the HIF1-α expression via activating the NF-kB signalling in the tumor microenvironment (TME) of oral cancer (Figure 7) and thus maintain the inflammatory stemness state (Yeh D-W *et al.*, 2016). The NF-kB (Salgado R, 2021)

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and β-catenin (Reya and Clevers, 2005) pathways were found to induce the HNSCC CSC stemness pathway (Figure 7). It leads to the expression of the stemness genes required for the maintenance of the CSCs and aids in malignancy progression (Han S *et al.*, 2016).

1.9 The signalling pathways involved in cancer dormancy and recurrence

Cancer dormancy is a complex step in the development of both primary tumor and cancer recurrence (Aguirre-Ghiso, 2007; Endo and Inoue, 2019). Cancer dormancy can be classified into angiogenic dormancy, immunomediated dormancy and cellular dormancy (Aguirre-Ghiso, 2007). Reduced blood vessel density in dormant tumor (Holmgren, O'Reilly and Folkman, 1995) suggests that the failure to induce an angiogenic response may be responsible for dormant state of cancer (Gimbrone *et al.*, 1972). Therefore angiogenic switch triggered by hypoxia or inflammatory response (Carmeliet and Jain, 2000) and production of angiogenic factors by heterogenous tumor cells (Relf *et al.*, 1997) may lead to escape the tumor from dormancy and cause recurrence, and or metastasis. Studies reported that even a short term angiogenesis induced by angiogenic factors can transform dormant tumor into progressive tumor (Indraccolo *et al.*, 2006).

The immunomediated dormancy can be referred as the maintenance of equilibrium state of proliferating tumor cells by immune system (Koebel *et al.*, 2007; Teng *et al.*, 2008). Researchers showed involvement of adaptive immune components, including CD4/CD8 T cells, interferon (IFN)-γ and interleukin (IL)-12 in maintaining tumor dormancy in a mouse model of sarcoma (Koebel *et al.*, 2007). Several other studies also showed significant role of CD8+T cells in maintaining the equilibrium state between cancer cell and immune cells in a dormant tumor (Lengagne *et al.*, 2008; Eyles *et al.*, 2010). There are different other immune factors involved in cancer dormancy such as several cytokines, Treg cells, myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells and MHC class I surface protein (Wang *et al.*, 2019). Immunosuppressive cytokines and activation of immune checkpoints lead the tumor to escape dormancy and cause tumor progression (Baxevanis and Perez, 2015).

The cell autonomous mediated or cellular dormancy is mediated by intracellular signalling pathways and cell cycle machineries. Cellular dormancy can be referred as the quiescence state of the tumor cells (Teng *et al.*, 2008), whereby cancer cells enter reversible G0 cell cycle arrest (Pelayo *et al.*, 2006). There are different pathways that maintain cellular

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dormancy in cancer cells such as p38 MAPK/ERK signalling pathway (Aguirre-Ghiso *et al.*, 2003; Yu-Lee *et al.*, 2018), TGF-β signalling (Yumoto *et al.*, 2016) and PI3k/AKT pathway (Jo *et al.*, 2008). Activated p38 MAPK pathway further activate p53 and p16 signalling as well as down-regulate cyclin D1, which could induce dormant disseminated tumor cells (DTC) to enter into growth arrest (Sosa *et al.*, 2011).

These slow cycling dormant tumor cells within the primary tumor can cause resistance to cancer drugs (Recasens and Munoz, 2019). For example, dormant glioblastoma cells were identified to cause resistance to temozolomide (Chen *et al.*, 2012) and dormant medulloblastoma cells cause resistance to cytarabine and vismodegib (Vanner *et al.*, 2014). Importantly, these DTC are mostly found in bone marrow (BM) (Sosa, Bragado and Aguirre-Ghiso, 2014) and in the BM TME, they interact with different components of TME including HSCs to establish pre-metastatic niche (Sosa, Bragado and Aguirre-Ghiso, 2014; Gao *et al.*, 2017). Bliss et al have shown that DTCs instruct MSCs in the BM-TME, to release exosomes with distinct miRNAs, such as miR222/223 and miR23b which lead cycling dormancy of certain DTCs (Bliss *et al.*, 2016), thus maintaining metastatic cancer dormancy. Metastatic cancer dormancy is the extensive period between the initial therapy and metastatic recurrence, in which patients remain clinically asymptomatic after successful treatment of the primary tumor (Wang *et al.*, 2019). Metastatic cancer dormancy leads to cancer recurrence and metastases, which are responsible for majority of cancer related deaths (Gao *et al.*, 2017). Therefore, DTC and its interaction in the TME need more attention to understand the mechanism of metastatic cancer dormancy to cancer recurrence. Studies showed that DTC in the BM-TME undergo an epithelial-to-mesenchymal transition (EMT) to obtain a stem cell-like phenotype, can be termed as CSCs. These CSCs are with increased property of migration and invasion (St John *et al.*, 2009; Kupferman *et al.*, 2010; Zuo *et al.*, 2011). These findings suggest that CSCs are unique and more potential factor for cancer recurrence and metastasis.

1.10 The cancer stem cells and their cross talk with the components of tumor microenvironment

Cancer stem cells (CSCs) are the rare and most aggressive cancer cells, endowed with self-renewal capacity and resistance to therapy (Kaplan *et al.*, 2005; Karnoub *et al.*, 2007). Initially, CSCs were identified in acute myeloid leukaemia in the year 1994 and later, CSCs were

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identified in different solid tumours including breast, brain, colon, pancreas, lung, prostate, head and neck, melanoma, and glioblastoma. CSCs reside in the specialised hypoxic niche in the TME (Das B *et al.*, 2008) in quiescent state and maintain a steady proportion across generations (Das *et al.*, 2019). CSCs exhibit drug efflux mechanism such as ABC transporter (Chaudhary and Roninson, 1991), which result chemotherapy resistance and increased DNA repair capacity and make CSCs resistant to radiotherapy (Bao *et al.*, 2006). Normal cells or normal stem cells undergo malignant transformation leading to the generation of CSCs. Various oncogenic pathways are involved in the malignant transformation process (Figure 8).

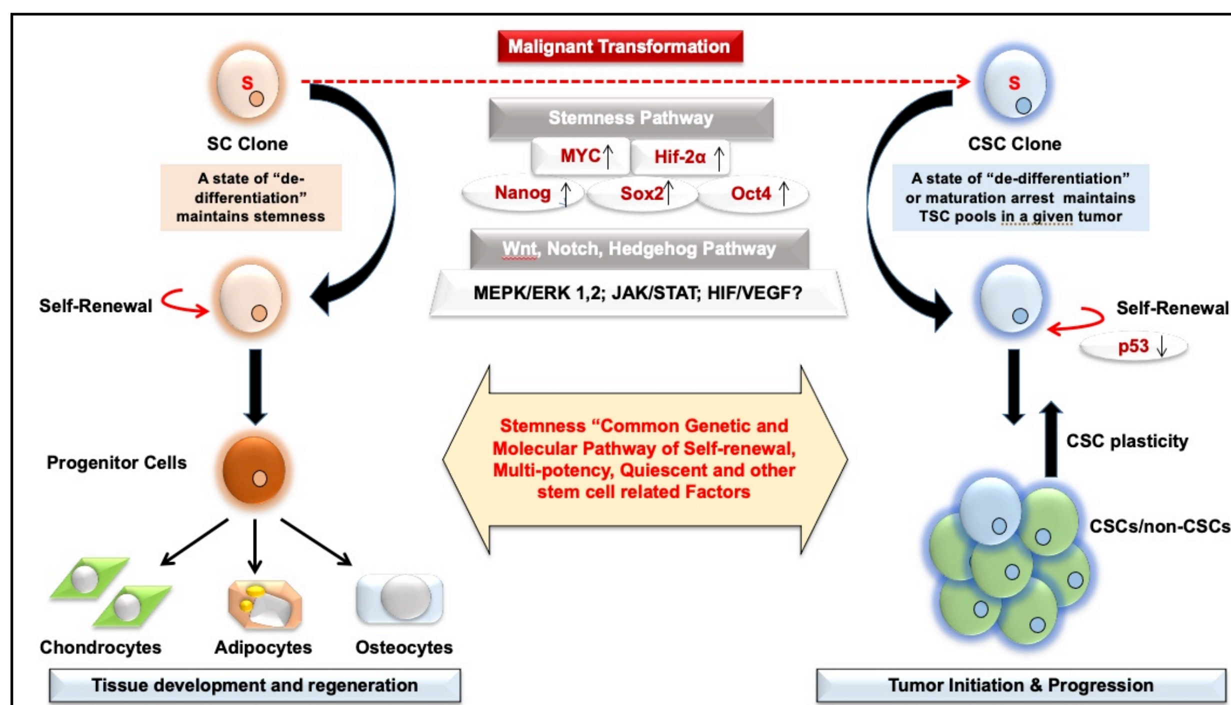

***Figure 8**: A schematic diagram showing the generation of CSCs from a normal stem cell clone.* *The stemness program involved in the generation of CSCs from a normal stem cell clone constitutes core transcriptional regulators, developmental pathways and growth factor signalling pathways. These pathways may also be involved in stem cell niche defense.*

The CSCs reside in the TME and exhibit plasticity i.e. stages of stemness, such as niche dependent stemness corresponding to the dormancy state, and the niche independent state corresponding to proliferating state. Different pathways including Notch (Fan *et al.*, 2006), Wnt/ β-catenin (Reya and Clevers, 2005) and Sonic Hedgehog pathways (Yauch *et al.*, 2008) that

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regulate self-renewal of normal stem cells were shown to regulate CSC as well. Researchers have been extensively studying to target these pathways to target CSCs. However, efficacy of this pathway targeting therapy is limited as yet. As these pathways are also important for regulation of normal stem cell, anti-CSC therapy targeting this pathway may be toxic for normal stem cell (O'Flaherty *et al.*, 2012). Therefore, there is growing interest among researchers to study the CSC niche so that these CSC can be targeted in their niches.

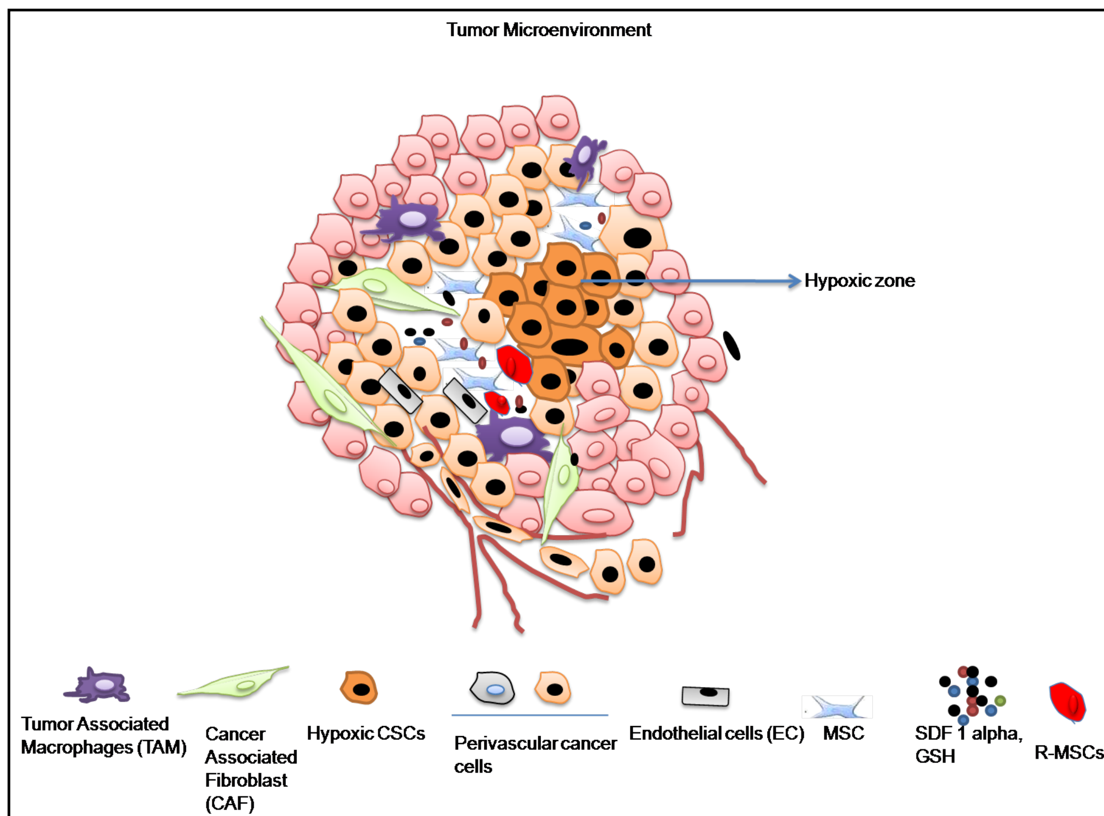

Figure 9: The tumor microenvironment with its components. *A tumor microenvironment (TME) constitutes off both cancer and non-cancer cells. The non-cancer cells are immune cells, Endothelial cells (ECs), Cancer associated fibroblasts (CAFs), Mesenchymal stem cells (MSCs). The TME also comprises of extracellular components like SDF1α, GSH secreted by ECs and CAFs. The figure shows the hypoxic zone of a tumor where cancer stem cells (CSCs) may localise. The interaction between CSC and MSC in the TME may reprogram MSCs to an altered state called reprogrammed MSC or R- MSCs.*

Table 3: The pathways and factors involved in MSC-CSC interaction for tumor growth.

Sl no	Factors/pathways involved	References
1	Inflammatory factor;IL-6	Rattigan *et al.*, 2010
2	Inflammatory factor; IL-1β	Tu *et al.*, 2008
3	Growth factor; transforming growth factor-β1 (TGF-β1)	Quante *et al.*, 2011
4	Growth factor; SDF-1α	Gao *et al.*, 2009
5	IL-6 and CXCL8	Wu *et al.*, 2016
6	CXCR2 ligand	Liu *et al.*, 2011
7	JAK2-STAT3 pathway	(Tsai *et al.*, 2011
8	Altered bone morphogenic proteins (BMP) production	McLean *et al.*, 2011

The study of CSC niche requires a detail understanding of TME. The TME comprises of cancer cells, CSCs, MSCs, endothelial cells (ECs), immune system cells, cancer-associated fibroblasts (CAFs), cytokines and growth factors (Hanahan and Coussens, 2012) (Figure 9). The CAF secrets various cytokines including Platelet-Derived Growth Factor, Vascular Endothelial Growth Factor (VEGF) and Hepatocyte Growth Factor which promote tumor progression (Polanska and Orimo, 2013). High-mobility group box 1 (HMGB1), a proinflammatory cytokine has been reported as a potent regulator of TME in different cancers (Lee MJ *et al*., 2023, Chen R *et al*., 2022, Hong B *et al*., 2018). Additionally, hypoxia is an important part of TME. Hypoxia activates two key transcription factors, the Hypoxia-inducible factor-1alpha, and Hypoxia-inducible factor -2alpha (HIF1α and HIF2α); these transcription factors induce self-renewal potential of CSCs and maintain the stemness of CSCs by inducing the expression of Oct-3/4,

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Nanog and Sox-2 (Mimeault and Batra, 2013; Philip *et al.*, 2013; Polanska and Orimo, 2013). Hypoxia induces the CSCs to survive in oxygen free environment, which promote tumor progression and metastasis (Hanahan and Weinberg, 2011).

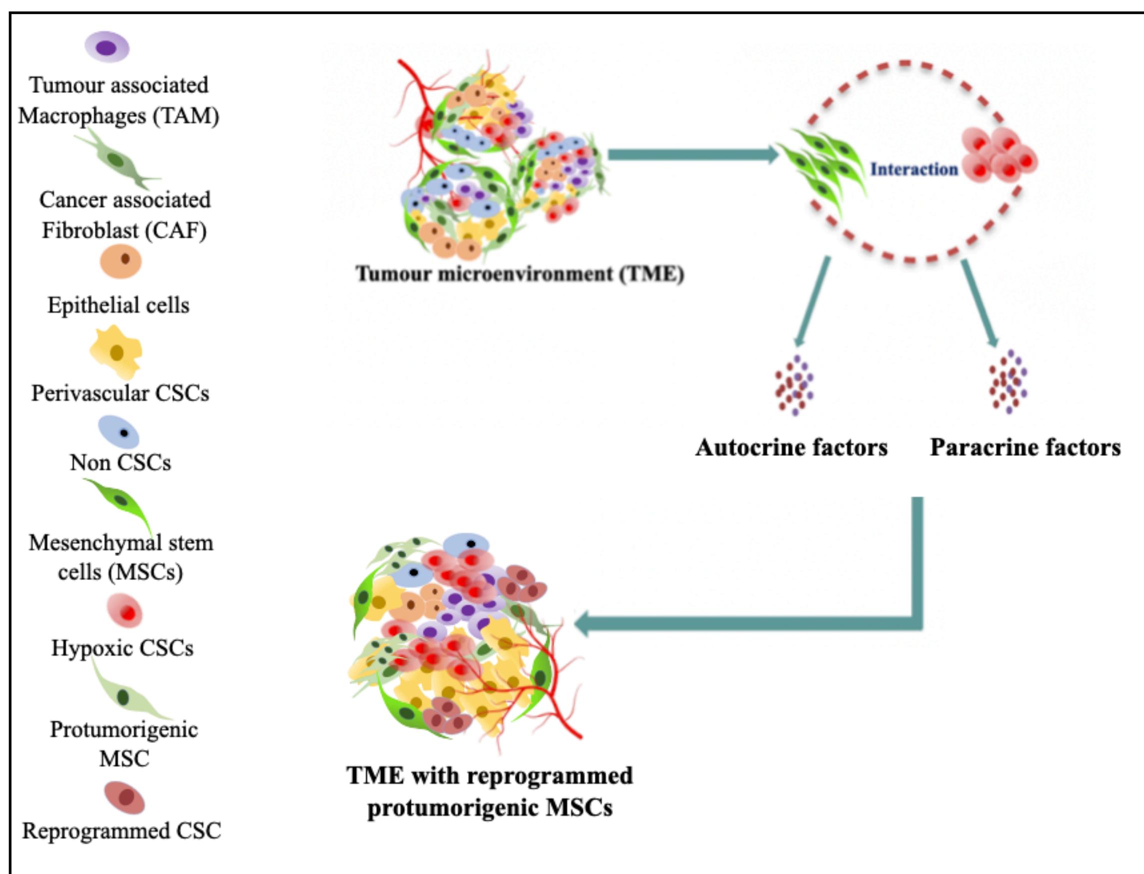

Figure 10: The CSC-MSC interaction leads to expansion of protumorigenic MSCs. *A tumor comprises of both CSCs and non-CSCs. The hypoxic CSCs secrete autocrine factors like HMGB1, VEGF, SDF 1α. These factors may be involved in the reprogramming of BM-MSCs to protumorigenic MSCs. This CSC-MSC interaction may lead the secretion of paracrine factors like GSH, SDF1 α in the TME which may further reprogram the non CSCs to CSC, and CSCs to more aggressive reprogrammed CSCs.*

MSCs also play key role in TME mediated CSC maintenance. A large number of BM derived MSCs in the TME are recruited by CSCs as reported by different studies (Hall, Andreeff and Marini, 2007, Xinyu Li *et al.,* 2022, Yuanming Jing *et al.,* 2022). These MSCs favour tumor growth via secreting chemokines or inflammatory factors such as IL-6 (Rattigan *et al.*, 2010),

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IL-1β (Tu *et al.*, 2008) and growth factors such as transforming growth factor-β1 (TGF-β1) and SDF-1α (Gao *et al.*, 2009; Quante *et al.*, 2011) (Table 3). These growth factors and inflammatory factors constantly attract BM-MSCs into TME under hypoxic condition (Rattigan *et al.*, 2010). Studies reported that MSC can increase the CSC population to promote tumor progression by altered bone morphogenic proteins (BMP) production (McLean *et al.*, 2011). MSCs were found to secret specific cytokines such as IL-6 and CXCL8, which further induce expression of stemness genes, such as, Oct4 and Sox2 in colorectal cancer cells (Wu *et al.*, 2016). MSCs were shown to increase ALDH expressing breast cancer cells by secretion of CXCR2 ligand (Liu *et al.*, 2011), CD133 expressing colorectal cancer cells by activating the JAK2-STAT3 pathway (Tsai *et al.*, 2011). Several studies reported that cancer cells may reprogram BM-MSCs into pro tumorigenic phenotype (Moskovits *et al.*, 2006; Bar, Moskovits and Oren, 2010; Chaturvedi *et al.*, 2013; Jung *et al.*, 2013). These studies indicate that CSC-MSC interaction plays a significant role in tumor progression or cancer recurrence (Figure 10).

1.11 Cancer stem cells in HNSCC progression

In 2007, Prince *et al.* showed CSC characteristic in CD44+ population of HNSCC cells (Prince ME *et al.*, 2007). Later, ALDH (Chen YC *et al.*, 2009, Clay MR *et al.*, 2010), CD133 (Zhou L *et al.*, 2007, Canis M *et al.*, 2012), CD24 (Han J *et al.*, 2014) and CD10 (Fukusumi T *et al.*, 2014) were also reported as putative CSC marker for HNSCC. Several studies reported the presence of CSC population in metastatic sites and in recurrence cases of different cancer types (Marzagalli *et al.*, 2021). ABCG2, the drug efflux pump (Shen B *et al.*, 2011) is a CSC marker, highly expressed in CSCs of cisplatin resistant HNSCC cell line; HSC-3 (Murakami K et al, 2022), and HNSCC metastatic cell lines; M3a2 and M4e (Song J et al, 2010). EpCAM is another CSC marker (Munz M *et al.,* 2009) which is shown to be highly expressed in cisplatin resistant HNSCC cell line (Noman ASM *et al.*, 2020). Enhanced expression of OCT 4 in CSCs increases the tumorigenic potential of oral squamous cell carcinoma, a type of HNSCC (Shin KH *et al.,* 2018). Several studies have reported the significance of CSC-MSC interaction in HNSCC progression (de Miranda MC *et al.*, 2021). The BM-MSC was reported to enhance the migratory ability, drug resistance and expansion of HNSCC cells when co-cultured with SCC-25 cells (Liu C *et al.*, 2021). The HNSCC patient's tumor derived MSCs were shown to produce high amount of tumor promoting factors such as stromal cell-derived factor (SDF)-1α, interleukin (IL)-6, and

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IL-8. The MSC's stromal support for tumor growth was also shown in a HNSCC xenograft model by this group (Kansy B. A. *et al*., 2014). Another HNSCC cell line; FaDu was treated with conditioned media (CM) obtained from MSC culture under hypoxic stress. This hypoxia exposed MSC-CM was found to release increased IL-6, which further increased the proliferation rate of FaDu cells post 48 hours of treatment (Wilhelm C *et al*., 2020). These interesting findings indicate a strong significance of CSC-MSC interaction in HNSCC progression.

The reawakening of dormant or quiescent CSCs in the TME may initiate the HNSCC recurrence. During chemotherapy induced cell death, urothelial carcinoma cells were reported to release proinflammatory factors such as PGE2. These PGE2 stimulated reawakening of quiescent CSCs into cell division for tumor repopulation (Kurtova AV *et al*., 2014, Chan KS, 2016). In a colorectal cancer patient derived xenograft model, it was demonstrated that although chemotherapy treatment reduced tumor burden, the absolute number of marked clones were not changed (Kreso A *et al*., 2013). This result indicates that the tumor may have CSC population which show resistance to chemotherapy and have the ability to oscillate between dormancy and recurrence. Several invivo models of bladder urothelial carcinoma (Kurtova AV *et al*., 2014), glioblastoma (Chen J *et al*., 2012), medulloblastoma (Vanner RJ *et al*., 2014) and T-cell acute lymphocytic lymphoma (T-ALL) (Das B *et al*., 2019) demonstrated the existence of quiescent CSCs. Interestingly, chemotherapy could not target these quiescent CSCs of the glioblastoma and these cells were identified to propagate glioblastoma cells post chemotherapy (Chen J et al., 2012). Similarly, quiescent CSCs lead relapse in sonic hedgehog subgroup medulloblastoma post chemotherapy (Vanner RJ *et al*., 2014). Therefore, understanding the biology of dormancy to reawakening is important to target these quiescent CSCs (Park SY and Nam JS 2020). Different stemness pathways such as the Notch and Wingless (Wnt) pathways were proven to promote cancer cell reawakening in different solid tumours (Sistigu A *et al*., 2020). However, the dormancy to reawakening mechanism is not well studied in HNSCC.

1.12 Stem cell niche defense mechanism and its role in cancer recurrence

The BM niche has a hypoxic microenvironment that maintains the quiescent state of the stem cells (Reagan and Rosen, 2016). These stem cells have the potential to self-renew (Aria F, 2008; Jones and Wagers, 2008) and can migrate to the area of inflammation (Crosby and Waters, 2010; Spaeth, Kidd and Marini, 2012). In the BM, the specialised niche may maintain the

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stemness of MSCs and HSCs (Pal and Das, 2017). Stemness in the niche may be of three types: niche-dependent, niche-independent and niche-modulatory. The niche dependent stemness can be referred as extrinsic when a given stem cell population requires paracrine support from niche cells to maintain stemness. Whereas, niche independent stemness is intrinsic and self-sufficient, where stem cell population activates autocrine pathways to maintain stemness. Stem cells with intrinsic stemness may be maintained in vitro without serum or any growth factor support (Pal and Das, 2017; Das B, 2014). Higher level of intrinsic stemness can lead to a state of "enhanced stemness" phenotype of higher fitness i.e. ability to not only survive in extreme microenvironment, but also secrete growth factors and anti-oxidants to modulate or defend the niche. As previously mentioned, our lab has first demonstrated this unique feature of niche-modulatory stemness in human ES cells (Das B *et al.*, 2012), where a sub-population of hESCs reprogram to ASC phenotype (Figure 5). This ASC phenotype showed higher expression of the stemness genes; Oct-4, Nanog and Sox-2. Interestingly, the Oct-4 expressing MSCs in the BM niche when exposed to platinum-induced oxidative stress, secreted anti-oxidant glutathione and squalene (Das B *et al.*, 2008). This finding indicated that MSCs may also exert a niche-defense mechanism by reprogramming to the ASC phenotype like hESCs. In this context, CSC may exploit the stem cell's niche defense potential to reprogram the niche in the favor of CSC proliferation and thus, initiate cancer recurrence (Figure 10).

We previously showed that a highly tumorigenic side population (SP) of osteosarcoma (HOS), neuroblastoma (SK-N-BE2) and rhabdomyosarcoma (RH-4) cell lines exhibit higher expansion and invasion when exposed to hypoxia or cisplatin treatment (Tsuchida R *et al.,* 2008). This SP cells were later reported to contain CSC like population in many cell line including HNSCC cell lines; SCC 25 (Bhuyan S *et al.*, 2022), M3a2, M4e and 686LN (Song J *et al.,* 2010). In neuroblastoma cell line, the highly expanded tumorigenic CSCs exhibited higher expression of stemness genes; Nanog, Sox2 and Oct4 when exposed to hypoxia and chemotherapy stress (Das B *et al*, 2008). These findings indicated that similar to MSCs and ESCs, a population of CSCs also reprogram and exhibit niche modulatory altruistic behaviour when exposed to stress. These highly invasive phenotype of CSCs are termed as tumor stemness defense (TSD) phenotype (Figure 11) (Bhuyan S *et al.*, 2022, Das B, 2009, Saikia PJ *et al.,* 2023). These findings indicate that the therapy associated stress may induce TSD reprogramming of CSCs and ASC reprogramming of MSCs in the TME.

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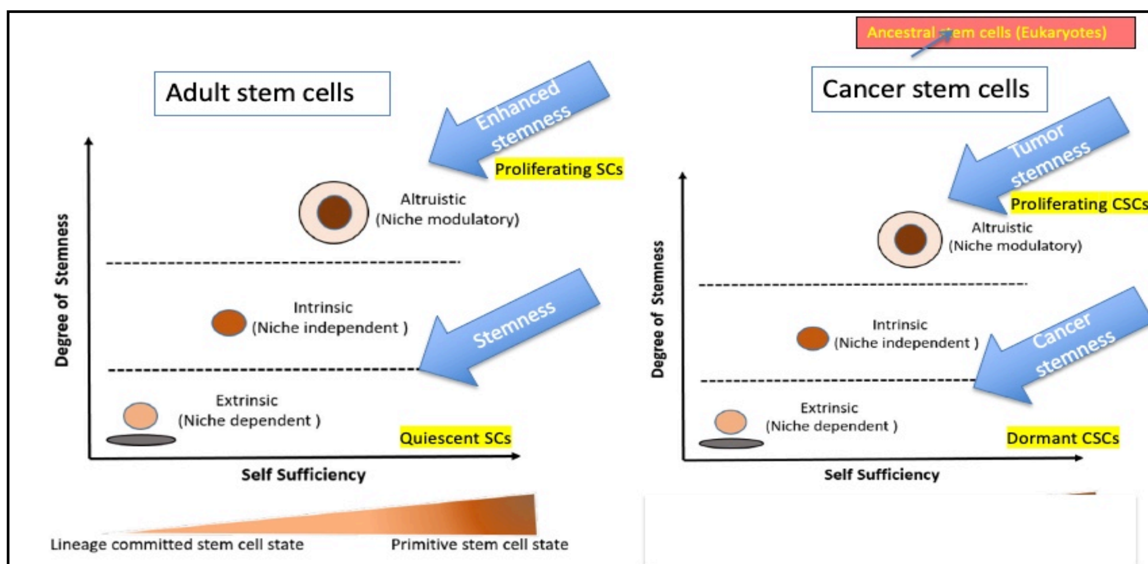

Figure 11: The relation between stemness, adult stem cell and cancer stem cell. *On the left, the normal stem cells (SCs) in their niche. On the right, CSCs in their niche. The niche dependent stemness state corresponds to dormancy, where the niche cells maintain the quiescence state of SCs or CSCs. In contrast, the niche independent and niche modulatory stemness state represent the proliferating state of SCs or CSCs.*

Importantly, MSCs were reported to maintain the stemness state of the CSCs and also induce the self-renewal of quiescent CSCs (Mimeault and Batra, 2013; Philip *et al.*, 2013) to promote tumor progression and cancer recurrence (Hall, Andreeff and Marini, 2007; McLean *et al.*, 2011; Wu *et al.*, 2016). BM-MSCs were found to promote proliferation, migration and invasion of cancer cells via TGF-β growth factor (Ye *et al.*, 2012). It has been found that MSCs can also serve as a home to tumor sites, increase the metastatic potential of tumor cells and thus, play an important role in creation of metastatic niche at the secondary tumor site (Karnoub *et al.*, 2007). Researcher found that co-injection of BM-MSC and triple negative breast cancer cell line inhibits the primary tumor, but increase invasion and metastasis in mice (Lacerda *et al.*, 2015). Similarly, MSCs obtained from tumor of HNSCC, gastric cancer and glioma cancer when injected with tumor cells to mice, it increased tumor growth (Kim *et al.*, 2013; Kansy *et al.*, 2014; Hossain *et al.*, 2015). Interestingly, the MSCs obtained from tumor cells were found to stimulate the growth of cancer cells than the MSCs derived from non-cancerous BM. The tumor derived MSCs were found to secret more tumor promoting factors including VEGF, TGF-β, IL-6 and IL-8 (Li *et al.*, 2015). Interestingly, several studies also reported anti- cancer potential of

MSCs (Hmadcha *et al.*, 2020; Kostadinova and Mourdjeva, 2020). MSCs were found to secret cytotoxic agents and apoptosis inducing factors such as TNF-Related Apoptosis-Inducing Ligand (TRAIL) that selectively induces apoptosis in different types of cancer (Wiley *et al.*, 1995; Takeda *et al.*, 2001; Akimoto *et al.*, 2013). These findings indicated the niche defense potential of MSCs. Considering these facts, it would be interesting to study how CSCs escape the niche defense mechanism of MSCs and reprogram the MSCs to modulate the niche that favour tumor growth, but suppress anti-tumor potential of MSCs (Figure 10).

1.13 Tuberculosis and Cancer in rural population: The challenge in public health care

Tuberculosis and Cancer are affecting the public health development in India, especially in rural population. Although, the burden of TB disease is considered to be lower in rural area than urban due to low population (Olson *et al.*, 2012), whether a difference exists between the prevalence of recurrent TB in rural and urban is not known (Mutembo *et al.*, 2019). Importantly, in India, TB occurrence is 100 times higher in certain indigenous population located in remote rural areas (Tollefson *et al.*, 2013). However, the availability of nurses and physicians in the area is less than 38% and 25% respectively (Heinemann E, Prato B, 2011). The Revised National TB Control Program (RNTCP) significantly reduced the TB prevalence and mortality rate (Singh and Ramamohan, 2020). However, RNTCP or any other TB health approach has not adequately analyzed the reason of MDR TB prevalence and reactivation of TB in rural population. A study conducted in high TB prevalence rural area of India demonstrated that most of the TB cases were due to reactivation (Narayanan *et al.*, 2002), which suggests the need in development of rural health policy to control TB reactivation. Next, this research group recently performed spoligotyping among the TB patients and identified East African Indian (EAI) strain as most predominant strain even after the presence of Beijing strain in the rural population for 2 decades (Siva Kumar *et al.*, 2020). Predominant strain EAI leads the rural population with 2-3% less MDR-TB than national average (Siva Kumar *et al.*, 2020). Such understanding of the disease is required to control the transmission of TB in rural India. Therefore, to control TB in rural population, there needs to be disease specific research, understanding and public health approach.

Similarly, in the context of cancer care, there is no or few cancer facility in rural area, while cancer mortality is double in rural than urban area (Banavali, 2015). Even in developed

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countries like America, cancer death rates are higher in rural areas (180 deaths per 100,000 persons) compared with urban areas (158 deaths per 100,000 persons) (https://www.cdc.gov/media/releases/2017/p0706-rural-cancer-deaths.html). Several studies have shown the inferior survival and treatment failure of rural cancer patients (Onega *et al.*, 2008; Olson *et al.*, 2012; Weaver *et al.*, 2013). Lack of awareness, poor accessibility to health care leads late diagnosis and higher number of recurrence cancer cases in rural population (Pandey *et al.*, 2016), despite curative therapy. Therefore, it is necessary to conduct long term monitoring and research amongst the rural cancer patients to understand cancer recurrence amongst this population.

1.14 The telemedicine approach: To reach TB reactivation and cancer recurrence subjects for rural public health development

Rural public health approach is an important element for rural health development. Rural populations have poorer levels of health status than urban population (White, 2013). Rural areas tend to have shorter life expectancy and high mortality rates due to infectious diseases (DesMeules *et al.*, 2006) like TB and other deadly diseases like cancer. In India, 72.2% people reside in rural area, while only 40.8% health workers are available for rural area (Anand S, 2016). Thus, it is necessary to develop improved public health approaches to manage the rural population in developing countries like India. The current rural health care system of India is a three tire system which comprises of Sub-centres (SC), primary health centres (PHC) and community health centres (CHC). There is RNTCP to manage TB in India (Singh and Ramamohan, 2020) and Tertiary Cancer Center (TCC) for cancer care (Banavali, 2015). However, these programs do not include the post therapy management. Therefore lack of post therapy monitoring may promote recurrence in rural TB and cancer patients. Additionally, due to lack of appropriate cancer care facility in the rural area, the rural cancer patients visit TCC in the city in an advanced stage, thus contributing to treatment failure and recurrence (Banavali, 2015). Importantly, TB and cancer require consistent monitoring and follow up even after successful therapy to avoid recurrence. Therefore, novel approach is needed to be developed which can be easily accessible, affordable and most importantly, which can provide consistent monitoring to the TB and cancer patients from rural population.

In this context, we speculate that telemedicine could be an excellent rural public health approach to manage and monitor TB and cancer patients in the rural area. Indeed, recent research

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suggests that tele-health can play a sustainable rural public health approach (Singh *et al.*, 2010). Although, the telemedicine, an electronic health approach was identified and implemented by WHO in the year 2005 (WHO report, 2005), before covid19 pandemic imposed lockdown the telemedicine health approach was not widely accepted (Zon *et al.*, 2021). As the telemedicine health approach is now under rapid adoption globally, this could be a potential public health approach for rural population. Indeed, KaviKrishna telemedicine care (KTC) has been operating for last two decades in providing health care and conducting clinical research in the Kamarup district on TB (Das *et al.*, 2020), cancer progression (Das *et al.*, 2019) and cancer care (Pathak *et al.*, 2019). Utilizing this rural telemedicine based approach; our lab has investigated the stem cell's role in *Mtb* dormancy (Das B *et al.*, 2013), *Mtb* transmission (Das *et al.*, 2020) and cancer progression (Das *et al.*, 2019). Therefore, we suggest that the telemedicine based approach may also be an appropriate tool to conduct clinical research on TB reactivation and cancer recurrence.

1.15 A rural telemedicine based clinical research approach to study stem cell's role in TB reactivation and cancer recurrence

There isn't research conducted on TB among indigenous people, a fact which has hampered providing the appropriate TB control program for this population (http://www.stoptb.org/assets/documents/resources/publications/acsm/kp_rural_spreads.pdf). The Revised National TB Control Program (RNTCP) significantly reduced the TB prevalence and mortality rate (Singh and Ramamohan, 2020). However, study demonstrated that RNTCP's performance in case detection and successful TB treatment amongst rural indigenous population is poor (Muniyandi *et al.*, 2015). The poor case detection and poor TB cure may lead to TB reactivation in the future. Thus, we need to conduct research among indigenous populations to understand the TB reactivation mechanism. There are very less, but successful examples of TB research conducted in rural population (Narayanan *et al.*, 2002), (Siva Kumar *et al.*, 2020) (Das *et al.*, 2013) (Das B *et al.*, 2020). Studies conducted in a rural population reported the prevalence of TB reactivation subjects in the year 2002 (Siva Kumar *et al.*, 2020) and then in the same population in the year 2021, researchers reported 2-3% less MDR-TB cases than national average of MDR-TB cases (Siva Kumar *et al.*, 2020). Our lab conducted a TB dormancy study among the Idu-Misimi rural indigenous tribe population of Arunachal Pradesh and identified for the first time that dormant *Mtb* reside inside BM-MSCs of successfully treated TB patients for

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decades (Das B *et al.*, 2013). Furthermore, using a satavata-tarka process, as described (Das B *et al.*, 2020), we visualized the transmission strategy of *Mtb* via smear negative PTB (SNPTB) subjects in the community (Das B *et al.*, 2020, Baishya T *et al.*, 2024). Thus, we conducted a contact TB investigation study of 15 years in a rural population including the SNPTB subjects and their contacts in a satra or temple network (Das B *et al.*, 2020). Using this satra network, we then developed a clinical scale to measure the quality of life of TB patients (Baishya T *et al.*, 2024). The study subjects from both of the studies are now under KTC monitoring to evaluate future TB reactivation or re-infection. These studies indicate the importance of rural population based research, especially on TB reactivation.

The incidence and mortality rate of cancer is higher in rural India (Dikshit *et al.*, 2012) (Das and Patro, 2010). The reason for higher cancer related deaths in rural area is analyzed in the context of lack of appropriate public health approach for cancer management and prevention in the rural area (Banavali., 2015). A research group reported that an ovarian cancer (OC) survivor from a rural area may have higher fear of cancer recurrence (FCR) (Galica *et al.*, 2020) as they have higher psychological morbidity (De Sousa, Mohandas and Javed., 2020). Our lab has been also working with a rural population from Sualkuchi, Assam, India for 20 years via KTC to evaluate the cancer health disparity in rural India (Pathak *et al.*, 2019). However, the basic biology behind the higher cancer related deaths in rural population has not yet been investigated. Moreover, there is minimal research conducted on the rural cancer patients. Our lab's anecdotal experience of 30 years with rural cancer patients via KTC suggests that most of rural cancer patient progress to cancer recurrence leading to early death. Evidence also suggests that rural cancer patients seek medical help only during emergency, thus are diagnosed with recurrence in an advanced stage (Dobson *et al.*, 2020). In this context, a rural cancer patient population may be appropriate and necessary for a post-therapy long term monitoring study to understand and diagnose early cancer recurrence. Previously, our lab obtained CSC population from cancer recurrence subjects with acute lymphoblastic lymphoma from rural area to investigate the self-renewal potential of these CSCs (Das B *et al.*, 2019). Indeed, our lab found a 2000 fold higher self-renewal capacity of CSCs versus non-CSCs obtained from recurrence subjects. This finding was possible due to the consistent follow up with the patients via telemedicine approach and collection of the blood samples immediately after the diagnosis of recurrence (Das B *et al.*, 2019). These findings provide the significance of conducting fundamental research among rural

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population to understand the biology of TB reactivation and cancer recurrence. In summary, utilising this easily accessible telemedicine based approach, long term clinical investigation may be conducted among a less mobilised rural population to reveal the underlying mechanism of TB reactivation or cancer recurrence.

1.16 The historical background of the evolution of KaviKrishna's unique rural telemedicine approach

KaviKrishna's IKS based rural telemedicine network was reported to use in conducting clinical investigations previously. Since 1994, KaviKrishna's rural clinical team of KaviKrishna Telemedicine Care (KTC) has been providing free medical care and consultancy to the rural people of India's North East (https://kktel.org). Accordingly, applying the community based participatory action research (CBPAR) through the KTC, between 1994-2000, KaviKrishna have harnessed the IKS of the weaving and artisan or Shudra community of Sualkuchi-Hajo cultural complex of rural Kamrup, a district of Assam, the northern eastern state of India. This effort led us to recover almost the extinct, Vedic Jiva Upakara Cikitsha Tantra, in short Jiva Upakara Tantra (JUT) that could translated to English as Vedic Altruism, as the tantra prescribes an altruistic mechanism of healing probably originated during the Vedic age in Kamarupa, Assam (the Sunga-period, 200 BC-100 AD; https://assamtribune.com/scripts/details.asp?id=may1109/City8). BD, the main clinician of KTC has utilized this Vedic altruism-based biosocial medicine approach to manage chronic lung diseases including COPD, PTB, and cancer related lung diseases. While managing PTB subjects in rural India through the KTC, BD has serendipitously discovered the strength of Vedic altruism based philosophy of medicine, and it"s possible application in rural tele-health, a rapidly emerging field in rural health (Das B 2019). BD has also uncovered an indigenous Kamarupa information network (IKIN) prevalent in the rural Kamarupa, which served as a social capital for the IKS mediated medical practice along the Vedic Silk Road (Das B 2019).

In the JUT or Vedic altruism based tantric healing method, the healing practitioners or ojash would use the JUT teachings and methods to manage smallpox, tuberculosis as well as various other ailments believed to be caused by both perceptible and imperceptible types of krimis (microscopic worms akin to the metaphysical creatures mentioned in the Rigveda, Atharvaveda, and Jain sacred texts). The treatment approach involved using special tantric

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rituals to control the behaviour of krimis. Community-based healing in the form of Nam kirtan (chanting yoga) based air-inoculation, along with herbal extract, Nigudah yoga and an ancient crude method of blood-based inoculation treatment resembling a basic form of modern plasma therapy, were all a part of the approach. They also used fecal materials, and decomposed plants and soil found in numerous caves of Suad Muni ashram of Sualkuchi (Pathak L *et al* 2023). Importantly, the tantric healers prepared herbal extracts from more than 36 plants that they harvested in and around the Suad muni ashram, and the cow pasture south of historic tantipara (weaver colony) located south of Hatisatra of Sualkuchi (Sandhya S *et al*., 2024). The urine and feces of cows fed in the pasture filled with the herbs were used for various ailments. Moreover, chanting, and Nigudah yoga treatment was orchestrated as a community medical care model among the temples in the Sualkuchi-Hajo cultural complex, through the IKIN network (Pathak L *et al.,* 2019). The goal of the community healing process involved the activation of the Sahasa-ojash, which then activates Avatar Kosha and vice versa (Das B 2019).

A key metaphysics of JUT involves the Vidhata-ojash, a putative, altruistic social defense mechanism that is triggered during times of environmental stress due to the harmful nigudahs or krimis (microscopic life forms as per Vedic and Jain scriptures; https:// en.wikipedia.org/wiki/Nigoda). From the Vidhata-ojash"s activation, the Vidhatha Kshetra, (an Avatar of the biofield/environment) transiently emerges to destroy the harmful krimis, thereby protecting Prakriti through altruistic behaviour. The Vidhata-ojash activation occurs through the temple-complex based social network, the IKIN (Chapter 2.7, page 80-86, Das B 2019). Analogous to JUT"s metaphysic global health concept of “Vidhata-Kosha” is JUT"s concept of the metaphysics of individual health in the pancha-kosha system, the Anna Moya, Prana Moya, Mono Moya, Vigana Moya and Ananda Moya kosha. As per JUT, during stress, a transient sixth Kosha arises, the Avatar Kosha having healing energy to restore the balances of the Pancha kosha. An individual"s Avatar Kosha arises through an interaction between a yantra (a complex healer"s instrument), the patient and his/her healer through their combined Nadi (energy network). Through this interaction, the bad-nigudah can be transformed in the Svatantra kshetra (a more independent, stronger version of Vidhata Kshetra) to become good-nigudah, which then activates a transient Avatar Kosha in the inner layer of the Pancha kosha system. The emergence of Avatar Kosha will potently increase the flow of Sahasa-ojash (the

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subtle healing energy mentioned in Bhagvat Gita), leading to the activation of Vyadhikshamatva, the immune system of the body. As a result, all the bad-nigudah will transform into good- nigudah, and the person will then be able to spread this good-nigudah to their community. This idea of the transformative power of Avatar Kosha stem from the Vyhua sutra and Madhu Vidya of Vedic metaphysics related to social buffering against stress. (Chapter 2, Das B 2019). The transformation involves the activation of a powerful self-esteem image of healing, the "Sahasa-Ojash" in the mind of a patient by an able healer or Sadhak, so that the patient"s Avatar-Kosha can be activated. At the heart of the Avatar kosha practice was a breathing yoga, the Nigudah Yoga (https://www.facebook.com/permalink.php?story_fbid=128555009421171&id=109061731370499; https://www.youtube.com/watch?v=9jphxniVQD0), proper nutrition including local herbs, and a self-care practice called Shraddha. For the Avatar-kosha to be effective in the community, a temple network is required, and thus, many of the temples of Sualkuchi-Hajo area were incorporated into this network, the Indigenous Kamarupa Information Network (IKIN).

How do we integrate IKS-based healing methods with Western medical practice for the evolution of an integrated modern medical system which is people centric, and reduces health disparity of rural world? KaviKrishna's CBPAR approach through the KaviKrishna Telemedicine Care to address the issue of integration do not allow to exploit the IKS as was done during colonialism, but, instead, enhance and keep the identity of the IKS intact, plus recognise it's contribution to our scientific growth. In this context, KaviKrishna has made tremendous progress during 1994-2022 by integrating these two different systems of medical care approach to develop clinical and biomedical research programs. Also, KaviKrishna applied and sustained the idea of operating a telemedicine network based on the metaphysics of Vedic altruism based biosocial healing.

Thus, a unique process of CBPAR based approach centered around KaviKrishna organization of Sualkuchi has started to take shape between 1994 through 2012, leading to the establishment of the KaviKrishna Laboratory in the IIT-Guwahati campus. The metaphysics components of Vedic altruism to develop a research program with the core idea of studying the oxidative stress component of disease pathogenesis was initially mentioned in the book, "the Science Behind Squalene" by BD in the year 2000 (Das B 2000). The core theme of the book is the role of antioxidant supplements such as squalene in keeping our body healthy.

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This idea and passion came to BD's mind, during the early period of CBPAR, when BD transformed the philosophy of JUT philosopher late Krishna Ram Das' poem, "Sonali Nakhar Jui" to testable experimental hypothesis to study stem cell altruism (Figure 14, Das B 2000) and in the Elsevier press release (https://www.eurekalert.org/news-releases/696977). The idea and evidences on the stem cell niche defense is now the research-core of KaviKrishna Laboratory and Thoreau Laboratory's biomedical research program in understanding the fundamental pathogenesis of cancer, TB, as well as possible impact of pollution and climate change in human body. Whereas, the idea of Vidhatha-kosha based biosocial medicine idea is the core of KTC's artificial intelligence (AI) driven digital health initiative. Utilising this approach via KTC, researchers of KaviKrishna Laboratory have completed 2 major clinical studies on TB during 2012-2024 (Das B *et al*., 2013, Das B *et al*., 2020). This approach was also used to provide care to more than 1500 covid19 patients during the 2020-21 pandemic.

Taken together, based on more than nearly three decades of CBPAR initiative in rural Assam of India through the KTC, KaviKrishna Lab is proposing a new public health approach that combines the ancient ideas of the Indigenous Knowledge System (IKS) of Assam and modern medical care to develop a novel model of rural health care system.

1.17 Conclusion

TB and cancer are two fatal diseases associated with reactivation or recurrence. In both the diseases, causative agents have the potential to remain dormant for years either before or after therapy. Then, the dormant causative agents cause reactivation or recurrence during favourable condition. The current TB control program or cancer management do not have any approach to tackle this challenge. Therefore, there is a novel insight required to understand the underlying mechanism of dormancy and reactivation. Stem cell's niche defense is a novel idea, proposed in the field of dormant *Mtb* and cancer cells dormancy (Pathak L and Das B, 2021, Saikia PJ *et al.,* 2023). This research proposes to study the stem cell niche defense mechanism in TB reactivation and cancer recurrence. The clinical significance of the mechanism would be evaluated in TB and cancer patients screened by a telemedicine based network (Das *et al*., 2020; Pathak L *et al*., 2019). Importantly, among all cancers, HNSCC is one of the commonest cancers with poor outcomes, late diagnosis and poor survival rate in India. Therefore, for cancer recurrence study, HNSCC cell line and subjects will be selected.

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Hypothesis

Preliminary study conducted by the present research group indicates that stem cell has a potential niche defense mechanism against oxidative stress and pathogen invasion. The stem cell niche defense was first reported in embryonic stem cells (ESCs), where a population of ESCs reprograms into an 'enhanced stemness' phenotype in the oxidative stress microenvironment by activating HIF2α and transiently suppressing p53. These reprogrammed cells sacrificed their self-fitness to enhance the fitness of neighboring cells under stress by secreting glutathione (Das *et al.*, 2012). We speculate that this altruistic behaviour or niche modulatory potential of stem cell to defend their niche could be hijacked by *Mtb* and CSC to enhance their own fitness during inflammation. It was already demonstrated how *Mtb* hijack the stem cell's niche defense mechanism to remain dormant inside the BM-MSCs escaping the anti-TB drugs (Das B *et al.*, 2013, Garhyan J *et al.*, 2014, Beamer *et al.*, 2015). The *Mtb* was found to remain dormant inside the CD271+ MSCs of mice and successfully treated TB subjects (Das B *et al.*, 2013). We hypothesised that inflammation such as acute respiratory tract infection (ARI) induced stress may causes mobilization and homing of d*Mtb* harboring BM-MSCs to the lungs (Pathak L and Das B 2021). Inside the lungs, d*Mtb*-BM-MSCs may reprogram from MSCs to transient altruistic stem cells (ASCs), which undergo clonal proliferation and become permissible for intracellular replication of d*Mtb*. When the transient ASCs undergo apoptosis or differentiation, the replicating intracellular *Mtb* may exit ASCs into extracellular space to infect alveolar macrophages and thus may reactivate the TB disease. We further hypothesise that, this stem cell niche defense may also contribute in multi drug resistance (MDR) evolution..

Similar to *Mtb*, CSCs were reported to reside in quiescent state in the hypoxic BM-TME (Das B *et al.*, 2008). We previously reported that hypoxia or cisplatin treatment enhances the expansion, invasion and stemness gene expression of EpCAM+/ABCG2+ CSCs of neuroblastoma, sarcoma, lung cancer cell line (Tsuchida R *et al.*, 2008, Das B *et al.,* 2008) and lymphoma patient derived tumours (Das B *et al.*, 2019). This finding indicated that a specific population of CSCs activates the stem cell niche defense mechanism when exposed to drug or hypoxia stress to defend their niche. This aggressive and highly invasive phenotype of EpCAM+/ ABCG2+ CSCs is termed as tumor stemness defense (TSD) phenotype (Das B 2009, Bhuyan B *et al.*, 2022, Saikia PJ *et al.*, 2023). Thus, the specific CSC population reprogrammed to a higher

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stemness phenotype; TSD and exhibit altruistic behaviour. Therefore, we hypothesise that the cisplatin treatment may also activate therapy associated stress induced TSD phenotype in the EpCAM+/ABCG2+ CSCs of HNSCC subjects, thus cause therapy failure followed by HNSCC recurrence. It can be speculated that the TSD phenotype of CSCs may interact with the other cells in the TME to establish a tumor favouring niche. Considering the previous findings of MSC reprogramming to ASCs when exposed to stress, we further speculate that the TSD phenotype of CSCs may reprogram the MSCs into ASCs to establish a niche modulatory, immunosuppressive and protumorigenic TME to favour tumor growth.

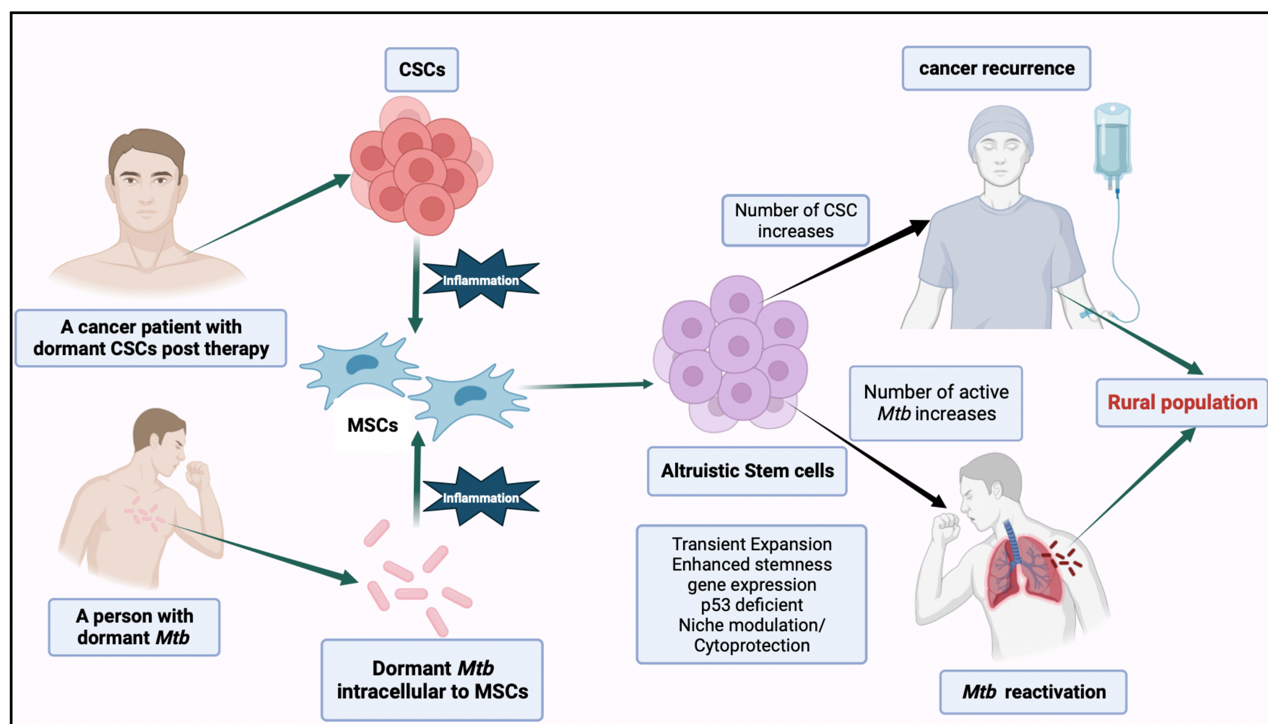

Figure 12: The hypothetical representation of Mtb and CSC activating stem cell niche defense mechanism leading TB reactivation and cancer recurrence. *Using invitro and invivo model, this hypothesis would be investigated. The hypothesis will be tested in the selected TB and HNSCC subjects. The study subjects were selected from a rural population and monitored for 5 years to understand the mechanism of dormancy to reactivation or recurrence.*

Specific Aim: Insight into stem cell niche defense mechanism in TB reactivation and head and neck cancer recurrence

TB reactivation and HNSCC recurrence are 2 un-noticed rural health problems. To tackle this problem, we need a novel insight about the cellular mechanism of TB and HNSCC reactivation or recurrence. The present research team of the lab has developed a novel stem cell niche defense based approach to study TB transmission and cancer progression (Das B *et al.*, 2013, 2019, 2020). To understand this novel mechanism, it is needed to conduct clinical investigation in an appropriate population. The lab has developed a telemedicine based public health approach for rural population via KaviKrishna Telemedicine Care (Pathak L *et al.*, 2019). Utilising this telemedicine network, a long term contact TB investigation study was successfully conducted (Das B *et al.*, 2020). The rural population is an appropriate study population due to its low mobility feature, affordable and accessible monitoring for longer duration. Therefore, this rural population was selected to test our hypothesis of stem cell niche defense in TB reactivation and HNSCC recurrence.

To test the hypothesis of stem cell mediated TB reactivation, the previously established stem cell mediated mice model of *Mtb* dormancy (Das B *et al.*, 2013) will be used. We sought to find out if a viral infection causing inflammation can reactivate the d*Mtb* by inducing reprogramming of d*Mtb* harboring MSCs to ASCs. A mouse corona virus strain, the murine hepatitis virus-1 (MHV-1) will be used to induce ARI causing inflammation in the stem cell mediated mice model of *Mtb* dormancy. Next, we will investigate the role of stem cell niche defense mechanism in clinical subjects. It has been reported that 10% of treated TB subjects have the potential to recur (Azhar, 2012, Global TB report by WHO, 2022) and about 18% of the recurred cases exhibit multi drug resistance (MDR) phenotype (Sharma N *et al.*, 2020, Shivekar SS *et al.*, 2020, Global TB report 2022). In this context, we speculate that the stem cell may provide the niche to *Mtb* strain to evolve as the MDR-*Mtb* strain and thereby contribute in recurrence. Utilizing the rural telemedicine approach, we will screen PTB subjects will obtain their sputum and blood samples. We will isolate the blood derived MSCs and will evaluate whether intracellular *Mtb* acquire the fitness of MDR strain. Utilising the telemedicine approach, we will also monitor these TB subjects for potential recurrence in future.

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To test the hypothesis of CSC niche defense in HNSCC recurrence, the previously established hypoxia indued CSC niche defense model of SCC-25 cell line (Bhuyan S *et al*., 2022) will be used. Instead of hypoxia, we will use cisplatin treatment to evaluate the therapy associated stress induced CSC niche defense in SCC-25 cells. The cisplatin treated SCC-25 cells will be sorted for EpCAM+/ABCG2+ CSCs and evaluated for TSD phenotype. We will further investigate the cross talk between TSD exhibiting CSCs and bone marrow derived MSCs by treating the MSCs with the conditioned media (CM) of TSD exhibiting CSCs. We speculate that the CM may reprogram the MSCs into protumorigenic and immunosuppressive ASC phenotype. The CM of "ASC" will be exposed to SCC-25 cells and evaluated whether ASC-CM can enhance the resistance of SCC-25 cells against cisplatin treatment. We sought to test our hypothesis of TSD and ASC mediated HNSCC recurrence in clinical subjects. Utilising the telemedicine approach, we will screen HNSCC subjects under cisplatin treatment and obtain blood samples to evaluate the presence of circulating ASC and TSD phenotype in the blood. We will further monitor these subjects for potential future recurrence.

Altogether, this proposed study may provide a novel insight of stem cell's niche defense mechanism in disease reactivation or recurrence with TB and HNSCC as experimental diseases.

Objectives:

Objective 1: To investigate the role of stem cell niche in dormant TB reactivation using a mice model of *Mtb* dormancy.

Objective 2: To study the role of stem cell niche in TB recurrence in the rural population of NE region.

Objective 3: To investigate the role of stem cell niche defense in head and neck cancer recurrence using an in vitro model of MSC and CSC interaction.

Objective 4: To investigate the role of stem cell niche defense in head and neck cancer recurrence with clinical samples.

Chapter 2.0.0

To investigate the role of stem cell niche in dormant TB reactivation using a mice model of *Mtb* dormancy

Abstract

Background: The efficient strategy of *Mycobacterium tuberculosis* (*Mtb*) is to remain dormant inside the infected host, cause reactivation during favourable environment and then transmit the pathogen to the community via aerosol. Studies have reported a rare fraction of the bone marrow mesenchymal stem cells (BM-MSCs); CD271+BM-MSC as a protective site for dormant *Mtb* in both LTBI and post successfully treated TB subjects. Utilising a mice model of *Mtb* dormancy, it was demonstrated that d*Mtb* intracellular to CD271+BM-MSC can reactivate the d*Mtb*, when injected to secondary recipient mice. **Methodology**: We postulated that stem cell niche may exert a defense mechanism against external pathogen infection which may be exploited by the intracellular d*Mtb* for reactivation. Therefore, the mice model of *Mtb* dormancy was infected with murine hepatitis virus-1 (MHV-1) to cause lung infection. Indeed, MHV-1 infection activated the stem cell niche defense mechanism in alveolar niche, thereby led to reprogramming of lung CD271+ MSCs into an enhanced stemness phenotype. This stem cell niche defense mechanism was characterised by transient expansion of CD271+MSCs for 2 weeks, higher expression of stemness genes followed by apoptosis. **Results**: Interestingly, the infected mice lung exhibited 20- fold lower viral loads than the *Mtb* free control mice on the third week of MHV-1 infection, and exhibited six-fold increase of reprogrammed MSCs. As these reprogrammed MSCs exert a unique altruistic defense against MHV-1, can be termed as altruistic stem cells (ASCs). Notably, the expansion of these ASCs facilitated intracellular replication of *Mtb*, and their extracellular release. In a invitro assay of MHV-1 infection, the conditioned media of mice lung derived ASCs exhibited cytoprotection against MHV-1 induced toxicity to type II alveolar epithelial cells by increasing their survival/proliferation and decreasing viral load. **Conclusion**: This result further confirmed the altruistic defense mechanism of MSCs, which is exploited by intracellular *Mtb* for TB reactivation.

2.1.0 Background

Previous reports suggest that tuberculosis is one of the key bacterial infections affected by viral pandemics (Low *et al*., 2004; de Paus *et al*., 2013; Alfaraj *et al*., 2017; Walaza *et al*., 2019). In 1918, the Spanish flu pandemic led to the rise of pulmonary TB incidence (Oei and Nishiura, 2012). In 2009, the influenza A (H1N1) pandemic led a worse prognosis of influenza in patients with TB or multi-drug-resistant TB (Park *et al*., 2014). Interestingly, SARS-CoV-1 and Middle East respiratory syndrome coronavirus infected patients were reported to develop pulmonary TB (Low *et al*., 2004; Alfaraj *et al*., 2017). Strikingly, one quarter of the world population is already infected with dormant TB. If SARS-CoV-2 or any other virus infects these dormant TB populations, it may cause severe impact in global health and economic by causing both viral infection and dormant TB reactivation. Thus, there is an urgent need to study the association of viral infection with dormant TB reactivation to avoid a later global TB pandemic. Literature review suggests that 10% of dormant TB population and 10% of treated TB population may reactivate the disease in their lifetime (Global TB report 2023). In this context, the *Mtb* is required to remain dormant inside a immunosuppressed host cell type with viable state. Several studies have proposed different sites for *Mtb* dormancy including apical part of lung, macrophages, fibroblast and ghon complex (Pathak L and Das B 2021). However, viable Mtb was not recovered from any of the proposed sites during LTBI period. Therefore, to understand the underlying mechanism of dormant TB reactivation, a novel insight is required.

The present lab has identified that *Mtb* remains dormant inside CD271+BM-MSC for decades in successfully treated TB subjects. Tornack et al. reproduced the findings in LTBI subjects by recovering viable *Mtb* from CD271+BM-MSCs of LTBI subjects (Tornack *et al.*, 2017). Using a mice model of m18b-*Mtb* (a mutant strain of 18b, dependent on streptomycin), the present lab has demonstrated that *Mtb* remains in a non-replicating, viable state inside the CD271+BM-MSCs for 6 months (Das B *et al*., 2013). When these *Mtb* harboring CD271+BM-MSCs injected into recipient mice, it showed granuloma formation in lung. Therefore, we hypothesize that the d*Mtb* harboring CD271+BM-MSCs may migrate to lung during any infection and activate the stem cell niche defense mechanism against the infection. Like human embryonic stem cells, the d*Mtb* harboring MSCs may also reprogram into an “enhanced stemness” phenotype, called ASCs to defend their niche against the infection induced

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inflammation (Das B *et al.,* 2012, Pathak and Das., 2021). The d*Mtb* inside the MSCs may exploit that stem cell niche defense mechanism to induce intracellular replication of d*Mtb*. We further speculate that while these ASCs sacrifice their enhanced fitness by undergoing apoptosis and secreting cytoprotective molecules to protect the neighbor cells, the intracellular *Mtb* may release in the extracellular space and infect the other cells of lung including macrophages, thus reactivate the d*Mtb*. To test this hypothesis, a mouse coronavirus strain; murine hepatitis virus-1 (MHV-1) will be used to induce inflammation in the already established stem cell mediated mice model of *Mtb* dormancy (Das B *et al*., 2013) (Figure 13).

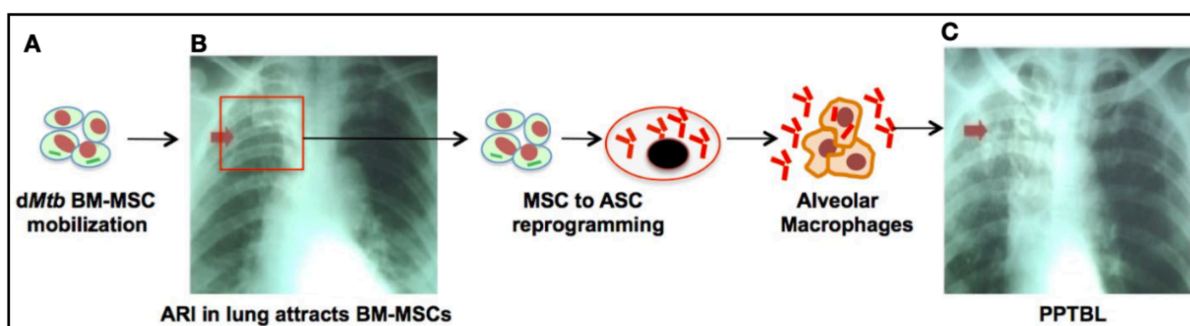

***Figure 13**: **Hypothesis on BM-stem cell mediated Mtb reactivation.** The dormant Mtb intracellular to BM-MSC may migrate to lung via circulation during ARI causing inflammation and may reprogram the MSCs to ASCs. ASC reprogramming will facilitate the dMtb reactivation and PPTBL development.*

The MHV-1 represents clinically relevant human coronavirus SARS- CoV-1 infection, and was known to cause acute lung inflammation by inducing ARI (De Albuquerque et al., 2006, Khanolkar et al., 2009 and 2010). We speculate that the MHV-1 infection in the lung may initiate a stem cell niche to niche interaction to repair the area of injury. This process may trigger migration of d*Mtb* harboring MSCs from BM niche to the alveolar niche via circulation. Further, the MSC may activate ASC based niche defense mechanism against the MHV-1 infection to defend the niche which could be exploited by the intracellular *Mtb* for reactivation (Figure 14).

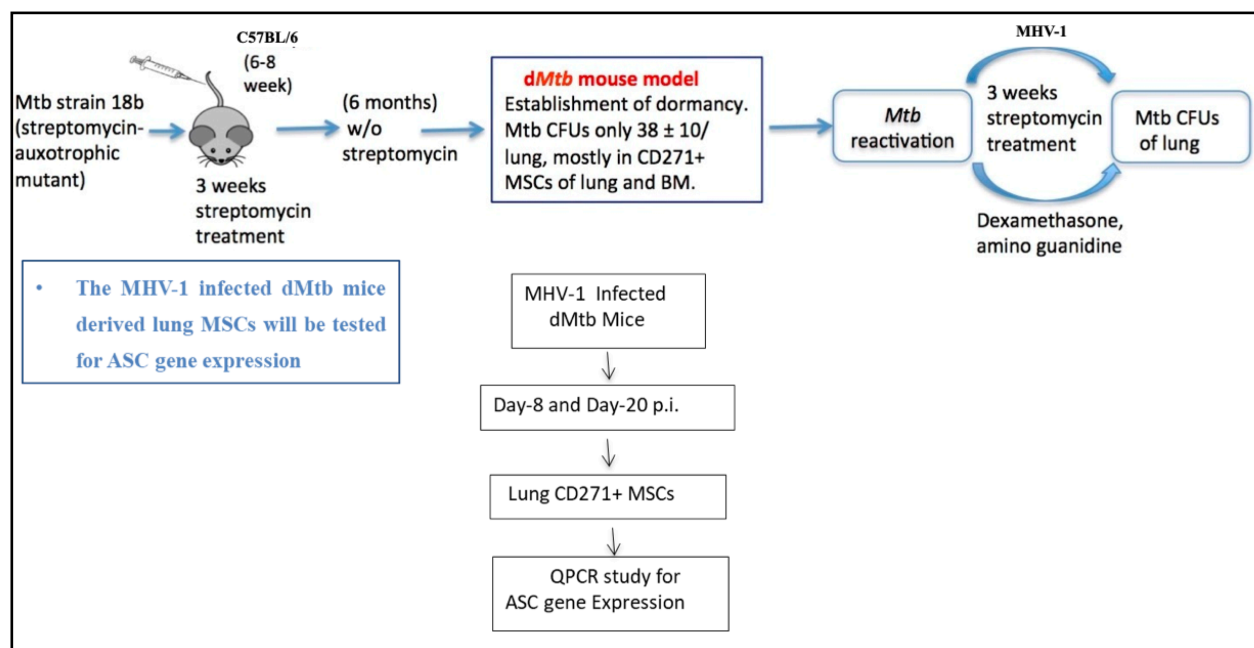

***Figure 14**: Experimental design of stem cell mediated TB reactivation study in a mice model of dormancy.* *The C57BL/6 mice were infected with m18b Mtb strain with 3 weeks of streptomycin treatment to induce lung granuloma formation. After 6 months of streptomycin withdrawal, these mice were found to contain dormant (non replicating and viable) Mtb inside the CD271+BM-MSC and few in the lung MSC. The mice will be infected with MHV-1 infection and the mice lung MSCs will be evaluated for inflammation induced ASC reprogramming and dMtb reactivation in the mice lung. The above experimental design was conceptualised through the application of satavata tarka based FGD session with Dr. Bikul Das and the research team.*

2.2.0 Methodologies

All experimental procedures were conducted following the approved protocols within a Biosafety Cabinet class II (BSC II) facility, adhering strictly to the guidelines set forth by the Institutional Biosafety Committee (IBSC) of the KaviKrishna Laboratory. The animal-related experiments received the necessary approvals from both the Institutional Animal Ethics Committee at Gauhati University and the Institutional Ethics Committee at KaviKrishna Laboratory. The streptomycin auxotrophic mutant *Mtb* strain18b, generously provided by Prof. Stewart T. Cole from Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland, was

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cultivated on 7H11 agar plates (BD Bioscience) supplemented with glycerol and an antibiotic cocktail from Hi Media, in addition to 50 mg/ml of streptomycin sulfate. The strain was meticulously maintained under controlled conditions at 37ºC and 5% CO^2.

2.2.1 Creating a Mouse Model of *Mtb* Reactivation Using Stem Cells: Female C57BL/6 mice, aged 6 to 8 weeks, were procured from the National Institute of Nutrition (Hyderabad, India) and housed in the pathogen-free animal facility at Gauhati University, following established protocols (Das B *et al.*, 2013). The invitro culture of m18b-*Mtb* was used to determine the *Mtb* colony forming unit (*Mtb*-CFU) after a two-week culture period. A cell suspension was prepared using Tween 80 (0.05%). Subsequently, each mouse (n=10) received an intravenous injection of 2×10^6 CFUs. To initiate infection, streptomycin was administered daily for three weeks. After this phase, mice were kept under observation for six months without streptomycin treatment to establish bacterial dormancy. As previously observed (Das B *et al.*, 2013), a minimal number of *Mtb*-CFUs (38±10 *Mtb*-CFU/lung) were detected exclusively in CD271+ MSCs, confirming the establishment of a mouse model of *Mtb* dormancy. For reactivation, streptomycin was intraperitoneally injected (3 mg/mouse in 200 mL of normal saline) daily for three weeks, with or without MHV-1 infection (De Albuquerque *et al.*, 2006, Khanolkar *et al.*, 2009b). In a separate experiment, streptomycin-treated mice were subjected to additional treatment with immunosuppressive agents: dexamethasone (0.08 mg/day, 6 times a week) or amino guanidine (2.5% wt/vol in drinking water) to induce d*Mtb* reactivation (Scanga *et al.*, 1999). The treated mice were monitored for the specified duration and then euthanized to collect lung tissues. The *Mtb*-CFU count in the lung tissues was evaluated to assess *Mtb* reactivation.

2.2.2 Isolation and Culture of CD271+ MSCs: The procedure involved in obtaining CD271+ MSCs from mouse lung tissues comprised several steps. Initially, the tissues underwent dissociation using collagenase/lipase, followed by cell sorting through magnetic techniques to specifically isolate CD271+ MSCs, as previously described (Das B *et al.,* 2013, Garhyan J *et al.,* 2015). The bone marrow (BM) cells were collected from mice femur (~2×10^7 cells) in 2 ml of modified Dulbecco's medium as previously described (Das B *et al.*, 2008). For the isolation of circulatory CD271+ MSCs, 5-10 ml of peripheral blood was collected from mice in stipulated period. The collected blood samples were subjected to ficoll (17144002, Ficoll Paque Plus, Cytiva, Sweden) based separation of peripheral blood mononuclear cells (PBMCs). To start the

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isolation process for BM, lung and circulatory CD271+ MSCs, CD45- cells were separated using the Ter119/CD45- depletion kit (number 19771; Stem Cell Technologies, Vancouver, BC, Canada). Subsequently, the CD45- cells underwent further magnetic sorting to specifically isolate CD271+ cells. Firstly, a mouse CD271 antibody (mouse clone ME20.4; catalog number Ab8877; Abcam, Cambridge, MA) was conjugated with phycoerythrin using the SiteClick antibody labelling kit (catalog number S10467; Life Technologies, Grand Island, NY). Then, the EasySep phycoerythrin sorting kit (catalog number 18554; Stem Cell Technologies) was utilized to separate CD271+ cells from the CD45- cell population. The cells were cultured in serum-free media without any growth factors for 4 to 8 hours for *Mtb*-CFU assay. In the cytoprotective assay, lung MSCs isolated on day 8 following infection were cultured at a concentration of $1x10^7$ cells/mL in Dulbecco's modified Eagle's medium/F12 media without serum for 24 hours to collect the conditioned media (CM).

2.2.3 *Mtb*-CFU Assay: The *Mtb*-CFU assay was conducted following previously outlined methodologies (Das B *et al.,* 2013, Garhyan J *et al.,* 2015). To perform the whole lung *Mtb*-CFU, lung tissues were aseptically retrieved from sacrificed mice and homogenized in phosphate-buffered saline with 0.05% Tween 80. Subsequently, the homogenized tissue was subjected to the CFU assay (Garhyan J *et al.,* 2015). For the *Mtb*-CFU assay involving immunomagnetically sorted lung and circulatory MSCs, the cells were first cultured in serum-free media without any growth factors for 4 to 8 hours. The conditioned media of cells were kept for extracellular *Mtb*-CFU count. For intracellular *Mtb*-CFU count, the cell pellet was lysed using 1 mL of 0.1% Triton X-100 for 15 minutes, followed by vortexing for 30 seconds. Serial 10-fold dilutions were then prepared in Middlebrook 7H9 broth. The diluted samples, either from the whole lung or the cell lysate, were separately plated onto Middlebrook 7H10 agar plates (BD Biosciences; number 295964), supplemented with streptomycin (50 mg/mL) to facilitate the growth of the m18b strain. The agar plates were subsequently incubated for 3 to 4 weeks at 37°C and 5% CO^2, after which CFUs were enumerated. The results were represented as the mean log10 CFUs per lung or per 10^7 cells.

2.2.4 Real-Time Quantitative PCR Assay: In brief, the mRNA isolation process involves using superparamagnetic Oligo (dT) Microbeads within a magnetic column, specifically targeting the poly RNA tail present in mammalian RNA. This results in the mRNA binding to the magnetic column while bacterial and stem cell DNA pass through in the lysate. Subsequently, the mRNA

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is transformed into cDNA on a heated magnetic bar, following the manufacturer's instructions. The produced cDNA is then utilized for real-time quantitative PCR analysis using the TaqMan Gene expression assay from Miltenyi Biotech. The used TaqMan primers were: mouse: CD271 (Mm 00446296_m1), octamer-binding transcription factor 4 (Mm00658129_g1), CD45 (Mm 01293575_m1), CD44 (Mm01277163_m1), p53 (Mm01731290_g1), HIF-2α (Mm01236112_m1), ATP binding cassette sub- family G member 2 (ABCG2) (Mm00496364_m1), Nanog (Mm02019550_s1), sex-determining region Y- related HMG box 2 (Mm03053810_s1), HIF-1α (Mm00468869_m1), glyceraldehyde-3-phosphate de- hydrogenase (Mm99999915_g1), p21 (Mm01332263_m1), MDM2 (Mm01233136_m1), gluta- mate cysteine ligase (GCL; Mm00802655_m1) and CD73 (Mm00501915_m1). The RNA was quantified by the delta-delta CT method using Q-Rex software version 1.1 (Rotor-Gene Q-Qiagen, New Delhi, India).

2.2.5 MHV-1 Infection into Mice: MHV-1 infection was introduced into mice using the parental virus strain obtained from ATCC (Manassas, VA) and cultured within the BSC class II facility at KaviKrishna Laboratory, following approval by the IBSC and IEC of KaviKrishna Laboratory. The animal protocol was approved by the Institutional Animal Ethics Committee (IAEC) of KaviKrishna Laboratory and Gauhati University. The MHV-1 strain was propagated on L2 cells (ATCC HCCL-149), purified via sucrose gradient centrifugation, and assessed for viral stock titration using an endpoint dilution assay on L2 cells, as detailed in previous descriptions (Leibowitz *et al*., 2011). Mice, either C57BL/6 previously infected with the mutant 18b *Mtb* strain followed by 6 months of streptomycin starvation, or the control group comprising healthy 25 to 26-week-old mice, were intranasally infected with 5000 plaque-forming units (PFUs) of MHV-1 per mouse, according to De Albuquerque *et al*. (2006) and Khanolkar et al. (2009b). The animals were anesthetized intraperitoneally with ketamine, followed by intranasal administration of 50 mL of MHV-1 in phosphate-buffered saline. This specific dosage is known to induce acute respiratory infection in this strain of mice, as documented in previous studies by Khanolkar *et al*. (2010) and Khanolkar *et al*. (2009a). Assessment of lung infection involved a viral load study and measurement of TNF-a on days 0 and 6, employing the methodologies as described by De Albuquerque *et al*. (2006) and Khanolkar *et al*. (2009a).

2.2.6 Viral plaque forming unit (PFU) Assay: The viral PFU assay was conducted following previously established protocols (Leibowitz J et al 2011). Animals were euthanized at specific

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time points, and their entire lungs were collected. Subsequently, the lung tissue was homogenized using the method described in Das B *et al.,* 2013, and the resulting supernatant was stored at -80°C for further analysis. One day prior to initiating the PFU assay, L2 cells were seeded to ensure reaching confluence on the day of the assay. The supernatant obtained from serially diluted lung homogenate was applied to the monolayer of L2 cells, followed by a 1-hour incubation at 37°C. After removal of the inoculum, the cells were overlaid with methylcellulose media and incubated for 48 hours at 37°C. Subsequently, the plaques formed were counted, and the virus titer was calculated using the formula: titer (PFU/mL) = [(number of plaques/well) / (volume of inoculum/well)] × dilution factor.

2.3 Results

2.3.1 MHV-1 Infection Leads to Reactivation of d*Mtb* in C57BL/6 Mice

MHV-1 infection was investigated for its potential to reactivate dormant *Mtb* (d*Mtb*) within lung-resident CD271+ MSCs in C57BL/6 mice. Intranasal administration of MHV-1 ($5x10^3$ PFU) was performed on total of 60 mice, as described by De Albuquerque *et al*. (2006) and Khanolkar *et al*. (2009b). These mice contained the streptomycin-dependent mutant *Mtb* strain 18b in a non-replicating dormant state for six months, as documented by Khanolkar *et al*., (2009b) and Kashino *et al*. (2006). Concurrently, streptomycin was administered as the mutant 18b strain requires this antibiotic for replication (Kashino *et al*., 2006). During the 20 day duration of MHV-1 infection, groups of animals (n=5 for each stipulated period for each group) were sacrificed at stipulated intervals. Lung samples were collected, and both viral titers and *Mtb*-CFUs in the lung tissues were assessed (Figure 15, B and C). MHV-1-infected mice served as the control. In a subset of the control group, streptomycin was included to assess its impact on viral load. In both control groups (MHV-1 minus streptomycin - not treated with streptomycin, and MHV-1 alone - treated with streptomycin), MHV-1 infection led to an initial increase in viral titers in the mouse lung for the first 4 days, followed by a gradual decrease within the subsequent 2 weeks (Figure 15B), aligning with prior findings in C57BL/6 mice (Khanolkar et al., 2009b). Notably, streptomycin did not affect viral titers. MHV-1-infected mice containing d*Mtb* and treated with streptomycin (referred to as the d*Mtb*MHV-1 group) exhibited a similar initial increase in viral load for the first 4 days as observed in the control. However, this load rapidly

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declined after 2 weeks, reaching a level 20-fold lower than the control group (P < 0.004) (Figure 15B). Additionally, evaluation of *Mtb*-CFUs in the lungs of the d*Mtb* alone versus the d*Mtb*MHV-1 group was conducted. Both groups (n=additional 5 mice for each stipulated period for each group) received daily streptomycin treatment for mutant strain replication. By day 8 of infection, *Mtb*-CFUs were sixfold to sevenfold greater than the *Mtb*-CFU count before the infection in both groups (P < 0.01) (Figure 15C). Between days 8 and 20 post-infection, the d*Mtb*MHV-1 group showed a 110-fold increase in *Mtb*-CFUs (P < 0.0001), while the d*Mtb* alone group displayed a 2.1-fold decrease (P < 0.004). These findings suggest that MHV-1 infection not only initiated but also sustained the *Mtb* reactivation process post-streptomycin treatment. In contrast, *Mtb* reactivation did not occur in the d*Mtb* alone group, as streptomycin treatment alone failed to increase *Mtb*-CFUs even after 3 weeks. Significantly, a remarkable 630-fold surge in *Mtb*-CFU counts (25000 *Mtb*-CFU/lung) was noted between days 0 and 20 in the d*Mtb*MHV-1 group, equivalent to the magnitude of a 1000-fold rise in *Mtb*-CFU observed in a dexamethasone- and aminoguanidine-induced *Mtb* reactivation model (Scanga *et al*., 1999).

Comparisons were made between MHV-1-induced reactivation of d*Mtb* and reactivation induced by dexamethasone or aminoguanidine, known to reactivate d*Mtb* in a drug-induced Cornell model by suppressing the immune system (Scanga CA *et al.,* 1999). Mice harboring d*Mtb* were treated with these drugs and streptomycin daily for a month, and lung MTB-CFUs were quantified. The *Mtb*-CFU levels following dexamethasone or aminoguanidine treatment increased threefold to fourfold, which was 400-fold lower than the MHV-1 infection group (P < 0.0001) (Figure 15D). Thus, MHV-1 infection was more effective than dexamethasone or aminoguanidine in inducing reactivation in the d*Mtb* model. These results suggest that mechanisms other than immunosuppression may underlie MHV-1 infection-mediated disease reactivation.

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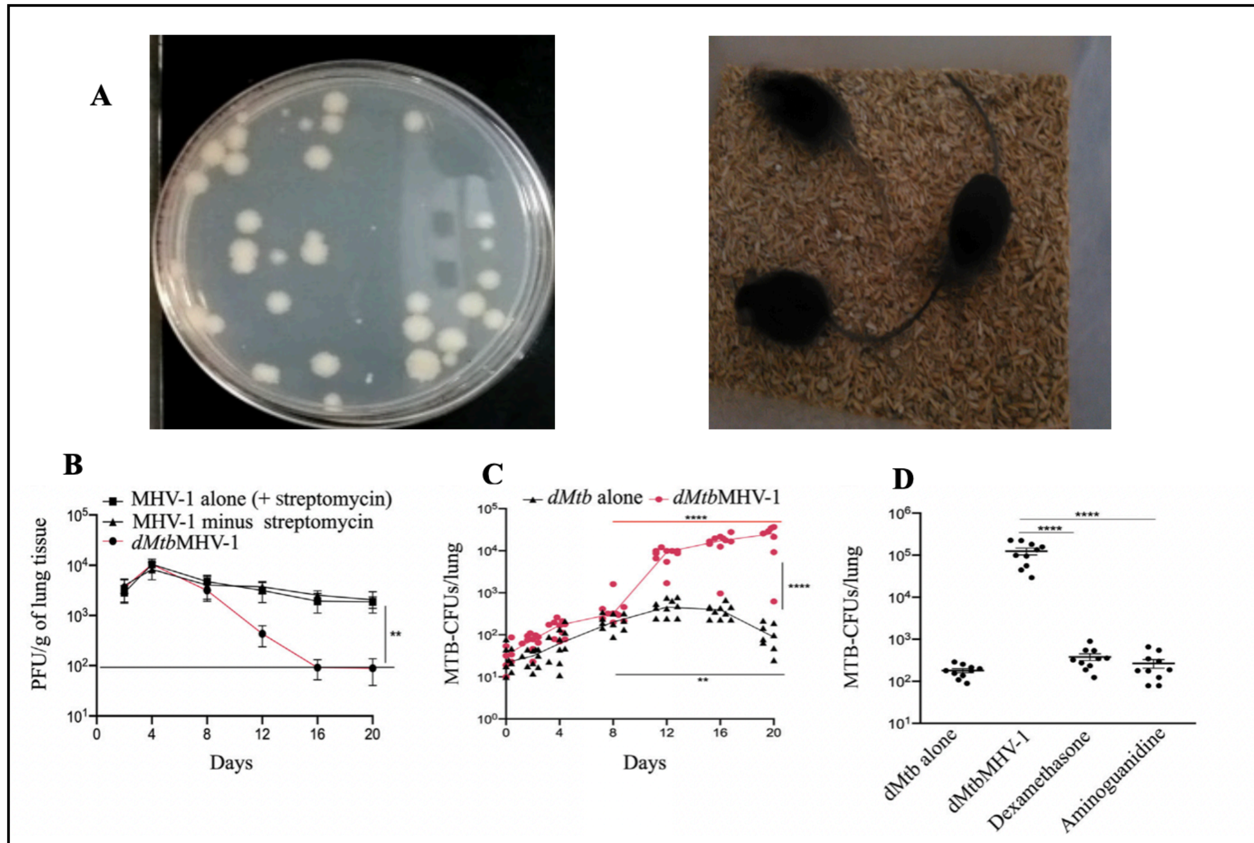

***Figure 15: Murine hepatitis virus-1 (MHV-1) infection induces the reactivation of dormant Mycobacterium tuberculosis (Mtb) in the lungs**. **A**: Cultivation of Mtb colonies of m18b strain on a 7H11 agar plate, along with the injection of m18b-Mtb CFU into the C57BL/6 mice to establish Mtb dormancy. The dormancy was confirmed as the minimal number of Mtb-CFUs (38±10 Mtb-CFU/lung) were detected exclusively in CD271+ MSCs of lung. **B** and **C**: Assessment of viral load [plaque-forming unit (PFU)] and MTB colony- forming units (MTB-CFUs) in the lungs of MHV-1 infected C57BL/6 mice at various time intervals following infection. **D**: Quantification of MTB-CFUs in the lungs of mice at day 30 post MHV-1 infection, or day 30 after treatment with dexamethasone or aminoguanidine. Approximately 25000 Mtb-CFUs were detected between days 0 and 20 in the lung of dMtbMHV-1 group, which was considered as Mtb-CFU count for Mtb reactivation. All of the mice received daily streptomycin treatment for 6 days per week for a duration of 3 weeks. Data presented as means ± SEM (B-D). n= 4 independent experiments (B-D). $^{**}P < 0.01$, $^{****}P < 0.0001$ (t-test).*

2.3.2 MHV-1 Infection Causes Expansion of circulating and lung CD271+ MSCs and Extracellular Pathogen Release in the lung

The lung CD271+ MSCs underwent analysis to access *Mtb* replication inside the cells and its subsequent release into the extracellular space. These specific MSCs were obtained by isolating CD271+/CD45- MSCs through immunomagnetic sorting following a defined period of MHV-1 infection. Viable cell count was determined using trypan blue to ascertain the proliferation of these cells within the specified timeframe. The control group is referred to as d*Mtb*. To investigate the potential impact of the MHV-1 virus on lung MSCs, potential expansion of CD271+ MSC was examined in mice infected with MHV-1. The sorted cells were then cultured in serum-free media for 8 hours to observe both the intra- cellular and the extracellular *Mtb* release. This was measured by assessing *Mtb*-CFUs in both the cell pellet and supernatant, respectively. The results demonstrated a transient 12-fold increase ($P < 0.001$) (Figure 16.1A) in CD271+ MSCs between days 0 and 8 in d*Mtb*MHV-1 group. Intriguingly, the group infected solely with MHV-1 exhibited a 4.5-fold increase ($P < 0.01$) in CD271+ MSCs during the same period; albeit a sixfold lower rate than the d*Mtb*MHV-1 group (Figure 16.1A). However, the d*Mtb* alone group did not display any expansion of the MSCs (Figure 16.1A). Remarkably, in the d*Mtb*MHV-1 group, alongside the MSC expansion, there was a corresponding 27-fold increase ($P < 0.001$) (Figure 16.1B) in the number of intracellular *Mtb*-CFUs. During the initial period (days 0 to 8), the supernatant of d*Mtb*MHV-1 group did not show significant rise in extracellular *Mtb*-CFUs compared to the d*Mtb* control (Figure 16.1C). However, between days 8 and 12, there was a substantial 40-fold increase of *Mtb*-CFU observed in the supernatant of the d*Mtb*MHV-1 group ($P < 0.001$) (Figure 16.1C). This suggest that the transfer of *Mtb* from CD271+ MSCs to the rest of the lung cells in the d*Mtb*MHV-1 group likely occurred between days 8 and 12, coinciding with the sharp rise in the supernatant. Notably, there was a corresponding 15-fold increase of intracellular *Mtb*-CFUs in the rest of the lung cells in vivo ($P < 0.001$), as depicted in Figure 16.1D. As anticipated, the d*Mtb* alone group did not exhibit significant evidence of CD271+MSC expansion, intracellular *Mtb* replication, or their extracellular release (Figure 16.1). Notably, there was similar expansion of CD271+ BM-MSCs ($P < 0.01$, Figure 16.2A) in the d*Mtb*MHV-1 group. Also, there was associated expansion of the circulating CD271+MSCs (CD271+ c-MSCs) containing intracellular *Mtb* (Figure 16.2B-D).. The expanded CD271+ MSCs and the intracellular *Mtb* in the circulation reduced during day 8-16 (Figure 16.2B-C),

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whereas CD271+ MSCs in the lung increased during these days (Figure 16.1A). Almost no *Mtb*-CFU in the extracellular space in circulation confirms that reduction of CD271+ c-MSCs was not due to apoptosis of these cells and extracellular release of *Mtb*. Rather, it may be due to the potential migration of the CD271+ MSCs containing intracellular *Mtb* from BM to circulation and then to lung. The result of expansion of CD271+ BM-MSCs, CD271+ c-MSCs and CD271+ lung MSCs strongly indicate the activation of stem cell niche to niche interaction during MHV-1 infection. In summary, the MHV-1 infection induced the expansion of CD271+MSCs containing *Mtb* in the lung and subsequent release of the pathogen into the extracellular space of lung to infect the non-CD271+ lung cells.

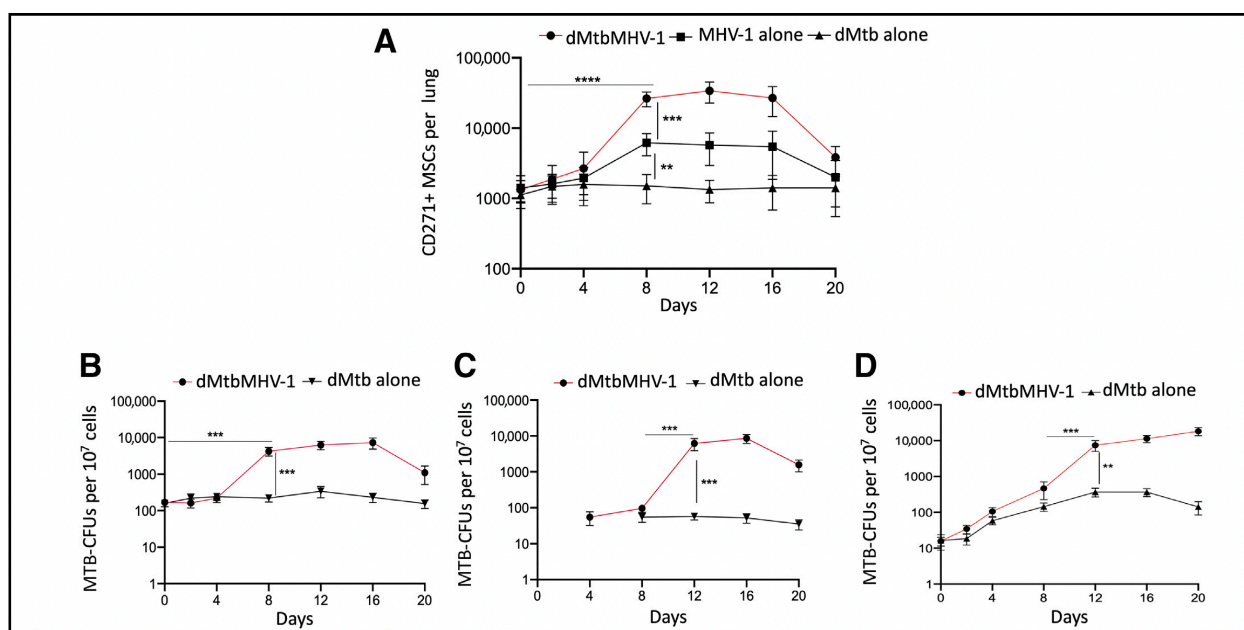

***Figure 16.1: Mtb enhances the MHV-1 mediated expansion of lung CD271+MSCs**. **A**: Transient expansion observed in CD271+CD45+ MSCs within the lungs of both MHV-1 infected and non-infected dormant Mtb (dMtb) mice. **B** and **C**: Assessment of intracellular (B) and extracellular (C) Mtb colony-forming units (CFUs) derived from CD271+ MSCs isolated from dMTB mice and cultured in vitro for 8 hours. **D**: Determination of intracellular Mtb-CFU/10^{7} in non-CD271+ lung cells (remaining lung mononuclear cells after obtaining CD271+/CD45- MSCs through immunomagnetic sorting). The data presented as means ± SEM (A-D). n=4 independent experiments (A-D). **P < 0.01, ***P < 0.001, and ****P < 0.0001 (one-way analysis of variance with the Dunnett post hoc test).*

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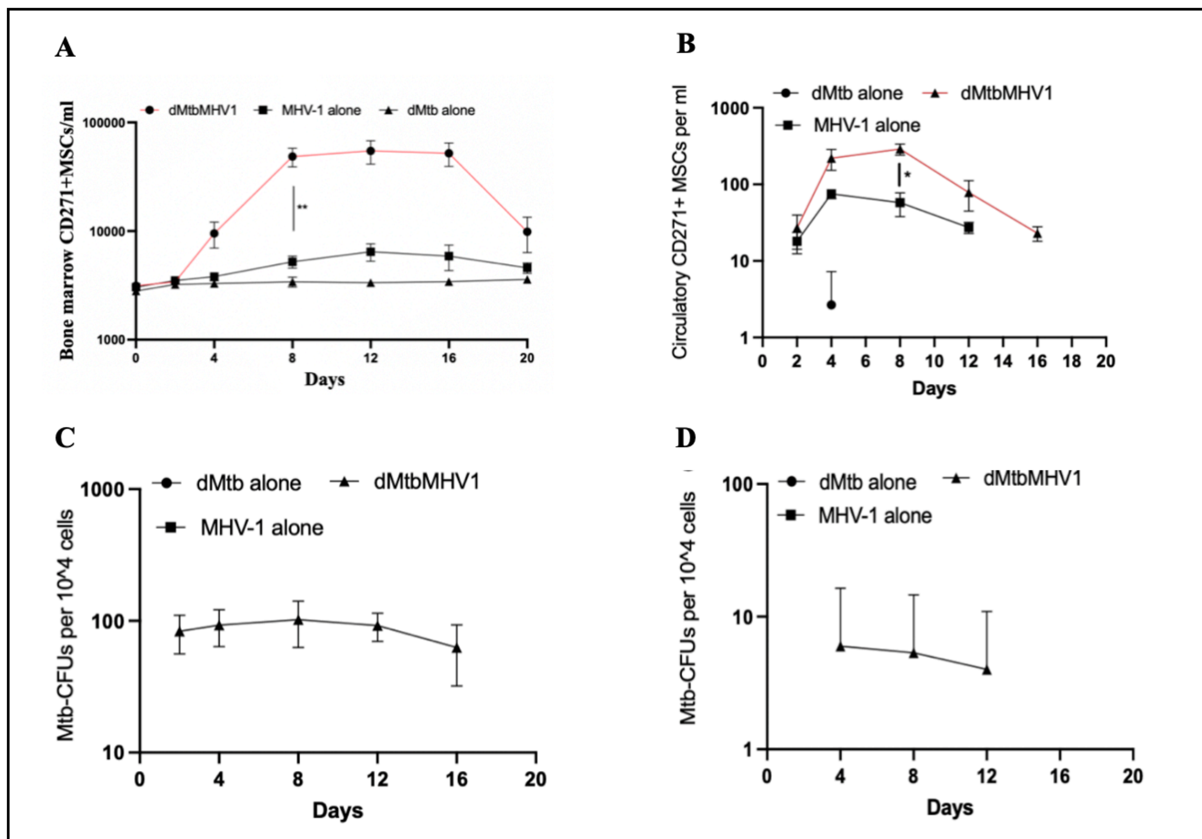

***Figure 16.2**: **Mtb enhances the MHV-1 mediated expansion of bone marrow and circulating CD271+MSCs**. **A**: Transient expansion of CD271+CD45- MSCs in the bone marrow of both MHV-1 alone and dMtbMHV-1 group was observed. **B**. Transient expansion observed in CD271+CD45- MSCs in the peripheral blood of both MHV-1 alone and dMtbMHV-1 group. **C** and **D**: Assessment of intracellular (B) and extracellular (C) Mtb colony-forming units (CFUs) derived from CD271+ MSCs isolated from the peripheral blood and cultured in vitro for 8 hours. The data presented as means ± SEM (A-D). n=4 independent experiments (A-D). *P < 0.05, **P < 0.01 (one-way analysis of variance with the Dunnett post hoc test).*

2.3.3 MHV-1 Infection Activates the Altruistic Stem Cell Mediated Innate Defense Mechanism

MHV-1 infection induced stress might trigger an altruistic stem cell (ASC) mediated innate defense mechanism against the infection. Therefore, the observation of MHV- 1 causing a transient expansion of CD271+ MSCs might be linked to this ASC defense mechanism against the virus. To explore this, the real-time quantitative PCR was employed to access the expression

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of genes associated with enhanced stemness phenotype or ASC reprogramming (HIF1α, HIF2α, Sox2, Oct4, Nanog, ABCG2, MDM2, GCL, p53, and p21) in the MHV-1 alone and d*Mtb*MHV-1 mice derived day 8 CD271+ MSCs that showed expansion of the MSCs (Figure 16A). Control group included d*Mtb* alone (d*Mtb* mice treated with streptomycin, but not infected with MHV-1), streptomycin alone (control mice with streptomycin treatment) and d*Mtb* minus streptomycin (not treated with streptomycin and MHV-1). Additionally, the expression of genes specific to MSCs (CD73, CD44, and Sca-1) and HSCs (CD45) was also evaluated (Figure 17, B and C). In the CD271+ MSCs from d*MTB* mice, certain genes like HIF1α, ABCG2, Sca-1, and MSC related genes, CD44, CD271, and CD73 remained highly expressed (Figure 17B). Streptomycin treatment resulted in 1.6-fold increase in HIF1α expression ($P<0.05$) (Figure 17B) in the CD271+ MSCs of d*Mtb* mice (d*Mtb* alone), but had no impact on the control mice (streptomycin alone). Notably, MHV-1 infection in both the streptomycin alone (MHV-1 alone) and d*Mtb* alone groups (d*Mtb*MHV-1) led to 3-7 fold significant induction of genes associated with the ASC phenotype in the CD271+ MSCs (Figure 17C). Interestingly, in the d*Mtb*MHV-1 group, the expression of ASC reprogramming-related genes (Oct4, GCL, and ABCG2) was increased by 2-3 fold ($P < 0.05$) (Figure 17C) compared to the MHV-1 alone group. These CD271+ MSCs expressed the MSC markers (CD73, CD44, and Sca-1), and lacked CD45 expression (Figure 17C), indicating their stemness.

The results suggest that MHV-1 infection induced ASC reprogramming of CD271+ MSCs was observed in both MHV-1 alone and the d*Mtb*MHV-1 group. Increased up-regulation of p53- upstream genes involved in apoptosis, such as *bax*, *p21*, *PUMA*, and down-regulation of survival genes, such as *HIF-2α*, on day 20 (Figure 17D) was observed. The expression of *Sca-1* was down-regulated in these cells on day 20 (Figure 17D), indicating differentiation. These results further confirm the ASC reprogramming of these MSCs. Consequently, the transiently expanded CD271+ MSCs underwent differentiation and apoptosis on day 20 of MHV-1 infection, confirmed by caspase-3/7 activity assay for apoptosis (Figure 17E). Notably, streptomycin treatment in either control (streptomycin alone) or in d*MTB* mice (d*Mtb*alone) did not induce apoptosis in CD271+ MSCs (Figure 17E). The p53 upstream genes were activated, thus induced apoptosis on day 20. This result indicated the activation of the proposed ASC-based innate defense mechanism in infected CD271+ MSCs against MHV-1.

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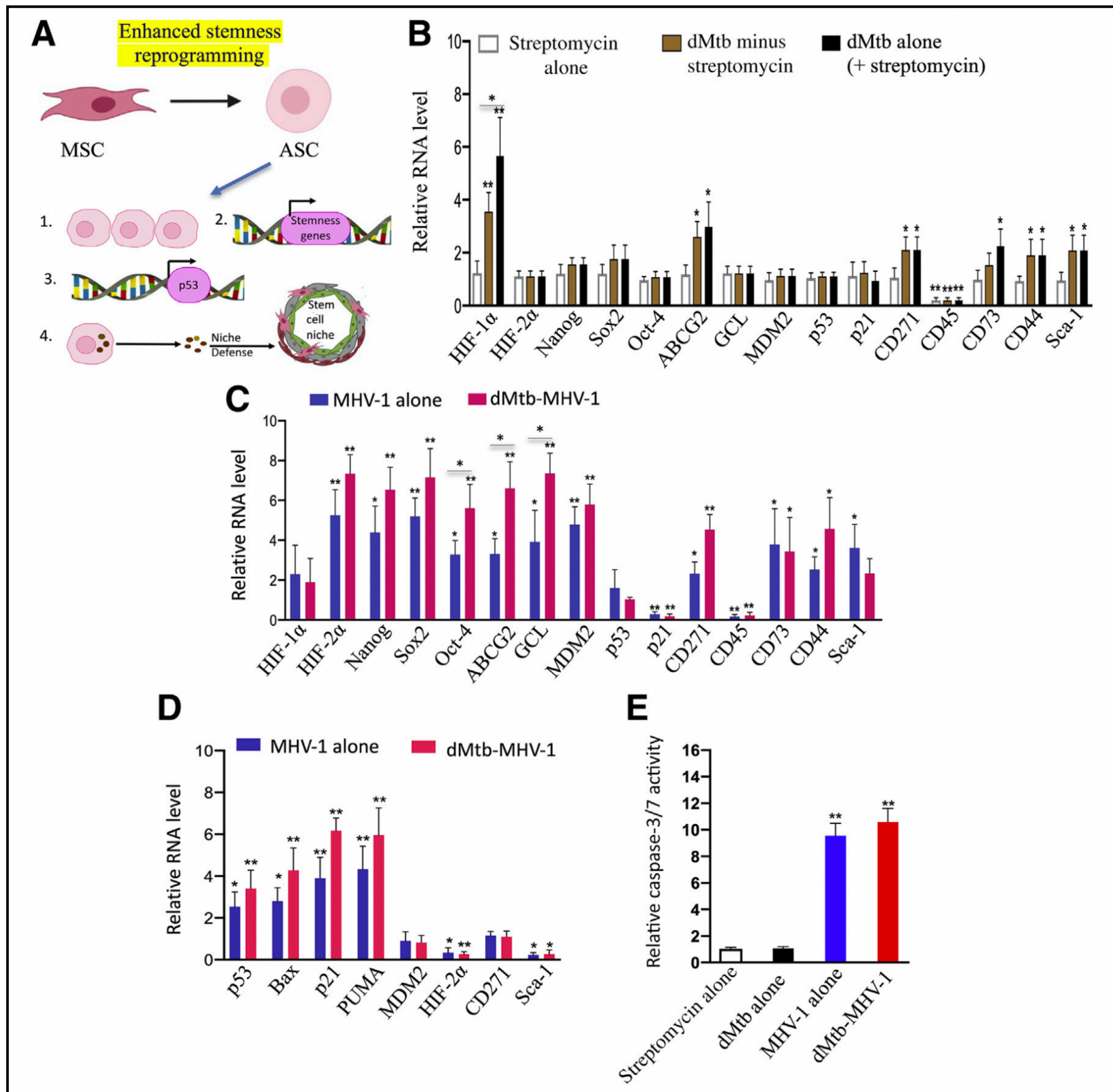

Figure 17: MHV-1 infection triggers the transformation of CD271+ MSC into ASC phenotype. ***A***: *Characteristics defining ASCs 1. Transient expansion of ASCs. 2. Expression of enhanced stemness state associated genes. 3. Activation of p53-induced genes related to differentiation/ apoptosis after 2 weeks. 4. Cytoprotection or stem cell niche-defense.* ***B***: *Histogram illustrating the expression of ASC genes in the CD271+ MSCs of day 8.* ***C***: *Histogram demonstrating the induction of ASC genes in the lung CD271+MSCs of day 8 following MHV-1 infection.* ***D***: *Histogram indicating the upregulation of p53-related genes associated with apoptosis/ differentiation in lung CD271+ MSCs on day 20 following MHV-1 infection. The qPCR data are presented as fold changes compared with the MHV1 alone group.* ***E***: *Increased caspase-3/7 activity in lung CD271+ MSCs on day 20 compared to day 8 following MHV-1 infection. Data presented as means ± SEM (B-E). n= 4 independent experiments (B-E).*

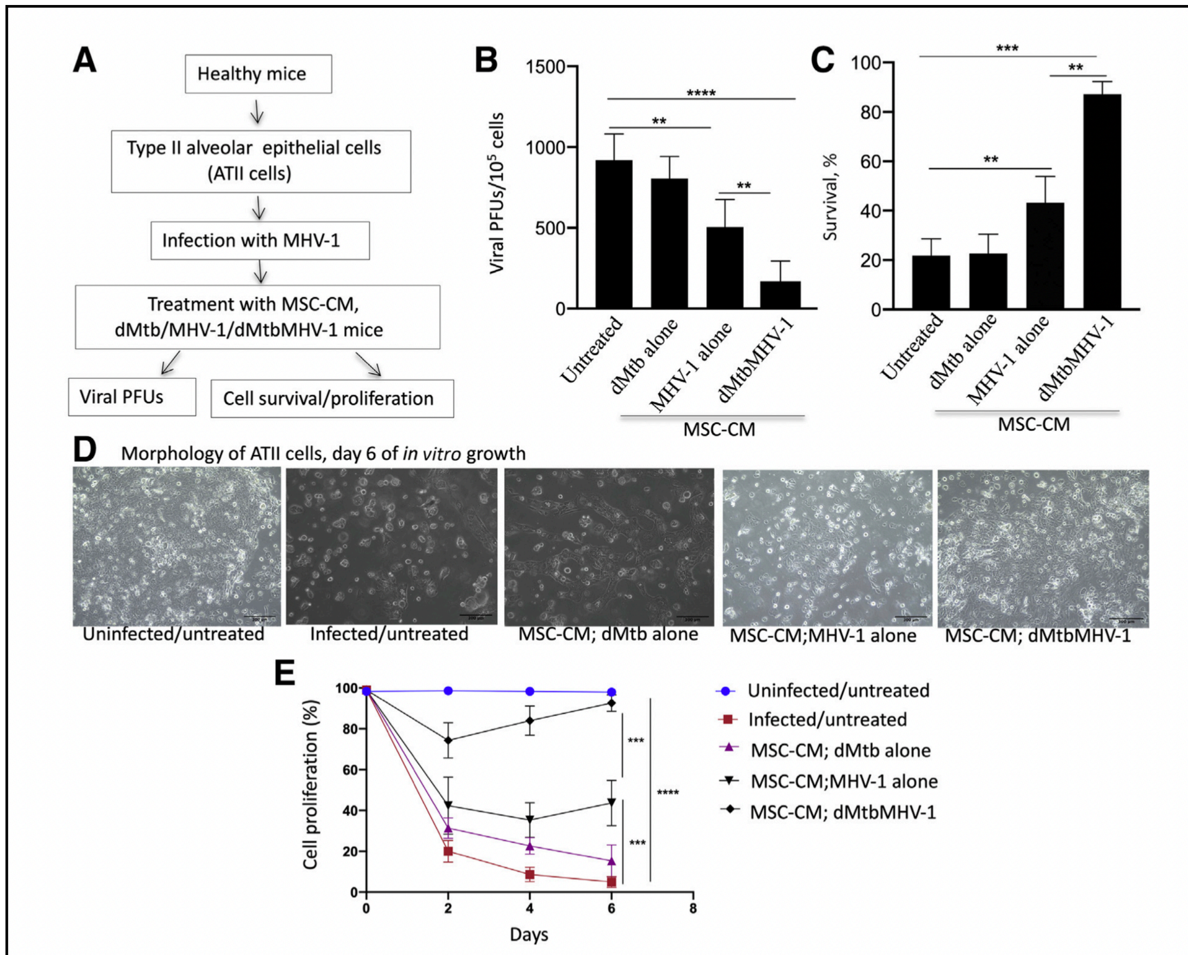

***Figure 18**: Evidence supports the presence of soluble factors inhibiting murine hepatitis virus-1 (MHV-1) infectivity and promoting cytoprotective effects within the culture supernatants derived from reprogrammed CD271+ MSCs. **A**: Illustration outlining the experimental design for showcasing the defense mechanism of altruistic stem cell against MHV-1. **B**: Assessment of viral load in type II alveolar epithelial (ATII) cells post treatment with conditioned media (CM) from lung CD271+MSCs. The CM from lung CD271+ MSCs collected on day 8 after infection was gathered from all of infected groups. **C**: Evaluation of cell viability in the treated cells using trypan blue staining. Untreated ATII cells not treated with CM served as the control. The CM obtained from CD271+ MSCs of dMtbMHV-1 exhibited the lowest MHV-1 PFUs in the ATII cells and the highest count of live cells. **D**: Observation of ATII cells under a phase-contrast microscope after 6 days of treatment with CM derived from CD271+ MSCs obtained from mice lung. **E**: Assessment of ATII proliferation through Alamar blue assay results.*

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The ASC-based defense against MHV-1 induced viral load and cellular toxicity was confirmed in an in vitro viral infection assay (Figure 18A). The CM of MHV-1 alone group derived CD271+ MSCs on day 8 exhibited significant 2-4 fold cytoprotection of ATII cells against MHV-1 infection (Figure 18, E), indicating the evidence of ASC-mediated defense against MHV-1. Interestingly, the viral load was decreased by 3-fold (Figure 18B), and proliferation of the ATII cells was increased by 2-4 fold in the d*Mtb*MHV-1 compared to MHV-1 alone group (Figure 18, C and E). This result indicates that presence of d*Mtb* significantly boosted the ASC- based defense in the ATII cells against MHV-1. Therefore, these results further strengthen the proposed mechanism of ASC-based niche defense against MHV-1 and boosting of the defense in the d*Mtb* harboring mice.

2.4 Discussion

In this study, we provided a novel insight of stem cell niche defense mechanism in lung MSCs against viral infection induced inflammation and how an intracellular dormant *Mtb* can exploit that niche defense mechanism for reactivation. Using a mice coronavirus strain MHV-1 (De Albuquerque *et al*., 2006, Khanolkar *et al*., 2009 and 2010) to cause ARI in a stem cell mediated mice model of *Mtb* dormancy (Das B *et al.,* 2013), we showed that lung MSCs activate a putative ASC-based defense mechanism against MHV-1, which is augmented by d*Mtb* remaining inside the lung MSCs. Given that MHV-1 infection matches SARS-CoV-2 infection and the d*Mtb* mouse model reflects d*Mtb* illness in humans, this study's findings on MHV-1 and d*Mtb* co-infection, as well as the discovery of an ASC-based defense against MHV-1, may have substantial therapeutic implications in COVID-19 as well as other fatal viral diseases.

A streptomycin-dependent auxotrophic strain of mutant 18b mediated mice model was used to explore the interaction of dormant *Mtb* niche with coronavirus infection in the lung, where d*Mtb* could be recuperated from the CD271+ MSC compartment of the lung after 6 months of streptomycin starvation. The subsequent administration of only streptomycin for 43weeks did not cause d*Mtb* reactivation, as lung *Mtb*-CFUs in the non-MSC compartment did not significantly increase. Although there was a 20-fold rise in *Mtb*-CFUs in the non-MSC compartment during the first two weeks of streptomycin treatment, this was only for a brief period as *Mtb*-CFUs rapidly reduced over the next two weeks. As a result, the cumulative rise in

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Mtb-CFUs was only threefold ($P > 0.05$) (Figure 15D), which is insufficient to promote TB reactivation in the lung (Scanga CA *et al.,* 1999). However, infection with MHV-1 in the presence of streptomycin resulted in a 3500-fold rise in *Mtb*-CFUs in the non-MSC compartment of the lung by 30 days of infection (Figure 15D), indicating lung TB reactivation, as previously established (Scanga CA *et al.,* 1999). Expansion of the CD271+ c-MSCs containing intracellular *Mtb* (Figure 16.2B-D) for initial 8 days followed by reduction of CD271+ c-MSCs and the intracellular or extracellular *Mtb* in the circulation during day 8-16 have been observed. This result indicated the potential migration of the CD271+ MSCs containing intracellular *Mtb* from BM to circulation and then to lung. Indeed, the significant expansion of CD271+ MSCs in BM was observed during day 4-16 of MHV-1 infection (Figure 16.2A). Notably, the rise in *Mtb*-CFUs in the non-MSC compartment of the lung was preceded by transitory expansion and stemness reprogramming of lung CD271+ MSCs, an increase of intracellular *Mtb*-CFU, and their release into the extracellular area. These findings imply that activated d*Mtb* released by reprogrammed MSCs invaded non-MSC lung cells, causing TB disease reactivation. Interestingly, despite the fact that the non-MSC lung compartment is unlikely to play a role in d*Mtb* reactivation in this mouse model, a sevenfold increase ($P < 0.05$) (Figure 16.1 D) of *Mtb*-CFUs were observed in the non-MSC lung compartment in the first 4 days of MHV-1 infection. The presence of MSCs during immunomagnetic sorting due to cross contamination may have led to the initial increase in *Mtb*-CFUs. The d*Mtb* reactivation, however, was associated with an immediate reduction in MHV-1 virus loads in lung tissue, as well as direct antiviral activity of the culture supernatant of reprogrammed MSCs or ASCs. In addition, the presence of d*Mtb* in MSCs enabled MSC to ASC reprogramming, as evidenced by a threefold to fourfold increase in CD271+ MSC number and *Oct4*, *GCL*, and *ABCG2* gene expression (Figure 17). Following 2 weeks, these reprogrammed MSCs activated genes associated with differentiation/apoptosis (Figure 17), demonstrating a loss of fitness further confirmed the ASC phenotype of these cells. These findings suggest a considerable interaction between the two concurrent infections in the lung MSC compartment, which could shed light on MSC-mediated defense against MHV-1 infection and how d*Mtb* exploits potential altruistic defense mechanisms for reactivation.

The present findings reveal preliminary evidence of an ASC-based altruistic defense against viral infection of alveolar stem cell niches (Figure 19) composed of type II alveolar cells, MSCs, and endothelial progenitors. These ASCs showed cytoprotective or niche defense

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potential against MHV-1 mediated toxicity of lung alveolar epithelial cells (ATII). The conditioned media of ASCs (reprogrammed CD271+ MSCs) obtained from MHV-1 infected mouse lung demonstrated a direct antiviral and cytoprotective function on MHV-1 infected ATII cells by lowering viral load and enhancing ATII cell survival/ proliferation (Figure 18).

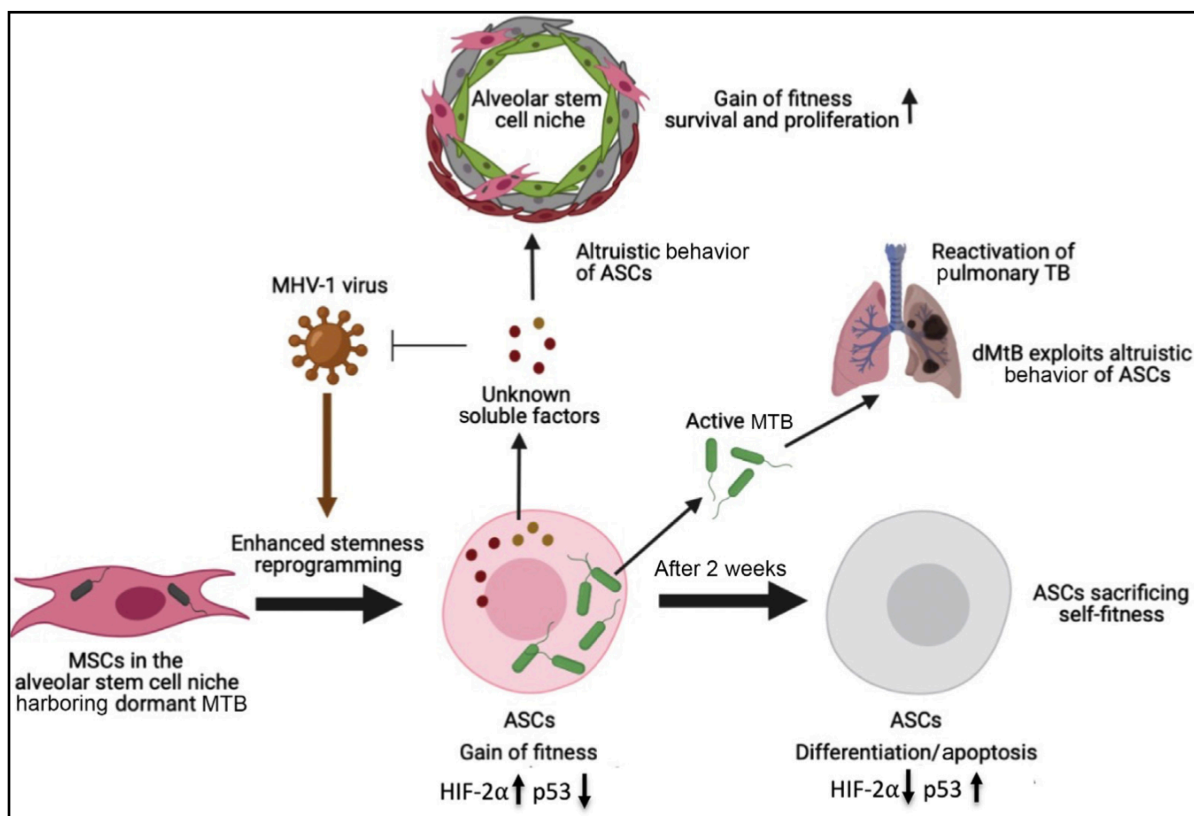

Figure 19: Evidence of an ASC-based altruistic defense against viral infection of alveolar stem cell niches using a mice model of Mtb dormancy. *The viral infection induces altruistic stem cell (ASC) reprogramming of lung mesenchymal stem cells (MSCs). These ASCs secrete yet unknown secretory molecules to enhance the group fitness of the type II alveolar cells reside in the alveolar stem cell niche. The ASCs sacrifice their higher fitness, thus undergo spontaneous apoptosis/differentiation by activating p53 upstream genes. The dMtb exploits this ASC- based stem cell niche defense to cause reactivation of pulmonary tuberculosis (PTB).*

Importantly, MSCs have been reported to have antiviral properties against many viral diseases (Rocha JLM *et al*., 2021, Teshima T *et al*., 2022). MSCs are resistant to viral infections

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compared to other differentiated cells due to the presence of IFN-stimulated genes (ISG) that can target at many stages during viral cycle (Bailey CC *et al*., 2014, Schoggins JW, 2019). The ISGs including PMAIP1, ISG15, SERPINE1, CCL2 and IFI6 can limit the viral infection such as ebola, SARS, influenza A and dengue (Bailey CC *et al*., 2014). MSCs release non coding miRNA with potent antiviral activity of targeting viral replication of hepatitis C virus (HCV) (Qian F *et al*., 2016). MSCs have the potential to modulate the function of immune cells (Naji A *et al*., 2019). BM-MSCs are showed to reprogram macrophages into an anti inflammatory profile and improve the survival in murine model of sepsis (Vasandan, A, B., *et al*., 2016, Shi Y *et al*., 2018). In this context, our findings of antiviral potential of reprogrammed MSCs or ASCs have significant impact in the field of vaccine development against viral diseases. Considering as entirety, this model of pathogen-induced stemness reprogramming of CD271+ MSCs in the lung could be used to explore stem cell altruism and its function in pathogen/host interactions.

Understanding the mechanism of stem cell niche defense against *Mtb* invasion may facilitate vaccine development against dormant *Mtb* (Caño-Muñiz S *et al.*, 2017). Importantly, this chapter uses a stem cell mediated mice model of *Mtb* dormancy which showed that even a few number of dormant intracellular *Mtb* are enough to reactivate the disease during inflammation. This finding indicates the necessity of complete elimination of the intracellular *Mtb* to prevent reactivation. Different approaches have been reported to kill the intracellular *Mtb* including the potential of MSC in killing intracellular *Mtb* by secreting nitric oxide (Khan *et al.*, 2017, Bogdan, 2015) and by rapamycin via inducing autophagy (Fatima *et al.*, 2020). However, the efficacy of these approaches for complete elimination of *Mtb* from stem cell niche have not been tested. In this context, our model of *Mtb* dormancy may be utilized to evaluate the efficacy of these approaches for effective clearance of intracellular *Mtb*. A bone-homing PEGylated liposome nanoparticles (BTL-NPs) was used to load rifampicin (RIF) and isoniazid (INH) anti TB drugs for *Mtb* clearance in CD271+BM-MSC in a mice model. This approach showed decreased TB relapse rate in the mice (Garhyan J *et al*., 2020), but not complete clearance of d*Mtb*. Our stem cell mediated mice model of *Mtb* dormancy may be utilized to develop novel drugs against dormant *Mtb* for effective and complete clearance of d*Mtb* from the stem cell niche. Interestingly, researchers showed that anti-inflammatory drug celecoxib reduces the survival of *Mtb* intracellular to CD73+/Sca-1+ lung MSCs in a INH treated mice model of *Mtb* infection (Jain N *et al*., 2020). This finding suggests that managing inflammation by anti-

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inflammatory agents may reduces the dynamic interaction between stem cell niche and lungs granulomas and thus, reduce the probability of reactivation (Pathak L and Das B, 2020). This potential may be investigated by utilising our MHV-1 mediated inflammation induced stem cell based *Mtb* reactivation model of mice.

2.5 Conclusion

The findings suggest that stem cell altruism is a fitness defense strategy that shields vulnerable neighbours in their niche. Altruistic behaviour in bacteria has been reported as a defense strategy against antibiotic exposure; a few bacterium members sacrifice self-fitness to improve group fitness, comparable to kin selection observed in biological altruism. The findings of this study give the first indication of an ASC-based altruistic defense against viral infection of alveolar stem cell niches. It is observed that MHV-1 infection triggers d*Mtb* loaded MSCs to reprogram into ASCs, and MHV-1 load reduction. Thus, this work may aid in understanding how MSCs triggering ASC defense mechanisms may aid in battling viral load in the host, thereby aiding in the development of viable anti-viral treatment not only for COVID-19, but also for other fatal viral diseases.

Chapter 3.0.0

To study the role of stem cell niche in TB recurrence in the rural population of north-east region.

Abstract

Background: In India, the rural population of Northeast region faces a higher prevalence of TB recurrence and multi drug resistance (MDR) TB. Our lab's affiliated organization, the KaviKrishna Telemedicine Care (KTC) has initiated a community based participatory action research (CBPAR) to take care of these rural TB subjects in North East India. KTC has been taking care of these rural TB subjects' well being and long-term monitoring by utilizing an indigenous biosocial medicine approach. Using a cohort of this TB population, bone marrow-Mesenchymal stem cell (BM-MSC) was identified as host cells for dormant *Mtb,* the causative pathogen of TB. Recovering the viable *Mtb* intracellular to BM-MSC from the TB subjects successfully treated with anti-TB drugs, indicates that *Mtb* intracellular to MSCs can escape the anti-TB drugs. In this present work, we hypothesised that these intracellular d*Mtb* may evolve into MDR strain of *Mtb* and contribute to treatment failure and recurrence. We also hypothesised that the biosocial medicine approach, the Avatar-Kosha may reduce inflammation, as well as recurrence. **Methodology:** We have utilized the KTC network to select n=35 patients. From these patients we obtained circulatory MSCs from peripheral blood mononuclear cells (PBMCs) obtained from n=28, did *Mtb* culture, and *Mtb*-DNA extraction for whole genome sequencing (WGS). We also isolated *Mtb*-DNA from cultured sputum samples of same TB subjects. We also studied the biosocial medicine effect on recurrence by performing the Sahasa-ojash scale. **Results:** The live *Mtb* was harboured within circulating MSCs (c-MSCs) of 12 subjects, with WGS performed on samples from 7 subjects. Notably, one subject exhibited rpoB mutation within c-MSCs despite not displaying the mutation in sputum-derived *Mtb*. In three other subjects, c-MSCs contained *Mtb* with a compensatory rpoC mutation, absent in sputum-derived *Mtb*. Furthermore, the c-MSCs from these four patients displayed an altruistic stem cell (ASC) phenotype. Importantly, all four subjects exhibited disease recurrence within two years of the completion of therapy. The Avatar-kosha based intervention showed marked reduction in CRP, and ESR level, as well as sustained increase of sahasa-ojash scale. **Conclusion:** These findings indicate the potential role of MSCs in TB recurrence and MDR evolution.

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3.1.0 Background

Studies have reported the MSCs as protective site for *Mtb* to remain intracellular in dormant state. The findings of previous chapter suggest that dormant *Mtb* intracellular to MSCs exploits the ASC based niche defense mechanism to reactivate the disease. Viable *Mtb* in bone marrow CD271+MSCs (CD271+BM-MSCs), lung CD271+MSCs, and circulating CD271+MSCs (CD271+c-MSCs) during inflammation indicated the niche to niche interaction of the pathogen for reactivation of the disease. Previously, viable, but non replicating *Mtb* was isolated from CD271+BM-MSCs of the post successfully treated TB subjects (Das *et al.*, 2013). This finding indicates that BM-MSCs may provide site for *Mtb* strain to resist anti TB drugs and thus may contribute in MDR-TB strain evolution. Indeed, it was also showed that BM-MSC provides an antibiotic protecting niche for *Mtb* (Garhyan *et al.*, 2015). In a cornel mice model, it was demonstrated that after treatment of the mice with the antibiotics rifampicin and isoniazid, *Mtb* effectively clears from the lungs and spleen. In contrast, *Mtb* persisted in the BM of antibiotic-treated mice (Beamer *et al.*, 2014). Furthermore, using a satavata-tarka process, as described (Das *et al*., 2020), we visualized the process of d*Mtb* evolution to MDR intracellular to stem cells (Mitra *S et al.,* 2024). Therefore, here we hypothesize that BM-MSCs may also help the *Mtb* strain to maintain their MDR phenotype. Importantly, BM-stem cells were reported to migrate to the area of inflammation (Crosby and Waters, 2010; Spaeth, Kidd and Marini, 2012). Previously, we proposed that the BM-MSC harbouring d*Mtb* may migrate to lung via circulation during inflammation caused by acute respiratory infection (ARI) and initiate the d*Mtb* reactivation in the lung. Indeed, the chapter 2 findings of increased CD271+ MSCs with increased intracellular *Mtb*-CFU in mice BM and circulation indicated that MHV-1 infection may induce the migration of CD271+MSCs containing *Mtb* from BM to area of inflammation, thus the lung. Then in the lung, these CD271+ MSCs were expanded with *Mtb*-CFUs and caused reactivation of the disease. Considering these findings, it can be speculated that the presence of *Mtb* intracellular to CD271+ c-MSCs in TB subjects may indicate the potential of reactivation or recurrence of the disease in future. Therefore, in this chapter, we planned to evaluate the presence of *Mtb* intracellular to CD271+ c-MSCs in active TB subjects, and whether the c-MSC helps the *Mtb* strain to maintain their MDR phenotype. We monitored the TB subjects having c-MSC with *Mtb* to further evaluate whether these subjects show TB recurrence in future.

MDR-TB strains are resistant to at least isoniazid and rifampicin, the 2 most powerful first line anti-TB drugs. The RIF resistance is caused by mutation in RNA polymerase B (rpoB) region of the *Mtb* strain (Yue J *et al.*, 2003, Ma X *et al.*, 2006) and INH resistance is caused by mutation in katG and inhA region (Caws M *et al*., 2006 Click ES *et al*., 2020). Importantly, resistance to rifampicin (RIF) is used as a marker for MDR TB as 90% of RIF resistance *Mtb* strains are often show resistance to isoniazid (INH) (Atashi S *et al*., 2017, Katoch CDS *et al*., 2021). RIF-resistance is caused by mutations in the "hot spot region" of 81-bps between codons 507 to 533 of the *rpoB* gene. This region is known as the rifampicin resistance determining region (RRDR) (Yue J *et al*., 2003, Ma X *et al*., 2006). The most common rpoB mutation is in 516, 526, 531 (Yue J *et al.*, 2003, Shea J *et al*., 2021). Mutations in rpoB cause fitness cost of the *Mtb* strain (Napier G *et al*., 2023) which may impact in virulence and transmission potential. However, the *Mtb* strain still remain highly transmissible, indicates that rpoB mutated strain may undergo successful compensatory evolution (Meftahi N *et al*., 2016). Interestingly, a recent study of WGS identified a set of compensatory mutations in the rpoC and rpoA region of the 30% MDR-*Mtb* strains (Comas *et al*., 2011). However, the fitness mechanisms for the rest of the 70% MDR clinical isolates remain unclear. Amongst rpoA and rpoC, rpoC compensatory mutation was reported to be strongly associated with the most common rpoB mutation; S531L allele (Song T *et al.*, 2014). Therefore, it was speculated that the rpoC 'compensatory mutations' may contribute to increase the fitness of rpoB or MDR-*Mtb* strain (Brandis *et al*., 2012). There are many rpoC mutation, however few were tested for the MDR fitness gain, which can be referred as compensatory mutation. These compensatory mutations are found in the codon from 452 to 492 (Song T *et al.*, 2014). Importantly, it has been reported that 10% of treated TB subjects have the potential to recur and about 18% of the recurred cases exhibit rpoB mutation or MDR phenotype. Therefore, we hypothesised that infection mediated inflammation may activate the stem cell niche to niche interaction between lung and BM, thus *Mtb* with MDR phenotype may be recovered from the CD271+c-MSCs of TB subjects with inflammation. We speculated that the mutation occurs in rpoC region of *Mtb* intracellular to BM-MSC and may slowly evolve to mutation in rpoB. Recovery of viable *Mtb* with rpoB or rpoC mutation from CD271+c-MSCs of TB subjects may indicate the initiation of recurrence of the disease. Therefore, we planed to investigate the presence of *Mtb* intracellular to circulating MSCs in TB subjects and whether these intracellular *Mtb* exhibit compensatory rpoC or rpoB mutation (Figure 20). The rpoB and

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rpoC mutation was also evaluated in sputum samples of TB subjects. To investigate this possibility, we collected sputum and blood samples from the suspected PTB cases (n=50). First, viable *Mtb* intracellular to CD271+c-MSCs was evaluated as previously shown in BM derived CD271+MSCs (Das B *et al.*, 2013). Next, the presence of rpoB and compensatory rpoC mutation was evaluated in the *Mtb* strain obtained from sputum and CD271+c-MSCs as previously described (Comas *et al.*, 2011).

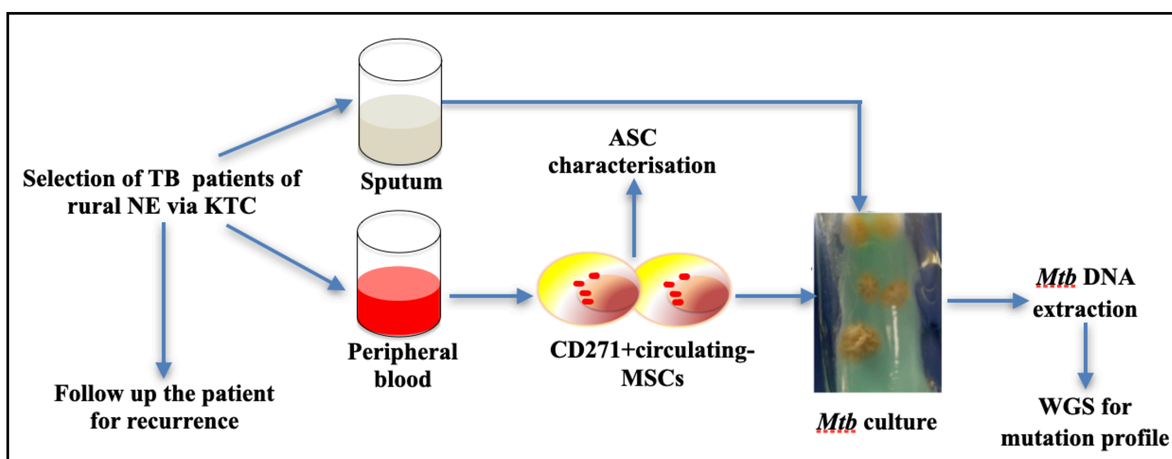

***Figure 20**: **Experimental design of investigating the role of stem cell niche in TB recurrence and MDR evolution**. Peripheral blood and sputum will be collected from the selected TB subjects and 1. evaluate the presence of Mtb intracellular to c-MSC, 2. Evaluate drug resistance related mutations of intracellular Mtb and sputum derived Mtb strain.*

3.2.0 Methodologies

3.2.1 Establishment of Rural Telemedicine Network for the patient management: To develop a novel rural public health approach, researchers need to conduct clinical research amongst the rural population. This study is proposing an Indigenous Knowledge System (IKS) based biosocial medicine approach, where we have uncovered an indigenous Kamarupa information network (IKIN) prevalent in the rural Kamarupa (Das *et al.,* 2020); Jiva Upakara Cikitisha Tantra (JUT). This biosocial medicine based Jiva Upakara Cikitisha Tantra approach emphasizes the interconnectedness of biological, psychological, and social factors in influencing health and well-being. The key elements of this approach include: 1) Community-Based: The system emphasizes the role of community-based healing practices and the importance of social

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networks in promoting health – the Vidhata kosha. 2) Holistic Assessment: Practitioners consider the physical, mental, and social aspects of an individual's health when providing care. 3) Personalized Treatment: Treatment plans are tailored to the specific needs of each patient, taking into account their individual circumstances and cultural background. 4) Integration of Traditional and Modern Medicine: The system incorporates traditional healing practices with modern medical interventions, providing a comprehensive approach to healthcare. 5) The Avatar Kosha: The Avatar Kosha is a central concept in the Jiva Upakara Cikitisha Tantra. It represents the dynamic interaction between an individual and their environment, encompassing both physical and non-physical aspects. The Avatar Kosha is influenced by various factors, including biological, psychological, social, and spiritual elements. 6) The Satvata-tarka: this is a systematic method of reasoning and debate that is central to the Jiva Upakara Cikitisha Tantra based training for tantric healers. It involves several distinct stages: a) Iccha-Prataskhya (Observation of Desire). This initial stage involves observing and understanding the underlying desires or motivations that drive an individual's actions and beliefs. It is essential to identify the root causes of problems or challenges that individuals may be facing. b) Prataskhya (Observation). This stage involves careful observation and analysis of the situation or problem at hand. It requires gathering information, identifying patterns, and understanding the underlying factors that contribute to the issue. c) Satavata/Purvavat-Anuman (Inference Based on Prior Knowledge). This stage involves drawing inferences based on existing knowledge and experience. It requires applying logical reasoning and critical thinking to understand the implications of the observed phenomena. d) Yukti-Tarka (Suppositional Reasoning). This stage involves exploring different possibilities and considering alternative explanations. It requires creativity and imagination to generate new ideas and perspectives. e) Samasya-Lakshana (Special Feature of Doubt). This stage involves identifying the specific areas of uncertainty or doubt that need to be addressed. It requires careful analysis and critical thinking to pinpoint the key questions that need to be answered. f) Pariksa-Dharana (Examination of a New Idea). This stage involves examining and evaluating new ideas or hypotheses. It requires careful consideration of evidence, arguments, and counterarguments. g) Pragna-Agan (Knowledge Emergence). This final stage involves the emergence of new knowledge or understanding as a result of the reasoning process. It represents the culmination of the Satavata-tarka method, where the initial observations and analysis lead to new insights and perspectives. By following these stages, an Ojash or tantric healer can engage

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in a systematic and rigorous process of reasoning and debate about sickness, leading to deeper understanding and the emergence of new knowledge of healing. 7) The Role of the IKIN temple network: The IKIN provides access to temple based clinics, lifestyle coaching, and disease monitoring tools, the IKIN enhances the reach and effectiveness of the program. 8) Nigudah Yoga and herbal nutrition. The special breathing yoga, indigenous whey protein supplements, and 36 herbal plants (Sandhya S *et al*., 2024) are the key elements of physical supplements to nurture the mind and body for effective healing. Thus, JUT is a traditional Kamrupian Healing art.

We speculated that IKS based focused group discussion (FGD) or guided interaction between patients, their family members and health practitioners can be applied to assess the impact of the Jiva Upakara Cikitisha Tantra on individuals' well-being in a community based participatory action research (CBPAR). Therefore, the Sahasa-Ojash scale was developed to measure the central element of JUT; Avatar-kosha in an individual. This scale measures the biosocial effects of the interventions, focusing on: Physical, Mental and Social Well-being. By measuring these various dimensions, the Sahasa-Ojash scale provides a comprehensive assessment of an individual's overall well-being.

3.2.2 Measurement of biosocial medicine effect by Sahasa-ojash scale: KaviKrishna Telemedicine Care (KTC) is situated in Sualkuchi-Hajo area of Assam, which is situated in Kamrup district with total population of 50000 and very high population density of 3000/km2. We conduct FGD every week amongst the devotees of different temples located in the area, school/college students, health practitioners, as well as patients family members at KTC. This led the "knowledge emergence" on understanding the disease and associated information amongst them. The telemedicine coordinators bridge the gap between the rural patients and their respective hospitals/Physicians via tele-health platform. Also they keep the records in every FGD (Vaishya T *et al.*, 2024). The selected volunteers are trained to collect information on disease prevalences in their specific area and a mapping is done along with the population for each locality. For the stem cell based MDR-TB evolution and TB recurrence study, we have screened the TB subjects utilizing this IKIN network. Then, the Sahasa-ojash scale of selected TB subjects was measured. The Sahasa-Ojash scale is a comprehensive measure of biosocial well-being developed by KaviKrishna. It assesses various dimensions of an individual's health, including physical, mental, and social factors. This scale measures the biosocial medicinal effects of

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Avatar-kosha. Components of the Sahasa-Ojash Scale: 1) Physical Health: Measured using the Health-Related Quality of Life (HRQOL) scale, which assesses physical function, role limitations, general health, vitality, pain, and energy. 2) Mental Health: Measured using the Rosenberg Self-Esteem Scale, which assesses an individual's self-worth and self-confidence. 3) Social Engagement: Measured using the SACS 0-7 scale, which assesses an individual's perceived collaboration skills and social engagement, capturing the essence of "Nadi" in the Vedic framework. 4) Anxiety: Measured using the Hamilton Anxiety Rating Scale, which assesses the severity of anxiety symptoms. The Sahasa-Ojash score is calculated using the following formula:

Sahasa-Ojash score = (HRQOL score) x (Rosenberg self-esteem score) x (Collaboration score) / (Hamilton anxiety score).

To assess the Sahasa-Ojash scale, study subjects complete questionnaires for each of the component measures. Utilising the telemedicine platform and following all the safety protocols of TB transmission, we have obtained the answers of the questionaries from the selected study subjects. The scores are then combined using the formula above to calculate the overall Sahasa-Ojash score. A higher Sahasa-Ojash score indicates better overall well-being, reflecting a balance of physical, mental, and social factors. A lower score may suggest areas where interventions or support are needed to improve an individual's quality of life (Das B 2019).

3.2.3 Participant selection and monitoring for the study: The study is conducted as per the Institutional Ethics Committee and Institutional Biosafety Committee guidelines. We also have the approval from RNTCP, Assam for collection of the TB patients' sample. The study protocol is also approved by the institutional committee for stem cell research, KaviKrishna. Participants were selected via KTC, utilizing the IKIN, which was previously also used to conduct clinical studies in TB (Das B *et al*., 2013, 2020) and cancer (Das B *et al*., 2019). These studies were also approved by the ethical committees of KaviKrishna. The newly diagnosed PTB patients (age 40-70, both sex without any major co- morbidity such as cancer, heart or kidney disease) were included in the study after obtaining informed consent from them. All study subjects were identified by observing the clinical symptoms for TB, as well as sputum-examination reports obtained from the local hospital. As per our hypothesis, our study included TB patients with inflammation. To select inflammation having TB patients, we looked for patients with persistent

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cough and chronic obstructive pulmonary diseases (COPD). Selected patients were monitored weekly by measuring their breathlessness: 6 minute walking test and spirometry test, as well as weight.

3.2.4 Sputum and blood sample collection for *Mtb* culture and identification: We have obtained sputum (n=50) from TB patients of north east region at KaviKrishna Telemedicine Care (KTC), Sualkuchi as per RNTCP guidelines of sample collection. 35 out of these 50 subjects were having productive cough, which indicated inflammation in the patients. From these 35 subjects, peripheral blood samples of 7-10ml/subject were collected in anticoagulant EDTA tubes. The samples were transported to KaviKrishna laboratory in a cold transport box on the same day of collection. The sputum samples were decontaminated with NALC/NAOH method and subjected to Zn staining for Acid Fast Bacilli (AFB). 100ul of processed sputum was used to isolate *Mtb* DNA by Qiagen kit method to confirm the *Mtb* and MDR status by PCR. The AFB positive, qPCR based *Mtb* confirmed processed sputum samples were cultured using LJ slant traditional method of *Mtb* culture and incubated at 37°C for 2-6 weeks until the colonies appear. Part of processed sputum was stored in 10% glycerol. The isolates were tested for susceptibility to first line drugs; RIF (40ug/ml) and INZ (2ug/ml). The revived *Mtb* colonies were subjected to duplex PCR to rule out NTM contamination. *Mtb*-DNA was extracted from the confirmed *Mtb* colonies and selected samples were sent for WGS in a reputed company. The various steps of the methodology is presented in Figure 21.

As per our hypothesis, we supposed to recover intracellular *Mtb* in the peripheral blood mononuclear cells (PBMCs) derived CD271+MSCs of TB subjects with higher inflammation. Therefore, the higher inflammation rate was determined by evaluating the c-reactive protein (CRP) (>30 mg/dL) and erythrocyte sedimentation rate (ESR) (>45 ml/hour) in the collected blood samples (Figure 25A). The CRP was measured using human CRP ELISA kit (RAB0096, Sigma Aldrich) and ESR was determined by the standard Westergren method as previously described (Westergren A, 1926). The TB subjects with higher CRP and ESR were subjected to ficoll (17144002, Ficoll Paque Plus, Cytiva, Sweden) based separation (n=28) to obtain PBMCs and stored in 10% DMSO for future use. During experiment, one drop of stored PBMCs was used to prepare the smear slide and checked AFBs under microscope. 100ul PBMCs were used to isolate *Mtb* DNA using Qiagen kit to do the PCR for *Mtb*, and rpoB mutation to confirm MDR status.

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3.2.5 PCR for *Mtb* and MDR confirmation: For *Mtb* confirmation, the MTB 16s specific region primer was used; (Forward: 5′ ACG GTG GGT ACT AGG TGT GGG TTT C 3′, Reverse: 5’ TCT GCG ATT ACT AGC GAC TCC GAC TTC A 3’ (Huard RC et al 2003). The confirmed *Mtb* samples were further subjected to nested PCR (make: Qiagen Rotor Gene- Q and software: Rotor-Gene Q 2.1.0.9) using MDR-TB specific primer (rpoB-516, 526 and 531 region) to confirm the MDR-*Mtb* strain. The thermal profile was applied as published previously (Tevere VJ *et al*., 1996)

3.2.6 Isolation of circulating mesenchymal stem cells (c-MSCs) and intracellular *Mtb*: The stored PBMCs of TB subjects were thawed and subjected to immunomagnetic sorting for CD271+ MSCs as previously described (Das B et al 2013). The cells were first subjected to negative depletion by using CD45 (#18259), CD33 (#18287HLA) and CD11b (#18770). The CD271+c-MSC population was obtained from these depleted cells using the EasySep Human CD271 positive selection kit (#18659). The sorted cells were cultured in serum-free defined media with the growth factor cocktails (TPO, SCF and Flt3 ligand) that allow the *Mtb* harbouring stem cells to expand, and the intracellular *Mtb* to replicate for the recovery of enough viable *Mtb* for culture. After a week of culture, the cells were lysed and subjected to LJ culture (n=28) to recover viable *Mtb* colonies. The revived *Mtb* colonies were subjected to duplex PCR to rule out NTM contamination. *Mtb*-DNA was extracted from the confirmed *Mtb* colonies and selected samples were sent for WGS in a reputed company. The various steps of the methodology is presented in figure 22.

3.2.7 Evaluation of ASC gene expression of CD271+c-MSCs: The sorted CD271+c-MSCs were cultured in serum-free media for 2 weeks and evaluated for expression of ASC genes by real-time quantitative PCR. The qPCR was performed using TaqMan primers at 40 cycles with 100 ng of starting cDNA. GAPDH was used as an endogenous control. The RNA was quantified by the delta-delta CT method using Q-Rex software version 1.1 (Rotor-Gene Q-Qiagen, New Delhi, India). The following TaqMan gene expression primers were used: human: Nanog (Hs02387400_g1), ABCG2 (Hs00184979_m1), HIF -1α (Hs00153153_m1), HIF -2α (Hs01026149_m1), Oct 4 (Hs03005111_g1), Sox2 (Hs00602736_s1), MDM2 (Hs01066930_m1), p53 (Hs01034249_m1), GAPDH (Hs00266705_g1), and CD133 (Hs01009250_m1).

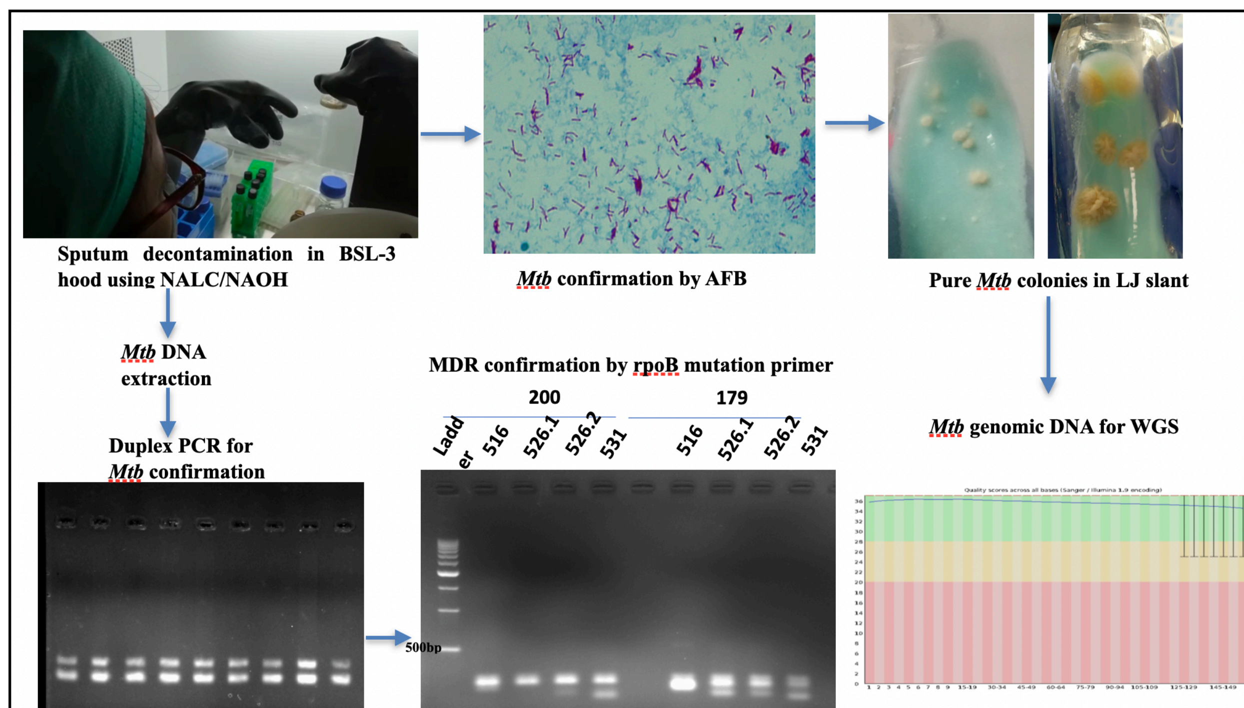

Figure 21: Methodology for extraction of Mtb DNA for WGS from Mtb colonies obtained in LJ slant of TB patient sputum samples*. The first image is showing the sputum decontamination inside BSC-III hood, followed by the AFB of a MDR-TB subject under a compound microscope, magnification 100X. The third and forth steps are Mtb detection and MDR detection using specific primer respectively. The fifth step is solid culture of decontaminated sputum followed by Mtb-DNA extraction and WGS*

3.2.8 *Mtb* DNA isolation for whole genome sequencing: For *Mtb* and MDR identification by PCR, we used Qiagen kit method for *Mtb* DNA extraction. For WGS, the *Mtb* colonies of the samples were subjected to DNA extraction using the sucrose lysis buffer followed by PCI and ethanol based extraction method (Käser M *et al.,* 2009). The isolated DNA were quality checked and quantified by Nanodrop spectrophotometer (model : Implen NP80) and 0.8% Agarose Gel Electrophoresis (Käser M *et al.,* 2009). The LJ slant colony derived *Mtb* samples were subjected to duplex PCR to confirm *Mtb*, not NTM. Once confirmed, the *Mtb*-DNA was sent to a reputed company for evaluating compensatory mutation by WGS using Illumina-Seq technology.

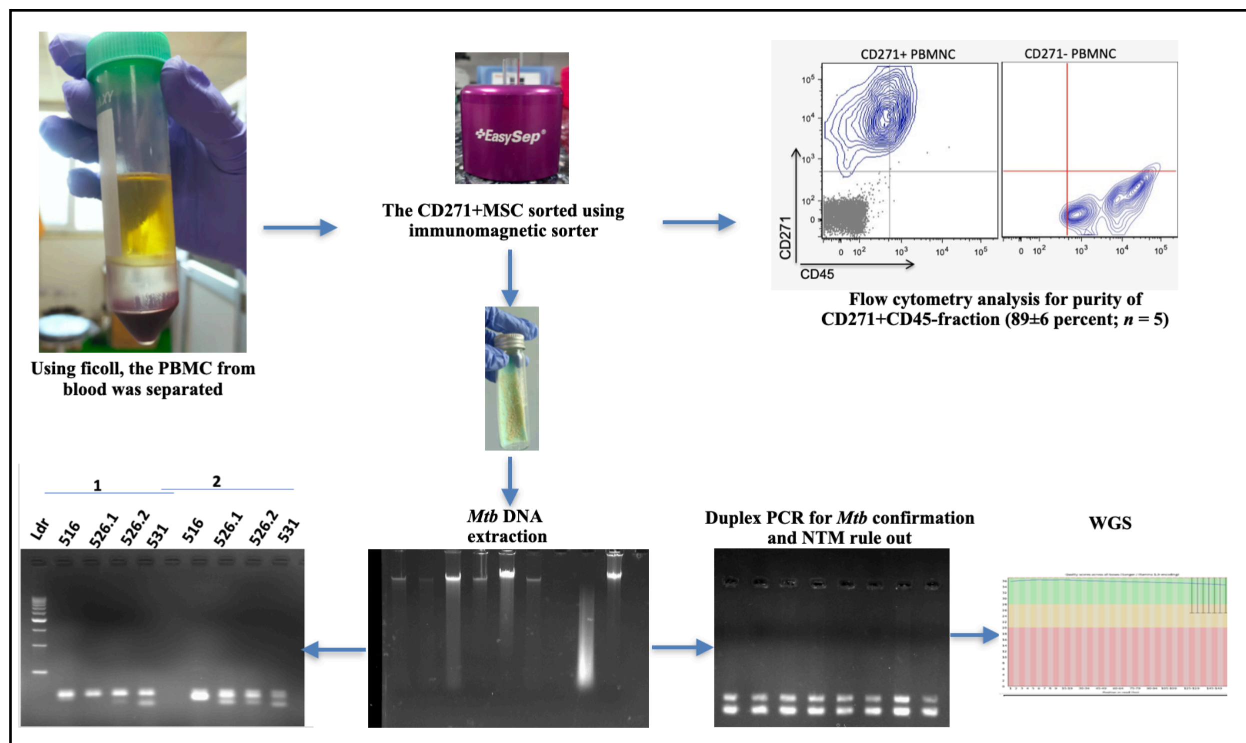

Figure 22: Methodology for extraction of Mtb DNA for WGS from Mtb colonies obtained in LJ slant of TB patient's PBMNC derived CD271+ c-MSCs. *The first image is showing PBMC layer in a 50ml falcon tube, obtained after ficoll based separation from peripheral blood of TB patient. The PBMCs were used to sort the circulating CD271+MSCs followed by intracellular Mtb isolation.*

3.2.9 *Mtb* DNA confirmation by duplex PCR: Amplification of the regions of genomic DNA from the controls, sputum derived *Mtb* DNA and LJ slant colony derived *Mtb* DNA isolates was performed with the primer set HSP N3 and HSP N4, amplifying a 300 bp region of the hsp65 gene (Varma-Basil M *et al.,* 2013). Second primer set was used to amplify a 190 bp region of the gene Rv1458c using the primer pair ABC T1 (5-GCAGCATTGAGGTACTTGGAC-3) and ABC T2 (5- TCGGTGAGACCCAAGGTGTC-3). *Rv1458c* is a gene encoding a putative ATP binding ABC transporter protein (tuberculist.epfl.ch). A total of 2.5 μl of the extracted DNA diluted with 2.5ul of nuclease free water was used as a template for PCR. The 20 μl reaction mixture consisted of 10micomoles of each primer of *hsp65* gene, 10 micromoles of each primer of gene *Rv1458c* and mastermix (2 x concentrations from Promega). The thermal profile consisted of an initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s,

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annealing at 60°C for 30 s and primer extension at 72°C for 1 min. Final extension was performed at 72°C for 10 min. The PCR product was analysed by electrophoresis on a 1.5% agarose gel. A 50 bp DNA ladder (Hi Media, Maharastra, India) was used as a molecular size marker.

3.2.10 Library preparation, sequencing and analysis: Genomic DNA quality was checked by 0.8% AGE (Käser M *et al.,* 2009) and quantity was checked in Nanodrop spectrophotometer (model : Implen NP80). Clear band image and 50ng/1ul concentration of DNA was selected for WGS. The isolated *Mtb* DNA were outsourced to a company for library preparation and whole genome sequencing using the Illumina Hi-Seq 2500 platform (100X coverage) on a 150*2 paired-end reads basis. DNA-Seq library was prepared according to Illumina instruction. Library DNA was checked for concentration and size distribution before sequencing with an Illumina HiSeq 2500 system according to the manufacturer's instructions (HiSeq 2500 User Guide). Two sequencing libraries were created: a fragment library (~150bp inserts) and jumping library (~3-5kb insert). Libraries were sequenced on Illumina HiSeq2500 instruments. Standard Illumina adapters were ligated to each DNA fragment and paired end reading was done. The raw sequenced data have been quality checked using the FastQC version v0.11.9 and were mapped with the reference genome of H37Rv (NC_000962.3) using the Burrows Wheeler Alignment tool (BWA-MEM) (Li Heng., 2013). The samples with below 90% mapping was excluded from the study. Now the aligned files were be further subjected to GATK analysis-variant calling (McKenna A *et al.,* 2010). The vcf files were then subjected for variant annotation using Annovar tool for identification of SNVs and Insertions/deletions (In/dels) using Pindel (Ye K *et al.,* 2009). The variant annotated file obtained was further analysed downstream for identifying the potential compensatory mutation sites.

3.3.0 Results

3.3.1 Characteristics of study population

Utilising the IKIN, we obtained sputum from TB patients of north east region (n=50) at via KTC (Figure 23). The 35/50 TB subjects were having productive cough, which indicated inflammation. The peripheral blood samples were collected from these 35 subjects. In 28/35 TB subjects, higher inflammation rate (80%) was further confirmed by evaluating CRP (>30) and ESR (>45) in the collected blood samples. From these 28 TB subjects, we isolated c-MSCs. We obtained the patient's TB history, family history of TB, history with other diseases, alcohol/ tobacco consumption status, persistent cough, weight loss and COPD status. We could recover intracellular *Mtb* from CD271+MSCs of 12 TB subjects (42.8%). DST result confirmed RIF resistance in sputum derived *Mtb* of 13 TB subjects (37%) (Table 4).

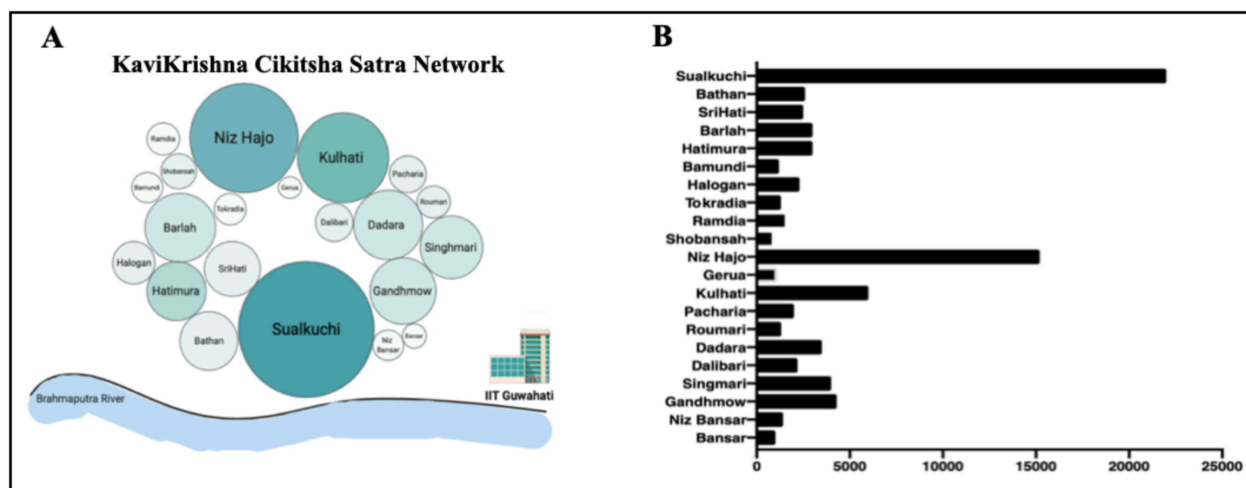

Figure 23**: The IKIN developed amongst rural Kamrup population under KTC supervision. A*. *The rural areas are presented as network.* ***B*. The population of each network in the Sualkuchi-Hajo area.*

Table 4: The table provides the clinical characteristics and laboratory diagnostic details of selected TB subjects.

	Productive cough	Fever	Family history of TB	MDR positive (PCR)	DST (RIF)	CRP (>30mg/dL)	ESR (>45ml/hour)
Gender							
Male: 22 Female: 13	24 11	10 10	2 0	6 2	8 5	16 12	16 12
Age Group							
30-50 years >50 years	14 21	8 12	0 2	2 6	6 7	15 13	15 13
Patients with COPD							
Yes No	19 16	8 12	1 1	3 5	8 5	17 11	17 11
Alcohol consumption							
Yes No	14 21	5 15	0 2	3 5	4 9	10 18	10 18
Tobacco consumption							
Yes No	22 13	6 14	2 0	2 6	4 9	10 18	10 18

35 TB subjects with higher inflammation were selected for the collection of peripheral blood and isolation of c-MSCs. Sputum and peripheral blood of these subjects were processed for Mtb culture. The table includes the laboratory diagnostic details obtained from the sputum samples of the TB subjects.

3.3.2 Isolation of viable *Mtb* from circulating MSCs (c-MSCs) of TB subjects

As we previously proposed that BM MSCs harboring d*Mtb* may migrate to lung during inflammation via circulation (Das B *et al.*, 2013), we wanted to evaluate the presence of viable *Mtb* intracellular to circulating MSCs in the active TB subjects. Also, we wanted to evaluate the

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association of c-MSC/*Mtb* with TB recurrence. To evaluate the hypothesis, the viable *Mtb* was first confirmed in smear microscopy of sputum. The AFB positive TB subjects by *Mtb*-CFU assay using traditional LJ based solid culture method. Good *Mtb* CFU was detected in 28/35 sputum in 2-5 weeks of culture. The CD271+MSCs were sorted from same subject's PBMNCs by immunomagnetic sorting, expanded in serum free media, lysed and cultured in LJ slant. The sputum sample derived LJ culture was used as control. The c-MSCs of 12/28 subjects showed *Mtb* CFU in 3-7 weeks of culture. Definitely, sputum derived *Mtb* culture showed more efficiency than c-MSC derived *Mtb* culture. This result confirmed that, viable *Mtb* can be recovered from circulatory MSCs of active TB subjects. The *Mtb* DNA extracted from both sputum and c-MSCs was sent for WGS. The sequenced data was mapped with the H37Rv (Rv0668) reference sequence (NC_000962.3) and obtained 90-99% mapping. The findings of intracellular *Mtb* detection indicates the possible future TB recurrence. Thus, we maintained follow up of all the TB subjects to identify TB recurrence.

3.3.3 Circulating MSC (c-MSC) of TB subjects provide the site for MDR evolution

We hypothesized that stem cell doesn't only provide niche for *Mtb* to reside, but also provide niche to resist the anti TB drugs. Thus, we speculated that intracellular *Mtb* may exhibit MDR associated mutations; rpoB and rpoC. As per the stem cell niche defense hypothesis, the intracellular *Mtb* of c-MSCs of non MDR TB subjects may exhibit mutation in rpoC region and may slowly evolve to mutation in rpoB. These potential compensatory mutations in turn may also enable the reactivation of the harbouring viable dormant *Mtb* within the patients.

The WGS was performed on *Mtb*-DNA of c-MSCs of 7 TB subjects. The raw data is deposited in NCBI-SRA database (PRJNA1054003). These subjects were confirmed as 3 MDR and 4 non MDR TB subjects by MDR specific PCR and DST for RIF/INZ. To validate the findings of tentative compensatory mutation sites in the *rpoC* gene region, we primarily focused on the non-synonymous mutations among all the other types of mutations, as reported in previous studies (De Vos M *et al.,* 2022, Wang S *et al.,* 2020). In one sample (KKL_4), we strikingly found 80% non-synonymous mutations (4/5) in the tail portion of *rpoC* gene region of *Mtb* DNA obtained from c-MSCs. Interestingly, this c-MSC derived *Mtb* DNA has also shown mutation in 531 codon of rpoB region. In this KKL_4, we also found some potential compensatory mutations in the rpoA-rpoC interaction site of the sputum *Mtb* DNA such as

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M475K, V486M, R481P, G483V, A492R. In another sample (KKL_5), there was about 54.5% (12/22) non-synonymous mutations obtained from across the *rpoC* gene region of *Mtb* DNA obtained from c-MSC. Amongst the mutations reported in KKL_5, we found some of the previously reported common compensatory mutations such as G483V, whereas no rpoC mutations were found in the sputum *Mtb* of this sample. There were no rpoB mutation in the c-MSC as well as sputum *Mtb* of this sample. The WGS result also showed rpoB mutation in the *Mtb* of sputum and c-MSC of 3 MDR subjects, which is consistent to PCR and DST result. Notably, one subject of 4 non MDR subjects showed rpoB mutation in *Mtb* intracellular to c-MSCs, without showing rpoB mutation in sputum derived *Mtb*. Intriguingly, in three other non MDR subjects, c-MSCs contained *Mtb* with rpoC mutation, independent of rpoC mutations in sputum *Mtb* (Figure 24). These findings indicate that the rpoB mutation evolves in *Mtb* intracellular to MSCs before it is diagnosed in sputum *Mtb*. The compensatory mutation in rpoC region of *Mtb* intracellular to c-MSC further indicates that the MSC provide site for compensatory mutation to compensate the low fitness of MDR, thus evolve from rpoC mutation to rpoB. This suggests the activation of niche to niche interaction and a probable TB reactivation with MDR evolution. Indeed, all these 4 TB subjects showed recurrence, 2 with MDR after 13-15 months of successful anti TB treatment, 1 active TB case was diagnosed as MDR after 2 months of first line treatment (Table 5). Importantly, the c-MSCs from these four subjects displayed the ASC phenotype (Figure 25B) when cultured invitro in serum free media, indicating the activation of stem cell niche defense. The ASC gene expression was positively correlated with higher inflammation, confirmed by higher CRP (>30 mg/dL) and ESR (>45 ml/hour) level (Figure 25A) in the blood samples of the TB subjects. In summary, the invitro and clinical results indicate the inflammation mediated activation of stem cell niche defense in the TB subjects, and its potential association with TB recurrence and MDR evolution.

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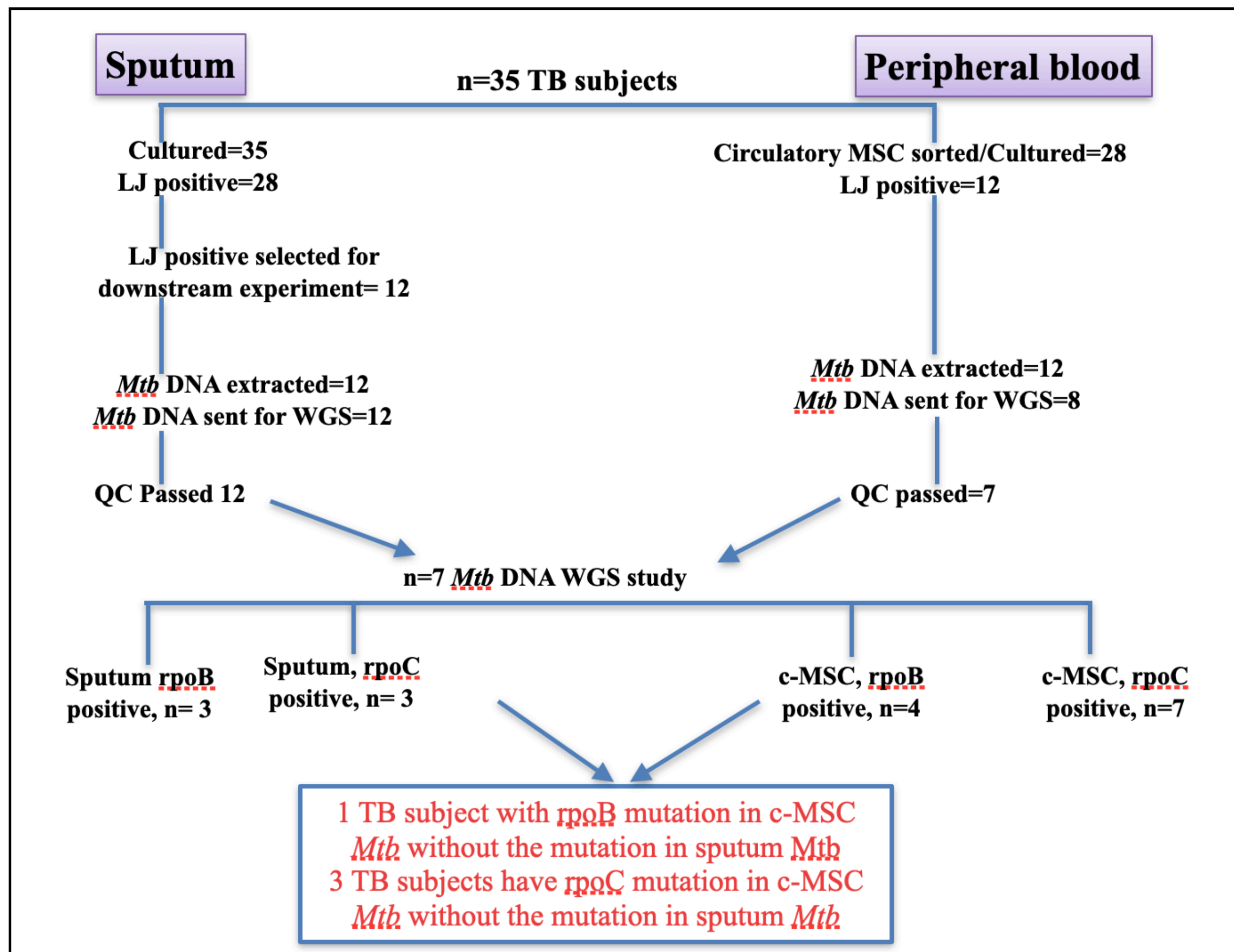

Figure 24: The rpoB and rpoC mutation analysis in sputum and c-MSC derived Mtb of TB subjects. *Genomic DNA of Mtb was isolated from Mtb colonies obtained from sputum and peripheral blood derived c-MSCs. The selected good quality Mtb DNA was sent for WGS to analyse the rpoB and compensatory rpoC mutation.*

Table 5: Association of rpoB/rpoC mutation of Mtb intracellular to c-MSCs with TB recurrence.

Patient number	RpoB mutation	RpoC mutation	Recurrence/MDR status
KKL_4	Sputum-No c-MSC-yes	Sputum-no c-MSC-yes	2 months after initial treatment with MDR
KKL_5	Sputum-No c-MSC-no	Sputum-No c-MSC-yes	13 months after completion of treatment with MDR
KKL_8	Sputum-No c-MSC-no	Sputum-No c-MSC-yes	28 months after completion of treatment without MDR
KKL_10	Sputum-No c-MSC-no	Sputum-No c-MSC-yes	15 months after completion of treatment with MDR
KKL_11	Sputum-yes c-MSC-yes	Sputum-yes c-MSC-yes	Passed away during treatment
KKL_13	Sputum-yes c-MSC-yes	Sputum-yes c-MSC-yes	Did not show recurrence during 3 years of follow up
KKL_15	Sputum-yes c-MSC-yes	Sputum-yes c-MSC-yes	Passed away 4 months after completion of therapy

The presence of rpoB and rpoC mutation of Mtb intracellular to c-MSC and Mtb from sputum of same TB subjects (n=7) were evaluated from the WGS data. The TB patients were monitored for 5 years to identify potential TB recurrence. Based on the WGS data and patient follow up, the association of c-MSCs with TB recurrence was analysed and summarised.

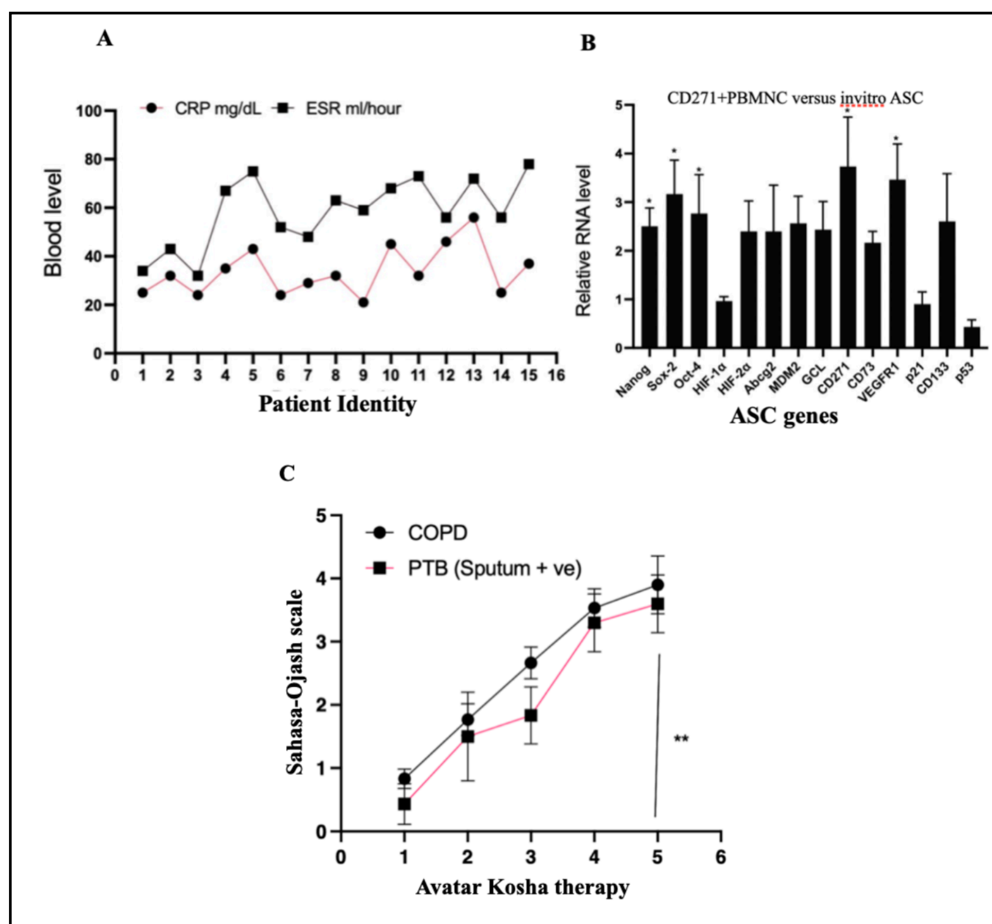

Figure 25: The Mtb harbouring c-MSCs of TB subjects with higher inflammation rate and lower Sahasa-Ojash scale exhibited gene expression of ASC phenotype. A*. The TB subjects with c-MSCs having Mtb-DNA were evaluated for inflammation by determining CRP and ESR.* ***B****. The TB subjects with higher CRP and higher ESR (n=16) exhibited the higher expression of ASC reprogramming genes in the CD271+c-MSCs after culturing for 2 weeks in serum free media. The relative RNA level of CD271+c-MSCs of TB subjects was compared with invitro ASCs.* ***C****. ANOVA analysis showed 16-fold increase in Sahasa-Ojash scale after five weeks of Avatar-kosha therapy. Data presented as means ± SEM (A-B). n= 4 independent experiments (A-B). *P < 0.05 (t-test), **P < 0.01 (ANOVA).*

3.3.4 Biosocial medicine effect on TB recurrence

We included all the 35 smear positive TB subjects to measure the Sahasa-ojash scale. We ensured these cases take anti TB treatment under NTEP. We gave home visit to these patients, and suggested the Nigudah care which included performing breath holding test, steam inhalation, and deep breathing. Patients were also given whey protein supplements, 20

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gm per day. Focused group discussion (FGD)through video chat was achieved each week of Nigudah care with the telemedicine team (Baishya T *et al.*, 2024). These patients were confirmed to be AFB negative for sputum (taken once weekly) and once confirmed, encouraged to participate in social function including Kirtan chanting in local temples and Namghars, as part of increasing social networking and participation. The 5 subjects with higher inflammation and ASC phenotype were excluded from the study as they did not show smear negative during the initial nigudah care of 4 weeks. Patients were suggested to follow the Avatar-kosha based psycho-social therapy for five weeks by engaging them in Satavata-tarka like FGD on a topic such as local culture, current political or economic situation in the area, and on any topic of their mutual interest. Each week, after the FGD session, Sahasa-ojash score was measured by taking the physical dimension score of SF-36 (0-100), Rosenberg self-esteem score (0-30), Self-Assessed Collaboration Skills (SACS) score (0-7), and Hamilton anxiety scale (0-56). Similar measurement was done for the COPD group as a positive control, as previous studies showed the benefit of Avatar Kosha therapy in enhancing the Sahasa-ojash score (Baishya T *et al*., 2024). For the COPD patients, before the therapy, the average physical dimension score of SF-36 was 48 with a SD of 22; the average Rosenberg self-esteem score was 7 with a SD of 3; the average SACS score was 2 with a SD of 1, and the average Hamilton anxiety scale was 32 with a SD of 6.

Therefore, we measured the average Sahasa Ojash score as 48x7x2/32 = 21. Since the final Sahasa-Ojash scale is calculated as the numerical score divided by 100, the average Sahasa-Ojash scale was 0.21. In a similar manner, the Sahasa Ojash scale of the PTB subjects (n=30) were also measured (Figure 25C). ANOVA analysis showed 16-fold increase in Sahasa-Ojash score after five weeks of Avatar-kosha therapy (Figure 25C). Next, we did quantitative assessment of breathlessness by measuring 6 minute walking test and spirometry test before and after the Avatar-kosha therapy. The cumulative results indicated the benefit of Avatar-kosha care to reduce the breathlessness and fatigue. A Fisher exact test was conducted to quantify the association between Avatar-kosha care and reduction of breathlessness. The results revealed a strong association, with an odds ratio of 3.04 (p=0.046), indicating that participants who received the intervention were less likely to experience

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breathlessness compared to those who did not. Interestingly, the study also demonstrated the close association between poor scores of sahasa-ojash and breathlessness. Furthermore, the Avatar-kosha therapy showed marked reduction in the CRP and ESR level at the end of the fifth week of therapy, in parallel to the rise of the Sahasa-ojash score. The subjects with higher inflammation and ASC phenotype showed less improvement in the self-efficacy with Sahasa-ojash scale (n=11 subjects), majority of these patients (n=9/11) did not show recurrence in 3 years of follow-up. The subjects with no ASC phenotype showed better improvement in the self-efficacy with Sahasa-ojash scale (n= 30-11= 19 subjects), they have fully recovered and none of them showed recurrence in 3 years of follow-up.

The findings of this study demonstrate the potential benefits of a CBPAR-based, holistic approach to healthcare for individuals with chronic diseases such as pulmonary TB. The results highlight the importance of addressing the social and environmental determinants of health, in addition to medical interventions, to improve overall well-being and to prevent recurrence.

3.4.0 Discussion

Stem cell niche defense mechanism plays a major role in reactivation of dormant Mtb intracellular to MSCs. This chapter was proposed to study the role of stem cell niche defense in TB recurrence in Northeast India. We speculated that in patients with pulmonary TB (PTB) both primary and recurrent PTB, the stem cell niche-to-niche interaction will be active between the bone marrow and alveolar stem cell niche. Therefore, we sought to isolate circulatory MSCs with intracellular *Mtb* in the patients with PTB. Importantly, we further hypothesized that MSCs may provide the niche to *Mtb* for MDR evolution. A novel rural telemedicine based approach has been utilised for screening and collecting sputum and blood samples from PTB subjects (Mitra S *et al.,* 2023). The blood derived PBMCs were subjected to immunomagnetic sorting for CD45-MSCs. The sorted cells were expanded, then lysed and placed in the LJ media to obtain enough *Mtb*. In the previous chapter, we isolated *Mtb*-CFUs from BM-MSCs, c-MSCs and lung-MSCs of MHV-1 infected mice. These findings of chapter 2 and the isolation of *Mtb*-CFUs in c-MSCs of 12 active TB subjects in chapter 3 suggested the potential niche to niche interaction of BM and alveolar stem cell in the TB subjects. We also hypothesized the inflammation induced

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migration of MSCs containing *Mtb* from BM to the circulation and then into the lung and cause recurrence of the disease. In this context, the 12 TB subjects with intracellular *Mtb* in c-MSCs were found having COPD or inflammatory state (high ESR and CRP) with weight loss. Also, only 2 patients of 12 TB subjects did not show recurrence in 2 years of follow up and rest of the patients exhibited treatment failure or recurrence. The invitro culture of these c-MSCs in serum free media showed the expression of ASC related genes. This result indicated a strong association of inflammation mediated ASC reprogramming of c-MSC containing intracellular *Mtb* with TB recurrence.

In PTB subjects, the treatment failure indicates the resistance of TB patients mainly to first line anti TB drugs; rifampicin and isoniazid. The current National Tuberculosis Elimination Programme (NTEP) protocol provides MDR treatment to TB patients positive for RIF resistance by detecting rpoB mutation in CBNAAT method (Khanna A *et al.*, 2022). We have also evaluated the rpoB status of intracellular *Mtb*. The results of WGS further confirmed the contribution of stem cell in *Mtb* being resistant to the anti-TB drugs. Due to low efficiency of LJ culture of intracellular *Mtb*, we could obtain WGS data of intracellular *Mtb* for 7 subjects. The rpoB mutation was confirmed in the intracellular *Mtb* strain of 4 PTB subjects, whereas the rpoB mutation was confirmed in sputum derived *Mtb* strain of 3 subjects. Thus, in one individual, while the c-MSCs showed MDR phenotype, sputum-derived *Mtb* did not show MDR. After 2 months, this particular patient showed the presence of MDR in the sputum despite anti tuberculosis treatment. Mutations in rpoB cause fitness cost of the *Mtb* strain (Napier G *et al.*, 2023), thus the presence of compensatory mutations in *rpoC* gene (Brandis *et al*., 2012, Li QJ *et al.*, 2016, Comas *et al*., 2011) was expected to be found in the intracellular *Mtb* to enhance the fitness of *Mtb.* There are few rpoC mutations tested for the MDR fitness gain; from 452 to 492 codon (Song T *et al.,* 2014). Considering these rpoC mutation as compensatory mutation, we found compensatory mutation in all the 7 intracellular *Mtb* strain. Whereas, the 3 corresponding sputum derived *Mtb* genome did not show compensatory mutations. Consistent to previous findings (Song T *et al.*, 2014), we also found the most common rpoB mutation; S531L allele in the 4 intracellular *Mtb* strain. Three of these 4 subjects were recruited in the study as MDR and one as non MDR. Notably, this one non MDR subject exhibited rpoB and rpoC mutation within c-MSCs despite not displaying the rpoB and rpoC mutation in sputum-derived *Mtb*. In other three non MDR subjects, c-MSCs contained *Mtb* with rpoC mutation, absent in sputum-derived

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Mtb. This suggests that MDR characteristics might manifest within MSCs before appearing in sputum samples. Furthermore, the c-MSCs from these 4 subjects displayed ASC phenotype, indicating the activation of stem cell niche defense, aligning with pre-clinical evidence of *Mtb*-induced MSC niche defense activation. Importantly, all 4 subjects exhibited disease recurrence within 1-3 years of the completion of therapy. These results not only confirm the role of MSC in TB recurrence, but also their ability to enhance the fitness of the intracellular *Mtb* strain by providing the niche to acquire rpoC mutation for evolution to rpoB in future.

The findings of the association of c-MSC having intracellular *Mtb* with TB recurrence has great implication in the field of TB management. Also, this study is ravelling a novel insight of stem cell niche in MDR evolution of *Mtb* strain in a TB patient cohort from north east region. Utilising the telemedicine approach, the association of c-MSC in TB recurrence and MDR evolution of *Mtb* may be conducted in a larger population in future. Our findings have shown expansion of *Mtb* harbouring c-MSCs in the drug sensitive and drug resistant TB recurrence subjects. In future, these c-MSCs may be evaluated for ASC phenotype with additional experiments including the niche modulatory and cytoprotective potential (Pathak L *et al*., 2021) to fully elucidate the role of ASCs in TB recurrence and MDR evolution. Importantly, there is no diagnostic method to detect the recurrence potential of *Mtb* post competition of anti TB treatment. In this context, exploring the ASC based niche defense mechanism in the TB recurrence subjects may provide biomarker for detection of potential TB recurrence. Even with the preliminary data, our findings on rpoC and rpoB mutations in c-MSC *Mtb* isolates potentially offer valuable insights into future recurrence with drug resistance phenotype of *Mtb*. Importantly, it is reported that 18% MDR TB cases are previously treated drug sensitive TB cases (Sharma N *et al.*, 2020, Shivekar SS *et al.*, 2020). Moreover, northeastern states of India are reported with higher rates of MDR-TB (Singhal R *et al*., 2014, Mudliar SKR *et al*., 2022). Therefore, increased transmission of MDR *Mtb* strain is a major challenge for NTEP in eliminating TB from India (Husain AA *et al*., 2021). In this context, our findings of stem cell niche in MDR-*Mtb* evolution in a TB patient cohort from north east region may provide a novel approach for early diagnosis of MDR TB and TB recurrence. Also, this knowledge could pave the way for future therapeutic strategies targeting the *Mtb* in stem cell niche to improve TB treatment outcomes.

As proposed previously (Pathak L and Das B, 2021), a dynamic interaction between BM stem cell niches and lung granulomas may contribute to TB progression and drug resistance.

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Studies suggest that both smoking and COPD are risk factors for TB initiation and recurrence. Urban air pollution and viral infections, such as ARI and Asthma, can increase COPD incidence and worsen lung inflammation. Therefore, pulmonary inflammation in subjects with COPD and viral infections may further enhance the interaction between BM stem cell and lung niche, leading to *Mtb* reactivation.

Does anti-inflammatory therapy influence the dynamic interaction between stem cell niches and lung granulomas? Can affordable anti-inflammatory interventions decrease the risk of PTB progression and drug resistance? In this context, KaviKrishna Telemedicine Care has observed anecdotal evidence suggesting that managing inflammation and improving nutrition in PTB patients may reduce disease development and recurrence aligning with published research. Especially, anecdotal data indicates that Avatar-kosha based therapy can improve healing and reduce recurrence of PTB subjects (Baishya T *et al*., 2024). The present work on the Avatar-kosha therapy as shown in the Figure 25C provides additional data to KTC's anecdotal evidences. Interestingly, the Avatar-kosha therapy including the Nigudah yoga and nutrition showed marked reduction in the CRP and ESR level at the end of the fifth week of treatment, in parallel to the rise of the Sahasa-ojash score. These patients, showed no recurrence even after three years of follow up, suggesting the potential benefit of the Avatar-kosha therapy in reducing the inflammatory states, and thereby reducing recurrence.

The qualitative phenomenology of the study participants showing enhanced Sahasa-ojash supports the key concept and processes of Avatar Kosha therapy, such as, 1) Dhata and Nadi: The interaction between the patient's self-efficacy (Dhata) and the community collaboration network (Nadi) leads to the emergence of Sahasa-Ojash, the biosocial healing force. 2) Kshetra and Vak-Bubudhan Prajna: The patient and healer interact in a subtle or virtual field (Kshetra), leading to the emergence of wisdom or knowledge (Vak-Bubudhan Prajna). 3) Shakti and Avatar Kosha: The power (Shakti) of this wisdom activates the body's systems, leading to the emergence of the Vak-Vidhata Avatar Kosha and Sahasa-Ojash, strengthening the Vyadhikhamatva (ability to resist disease). 4) The Yantra of JUT is a conceptual instrument or tool used to facilitate the interaction between the patient and healer. Interestingly, in a separate study, we found that the Yantra that includes the Dhata, Nadi, Kshetra, Vak-Bubudhan Prajna, Vyadhikhamatva, Pancha-padika education system and Satavata-tarka connect healers and the patients through education and dialogue for patient's empowerment and cancer disparity reduction (Pathak L *et al*., 2019).

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Thus, it appears that Yantra and Avatar Kosha framework emphasizes the importance of community-based healing and the role of social interactions in health.

Our work also provides new insight on the importance of biosocial medicine. The Yantra component of Avatar-kosha incorporates both physical and mental aspects of health, aligning with the principles of biosocial medicine. The Yantra proposes a process of knowledge emergence, suggesting that community-based healing practices can contribute to the development of new insights and solutions for public health issues, a key promise of CBPAR. The Avatar-kosha based therapy including the Yantra approach could be applied to address mental health challenges, as it emphasizes the importance of social connection and community support. The Yantra framework could be used to develop community-based digital health programs for managing chronic diseases, such as diabetes or heart disease. However, this work on the Avatar-kosha therapy is very preliminary, and require rigorous studies to evaluate the effectiveness of the Yantra approach in improving health outcomes. Hence, future work is needed to develop digital health tools for extensive studies. Moreover, it is important to validate the Sahasa-ojash score by using the large scale study. Exploring ways to scale up the Yantra approach to reach a wider population would be beneficial in this era of digital health. In this context, our preliminary findings described in Figure 25C can aid in developing digital health model in investigating how the biosocial medicine approach can be integrated into existing healthcare systems to enhance patient care. In conclusion, by further exploring and developing the Yantra approach of Avatar-kosha therapy, we can potentially harness its power to improve health and well-being in communities across the globe.

3.5.0 Conclusion

The potential role of stem cell niche defense in the TB dormancy and reactivation is not yet clearly known. Here, we have recovered viable *Mtb* intracellular to circulating CD271+ MSCs of subjects with active PTB. Importantly, the intracellular *Mtb* exhibited compensatory mutation in ropC, whereas the patient's sputum did not exhibit compensatory mutation. Considering that MSCs contain mostly d*Mtb*, these data indicate that the stem cell niche may provide site for d*Mtb* evolution to MDR. Although the above data does not directly confirm the role of stem cell niche defense in d*Mtb* evolution to MDR phenotype, our work indicates an association of the niche defense with MDR evolution. We are revisiting our model of c-MSC as

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it may be a viable marker for detecting early recurrence. The compensatory mutated *Mtb* may help survive the MDR low fitness within the stem cell niche. The mechanism of MDR-TB dispersion in a community is not yet clear, as the incidence of exogenous versus endogenous MDR-*Mtb* strain data are not yet found in the NE population. The future analysis of the WGS data set of *Mtb* of c-MSCs may provide novel insight about the exogenous versus endogenous spread of MDR-*Mtb*, and thus the dispersal of the TB recurrence and MDR-TB in a given community. Finally, the further scaling of Sahasa-ojash based health care assessment may benefit the TB control program in NE.

Chapter 4.0.0

To investigate the role of stem cell niche defense in head and neck cancer recurrence using an in vitro model of MSC and CSC interaction

Abstract

Background: CSCs reside in their niches and may activate a niche defense mechanism to protect the tumor from external threats such as oxidative stress, immune cells, and therapies. We hypothesised that chemotherapy may inadvertently activate niche defense in the CSC niche, which may lead the reprogramming of CSCs into an aggressive tumor stemness defense (TSD) phenotype. These TSD exhibiting CSCs may further reprogram the MSCs into an enhanced stemness phenotype; ASCs, thus promoting tumor recurrence. To develop the invitro model of CSC-MSC interaction, we used SCC-25 cell line as representative head and neck squamous cell carcinoma (HNSCC). Also, a pre-clinical model was developed involving cisplatin-treated CSCs and their interaction with MSCs. **Methodology:** The SCC-25 cell line was treated with cisplatin, then EpCAM+ABCG2+CSCs were isolated by immunomagnetic sorting. Higher CSC frequency and higher stemness gene expression of EpCAM+/ABCG2+ cells were tested to confirm the TSD characteristics. Then, the conditioned media (CM) of TSD exhibiting EpCAM+/ABCG2+ cells were collected and used for in vitro culture of human BM progenitor cells to study the cross talk between TSD phenotype and MSCs. The TSD-CM treated MSCs were analysed for ASC phenotype by evaluating the CM of treated MSCs for cytoprotection of murine HSCs and MSCs in a mouse model of carboplatin-induced BM toxicity and also by determining the cells' p53/ MDM2 oscillation state. **Results:** The TSD-CM treated EpCAM+/ABCG2+ CSCs reprogrammed into the TSD phenotype. The TSD-CM was found to secret inflammatory mediator HMGB1, along with various growth factors such as VEGF and SDF-1alpha. Further, the TSD-CM reprogram the CD271+ MSCs to ASC niche defense. The ASC-CM was found to secret the immunosuppressive cytokines; IL-6, IL-10 and TGF-beta. **Conclusion:** In this manner, we found that the TSD-CM reprograms the CD271+/CD45- MSCs into ASC phenotype. The ASC reprogramming was found to be mediated by HMGB1/TLR4 inflammatory pathway. Notably, ASC-CM was found to promote the expansion of the dormant EpCAM+ABCG2+CSC population within the SCC-25 cell line. Thus, an in vitro model has been developed to study the role of stem cell niche defense in the HNSCC recurrence.

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4.1.0 Background

HNSCC is one of the commonest cancers with poor outcomes, late diagnosis and poor survival rate. Most of HNSCC cancer are diagnosed at stage III or stage IV. Chemoradiation with cisplatin is the standard management for these locally advanced unresectable head and neck cancer cases (Strojan P *et al.,* 2016, Noronha V *et al.,* 2018, Irawan C *et al.,* 2022). However, despite surgery or chemo radiation, 50% of stage III or stage IV cases develop locoregional or distant recurrence. Also, approximately 35% patients need changes in treatment plan due to cisplatin induced toxicity (de Roest RH *et al*., 2022). The overall survival period of more than 50% HNSCC cases is less than 3 years. Poor survival and distant recurrence indicate the therapy failure and lack of proper treatment management of advanced HNSCC cases. Therefore, it is important to understand the mechanism of therapy failure in HNSCC.

In this context, our lab has previously shown that cisplatin treatment increases the proliferation of a highly tumorigenic side population (SP) of osteosarcoma (HOS), neuroblastoma (SK-N-BE2) and rhabdomyosarcoma (RH-4) cell lines (Tucshida R et al., 2008). These SP cells were later reported to contain cancer stem cell (CSC) like population in different cell lines (Hirschmann-Jax C *et al*., 2004, Patrawala L *et al.,* 2005, Chiba T *et al*., 2006, Das B *et al*., 2007), including HNSCC cell lines; M3a2, M4e and 686LN (Song J *et al*., 2010). CSCs are the most aggressive cancer cells with self-renewal capacity and resistance to therapy (Phi LTH et al 2018, Li Y *et al*., 2021). Different CSC markers are identified in HNSCC cells including CD44+ (Prince ME *et al*., 2007), CD133 (Canis M *et al*., 2012), and ALDH (Clay MR *et al*., 2010). Importantly, ABCG2 CSC marker was highly expressed in CSCs of cisplatin resistant HNSCC cell line; HSC-3 (Murakami K *et al*., 2022), and HNSCC metastatic cell lines; M3a2 and M4e (Song J *et al*., 2010). EpCAM is another CSC marker (Munz M *et al*., 2009) which is shown to be highly expressed in cisplatin resistant HNSCC cell line (Noman ASM *et al*., 2020). These reports indicate that though different markers are identified for HNSCC CSC, the ABCG2 and EpCAM have more significance in context of HNSCC cells' drug resistance and metastasis.

In osteosarcoma and neuroblastoma model of xenograft, cisplatin treatment was shown to activate a VEGF/FLt1 autocrine signalling in the SP cells, and thereby the SP cells exhibit a stemness phenotype (Tsuchida R *et al*., 2008). In neuroblastoma, hypoxia and chemotherapy stress enhance the expression of Nanog, Sox2 and Oct4 in SP cells (Das B *et al*., 2007). Cisplatin treatment was further found to induce stemness pathways; Bmi-1 and IL-6/STAT3 in oral cancer

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(Nör C *et al.,* 2014). These findings indicate that instead of reducing cancer cell population, cisplatin therapy may increase the cancer cell proliferation. However, whether cisplatin can enhance tumor stemness in CSCs of HNSCC is not yet investigated. We speculate that cisplatin stress may reprogram the ABCG2+/EpCAM+ CSC population of HNSCC cells into a highly invasive TSD phenotype by enhancing the stemness, thereby may induce therapy resistance, metastasis, and recurrence. The reprogramming of CSCs could be the defense mechanism of CSC niche against the cisplatin stress. We previously have reported the ASC reprogramming of MSCs as stem cell niche defense response of alveolar MSC niche against viral infection. Numerous studies have reported that cancer cells including CSCs may reprogram BM derived MSCs including CD271+ BM-MSCs to pro-tumorigenic phenotype (Moskovits *et al.*, 2006; Bar, Moskovits and Oren, 2010). Therefore, here we further hypothesised that the TSD phenotype of CSCs may interact with the MSCs in tumor niche to establish a protumorigenic TME.

BM-derived MSCs are one of the major components of tumor stroma (Joyce JA *et al.*, 2009). CSCs were reported to recruit BM derived MSCs in the TME (Hall, Andreeff and Marini, 2007) to establish immunosuppressive TME by secreting immunosuppressive cytokines or inflammatory factors such as IL-6 (Rattigan *et al.*, 2010) and IL-1β (Tu *et al.*, 2008). Another immunomodulatory component HMGB1 was reported to promote stemness and tumorigenicity in breast (Zhao XL *et al.*, 2017), and colorectal cancer (Zhu L *et al.*, 2014). Interestingly a recent study reported that cisplatin treatment increases the secretion of immunomodulatory HMGB1 from non small cell lung cancer cells (Gillespie KP *et al.*, 2023). Further, radiotherapy induced HMGB1/TLR2 signalling has been reported to enhance the stemenss of pancreatic cancer cells (Zhang L *et al.*, 2019) and HMGB1/TLR2/TLR4 signalling has been reported to enhance stemness of oral CSCs (Bhuyan S *et al.*, 2022). MSCs obtained from tumor of HNSCC, gastric cancer and glioma cancer when injected with tumor cells into mice, it increased tumor growth (Kim *et al.*, 2013; Kansy *et al.*, 2014; Hossain *et al.*, 2015). The tumor derived MSCs were found to secret more tumor promoting factors including VEGF, TGF-β, IL-6 and IL-8 (Li *et al.*, 2015). Therefore, we speculated that the cisplatin induced TSD phenotype may reprogram the BM-MSCs into ASCs as part of the niche defense mechanism and the ASCs may further promote the tumor growth, metastasis and recurrence.

To evaluate the hypothesis of cisplatin induced activation of CSC niche defense in HNSCC, we used SCC-25 cell line. SCC-25 cell line is an oral squamous cell carcinoma cell line and also

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one of the representative cell line for HNSCC. This cell line was already shown to contain EpCAM+ABCG2+ CSC population by our lab (Bhuyan S *et al*., 2022). Importantly, these EpCAM+ABCG2+ CSCs in the SCC-25 cell line were shown to expand when exposed to hypoxia stress for 24 hours followed by 48 hours of reoxygenation (Bhuyan S *et al*., 2022).

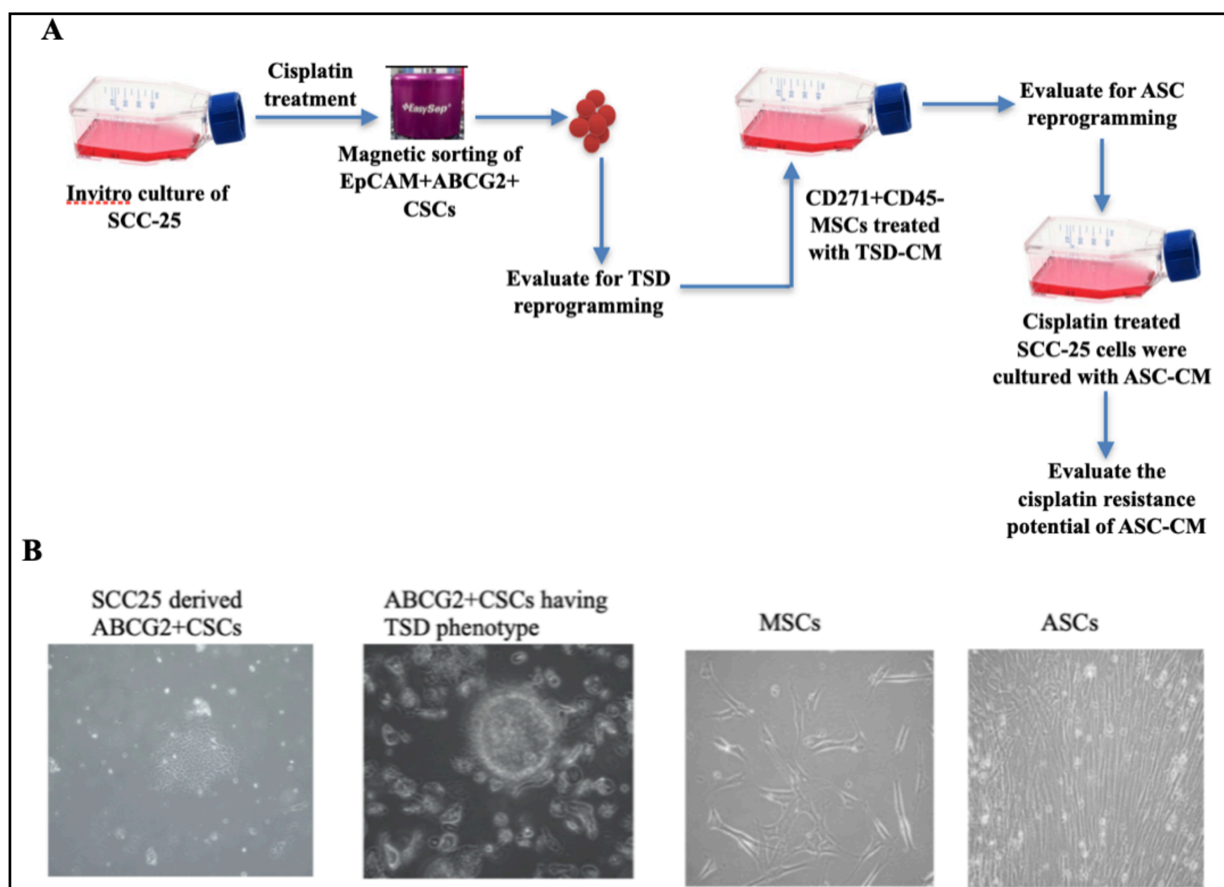

***Figure 26.1**: **Experimental design to evaluate the activation of CSC niche defense in an invitro model of HNSCC recurrence. A**. The SCC-25 cell line was used to represent HNSCC and the sorted CSCs were treated with cisplatin to evaluate the therapy induced CSC niche defense. CSC niche defense was determined by evaluating the TSD characterisation and the reprogramming of MSCs into ASCs while treated with TSD-CM. The ASC-CM was further evaluated for the potential of cisplatin resistance to SCC-25 cells. **B.** Images of SCC-25 derived ABCG2+CSCs, TSD phenotype exhibiting ABCG2+CSCs, MSCs and ASCs under Phase contrast microscope at 10X magnification. The above experimental design was conceptualised through the application of sarvatarka based FGD session with Dr. Bikul Das and the research team.*

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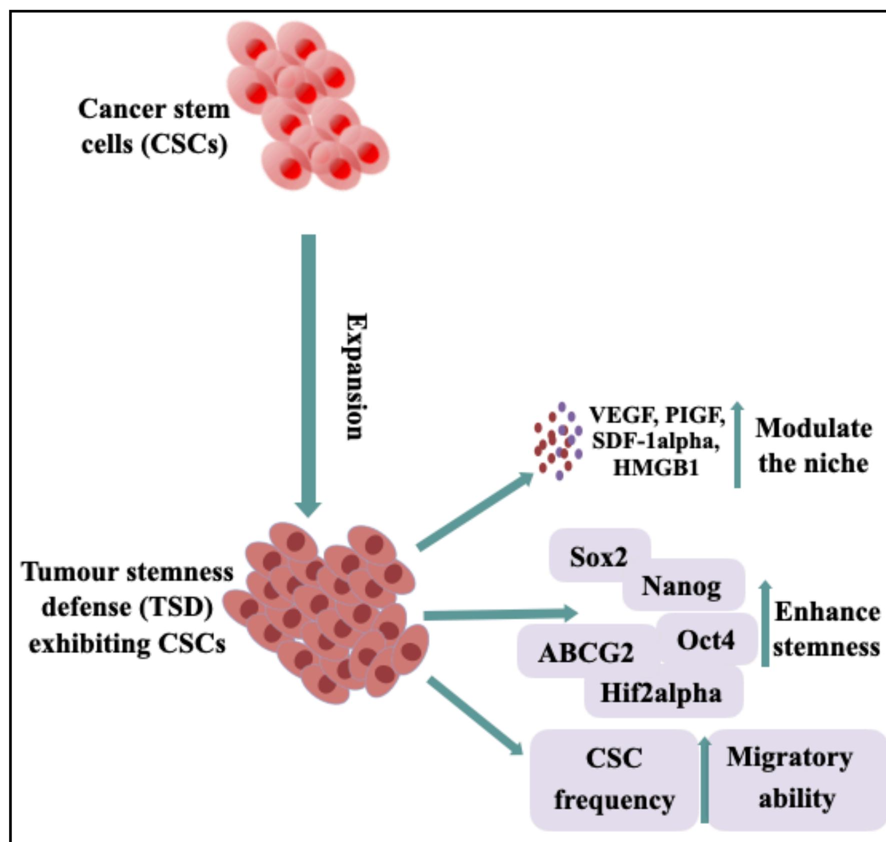

Figure 26.2: The schematic representation of characteristics of TSD phenotype. *Platinum exposure may activate the CSC niche defense. Some of the dormant CSCs may switch to an actively proliferating state, the TSD phenotype. The TSD phenotype activates the MYC-HIF-2α stemness pathway leading to the high expression of Nanog, Sox2 and Oct-4. The CSC with TSD phenotype highly expresses the ABCG2 cell surface marker. The TSD phenotype acquires a higher migratory and invasive phenotype. The xenograft derived from the TSD phenotype exhibits very high CSC frequency. The TSD phenotype is transient and secret cytokines, growth factors as well as inflammatory molecules such as HMGB1 to modulate the niche.*

We speculate that expansion of EpCAM+ABCG2+ CSCs is one of the characteristic of TSD phenotype. Therefore, we considered this SCC-25 cell line to investigate the cisplatin induced TSD reprogramming of EpCAM+ABCG2+ CSCs in HNSCC. Additionally, researchers used this SCC-25 cell line for CSC-MSC interaction in HNSCC. The BM-MSC was reported to enhance the migratory ability, drug resistance and expansion of HNSCC cells when co-cultured with SCC-25 cells (Liu C *et al*., 2021). These findings provide a strong rational of using the SCC-25

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cell line to investigate the activation of CSC niche defense by evaluating the TSD reprogramming of CSCs and CSC-MSC interaction. The SCC-25 cell line was sorted for CSCs using the EpCAM+/ABCG2+ CSC markers by immunomagnetic sorting. The sorted cells were treated with cisplatin (3-10µm) for 4 days to evaluate the TSD phenotype of CSCs (Figure 26.1-26.2). The CM of TSD exhibiting CSCs was used to culture CD271+CD45-MSCs to evaluate the reprogramming of MSCs with altruistic stemness potential. Thus, these reprogrammed cells can be referred as R-MSCs or ASCs. Further, the ASC-CM was used to culture SCC-25 cells treated with cisplatin and evaluated whether ASC could enhance the cisplatin resistance potential of SCC-25 cells.

4.2.0 Methodologies:

All of the necessary experimental procedures were approved and undertaken inside Biosafety Cabinet class II (BSC-II) facility in accordance with guidelines of Institutional Bio-safety Committee of KaviKrishna Laboratory.

4.2.1 SCC-25 cell culture: The SCC-25 was obtained from ATCC (ATCC CRL-1628) and cultured in Dulbecco′s modified Eagle′s medium containing Ham′s F12 (DMEM F-12) in the ratio of 1:1. DMEM F-12 is enriched with sodium bicarbonate (1.2 g/L), 2.5 mM L- glutamine, 15 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.5 mM sodium pyruvate (catalog no. 11330- 057, Gibco). The medium was supplemented with hydrocortisone (400ng/ml; catalog no. H0888, Sigma) and 10% fetal bovine serum (catalog no. 16000-044) and used as complete isolation media. The cells were maintained in a humidified atmosphere of 5% CO^2 at 37°C.

4.2.2 Development of invitro model of SCC-25 derived TSD phenotype: To obtain TSD phenotype of side population (SP) cells and EpCAM+ABCG2+ CSCs, the SCC-25 cells were treated with cisplatin (10 uM for 4 days) in serum free DMEM/F12 media, supplemented with hydrocortisone (400ng/ml), Epidermal Growth Factor (EGF) and Basic Fibroblast Growth factor (bFGF) (20ng/ml). For the SP cell isolation, the cisplatin treated cells were grown in injured conditioned media (ICM), pre-incubated with Hoechst 33342 (4.5µg/ml/10^6 cells) either alone or in combination with 50µM verapamil (Sigma Chemical, USA). Then, the SP cells were analysed and sorted using FACSAria II (BD Bioscience) as previously described (Das B *et al.*, 2008). At

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least 1M SCC-25 cells were sorted to obtain EpCAM+/ABCG2+ CSCs using immunomagnetic sorter. For hypoxia induced TSD phenotype, the sorted cells were exposed to 24 h of hypoxia followed by 72 h of reoxygenation (Das B *et al*., 2008) and then expanded for 7 days in spheroidal culture media [serum-free culture containing EGF and bFGF (20 ng/ml)] as previously described (Das B *et al*., 2008, 2019). The cells were evaluated for TSD phenotype from day 8 and the CM (TSD-CM) was collected (1M cells/ml) within day 8-10. The collected TSD-CM was stored at -20°C for 4-6 months for future use.

4.2.3 Preparation of injured conditioned media (ICM): The ICM was previously shown to induce stemness (Das B *et al*., 2008). The ICM was prepared from BM-MSCs obtained from healthy volunteers as previously described (Das B *et al*., 2008). Briefly, BM-MSCs expanded in serum rich DMEM/F12 media (10% horse serum, 10% FBS, 50 uM 2-mercaptoethanol, and 1 uM hydrocortisone) were exposed to extreme hypoxia (<0.1 % Oxygen) followed by re-oxygenation plus hydrogen peroxide for 3 days. The media was half changed with fresh media (serum free DMEM/F12 media enriched with HEPES, glutamine, sodium pyruvate, 400 ng/ml hydrocortisone and 50 uM 2-mercaptoethanol) and the cells were allowed to grow for 1 week. The total media (5 ml/10^6 MSCs) were collected, and SDF-1alpha was measured. The media batch having SDF-1 alpha concentration of <1.5ng/10^6 cells/ml was stored as ICM for future use.

4.2.4 CD271+BM MSC isolation and culture with TSD-CM: The CD271+ MSCs were obtained from human bone marrow using the EasySep Human CD271 positive selection kit (#18659) as per manufacture instructions (Das B *et al*., 2013). The sorted CD271+ cells were first grown in the serum-free StemSpan SFEM medium with growth factors Flt3 ligand, SCF and TPO for 4-5 days. These cells were then washed, and treated with fresh CM collected from TSD-CM. The cells were then cultured on fibronectin (5ug/ml) coated plates, and the CM was replaced with fresh TSD-CM twice weekly during two weeks of culture. The MSCs from day 8 culture was evaluated for reprogrammed MSCs (R-MSCs) with altruistic stemness potential. Thus, the R-MSCs with altruistic stemness characteristic are referred ASCs. The CM of these ASCs was collected (1M cells/ml) within day 8-10. The collected ASC-CM was stored at -20°C for 4-6 months for future use.

4.2.5 Evaluation of self sufficiency potential of ASCs: The ASCs were cultured in serum free media without any growth supplements for 2 weeks. Then, sustained level of low p53 was

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evaluated by western blot during these 2 weeks of culture to confirm the self sufficiency potential of ASCs. The cells were also subjected to CFU-F assay and expression of CD271 marker to confirm the undifferentiated and self sufficiency state.

4.2.6 Flow cytometry for MDM2 and cleaved caspase-3 expression: Briefly, the single-cell suspensions of R-MSCs were incubated on ice for 15 minutes with primary antibodies of MDM2 and caspase 3 (Abcam, Cambridge, UK) in staining media (Invitrogen). Then, washed thrice in staining medium, and incubated with Alexafluor secondary antibody for 30 minutes. After washing, the stained cells were suspended in complete culture media and analysed in using and FACSAria II (BD Bioscience).

4.2.7 ELISA: The CM of TSD+CSCs (TSD-CM) or ASC+MSCs (ASC-CM) from different groups was collected and subjected to ELISA to measure VEGF, PIGF, SDF-1alpha, GSH, HMGB1, IL-10, TGF-beta, TNF-alpha, IFN-gamma, IDO, PGE-2, HO-1 and CD20 (R&D ELISA kit) according to the manufacturer's protocols. The protein levels of HIF-2α, HIF-1α, p53, GSH, MDM2, TLR2 and TLR4 were measured by In Cell ELISA using a horseradish peroxidase (HRP)-conjugated detection reagent (In-Cell ELISA Colorimetric detection kit, ThermoFisher, #62200) and/or standard ELISA kits. The absorbance was measured at 450 nm using the iMarkMicroplate Absorbance Reader (Bio-Rad, Gurgaon, India).

4.2.8 Culturing CD271+BM MSC in TSD-CM containing neutralising antibody: The neutralizing antibodies against VEGF, PIGF, SDF-1α and HMGB1 were added to the collected TSD-CM for 24 hours incubation at 4^{0}C and then added to the CD271+ BM MSCs. The CM was stored at 4^{0}C during the incubation with neutralizing antibodies. The concentrations are: Anti-VEGF and Anti-PIGF (100 ng/ml); Anti-SDF-1α: (25 ug/ml), and Anti-HMGB1: (100 ng/ml). The antibodies are obtained from R&D Systems, MN, except anti-HMGB1 (#ST326052233; Tecan US, Inc. NC). The viability of CD271+BM MSCs post TSD+CSC-CM treatment with neutralising antibodies is evaluated by trypan blue cell viability test.

4.2.9 Real-Time quantitative-PCR (qPCR): Real-time quantitative PCR was performed using TaqMan primers at 40 cycles with 100 ng of starting cDNA. GAPDH was used as an endogenous control. The RNA was quantified by the delta-delta CT method using Q-Rex software version 1.1 (Rotor-Gene Q-Qiagen, New Delhi, India). The following TaqMan gene expression primers were used: human: ABCG2 (Hs00184979_m1), HIF -1α (Hs00153153_m1), HIF -2α (Hs01026149_m1), Oct 4 (Hs03005111_g1), Sox2 (Hs00602736_s1), CD44 (Hs01075862_m1)

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MYC (Hs00153408_m1), MDM2 (Hs01066930_m1), p53 (Hs01034249_m1), GAPDH (Hs00266705_g1), Nanog (Hs02387400_g1), CD45 (Hs00898488_m1), CD133 (Hs01009250_m1) and HMGB1 (Hs01923466_g1).

4.2.10 Evaluation of migratory cell numbers: A Boyden chamber invasion assay was performed to isolate the SPm and SPn population from the sorted SP population as previously described (Das B *et al.,* 2008). Briefly, polyvinyl membrane-based chambers (Corning Life Sciences, Lowell, MA) were coated with ice-cold Matrigel (BD Bio- sciences, San Diego) and incubated at 37°C for 4 hours. The sorted SP cells were added to the upper chamber, the lower chamber was filled with DMEM/F12 media and incubated at 37°C for 8–24 hours. The migratory cells were counted as SPm cells using crystal violet staining. The non-invading cells were also isolated and counted as SPn cells. Similar process was applied to count the migratory cells in SCC-25 derived cisplatin treated ABCG2+/EpCAM+ CSCs.

4.2.11. Western Blotting: Western blot (WB) analysis was done using a 4%–12% sodium dodecyl sulfate–polyacrylamide gel and polyvinylidenedifluoride membranes (Immobiolon-P, MilliporeSigma, cat. # IPVH20200), following standard protocol. 50µg of the clear protein was used. We have used the following antibodies: beta actin (# BB-AB0024S, Biobharti, Kolkata, India), ABCG2 (Cell Signaling technology, USA) and p53 (# BB-AB0100, Biobharti, Kolkata, India).

4.2.12 The gene and protein inhibition assay: The invitro inhibition of p53 was achieved by treating the CD271+MSCs (1x10^4 cells) with pifthrin alpha (Sigma-Aldrich; 2 µM) and MDM2 was by treating the cells with Nutlin-3 as per manufacture protocol. The accell siRNA was used to inhibit gene expression of p53 (GE healthcare Dharmacon Inc IL, A-003329-22-0005).

4.2.13 Cytoprotection assay: The in vivo BM stem cell cytoprotection assay was conducted in Balb/c mice as described (Das B *et al*., 2008). Briefly, 10-12 week old Balb/c mice were injected with the CM of R-MSCs i.p. three hours before the injection of high dose cisplatin (12 mg/kg). In 2 groups, R-MSCs CM was also subjected to BSO (buthionine sulphoximine) and NAC (N-acetylcysteine) treatment (100mg/kg body weight administered ip 30 minutes before injecting to mice). On day 5 after the injection, animals were sacrificed, BM was collected and subjected to magnetic sorting for both hematopoietic stem cells (HSC) and mesenchymal stem cells (MSCs) using the immunomagnetic sorting kits (EasySep kits #19756 for HSC, and #19771 for

MSCs; StemCell Technologies, Vancouver). The isolated cells were then subjected to CFU assay for both HSCs and MSCs (Das B *et al*., 2012, Das B *et al*., 2008). For the invitro cytoprotection assay, SK-N-BE (2), SCC-25 and H-146 cell lines were obtained from ATCC. The cancer cells were treated with cisplatin (3-10µm) with or without the CM of R-MSCs for three-days, and then plated in methylcellulose media to perform clonogenic assay.

4.2.14 Clonogenic assay: The treated cells (1x10^3) were seeded in methylcellulose media (Methocult M3134, Stem Cell Technologies, BC, reconstituted in DMEM/F12 with 10% FBS). The cells were seeded in 6 well plates, incubated at 37^0C and 5% CO^2, and colonies were counted after two weeks. To obtain the CSC frequency, we performed extreme dilution limiting assay (ELDA).

4.2.15 Immunofluorescence assay: The SCC-25 derived SP cells were fixed with 4% paraformaldehyde for 10-20 minutes on a coverslip and stained for EpCAM as well as ABCG2. Images were obtained using confocal microscopy.

4.2.16 Statistical analysis: All experiments unless otherwise stated, were repeated thrice and in triplicates. The data are presented as mean ± standard error of mean (SEM). The statistical calculations were performed with Graph-Pad Prism 10.0 (Hearne Scientific Software, Chicago, IL, USA) by using Student's t-test for cell survival assays. ELISA and qPCR data were analyzed using one-way ANOVA with Newman-Keuls post-hoc test. Statistical significance was set at P< 0.05%.

4.3.0 Results

4.3.1 Developing the invitro model of platinum-induced TSD phenotype in HNSCC cell line

Here, we have characterized a pre-clinical model, where a human HNSCC cell line, SCC-25 was treated with cisplatin in vitro. To develop this in vitro model, we revisited our previous work on cisplatin-induced reprogramming of dormant tumor side-population (SP) cells to a TSD phenotype characterized by the high expression of Nanog, Oct-4, Sox2 and VEGFR1 mediated autocrine pathway as well as migratory and invasive phenotype. The migratory SP cells (SPm cells) were previously shown to contain ABCG2+EpCAM+ population. Thus, in the present study, SCC-25 cells were exposed to cisplatin or hypoxia-reoxygenation for 4 days in injured conditioned media (ICM) to obtain the SPm cells, and evaluated for TSD characteristic.

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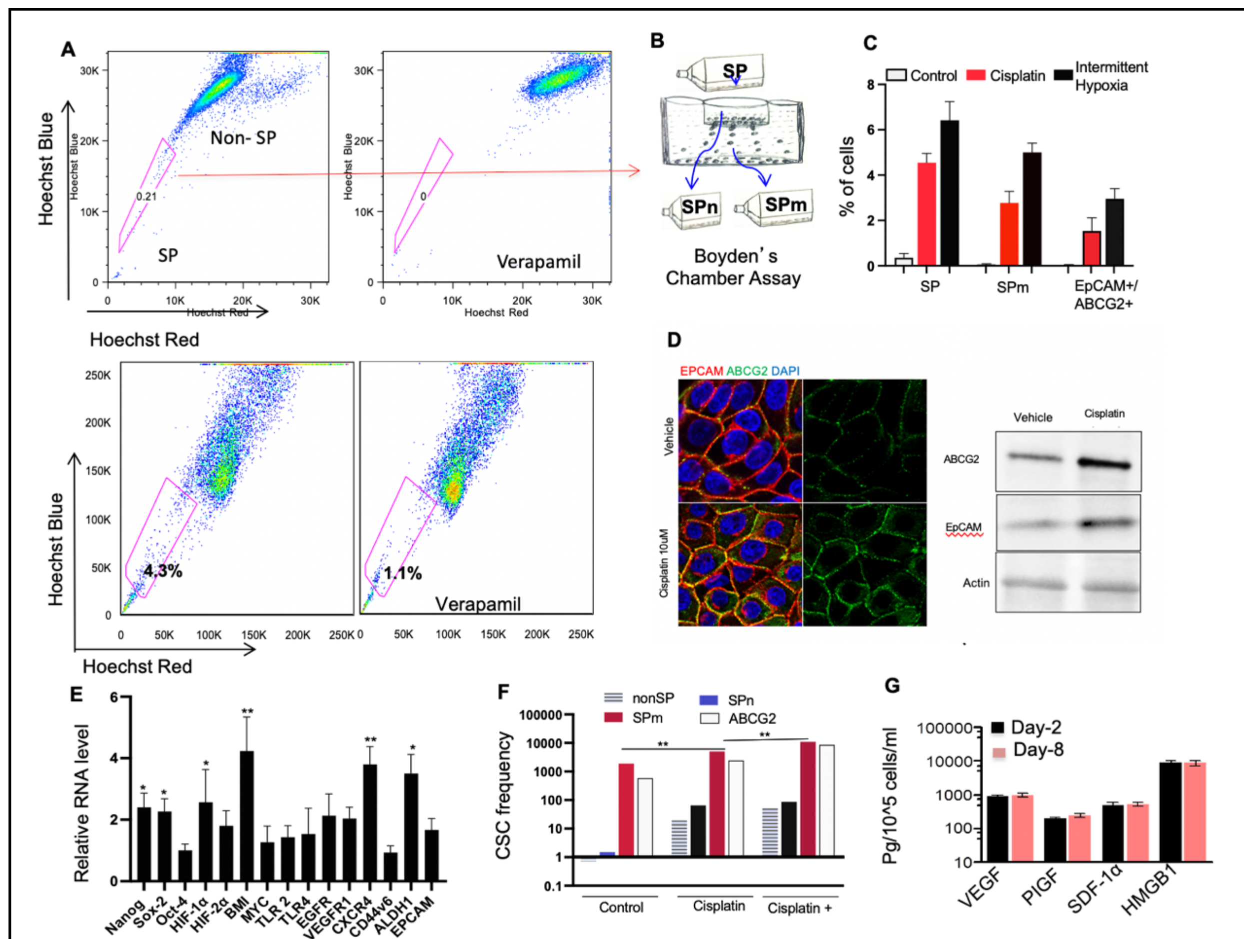

Figure 27: In vitro model of cisplatin-induced TSD phenotype of SCC-25 cells. A. *Top panels: Flow cytometry profile of the SP and non-SP fractions of SCC-25 cells. Lower panels: flow cytometry profile of cisplatin treated SP cells (10 uM for 4 days in serum-free media supplemented with 20 ng/ml of EGF and bFGF).* ***B.*** *Schematic of the Boyden chamber assay for the isolation of SPm cells* ***C.*** *Histogram showing the percentage of SP, migratory SP (SPm) and EpCAM+/ABCG2+ cell fraction in the cisplatin-treated vs untreated SCC-25 cells. Hypoxia/re-oxygenation treated SCC-25 cells served as positive control.* ***D.*** *EpCAM and ABCG2 expression of SPm cells were confirmed by immunofluorescence and western blot assay after grown in the ICM for 3 days.* ***E*** *qPCR analysis of stemness genes showing high expression in EpCAM+/ ABCG2+ (cisplatin) vs EpCAM/ABCG2+ (untreated) cells.* ***F****. CSC frequency of cisplatin treated SPm or EpCAM+/ABCG2+ cells as performed by the in vitro serial dilution assay.* ***G****. The EpCAM+/ABCG2+ cells from cisplatin treated SCC-25 cells when grown in serum-free media without growth factor maintained the secretory phenotype. For C, E and G data represent +/- SEM. C&D, Student t test, *p<0.05, **p<0.01; F: maximum likelihood analysis ***p<0.001.*

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We used hypoxia/re-oxygenation treated SPm fraction of SCC-25 cells that served as a likely positive control for the TSD phenotype. We first found that untreated SCC-25 contain a rare 0.21% fraction of SP cells, 20% of which is SPm cells, totalling only 0.08% of the parental cell line. Boyden chamber assay was performed for the isolation of migratory SP (SPm) cells. Notably, we obtained a similar 0.06% of EpCAM+/ABCG2+ cells in the parental population (Figure 27 A-C). IF and western blot confirmed that most of the SPm cells contains EpCAM and ABCG2 cell surface marker (Figure 27 D). Both cisplatin and hypoxia/reoxygenation treatment increased the SPm and EpCAM+/ABCG2+ cells, as well as protein by 20-25-fold. The cisplatin treated EpCAM+/ABCG2+ cells exhibited higher expression of stemness genes compared to EpCAM+/ABCG2- cells, suggesting that EpCAM+/ABCG2+ cells have the higher chance to reprogram to TSD phenotype than the counterpart, the EpCAM+/ABCG2- cells (Figure 27 E). Invitro serial dilution assay confirmed higher CSC frequency of SPm or EpCAM+/ABCG2+ cells. Post-cisplatin EpCAM+/ABCG2+ cells grown in serum-free media without growth factor maintained the secretory phenotype, which confirmed the self sufficient TSD characteristic of the cisplatin treated EpCAM+ABCG2+ cells (Figure 27 F-G).

4.3.2 The TSD phenotype exhibiting EpCAM+ABCG2+ CSCs reprogram MSCs to ASCs

Previously our lab demonstrated that Hif2α/Myc stemness pathway maintain the stemness in ABCG2+CSCs by asymmetric self renewal (Das B *et al.,* 2019). The conditioned media of hypoxia treated ABCG2+ CSCs were shown to further reprogram the MSCs into pro tumorigenic ASCs (Talukdar J, PhD thesis, 2017). In this chapter, the ASCs were characterised as previously described in the chapter 2 (Pathak L *et al*., 2021). We hypothesised that the cisplatin induced TSD phenotype may also reprogram the MSCs to ASCs. Indeed, we found that the CM of cisplatin induced TSD phenotype reprogram the MSCs into ASCs (Figure 28-31). The ASCs were shown to highly express stemness genes (Figure 28B) and maintained stemness for 2 weeks when grown in serum free media without growth factors by transient suppression of p53 (Figure 28A-D). The ASCs also sustained undifferentiated state by expressing CD271 and self-sufficient state by exhibiting significant CFUs for two weeks in serum free medium without growth factors supplements (Figure 28E). An important characteristic of ASCs; p53/MDM2 oscillation was demonstrated in ASC versus U-MSCs (Figure 29A, E).

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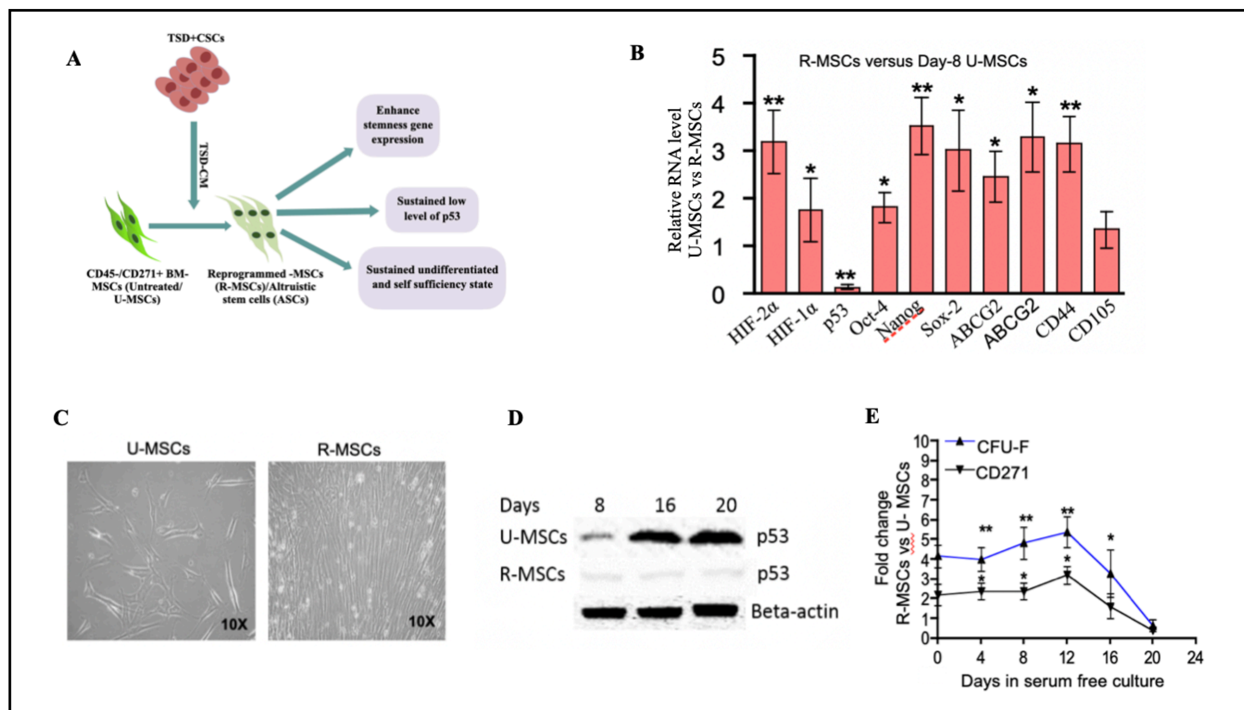

Figure 28: The CM of TSD phenotype (post cisplatin treated EpCAM+/ABCG2+ CSCs) reprogram CD271+ MSCs to ASCs. A*. The experimental design to assess the altruistic stemness characteristic of the CD45-/CD271+ MSCs cells post treated with TSD-CM.* ***B****. High expression of altruistic stemness associated genes in the R-MSCs vs U-MSCs.* ***C****. The rapidly proliferating R-MSC phenotype of CD45-/CD271+ MSCs post treated with TSD-CM. The CD45-/CD271+ MSCs grown in serum free media with growth factors without any treatment is referred here as untreated or U-MSCs. The images of both U-MSCs and R-MSCs were taken on day 8 under phase contrast microscope.* ***D****. Western blot showed sustained level of low p53 in the R-MSCs when grown in serum free media without any growth factors.* ***E****. The sustained undifferentiated and self-sufficient state of R-MSCs grown for two weeks in serum free medium without growth factors supplements. The data was compared with the day-8 U-MSCs grown in serum free medium with growth factor supplements, and exhibited CFUs at 1.1±0.8/10*^{3}*cells (n=5). *p<0.05, **p<0.01, student t test. Data represents ±SEM, n = 4 independent experiments.*

Also, the CM of ASCs exhibited GSH dependent and independent cytoprotection to murine HSCs and MSCs (CD45- cells) in a mouse model of carboplatin-induced BM toxicity (Figure 29B-C). Further, ASC-CM exhibited cytoprotective activity against cisplatin-induced toxicity (3 µM/ 3 days) of SK-N-BE(2), SCC-25 and H-146 cell lines. The ASC-CM was shown

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to increase ABCG2+ CSC population in these cells and also induced cisplatin resistance potential in the non CSC population of these cells (Figure 30A-C). Additionally, these ASCs were found to secret tumor promoting factors including HGF, SDF-alpha and VEGF, inflammatory cytokines including TNF-alpha, IFN-gamma, IL-6 and IL-10, as well as immunosuppressive molecules including TGF-beta, IDO, PGE-2, HO-1 and CD200 (Figure 31).

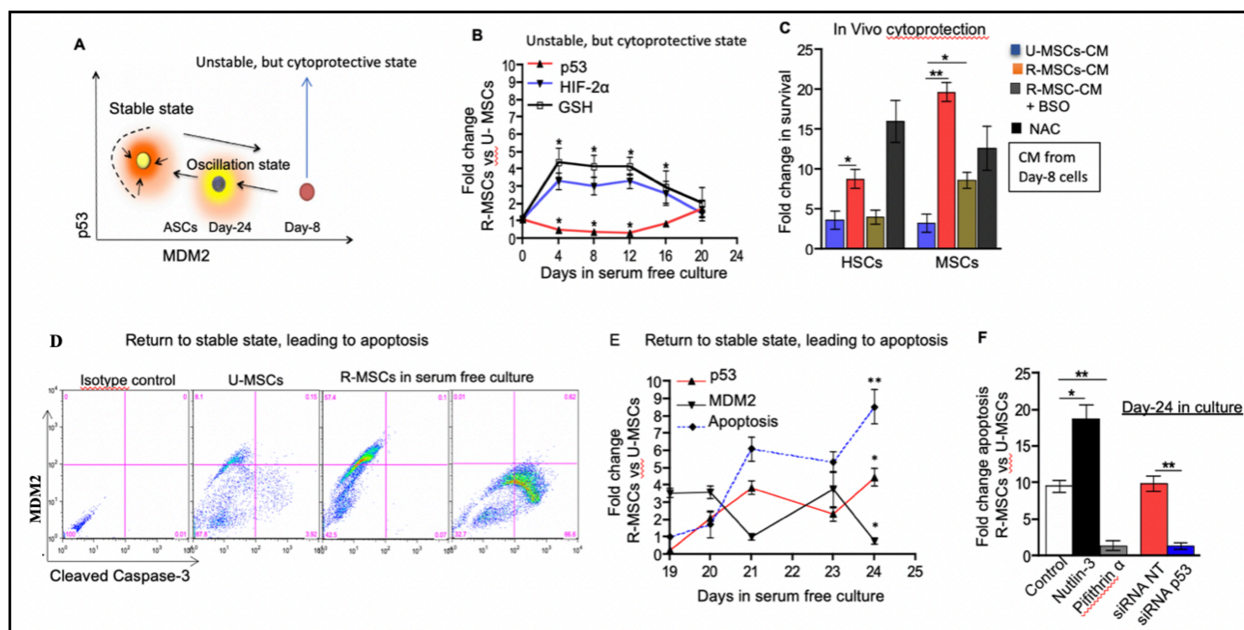

Figure 29: The R-MSCs exhibit cytoprotection, and then undergoes p53 dependent apoptosis. ***A.*** *Model showing the altered state of p53/MDM2 oscillation state of ASCs.* ***A*** *special architecture built into the p53/MDM2 feedback system permits p53 and MDM2 to undergo oscillation and then transiently acquire an unstable state of high MDM2 and low p53 (Das B et al 2012). This property of the p53/MDM2 system might function as a potential safety mechanism that is built into stem cells to prevent the enhanced stemness phenotype to undergo malignant transformation.* ***B****. The sustained undifferentiated and GSH secretory state of R-MSCs grown for two weeks in serum free medium without growth factors supplements. HIF-2α and p53 levels were measured by ELISA. GSH levels were measured in the CM obtained from R-MSCs. The data was compared with the day-8 U-MSCs grown in serum free medium with growth factor supplements.* ***C****. CM of R-MSCs exhibited GSH dependent and independent cytoprotection to murine HSCs and MSCs in a mouse model of carboplatin-induced BM toxicity. R-MSCs or U-*

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MSCs CM subjected to BSO (buthionine sulphoximine) and NAC (N-acetylcysteine) treatment protocols are given in the method section. ***D.*** *Representative flow cytometry panels showing the association of MDM2 with cleaved caspase-3 expression in Day-8 and Day-24 R-MSCs.* ***E.*** *The return of p53 from an unstable to a stable state and associated reactivation of p53/MDM2 oscillation. The fold change in the protein level of p53, MDM2 and cleaved caspase-3 in the third week (day 19 to 24) of serum-free culture with growth factors.* ***F****. Altruistic cell death is p53 dependent. The treatment of R-MSCs by nutlin-3 and pifithrin alpha was performed for two days (starting on day 22). The Accell siRNA NT and p53 treatment was done as described before (Das B et al., 2012) starting on day 20. *p<0.05, **p<0.01, student t test. Data represents ±SEM, n = 4 independent experiments.*

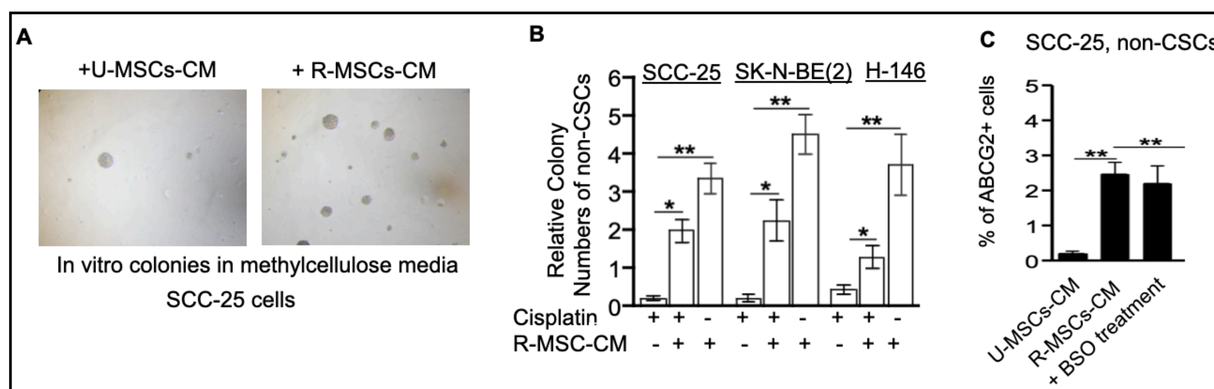

Figure 30: ASC-CM activates the cisplatin resistance potential in the non CSCs. A. *Representative colonies of SCC-25 non-CSCs with or without treatment with the CM of R-MSCs, after clonogenic assay.* ***B.*** *R-MSCs-CM exhibited cytoprotective activity against cisplatin-induced toxicity (3 µM/ 3 days) of SK-N-BE(2), SCC-25 and H-146 cell lines. The non CSCs were treated with cisplatin with or without the CM of R-MSCs for three-days, and then plated in methylcellulose media to perform clonogenic assay.* ***C.*** *R-MSCs-CM increased the percentage of ABCG2+ cells in non-CSCs of SCC-25 cells. *p<0.05, **p<0.01, student t test. Data represents ±SEM, n = 4 independent experiments.*

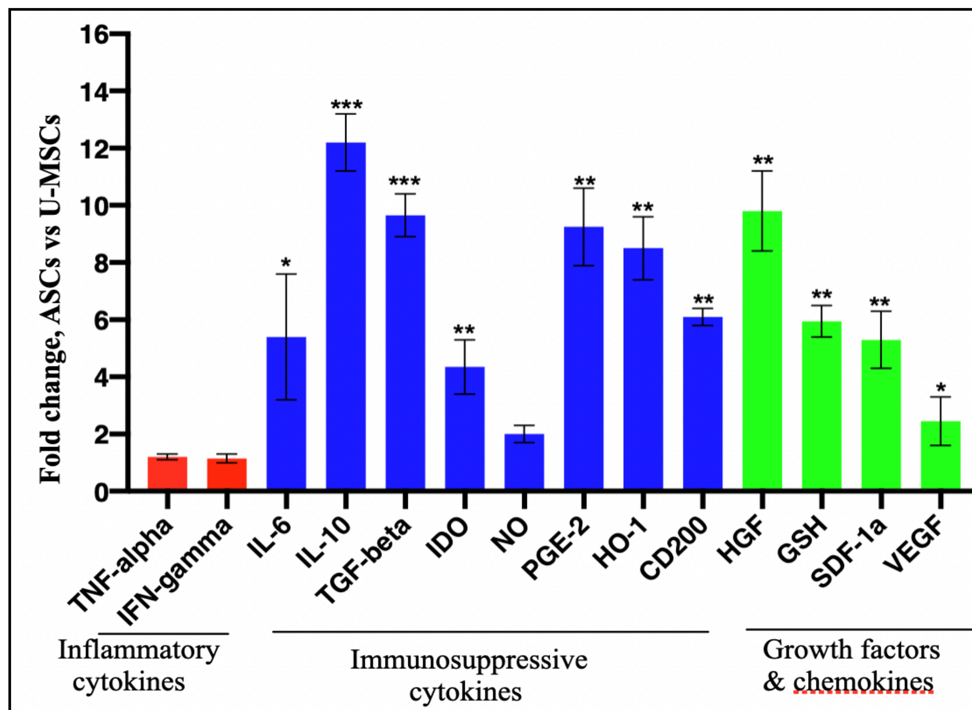

Figure 31: ASCs are immunosuppressive in nature. *The CM of ASCs induced by CM of TSD was evaluated for secretion of growth factors, inflammatory and immunosuppressive cytokines. The result is compared with the CM of untreated MSCs. The secretion of immunosuppressive cytokines was significantly higher. *p<0.05, **p<0.01, ***p<0.001, student t test. Data represents ±SEM, n=4 independent experiments.*

4.3.3 The TSD phenotype exhibiting EpCAM+/ABCG2+ CSCs activate HMGB1 dependent TLR4 inflammatory pathway in MSCs

We planed to evaluate the stemness pathway in the TSD+CSCs that may induce the reprogramming of MSCs into ASCs. The cisplatin induced TSD+CSCs were found to secret various factors; VEGF, PIGF, SDF1-alpha and HMGB1 (Figure 27.1G). Importantly, the inflammatory factor HMGB1 level was reported 100-fold higher than the rest of the growth factors in ABCG2+ cells. We speculated that these secretory factors may reprogram MSCs to ASCs.

Hence, the CD271+BM-MSCs were cultured in the CSC-CM containing neutralising antibodies for all these factors. We found that neutralising HMGB1 significantly reduces the expansion of CD271+ BM-MSCs, suggest that HMGB1 is the key secretary molecule in the CSC-CM that activate the MSC to ASC reprogramming (Figure 32A-B). Considering that TLR2 and TLR4 are well-known receptor for exogenous HMGB1, we reasoned that these two receptors

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may endocytose the HMGB1. Indeed, we found the higher expression of TLR4, but not TLR2 in the TSD-CM treated CD271+BM-MSCs during 12th to 16th days of treatment. Importantly, the Hif2α expression level is reduced by 4 fold when the TLR4 is neutralised in the CD271+BM-MSC culture (Figure 32C-E). This finding indicates that the TSD exhibiting CSCs activate a HMGB1/TLR4 inflammatory pathway that may activate Hif2α stemness pathway in the TME that reprogram the MSCs into pro-tumorigenic ASCs.

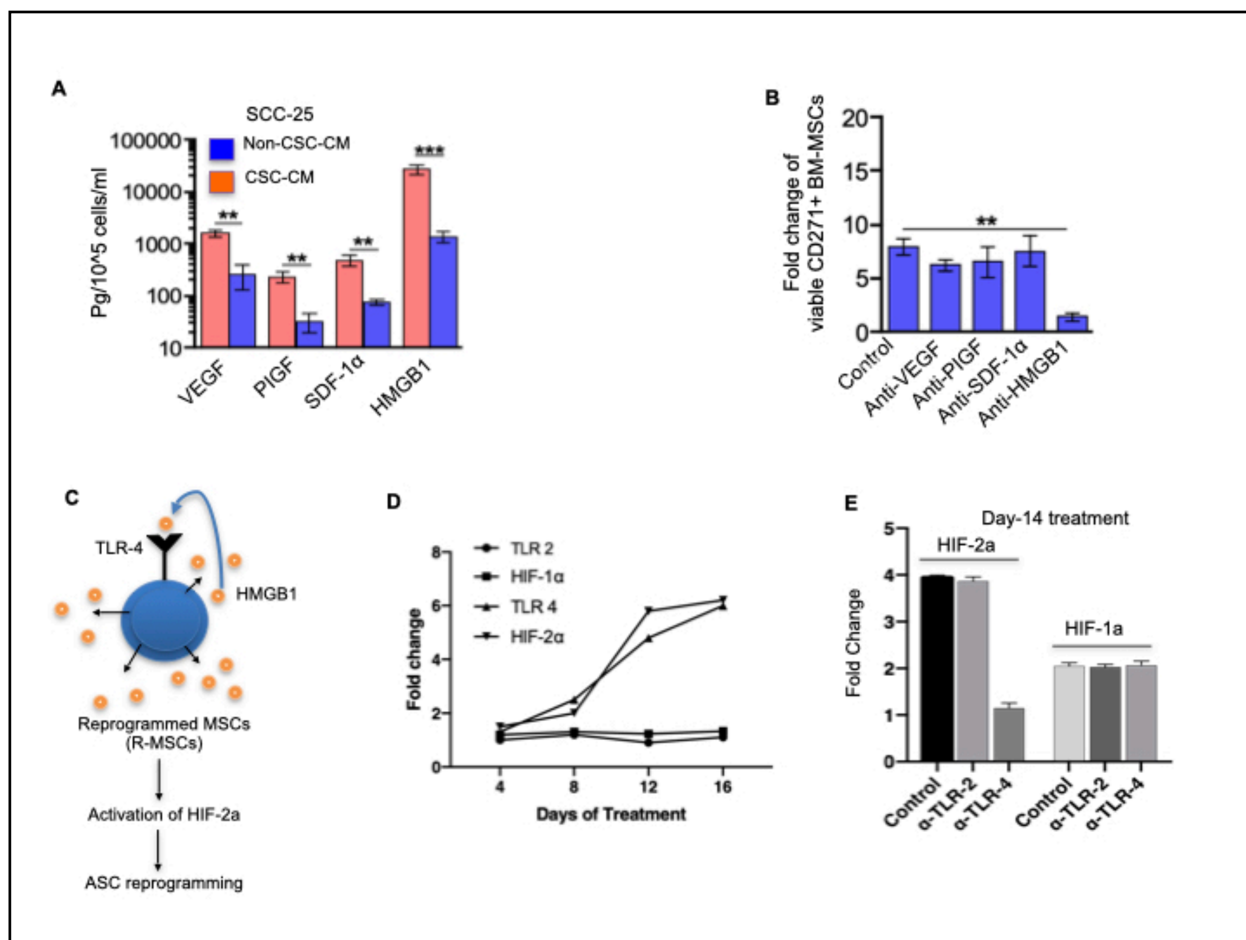

Figure 32: The reprogramming of MSCs to ASCs is mediated by HMGB1/TLR4 inflammatory pathway. A. *The secretion of VEGF, PIGF, SDF1alpha and HMGB1 in the CM of CSC, non CSC.* ***B***. *CM containing Neutralising antibodies are treated with CD271+MSCs and the viability of cells are counted using trypan blue assay.* ***C***. *Pictorial representation of HMGB1/TLR4 pathway activating HIF2α in ASCs.* ***D***. *Evaluation of HIF2α and TLR4 in the CD271+MSCs post CSC-CM treatment. E. HIF2α expression evaluation in MSCs after neutralising the TLR4. n=3, independent experiment.*

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4.4.0 Discussion

CSCs reside in their niches and may activate a niche defense mechanism to protect the "tumor organ" from external threats such as oxidative stress, immune cells, and therapies. Here, we demonstrated that cisplatin treatment may activate the niche defense mechanism in EpCAM+/ABCG2+ CSCs of SCC-25 cells by inducing the reprogramming of this CSC population into an invasive tumor stemness defense (TSD) phenotype. The cisplatin induced TSD phenotype was characterised by the enhanced stemness gene expression, higher CSC frequency and niche modulatory potential (Figure 26.2). The cisplatin treated EpCAM+/ ABCG2+ CSCs grown in serum-free media without growth factor maintained the secretory phenotype, confirmed the self sufficiency potential of these cells. Importantly, the conditioned media of the TSD phenotype reprogrammed the CD271+ BM-MSCs into the ASC phenotype, partially characterized by transient suppression of p53. We utilized a mixed BM stem and progenitor cell population; the CD271+/ CD133+ BM cells, to isolate the candidate stem cell population that could undergo ASC reprogramming. The reprogrammed CD271+ BM-MSCs designated as R-MSCs, showed the secretion of pro-tumorigenic and immunosuppressive factors. Unlike biased-stem cell competition, where stem cells acquiring self-sufficiency to eliminate neighboring cells, R-MSCs underwent altruistic cell death to secret cytoprotective molecules to protect neighbouring cells. These results are indicative of the potential role of stem cell altruism in the pathogenesis of cancer growth.

In the present study, we characterized the ASC phenotype in R-MSCs including the expression of ES cell transcription factors, Nanog, Sox-2 and Oct-4, as well as high HIF-2α and low p53 state. R-MSCs secreted GSH that exerted cytoprotection to HSCs and MSCs under oxidative stress. Furthermore, HIF-2α, which may form an autocrine loop with VEGFR1, appears to be involved in maintaining self-sufficiency, an important characteristic of the ASC phenotype (Das B 2014). R-MSCs then exhibited spontaneous return of p53/MDM2 oscillation leading to self-elimination of these cells. Considering the altruistic characteristics of these R-MSCs, they are further referred as ASCs. Interestingly, ASCs-CM exhibited cytoprotective activity against cisplatin-induced toxicity of SK-N-BE(2), SCC-25 and H-146 cell lines when grown these cells invitro with ASC-CM. These findings of ASCs favouring tumor growth indicates that activating ASC niche defines may be of great benefit for TSD+CSCs to initiate stem cell niche to niche interaction for metastasis and recurrence.

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Although the mechanism for induction of ASC reprogramming of CD271+ BM-MSCs by TSD+CSC is not yet clear, HMGB1 secreted by TSD+ CSC-CM was found to be involved in the initial expansion of ASCs. Considering that TLR2 and TLR4 are well-known receptor for exogenous HMGB1, we reasoned to find the activation of the TLR4 signalling pathways in ASCs. Previously also, HMGB1 dependent TLR2/4 signalling was found to enhance the stemenss of pancreatic cancer cells (Zhang L *et al.*, 2019) and oral CSCs (Bhuyan S *et al.,* 2022). We found the activation of TLR4 in the ASCs when treated with HMGB1 secreting TSD+CSC-CM. Future studies are required to unravel the role of TLR4 in the internalization of HMGB1 into the ASCs. In cancer, TLRs are reported to act as double edge sword in either enhancing stemness (Kaczanowska S *et al*., 2013, Sato Y *et al*., 2009) or apoptosis (Basith S *et al*., 2012). Interestingly, further evaluation showed higher expression of Hif2α in the ASCs only during the activation of HMGB1/TLR4 signalling pathway. These results provide a preliminary evidence on the Hif2α dependent HMGB1/TLR4 inflammatory stemness pathway as the underlying mechanism of ASC reprogramming. Although, the other downstream stemness pathway may also be investigated in association with HMGB1/TLR4 inflammatory pathway.

While both TSD and ASC contribute to tumor progression, identifying their unique characteristics and mechanisms will allow for more precise targeting. TSD arises directly from CSCs in response to stress, whereas ASCs originate from MSCs reprogrammed by factors secreted by TSD cells. Although both phenotypes may share some markers and signalling pathways, such as the activation of the HIF-2α stemness pathway, there are distinct molecular signatures associated with each, reflecting the distinct origin of these two cell types. Functionally, TSD may primarily focus on enhancing the survival and self-renewal of CSCs under stress, while ASCs might play a broader role in supporting the tumor microenvironment, including promoting angiogenesis, immune evasion, and CSC maintenance.

While both TSD-CM and ASC-CM may have the potential to influence CSC populations, the specific mechanisms and signalling pathways activated by each may vary. Although both TSD-CM and ASC-CM may influence CSC populations, they could activate different signalling pathways or utilise distinct mechanisms to exert their effects. Comparative analysis of their impact on CSCs could reveal unique contributions of each component. TSD-CM might primarily promote the survival and self-renewal of existing CSCs. In contrast, ASC-CM, originating from reprogrammed MSCs, could potentially induce the dedifferentiation of non-CSCs into

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CSCs, contributing to CSC expansion. It's also possible that TSD-CM and ASC-CM act synergistically to regulate CSC populations, highlighting the complex interplay between CSCs and the tumor microenvironment. In this context, the secretion of cytoprotective and immunosuppressive factors by ASC may provide synergistic defense along with TSD+ CSCs.

Investigating these differences and comparing the effects of TSD-CM and ASC-CM on CSCs in future studies could provide valuable insights into the specific factors and pathways involved in CSC regulation. This deeper understanding could contribute to the development of more targeted therapeutic strategies aimed at disrupting the CSC niche defense and overcoming therapy resistance.

Our work on the CBPAR based Avatar-kosha therapy to cancer patients in the rural setting is unique, and provide preliminary evidence to enhance not only general well being but also community based social well being, including the increase of social capital. The role of the Avatar-Kosha therapy in the cancer disparity reduction need further evaluation.

4.5.0 Conclusion

In this initial study of ASC reprogramming of BM stem cells, we chose a few selected cellular pathways including VEGF, and SDF-1α based on our previous findings where we characterized these pathways in SP cells. We have described a novel mechanism wherein a functional equivalent of aggressive and inflammatory CSCs, the TSD phenotype can induce reprogramming to a specific subpopulation of BM-stem cells, the CD271+ BM-MSCs that can promote cancer progression by secreting pro-tumorigenic and immunosuppressive factors. The reprogramming mechanism involves temporary suppression of p53 that allows these CD271+ BM-MSCs to reprogram to a higher stemness state with an acquired ability to provide cytoprotection to non-stem cancer cells against cisplatin-induced toxicity. Thus, we have found a unique TSD exhibiting CSC specific reprogramming of CD271+ BM-MSCs that endows stem cell niche defense. Our study indicates that CSCs and BM stem cell cross talk may lead to the activation of the ASC based stem cell niche defense; CSCs may exploit the niche defense for tumor progression.

Chapter 5.0.0

To investigate the role of stem cell niche defense in head and neck cancer recurrence with clinical samples

Abstract

Background: HNSCC is the commonest cancer in India and 50% of HNSCC cases cause local or distant recurrence. Cisplatin based chemoradiation is the only management for the recurrence and advanced staged HNSCC cases. In the chapter 4, using a HNSCC based preclinical model of CSC-MSC cross-talk, it was showed that chemotherapy may activate the niche defense mechanism in CSC, by which CSCs may be reprogrammed to TSD phenotype and TSD+CSCs may further reprogram MSCs into ASCs with protumorigenic nature. Therefore, it was hypothesized that chemotherapy-induced CSC niche defense may also be identified by isolating and characterizing circulating tumor cells (CTCs) with TSD phenotype and circulating mesenchymal stem cells (MSCs) with ASC phenotype in the HNSCC subjects. We also hypothesised that the biosocial medicine approach, the Avatar-Kosha may reduce inflammation, as well as recurrence. **Methodology**: We did a prospective observational study of HNSCC subjects managed with cisplatin based regimens (n=29). Peripheral blood samples were collected for Ficoll gradient separation to isolate mononuclear cells. The cells were cultured in a serum-free media supplemented with injured conditioned media (ICM) which is known to promote spheroid formation and then subjected to immunomagnetic sorting for EpCAM+/ABCG2+ CTCs. The CD271+/CD45- MSCs were also sorted using the method of immunomagnetic sorting. We also studied the biosocial medicine effect on recurrence by performing the Sahasa-ojash scale. **Results**: The EpCAM+/ABCG2+ CTCs were obtained from 7 patients which exhibited TSD-specific gene expression, and rapid expansion in the ICM. We found 21/29 cisplatin treated HNSCC recurred subjects with c-ASCs ($>1x10^{3}$), and they showed treatment failure eventually. Additionally, 8/29 subjects having lower number of c-MSCs ($<1x10^{3}$) responded to treatment. The 4/8 with more than $1x10^{2}$ c-MSCs showed recurrence after 7-15 months of completion of cisplatin based treatment. The subjects with less than $1x10^{2}$ c-MSCs showed treatment response. The Avatar-kosha based intervention showed marked reduction in CRP, and ESR level, as well as sustained increase of sahasa-ojash scale. **Conclusion**: These findings indicated the clinical significance of CSC niche defense, thus may be further explored to develop biomarker for therapy failure and poor prognosis in HNSCC Subjects.

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5.1.0 Background

Studies reported that cisplatin increases the CSC population in platinum resistant cells obtained from different cell lines (Thakur B and Ray P, 2017). Our invitro result has shown that cisplatin stress reprogram the EpCAM+/ABCG2+ CSCs into an aggressive highly proliferative TSD phenotype. Both ABCG2 and EpCAM have been found as CSC marker in cisplatin resistant HNSCC cells and advanced HNSCC cases (Murakami K *et al.,* 2022, Shen B *et al.,* 2011). We speculate that the cisplatin stress may also reprogram the EpCAM+/ABCG2+ CSC population of advanced stage HNSCC subjects into the TSD phenotype, thereby may contribute to therapy resistance, metastasis, and relapse. The invitro findings of previous chapter have confirmed the invasive and migratory potential of this TSD phenotype. Therefore, we hypothesised that the invasive TSD phenotype may migrate from the primary tumor niche to distant tissue via circulation to invade and metastasize a new niche. Therefore, we proposed to isolate first EpCAM+ABCG2+ circulating tumor cells (CTCs) from the selected study subjects and evaluate whether CTCs exhibit TSD phenotype. The quiescent cancer cells when enter the bloodstream to invade distant tissues, can be identified as CTCs (Kulasinghe A *et al.,* 2009). CTC is transient in nature and thus very challenging to isolate. Though, CTC has huge significance in the field of early detection of cancer. A unique characterisation of CTCs may provide a novel way to isolate CTCs and thus, may contribute in the field of early detection of cancer. Therefore, we sought to isolate CTCs and evaluate whether they exhibit specific TSD phenotype.

MSCs were reported to maintain the stemness state of the CSCs and induce the self-renewal of quiescent CSCs (Mimeault and Batra, 2013; Philip *et al.*, 2013, Jung Y *et al.*, 2013). MSCs were also found to secret specific cytokines such as IL-6 and CXCL8, which further induce expression of stemness genes, such as, Oct4 and Sox2 in colorectal cancer cells (Wu *et al.*, 2016). We further postulate that the TSD exhibiting CSCs may reprogram the MSCs into ASCs to favour tumor growth, where p53 is temporarily suppressed to enhance the secretory and cytoprotective capacity of stem cells. Unlike stem cell competition, the mechanism of stem cell altruism is characterized by the acquisition of higher regenerative capacity that benefits the neighboring cells. Hence, exploiting ASC niche defense mechanism may also help CSCs to enhance the regenerative capacity of BM stem cells so that these cells can convert non-stem cancer cells to CSCs. In the chapter 4, an invitro model of CSC-MSC interaction, we already

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have demonstrated how chemotherapy may activate the niche defense mechanism in CSCs. This niche defense mechanism led the reprogramming of CSCs into TSD phenotype and TSD+CSCs further reprogrammed the CD271+ BM-MSCs into ASCs. These ASCs secreted protumorigenic growth factors and immunosuppressive cytokines. Considering these findings, we speculated that similar to TSD exhibiting CTCs, ASC exhibiting MSCs may also migrate from BM to distant tissue to establish pre metastatic niche. Studies have reported the involvement of MSCs in developing inflammatory pre metastatic niche (Wang H *et al*., 2021). Therefore, in this chapter, we proposed to isolate ASCs from peripheral blood of cisplatin treated HNSCC subjects. The clinical subjects were screened and monitored for 2-4 years using a rural telemedicine clinic-based network for treatment failure or recurrence status (Figure 33).

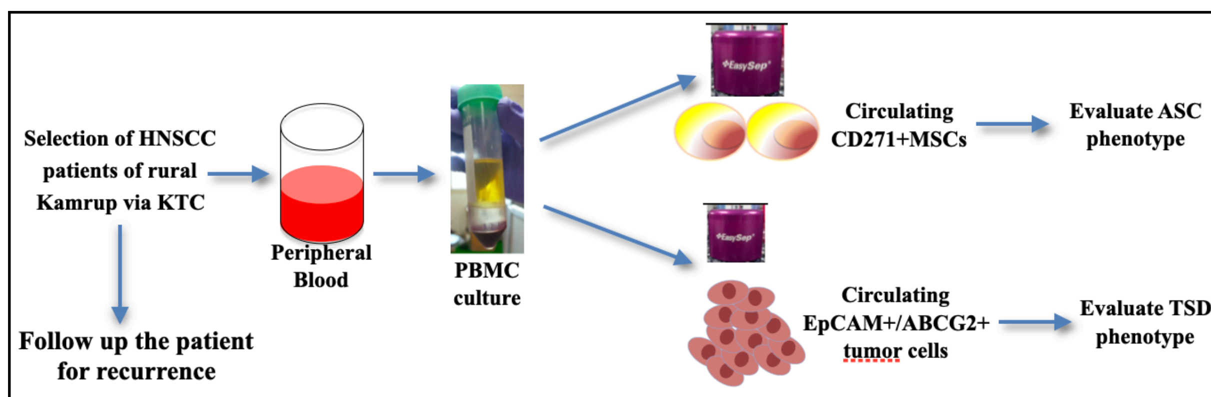

Figure 33: Experimental plan. *This schematic diagram depicts the experimental plan to evaluate the presence of circulating tumor cells with TSD phenotype and circulating MSCs with ASC phenotype in HNSCC recurrence cases.*

5.2.0 Methodologies:

All of the necessary experimental procedures were approved and undertaken inside Biosafety Cabinet class II facility in accordance with guidelines of Institutional Bio-safety Committee of KaviKrishna Laboratory.

5.2.1 Participant selection and study subjects: The study is conducted with the approval from the institutional ethics committee of respective institutions. The study protocol is also approved by the institutional committee for stem cell research, KaviKrishna. Participants were selected via KTC, utilizing the IKIN, which was previously also used to conduct clinical studies in TB (Das

B *et al.*, 2013, 2020) and cancer (Das B *et al.*, 2019). The approval providing institutes are: KaviKrishna Laboratory and Guwahati Medical College and Hospital (Guwahati, Assam, India).

Table 6: The table is presenting the clinical characteristics of 29 HNSCC subjects included in the study.

Clinical Characteristics	Cases, n (%)
All cases	29 (100)
Age	
35-49	17
50-65 years	12
Sex	
Male	18
Female	11
Clinical stage	
IIB-III	12
IV	17
Recurrence	
Non recurrent	11
Recurrent	18
Treatment	
Cisplatin+surgery	14
Surgery+cisplatin	15
Site	
Carcinoma tongue	8
Carcinoma buccal mucosa	12
Carcinoma oropharynx	4
Carcinoma larynx	5

Subjects with pregnancy, diabetes, heart diseases, low haemoglobin (<8mg/dl), and low WBC count (<3000 cells/ul blood) were excluded from the study. The newly diagnosed or recurrence HNSCC subjects (n=29, age 35-65) of stage II, IIIB and IV were included in the

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study. They were given cisplatin monotherapy either as neoadjuvant, adjuvant, or second-line therapy after recurrence. The patient information (Table 6) were obtained from the histopathology report, PETCT report, the treatment details card and certified by an in-house pathologist at KTC. The blood samples were collected after written informed consent from each patient. For the circulating tumor cell (CTC), 3 blood samples (n=21, 5-10 ml of blood/each time, 1 time/week/patient) were collected from each patient during the second cycle of cisplatin-based chemotherapy, starting from day 0. For the c-MSC study, 5-10 ml blood from each patient (n=29) were collected during the second cycle of cisplatin based chemotherapy on day 7.

5.2.2 Isolation of peripheral blood mononuclear cells (PBMCs): The collected blood was subjected to RBC lysis followed by Ficoll separation. RBC lysis was done as per manufacture instruction (RO75, Hi media, Maharastra, India). After RBC lysis, the cell pellet was diluted in PBS (pH-7.4, GIBCO Cat. No 10010-023) containing 2% FBS. In a 15 mL of polystyrene falcon tube (Corning), Ficoll-Paque Plus was pipetted followed by careful pipetting of the diluted defibrinated samples in 3:4 ratio. Centrifugation was done in break off condition at 400 × g for 30-40 minutes at 20°C. From the interface, the lymphocyte layer was collected and washed once for 5 min at 225 g and two additional times for 10 minutes at 120g at 18-20°C. The washed cells composed predominantly of lymphocytes with the remainder being monocytes thus comprising the mononuclear cells. Mononuclear cells were stained with trypan blue 0.4% (GIBCO, Cat No: 15250-061), and counted using a hemocytometer, which was estimated to be 0.5-3x10^6 mononuclear cells/ml of blood. Then, the cells were either stored with DMSO at -80°C or used for further experiment.

5.2.3. Isolation of EpCAM+/ABCG2+ CTCs from tumor spheroid culture: The isolated PBMCs were plated in ultra-low attachment dish and and then in vitro cultured in the "injured conditioned media" (ICM) (Das B *et al*., 2008) for 2 to 8 days for tumor spheroid formation. The method of ICM preparation is mentioned in chapter 4. The PBMC grown in the ICM (1.0 x 10^6 cells/4ml of ICM) facilitated the formation of tumor spheroids, and these spheroids subjected to flow cytometry to quantify EpCAM+ cell and phase contrast microscopy for tumor spheroid quantification. The tumor spheroid was dissociated and subjected to immunomagnetic sorting for EpCAM+ cells (Antibody #ab213500, Abcam, conjugated with Fluorescein isothiocyanate (FITC) by a SiteClick antibody labeling kit). The EpCAM+ cells were then expanded for 2-3 days in the ICM and subjected to immunomagnetic sorting for ABCG2+ cells by our established

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protocol as described (Das B *et al*., 2019, Garhyan J *et al*., 2015). Briefly, the EpCAM+ cells were stained with ABCG2 antibody conjugated with PE by a SiteClick antibody labeling kit (#ab 3380, Abcam) and then utilized for immunomagnetic sorting of ABCG2+ cells. The sorted EpCAM+/ABCG2+ cells were then further expanded for 7 days in ICM to study the TSD phenotype.

5.2.4 Isolation of circulating MSCs from PBMCs: The PBMCs were subjected to immunomagnetic sorting for CD45 negative cells (kit #18259). The CD45- cells were expanded in serum free StemSpan SFEM (#09600) media supplemented with Flt3 ligand, stem cell factor (SCF) and thrombopoietin (TPO) for 1 week. These cells were then subjected to CD271 positive selection using EasySep Human CD271 positive selection kit (#18659) as per manufacture instructions. These sorted CD45-/CD271+MSCs were subjected to evaluation for ASC phenotype.

5.2.5 Real time PCR study: The sorted circulating CD45-/CD271+MSCs were subjected to real time PCR to evaluate the ASC gene expression as described previously (Pathak L *et al*., 2021). Briefly, cells were re-washed in PBS & RNA isolation was done using Qiagen RNeasy mini kit followed by conversion of RNA to cDNA in thermal PCR cycler (Qiagen Rotor-Gene Q) using Sensiscript RT kit, Qiagen. c-DNA synthesis: the volume for PCR reaction mixture was 20 ul was made comprising of 5X cDNA synthesis buffer mix (8 µl), reverse transcriptase enzyme (1 ul), template RNA (5 µl) and NFW (6 µl). qPCR: the qPCR was also performed in Rotor Gene Q, Qiagen system using iTaq Universal SYBR Green Supermix at 50 cycles with 2ng of starting DNA as per instructions provided in the Primer Design Kit. RNA levels were normalized to GAPDH as an endogenous control and RNA was quantified using the ΔΔCt method using the software of Qiagen system. Primers for ASC genes; Hif2α, p53, oct4, sox2, nanog, ABCG2, CD271, CD44, VEGF, and SDF1α were designed using Primer 3 software and obtained from Bioserve, Bangalore, India.

5.2.6 In Cell ELISA: The protein levels of p53 was measured by In Cell ELISA using a horseradish peroxidase (HRP)-conjugated detection reagent (In-Cell ELISA Colorimetric detection kit, ThermoFisher, #62200) and/or standard ELISA kits.

5.2.7 Statistical Analysis: All experiments unless otherwise stated, were repeated thrice and in triplicates. Mean values ± SEM are shown. Student's t test was used for comparisons (Graph Pad Prism version 4.0a for Macintosh). Data are expressed as means ± SEM; *P < 0.05, **P < 0.001, ***P < 0.0001.

5.3.0 Results

5.3.1. Isolation of circulating tumor cells with TSD phenotype from cisplatin treated HNSCC subjects

The chapter 4 has shown the interaction between TSD+ CSCs and ASC+ MSCs in an invitro model of CSC niche defense against cisplatin induced stress. We speculated that cisplatin-induced CSC niche defense can be identified by isolating and characterizing TSD+ CTCs and ASC+ circulating MSCs (ASC+ c-MSCs) in the peripheral blood of cisplatin-treated HNSCC patients. To investigate this possibility, the PBMCs are cultured in the ICM for 1 week to obtain multicellular tumor spheroids and the number of spheroids formed was used as an indicator of the presence of TSD+ CTCs. The PBMCs were also subjected to flow cytometry to evaluate the presence of EpCAM+ population. The flow cytometry analysis of PBMC revealed the presence of 0.1-1.1 % of EpCAM+ cells in 14/21 patients throughout the chemotherapy weeks of 0 to 3 (Figure 34.1 A-B). When the tumor spheroids were dissociated and subjected to flow cytometry analysis after 1 week of culture in ICM, EpCAM+/ABCG2+ cells showed 2-20-fold expansion (Figure 34.1C). However, only 7/14 patients showed 2-16 spheroids per 1x10^5 PBMC (Figure 34.1 D-E) in the day-0. Among these patients, in the 1-3 weeks of chemotherapy, multicellular spheroids were recovered from all but the patient 4 (Figure 34.1E). Importantly, on flow cytometry analysis, these spheroids contained 8-18% of EpCAM+/ABCG2+ cells, and showed increase in the percentage of these cells from week-1 to 3 (Figure 34.1 A-C), suggesting either the increased number of circulating EpCAM+/ABCG2+ cells or the change in the phenotype of these cells leading to expansion in the in vitro ICM. The immunomagnetically sorted EpCAM+/ABCG2+ CTCs obtained from the spheroids exhibited TSD-specific gene expression, and rapid expansion in the ICM (Figure 34.2 A-B).

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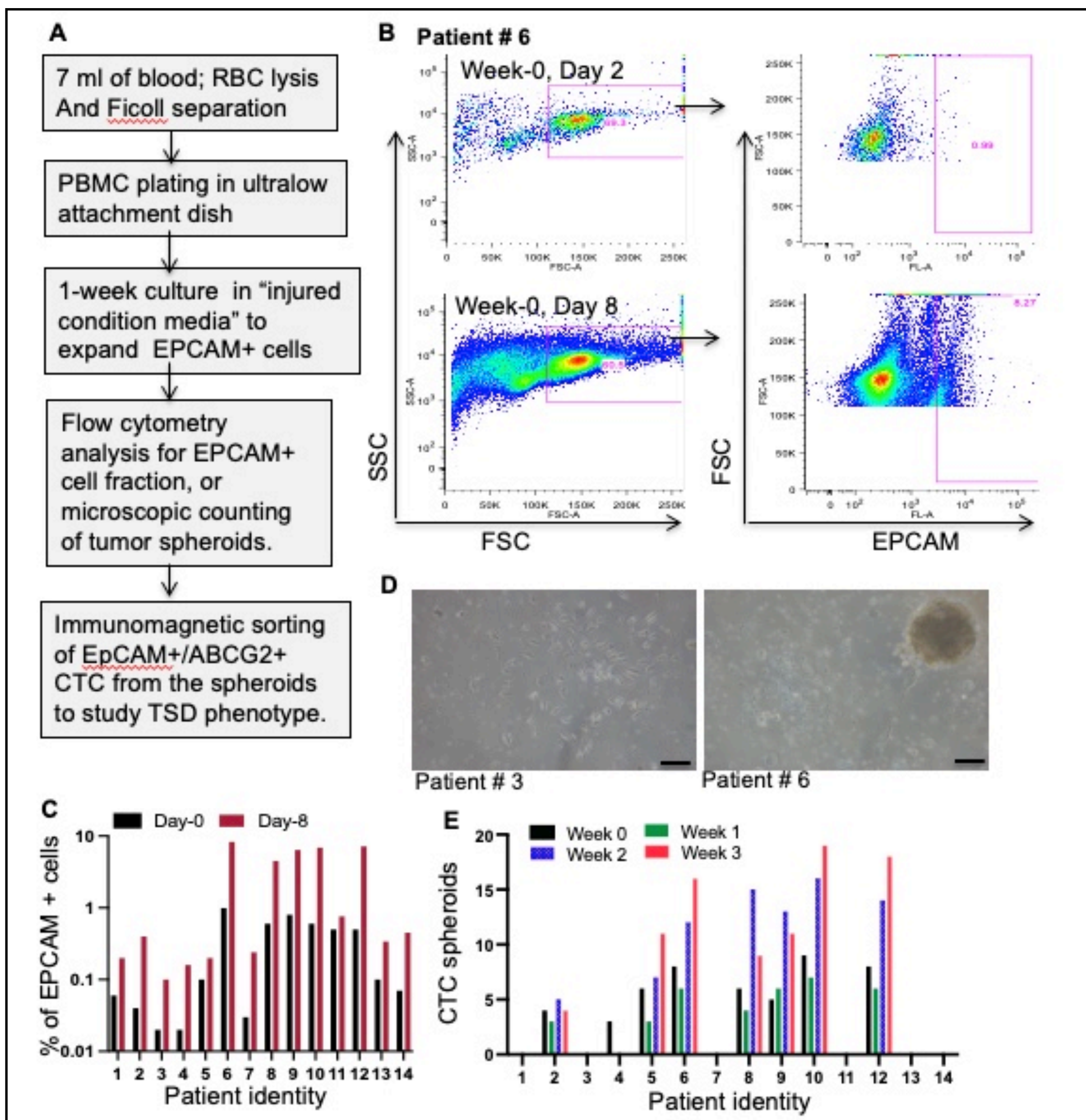
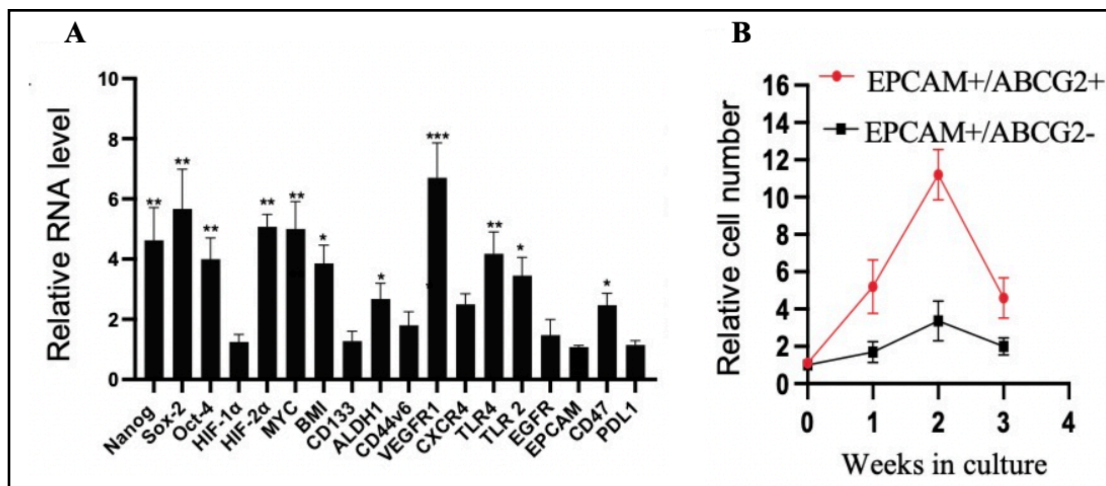


Figure 34.1: Isolation and expansion of circulating EpCAM+ cells enriched multicellular spheroids from HNSCC subjects undergoing cisplatin-based chemotherapy. ***A****. Experimental model.* ***B****. Representative flow cytometry panels showing the EpCAM+ cell population in the PBMC of Day-0, and Day-8 in vitro culture in the "injured conditioned media" derived from hypoxia-treated bone marrow stromal cells.* ***C****. Quantification of the EpCAM+ cells in the PBMC of selected patients.* ***D****. Phase-contrast images of PBMC culture showing multicellular tumor spheroids in the right image. Magnification 10X.* ***E****. Quantification of day-8 multicellular spheroids of PBMC (1.0 x 10^6 cells grown in 4 ml ICM). Week 0, is the day cisplatin 2nd cycle was started. Week 1-3, weeks after the chemotherapy dose.*



34.2 Characterisation of EpCAM+/ABCG2+ CTCs with TSD Phenotype. A: *Real-time PCR confirmed the expression of genes associated with the TSD phenotype in sorted EpCAM+/ ABCG2+ cells from multiple patients (n=5, patients 6, 8, 9, 10, 12).* ***B:*** *EpCAM+/ABCG2+ cells displayed increased growth compared to EpCAM+/ABCG2- cells when grown under low oxygen (2% O2) conditions in serum-free media.*

5.3.2 Isolation of circulating MSCs with ASC phenotype from HNSCC subjects

For the circulating ASCs, the potential presence of CD45-/CD271+ MSCs was first estimated in the circulation of selected HNSCC high grade cancer subjects being treated for platinum based chemotherapy with or without radiation (n=29). We found two clusters of patients; n=21 subjects showed 5-fold increase of circulating CD45-/CD271+ MSCs (CD45-/ CD271+ c-MSCs) as compared to the remaining n=8 HNSCC subjects (Figure 35A). Subsequently, we found that the n=21 subjects were non treatment responding, whereas the n=8 subjects responded to treatment. Next, we subjected the CD45-/CD271+ c-MSCs for in vitro culture and phenotype analysis. The sorted cells ($2.5x10^3$ cells/5ml blood) were first grown in the serum-free StemSpan SFEM medium with growth factors Flt3 ligand, SCF and TPO for 1 week for expansion. After a week, the CD45-/CD271+ c-MSCs of the n=21 cluster showed 50-65-fold expansion, and these sufficient number of the cells were subjected for phenotypic analysis. The qPCR results suggested the potential altruistic phenotype of the isolated CD45-/CD271+ c-MSCs among the n= 21 cluster (Figure 35B). ELISA confirmed the oscillatory phase of p53 in MSCs

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during 2 weeks of culture (Figure 35C). To measure the altruistic cell death, the expanded cells were continued to grow for another 1 to 2 weeks. The cells showed rapid decline in the next week of culture. These findings confirmed the ASC characteristics of CD45-/CD271+ c-MSCs obtained from HNSCC recurrence subjects.

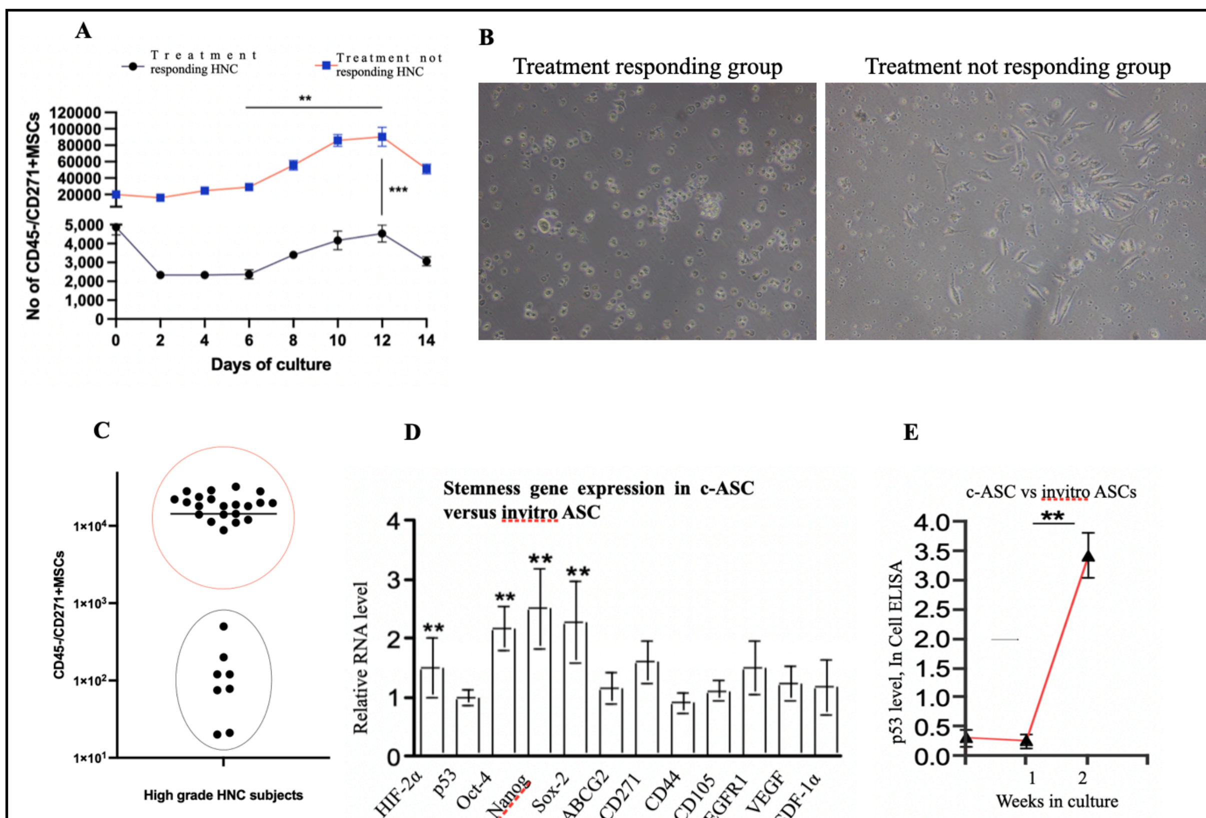

Figure 35: Evaluation of ASC phenotype of the isolated circulating MSCs from the HNSCC subjects. A*. Circulating CD45-/CD271+ MSC expansion in vitro in treatment responding versus not responding group.* ***B****. The phase contrast microscopy images of circulating CD45-/CD271+ MSC culture in treatment responding versus not responding group.* ***C****. Viability of circulating CD45-/CD271+MSCs in all the high grade HNSCC patients (n=29) using trypan blue* ***D****. Expression of ASC stemness genes using qPCR on day 8th culture of c-MSC versus invitro MSC.* ***E****. Evaluate the p53 level in the circulating CD45-/CD271+MSCs versus in vitro ASCs in 2 weeks of in vitro culture. *p < 0.05, **p < 0.01, ***p < 0.001.*

Table 7: The table is showing the association of circulating MSC and recurrence in 8 HNSCC subjects treated with cisplatin.

SL no	Patient number	CD45-/CD271+ c-MSCs	Recurrence status
1	**KKL/ASC/1**	**>100 MSCs**	After 8 months of completion of treatment
2	**KKL/ASC/2**	<100MSC	Not recurred yet
3	**KKL/ASC/3**	**>100 MSCs**	After 11 months of completion of treatment
4	**KKL/ASC/4**	**>100 MSCs**	After 7 months of completion of treatment
5	**KKL/ASC/5**	**>100 MSCs**	After 15 months of completion of treatment
6	**KKL/ASC/6**	<100MSC	Not recurred yet
7	**KKL/ASC/7**	<100MSC	Not recurred yet
8	**KKL/ASC/8**	<100MSC	Not recurred yet

The HNSCC subjects were monitored for 2-4 years post sample collection. The subjects with more than 100 c-MSCs responded to treatment for initial 8-15 months and then showed recurrence. HNSCC with less than 100 c-MSCs have responded to treatment and have not showed recurrence.

We found 21/29 subjects showed higher MSC expansion or ASC reprogramming ($>1x10^3$), whereas 8/29 subjects lower expansion of MSC expansion ($<1x10^3$) (Figure 35B). Importantly, 18/21 subjects were recurred subjects when included in the study and these subjects

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did not show respond to treatment, eventually passed away within 1 year of follow up. 3/21 subjects with ASC+ circulating MSCs were freshly diagnosed, stage III subjects. These subjects initially showed respond to treatment, then eventually deteriorated to stage IV in 4 months of starting the treatment. This result indicates that the presence of ASC+ circulating MSCs in the peripheral blood is directly associated with HNSCC treatment failure and recurrence. 8/29 subjects showed respond to treatment, however 4/8 subjects with more than 100 c-MSCs showed recurrence after 7-15 months of completion of cisplatin based treatment (Table 6). This result further strengthens the association of higher expansion of circulating MSCs or reprogrammed ASCs in the peripheral blood with HNSCC treatment failure and recurrence.

5.3.3 Biosocial medicine effect on HNSCC recurrence

We included all the 29 HNSCC subjects to measure the Sahasa-ojash scale. Through the KTC mediated CBPAR initiative (chapter 3), we gave home visit to these patients, and suggested the Nigudah care which included performing breath holding test, steam inhalation, and deep breathing, as well as 20 gm whey protein per day for three months during therapy. Satavata-tarka based FGD was performed to enhance the understanding of the care among the patients. We showed that such FGD can enhance social networking and reduce cancer disparity (Pathak L *et al*., 2019). Each week, after the FGD session, Sahasa-ojash score was measured by taking the physical dimension score of SF-36 (0-100), Rosenberg self-esteem score (0-30), Self-Assessed Collaboration Skills (SACS) score (0-7), and Hamilton anxiety scale (0-56). The results were analysed by ANOVA, which showed 20-fold increase in Sahasa-Ojash score after five weeks of Avatar-kosha therapy. Next, we assessed the degree of breathlessness before and after the Avatar-kosha therapy. The breathlessness was measured by 6 minute walking test and spirometry test. A Fisher exact test was conducted to quantify the association between Avatar-kosha care and reduction of breathlessness. The results revealed a strong association, with an odds ratio of 3.6 (p=0.032), indicating the efficacy of Avatar-kosha therapy in reducing breathlessness.

The findings of this study demonstrate the potential benefits of a CBPAR-based, Avatar-kosha therapy in managing rural cancer patients.

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5.4.0 Discussion

Chemoradiation with cisplatin is the standard of management in stage IIIB/IV and recurrence HNSCC cases (Noronha V *et al*., 2018, Irawan C *et al*., 2021). However, in 50% of advanced stage HNSCC cases, the standard management cannot prevent tumor progression or recurrence. In this chapter, we provided clinical evidence for cisplatin-induced CSC niche defense in HNSCC subjects. We used a rural telemedicine clinic-based network for selecting and monitoring 29 HNSCC subjects for the study. Here, we isolated the TSD phenotype exhibiting EpCAM+/ABCG2+ CTCs from 6 HNSCC subjects. We were further able to isolate the c-MSCs with ASC phenotype from these 6 subjects having TSD+CTC. Additionally, in 15 subjects, ASC+c-MSCs were isolated. Notably, all these 21 subjects were suffering from recurrence, and 18 of them passed away after a few months of recurrence despite treatment. On the other hand, the subjects having low numbers of circulating CD271+/CD45- MSCs (n=4, $<1x10^2$) showed treatment response. Another 4 subjects with more than 1x10^2 c-MSCs showed recurrence after 7-15 months of completion of cisplatin based treatment. The concurrent recovery of CTCs with the TSD phenotype and circulating MSCs with the ASC phenotype from the same HNSCC subjects undergoing cisplatin therapy strengthens the in vitro findings described in Chapter 4.

The findings of TSD+ CTCs and ASC+ c-MSCs from HNSCC subjects suggest potential role of TSD and ASC based defense mechanism in cancer progression. CTC in HNSCC has been extensively studied as prognostic marker and for personalized treatment strategy (Tinhofer I *et al*., 2018, Deng Z *et al*., 2022). However, CTC detection still remained as a challenging task due to its transient nature as they undergo epithelial-to-mesenchymal transition (EMT) to acquire a CSC like phenotype (St John, M. A. *et al.* 2009, Kupferman, M. E. *et al.* 2010). Though, most commonly used CTC marker in HNSCC is EpCAM (Baa AK *et al*., 2023), EpCAM based CTC detection method may not capture CTCs which have undergone EMT as they may not express EpCAM (Kulasinghe A *et al*., 2018). In such cases, vimentin, which is a widely used marker for EMT (Mendez MG *et al*., 2010) can be used as a CTC marker in HNSCC (Balasubramanian P *et al*., 2012, Mogre S *et al*., 2022). Importantly, a comparative study showed that CTC detection rate in HNSCC is higher with vimentin than EpCAM (Gap Y *et al*., 2021). Also, a recent study suggests that the presence of vimentin has more significance for prediction of poor survival and recurrence (Zhang X *et al*., 2024). However, to evaluate the TSD+CTCs in cisplatin treated

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HNSCC subjects, we used EpCAM as the marker for CTC as EpCAM has been found previously in cisplatin resistant HNSCC cells (Noman ASM *et al*., 2020). Also, we have demonstrated hypoxia induced TSD phenotype in EpCAM+/ABCG2+ CSCs of HNSCC cell line; SCC-25 (Bhuyan S *et al*., 2022). Therefore, to be specific, we first isolated the EpCAM population followed by ABCG2 population from the EpCAM+CTCs and evaluated the TSD phenotype. However, the vimentin specific findings indicated that Vimentin+ CTCs may also exhibit TSD phenotype.

Circulating MSCs were found in human subjects with diseases including cancer although their phenotype and pathological roles are not yet determined. Recent progress in the cancer pre-metastatic field indicates the potential role of circulating BM derived progenitor cells in the modulation of the pre-metastatic niche (Wang H *et al*., 2021, van der Velden DL *et al*., 2018) for tumor progression. Studies have shown the presence of circulating MSCs in the peripheral vein of patients undergoing bone surgery (Churchman SM *et al*., 2020). In patients of metastatic breast cancer, circulating cancer associated fibroblast (CAF) was identified by FAP and α-SMA co-expression (Ao Z *et al*., 2015). Researchers showed the presence of circulating CAFs in breast tumor mouse xenograft (Lu T *et al*., 2023). The circulating CAFs along with CTCs were also identified in prostate cancer (Ortiz-Otero N *et al*., 2020). However, c-MSCs along with CTCs were not identified before. One of the reasons could be the rare number of c-MSCs, which limit our ability to detect these cells. In healthy subjects, only 0.0018% of PBMCs are MSCs (Rebolj K *et al*., 2018), whereas in cancer patients, 0.005% of PBMC are found to be MSCs (van der Velden DL *et al*., 2018). The rare presence of c-MSCs limits our understanding about the significance of these cells in tumor progression. In this context, our findings of expansion of circulating MSCs in the cisplatin treated subjects may provide a novel insight in the field of significance of MSC in tumor progression and metastasis. Future study in a large population may identify ASC+ MSCs to complement TSD+ CTC as a clinically relevant biomarker to predict metastasis and recurrence. However, it is not yet clear, why the CD271+ MSCs are the target of CSC niche defense mediated MSC to ASC reprogramming. A possibility for specific targeting of the CD271+ MSCs may be their preferential location in the hypoxic niche of BM, a favorite site for cancer metastasis. Presence of TSD+ CTCs and ASC+ MSCs in the circulation also indicates the stem cell's niche to niche interaction during cisplatin induced stress. However, further study

has to be conducted to provide more evidence on interaction of primary tumor niche and circulating tumor niche.

Importantly, this study provided preliminary evidence on stem cell niche defense and their potential association with HNSCC recurrence. 18/21 subjects with c-ASC (>10^4 MSCs) were recurred subjects and did not show respond to treatment. Interestingly, 7/18 subjects showed the presence of TSD exhibiting CTCs. Additionally, out of treatment responding 8 subjects, 4 with more than 100 c-MSCs showed recurrence after 7-15 months of completion of chemo radiation.

This study demonstrated the clinical significance and molecular mechanism of CSC niche defense that can rewaken the dormant CSC to defend the niche from therapy. Future studies required to find out the immune component of these TSD phenotype and developing small molecular inhibitor that may target the stemness pathways to manage these 30% of OSCC that fail to respond to cisplatin therapy. In these population, having locally available herbal extracts with stemness inhibitory potential may provide affordable care of many rural patients who still rely on cisplatin monotherapy.

5.5.0 Conclusion

In summary, this clinical finding strengthens our in vitro work that CSCs with TSD phenotype can reprogram CD271+MSCs to ASC phenotype. Additionally, the results provided a preliminary data on isolation of CTC and ASCs from peripheral blood of cisplatin treated HNSCC subjects, which could be further explored to develop a treatment progress monitoring strategy for HNSCC subjects. Also, future work is needed to evaluate the usefulness of the ASC exhibiting MSCs and TSD exhibiting CTCs as the biomarkers of HNSCC progression, treatment failure and recurrence.

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Chapter 6.0

Discussion and Future Perspective

Stem Cells reside in their protective niches. The niches may exert altruistic defense mechanisms to protect the stem cells from external stress including pathogen invasions as well as cancer migration/metastasis. Pathogens and cancer cells may also hijack the stem cell niche defense for their own benefit including dormancy and reactivation. Previously, our lab showed that *Mtb*, the causative agent of TB may hijack CD271+ BM-MSCs for intracellular dormancy. MSCs were also found to cross-talk with cancer stem cells to maintain cancer dormancy. In this thesis work, the potential reprogramming of MSCs to p53 deficient altruistic stem cells (ASCs) by *Mtb* and CSCs has been studied. ASCs being a component of the altruistic stem cell niche defense mechanism, we speculated that both *Mtb* and CSCs may hijack ASCs for their own benefit. As hypothesized, the study has demonstrated that *Mtb* and CSCs may hijack the stem cell niche defense mechanism for recurrence/relapse following therapy.

The present findings reveal a unique defense strategy of stem cell niche, which could be exploited by the intracellular pathogen or cancer stem cells for disease progression and recurrence. The stem cells may exert altruistic behaviour as a defense strategy to protect their niches from external threats and internal stress conditions. We first reported this altruistic behaviour of stem cells by using an in vitro model of embryonic stem cell (ES cells) niche. In this in vitro model, human ES cell colonies were exposed to hypoxia/oxidative stress and then the surviving cells were characterized for stemness. In this manner, a sub-population of cells, the ABCG2+ cells were identified to confer transient suppression of p53, and exhibited altruistic behaviour i.e. sacrificing self-fitness to enhance group fitness. This observation was described as the mechanism of stem cell altruism (Das B *et al*., 2012), therefore, an example of altruistic defense. The stem cell altruism could be defined as a fitness-defense mechanism that protects weak neighbours in their niche (Pal B and Das B 2017), in contrast to stem cell competition, which is a fitness-sensing mechanism that eliminates weak neighbours in the niche (Di Gregorio A, *et al*., 2016). The idea of stem cell altruism is inspired by the Indian Vedic altruism theory of Jiva Upakarvada (Pathak L *et al.,* 2021). The underlying significance of further studying stem cell altruism is that it may be an evolutionary conserved innate defense mechanism of stem cell

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niche having potential similarity to microbial altruistic defense against stress (Das B, The Science Behind Squalene, Toronto University Press, 2000 and Das B, 2014).

6.1 The role of stem cell niche defense in TB reactivation or recurrence

The chapter 2 of this study provides the first evidence of the potential role of stem cell-based altruistic defense against viral infection of alveolar stem cell niches. The cytoprotective activity of the reprogrammed lung MSC-derived CD271+ ASCs was demonstrated in MHV-1-mediated toxicity of lung alveolar epithelial cells. The conditioned media of the ASCs recovered from the MHV-1-infected mouse lung decreased the viral load and increased the ATII cell survival and proliferation when the ATII cells were infected with MHV-1. After 2 weeks of invitro culture, these ASCs spontaneously activated apoptosis/differentiation-associated genes, and underwent apoptosis. This ASC-based defense mechanism may be utilized to develop novel therapeutics against infectious diseases. However, future research is required to validate the altruistic nature of these reprogrammed MSCs, including the secretion of cytoprotective biomolecules and antimicrobial peptides or miRNA that may shield the epithelial stem cell niche from viral toxicity or inflammation. Importantly, this ASC-based defense was exploited by the intracellular *Mtb* that remain inside the MSC for a prolonged period escaping therapy, as we have shown in this study. Thus, using a mice model of Mtb dormancy, this study has shown ASC reprogramming induces intracellular *Mtb* replication followed by extracellular release and infection to other cells of lung niche. The *Mtb* CFU in mice lung increased 630 fold on day 20 of MHV-1 infection, suggested that mice showed TB reactivation. Thus, this model of *Mtb* and MHV-1 co-infection in mice may be a useful model to study the role of ASC niche defense mechanism in pathogen/host interaction (Figure 36). This stem cell model of *Mtb* reactivation may be utilised to develop diagnostic to detect future dormant *Mtb* reactivation and also to develop therapeutics against viral infection.

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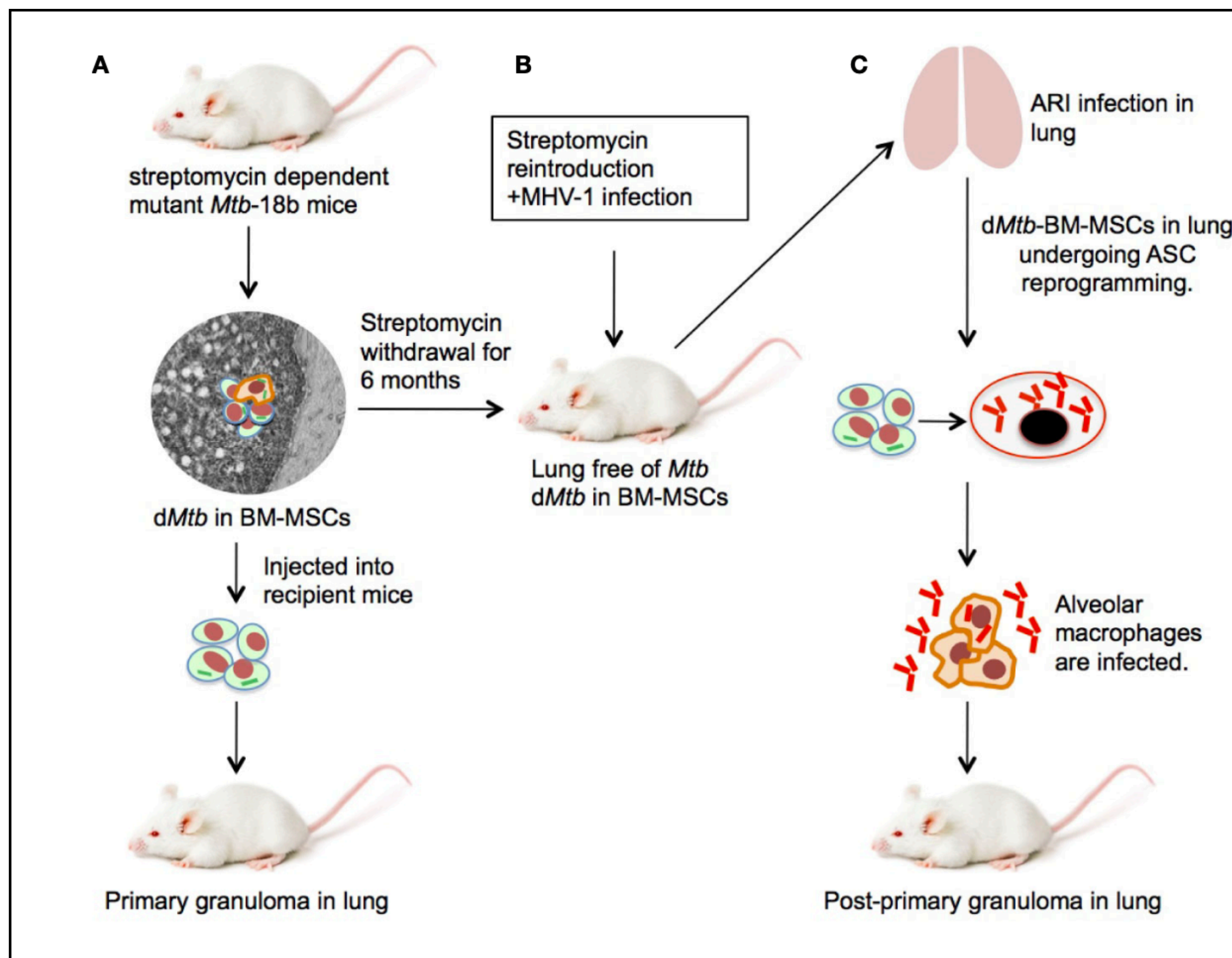

Figure 36: An experimental mouse model of stem cell mediated TB reactivation. *The MHV-1, mouse coronavirus strain was used to induce the ARI infection, which further activated the stem cell niche defense by reprogramming the dMtb harboring MSCs into ASCs. During ASC reprogramming, intracellular Mtb replicated, released and infected other cells of lung, caused TB reactivation.*

The stem cell niche defense may involve the cross-talk between BM and lung stem cell niche. We hypothesized that the BM-MSC harbouring d*Mtb* may migrate to the lung during MHV-1-induced inflammation and initiate the d*Mtb* reactivation. Therefore, we sought to find circulatory MSC having intracellular *Mtb* in the MHV-1 and d*Mtb* co-infected mice. Infected mice, and the isolated PBMC were subjected for immunomagnetic sorting and *Mtb* culture. Thus, we were able to recover circulating CD271+/CD45- MSCs in the *Mtb*/MHV-1 infected mouse, and then recover viable *Mtb* from the cell population. At the same time, we also recovered viable

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Mtb from the CD271+ BM-MSCs of the mouse BM. We noted that the circulating CD271+ BM-MSCs with intracellular *Mtb* appeared 2-3 days before the appearance of the *Mtb* harboring CD271+ BM-MSCs in the lung. These data indicate that peripheral blood could be a source to isolate *Mtb* undergoing dormancy to reactivation as per the BM and lung stem cell niche-to-niche interaction. In the chapter 3, we tested this hypothesis by obtaining the circulating CD271+ MSCs from subjects with PTB.

We reasoned that lung granulomas of active PTB subjects and the Bm stem cell niche may continue to maintain the niche-to-niche interaction as a part of stem cell niche defense against *Mtb* infection. We also speculated that the d*Mtb*/stem cell host-pathogen interaction may contribute to d*Mtb* evolution to MDR phenotype intracellular to stem cells. Therefore, we planned to isolate and culture *Mtb* of circulating CD271+ cells (which are enriched in both MSCs and HSC phenotype), and then perform WGS to study MDR evolution. We also planned to investigate the recurrence of PTB following successful treatment, and whether the recurrence is associated with the d*Mtb* evolution to MDR to be detected in the c-MSCs or BM-MSCs. Specially, we were interested to find compensatory mutation, as the MDR mutation (rpoB) of *Mtb* is associated with fitness loss, which is compensated by compensatory mutation in the rpoC region of *Mtb* genome. Thus, we planned to recruit subjects with PTB for our study including the isolation of c-MSCs as well as BM-MSCs, and do necessary follow-up for recurrence.

For the above purpose, a long-term follow up and sustained engagement of the patients and their families are necessary. In this context, our lab has also developed a community-based participatory action research (CBPAR) program through the KaviKrishna Telemedicine Care (KTC). It is located in rural area of Sualkuchi-Hajo cultural complex of Kamrup, district, Assam, India. KTC has been providing free consultations and follow up care to local TB and cancer patients since 1994, and started a CBPAR program in 1998 to continue with a TB contact investigation study (Das B *et al*., 2020), as well as nutraceutical study of dietary supplements (Das B, 2000). Nearly two and half decades of CBPAR led to the development of the KaviKrishna Cikitsha Satra network by implementing the philosophy of the indigenous knowledge system, the Vedic Jiva Upakara Tantra (Mitra S *et al*., 2024). The KaviKrishna Cikitsha satra network was not only utilized to screen for PTB subjects but also to monitor these subjects for TB recurrence (Baishya T *et al.*, 2024).

Indeed, we were able to isolate viable *Mtb* from circulating CD271+MSCs of 12 TB subjects and these subjects also showed association with TB recurrence with MDR evolution. We found rpoC mutation in the *Mtb* strain obtained from c-MSCs of TB subjects, which showed TB recurrence with mutation in the rpoB region of sputum derived *Mtb* after 8-15 months of completion of anti TB treatment. This result indicated that mutation may occur in rpoC region of *Mtb* intracellular to MSC and may slowly evolve into mutation in rpoB. Thus, identifying rpoC mutation in c-MSC *Mtb* DNA will indicate the subject's potential to become MDR in the future. We could also detect rpoB mutation in intracellular *Mtb* before it gets detected in sputum derived *Mtb*. Thus, future research is necessary to gain further insight into the role of the stem cell niche defense in MDR evolution.

Despite years of TB control program, the TB cases are increasing with increased incidence of MDR TB. The TB associated morbidity is also increasing. In 2021, 2.5% new MDR TB cases were diagnosed in India, where 13% are TB recurrent or previously treated cases. This is why, it is important to learn more about the MDR evolution during disease recurrence. Thus, the study of chapter 2 and 3 now provides a novel insight into the TB transmission i.e. relevance of c-MSCs in providing a protective niche for d*Mtb* reactivation and MDR evolution. The chapter 3 also helped us further consolidate the telemedicine based network for patient follow up in the entire North East India.

In the future, this network can be used to increase the study population to validate the role of stem cell niches in TB reactivation and MDR evolution. This work can be extended to develop diagnostic for identifying MDR TB cases and potential TB reactivation cases in early stage using their peripheral blood samples before it is diagnosed in sputum. Combining the Cikitsa satra network and MSC based diagnostic approach may lead to the development of a new public health tool for TB management that may contribute to preventing transmission of MDR TB in the community.

6.2 The role of stem cell niche defense in therapy resistance and HNSCC recurrence

The study hypothesized that cisplatin treatment would induce a tumor stemness defense (TSD) phenotype in CSCs, leading to the reprogramming of MSC into an altruistic stem cell (ASC) phenotype, which would in turn promote cisplatin resistance in cancer cells. The results largely support this hypothesis (Figure 37). The chapter 4 and 5 of this study demonstrated the

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potential role of stem cell niche defense and HNSCC recurrence. India has the highest HNSCC cases with more than 50% recurrence rate post-therapy. Using an invitro model of SCC-25 derived CSCs, it was observed that cisplatin treatment indeed led to the emergence of TSD characteristics in EpCAM+/ABCG2+ CSCs, and that conditioned media from these cells could reprogram MSCs into ASCs. This TSD phenotype exerts the same defense behaviour similar to ASCs for defending the niche when exposed to stress or drugs. This TSD phenotype is altruistic in nature with niche modulatory and self sufficiency potential. The conditioned media of TSD phenotype (TSD-CM) secreted protumorigenic factors that further reprogram the MSCs to ASCs by activating the inflammatory stemness pathway. Furthermore, the finding that ASC-CM enhanced cisplatin resistance in SCC-25 cells further strengthens the proposed model of CSC-MSC interaction driving therapy resistance. This alignment of results with the hypothesis underscores the potential significance of the TSD-ASC axis in tumor recurrence following chemotherapy. Thus, this invitro model of CSC-MSC interaction demonstrated how the invasive TSD phenotype of CSCs modulates the niche for tumor growth. This CSC-MSC interaction model may be used to study the behaviour of other cells in the tumor niche when treated with TSD-CM.

The findings of this study contribute to the growing body of evidence highlighting the dynamic interplay between CSCs and their microenvironment, particularly the role of MSCs in supporting CSC survival and therapy resistance. The concept of a "stem cell niche defense" mechanism, where CSCs actively modify their niche to enhance their own survival, aligns with previous research demonstrating that CSCs can interact with and influence various stromal cell types within the TME. The identification of the HMGB1/TLR4 pathway as a mediator of ASC reprogramming adds another layer of complexity to our understanding of CSC niche interactions. These findings emphasize the importance of considering the dynamic nature of the TME and its role in therapeutic resistance.

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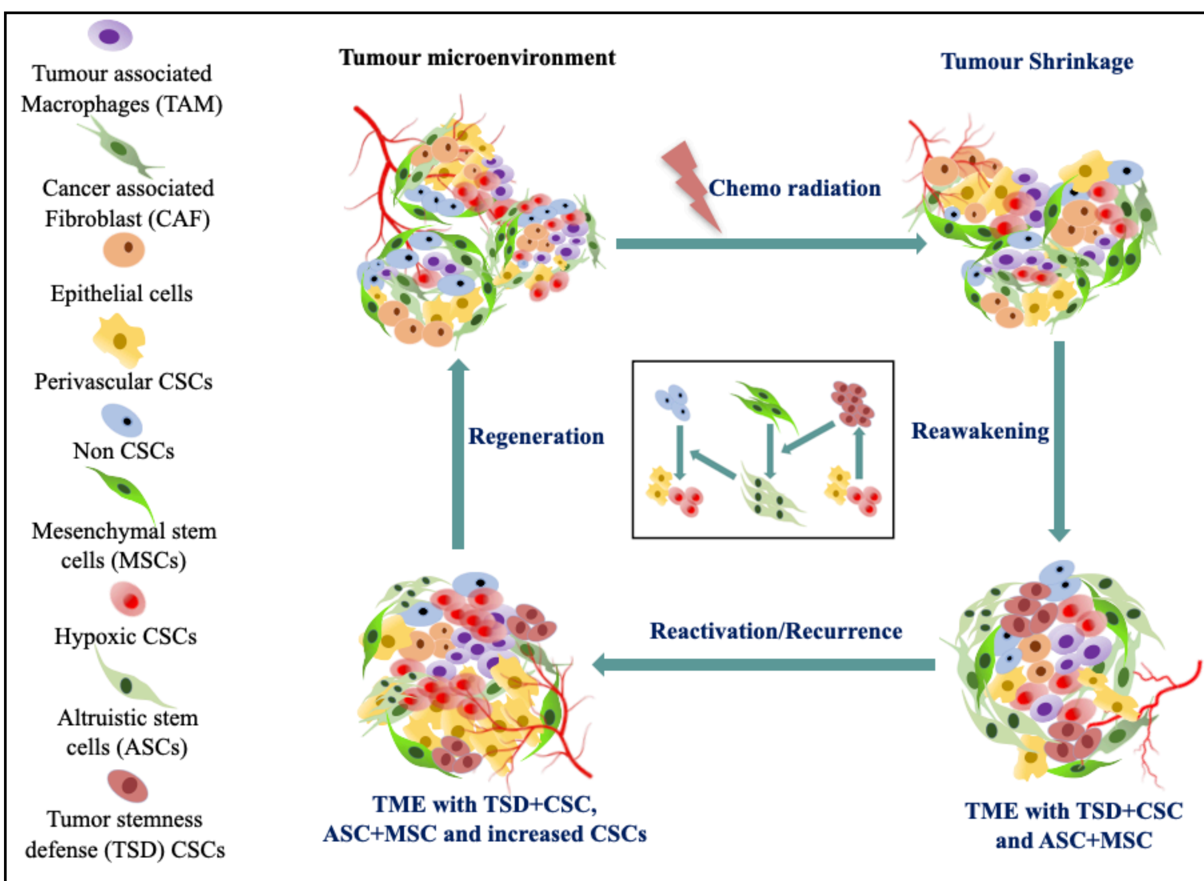

Figure 37: The activation of CSC niche defense lead the cancer recurrence. *This invitro model is tested with SCC-25 cell line, a representative HNSCC cell line. Next, evaluated the presence of TSD+CTCs and ASC+MSCs in HNSCC subjects and monitored the patients for recurrence.*

The in vitro study findings predicted that the CSC and MSC interaction in the TME may lead to the release of TSD exhibiting circulating tumor cells (CTCs) and ASC exhibiting MSCs in the circulation. Indeed we isolated TSD+CTCs and ASC+MSCs from the circulation of HNSCC subjects managed with cisplatin. Therefore, we performed a prospective study to find the association between the presence of TSD+ CTCs, circulating ASCs, and HNSCC recurrence. We found the association of c-ASC, TSD+CTCs and HNSCC recurrence. While the study strongly suggests a causal relationship between TSD+CTCs, c-ASC, and cisplatin resistance, alternative explanations or contributing factors should be considered. For example, other factors secreted by TSD phenotype or present in the TME might also influence MSC reprogramming and drug resistance. Additionally, the ASC phenotype might not be the sole mediator of therapy resistance; other mechanisms within the CSC niche could also contribute. Further investigations

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are needed to dissect the precise roles of different factors and mechanisms involved in CSC-mediated therapy resistance.

The above clinical findings strengthen the in vitro work that CSCs with TSD phenotype can reprogram CD271+MSCs to ASC phenotype. Indeed, in the 7/18 subjects we were able to recover the circulatory EpCAM+/ABCG2+ cells with TSD phenotype. The HNSCC subjects were also screened, and monitored via KaviKrishna telemedicine care (KTC), Sualkuchi utilising the KaviKrishna Cikitsha satra network. Considering the clinical significance of the findings, more study subjects may be enrolled utilizing this network in future to evaluate the presence of c-ASC in HNSCC subjects. Also, further work is needed to evaluate the usefulness of the circulating CD45-/CD271+ cells as the biomarker of HNSCC progression, treatment failure, and recurrence.

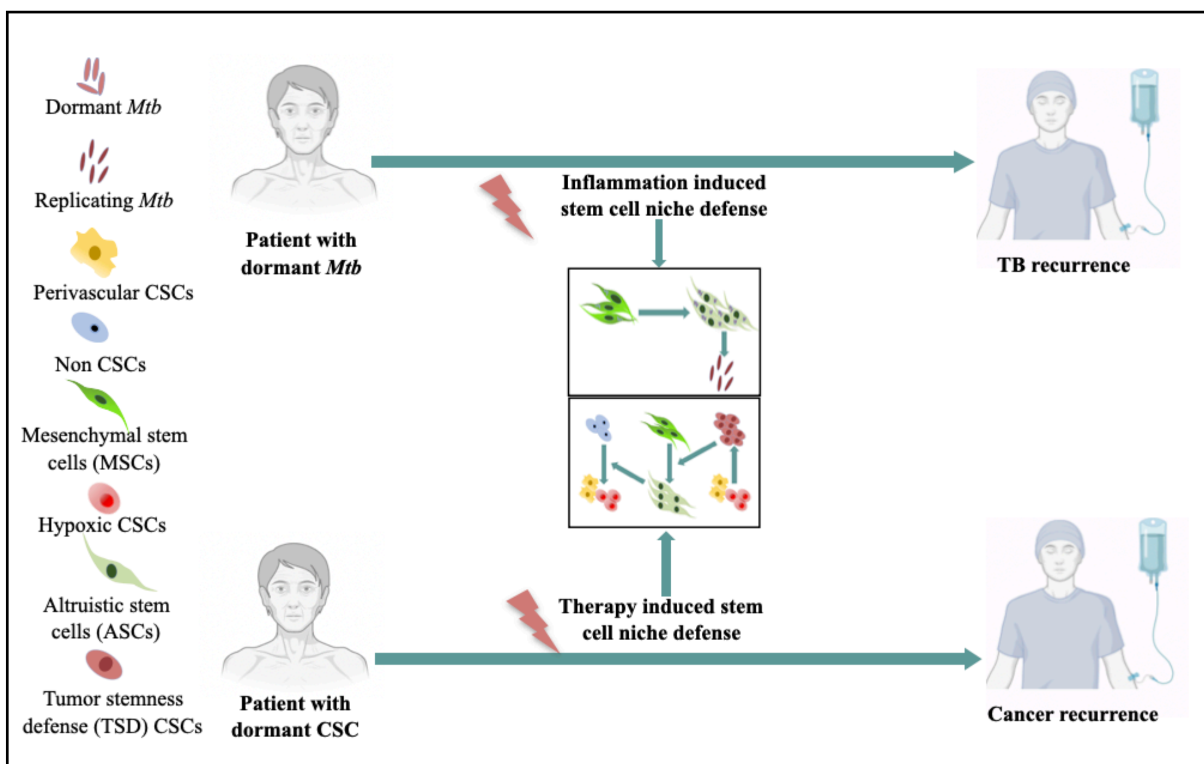

Figure 38: The summary figure of the PhD work. *The schematic figure is showing that stem cell niche defense mechanism may contribute to TB and cancer reactivation or recurrence.*

6.3 Rural Indigenous Knowledge Systems in Long-term Clinical Investigations

Conducting long-term clinical investigations within rural indigenous populations necessitates a unique approach that integrates indigenous knowledge systems into research practices. This approach respects the cultural context, values, and beliefs of the community, ensuring that research is conducted ethically and in a culturally sensitive manner. By leveraging an indigenous knowledge-based network system, researchers can establish a collaborative partnership with the community, fostering trust and promoting participation. This system draws upon the deep understanding of local health practices, environmental factors, and social dynamics within the community. It involves incorporating traditional healers, community leaders, and elders in the research process, from study design to data collection and interpretation. This collaborative approach ensures the research is relevant to the community's needs and priorities.

KTC's journey in community-based research began in 1994 with a TB contact investigation study and an examination of the role of dietary supplements in mitigating cancer chemotherapy side effects. These initial endeavors utilized a Community-Based Participatory Action Research (CBPAR) approach, emphasizing deep community engagement and the integration of Indigenous Knowledge Systems (IKS). This foundation laid the groundwork for KTC's subsequent work in TB control and cancer care, demonstrating a long-term commitment to culturally sensitive and community-driven healthcare interventions.

Through my involvement in KTC activities since 2018, and by employing a KTC-based CBPAR approach for my clinical studies (described in chapters 3-5) on both cancer and TB patients, I've gained firsthand experience of the strengths and limitations of this model.

Strengths are 1) Enhanced cultural relevance: By embedding indigenous knowledge systems into the research process, studies are more likely to address the specific health concerns and priorities of the community, leading to more meaningful and impactful outcomes. 2) Increased community participation: Engaging the community in all stages of the research fosters a sense of ownership and empowerment, leading to increased participation rates and improved data quality. 3) Strengthened trust and rapport: Building relationships based on mutual respect and understanding fosters trust between researchers and the community, facilitating long-term collaborations. 4) Improved study outcomes: Incorporating local knowledge and practices can

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enhance the effectiveness of interventions and improve the overall health outcomes of the community.

The limitations are 1) Time and resource intensive: Building and maintaining strong relationships with the community and integrating indigenous knowledge systems requires significant time and resources. 2) Potential for conflicting perspectives: Balancing scientific methodologies with indigenous knowledge systems can be challenging, and navigating differing perspectives may require careful negotiation and compromise. 3) Generalizability of findings: The unique cultural and contextual factors within indigenous communities may limit the generalizability of CBPAR methodologies to other populations.

Greater recognition of the value of indigenous knowledge systems in clinical research is needed, along with increased funding and support for community-based research initiatives. Training programs and mentorship opportunities for indigenous researchers are crucial for developing local research capacity and leadership. Finally, shifting power dynamics and promoting equitable partnerships between researchers and indigenous communities is essential for decolonizing research and ensuring ethical conduct.

Thus, integrating indigenous knowledge systems into long-term clinical investigations offers a promising approach to conducting culturally relevant and impactful research within rural indigenous communities. While challenges exist, the potential benefits of enhanced participant engagement, improved study outcomes, and strengthened community partnerships make this a valuable model for future clinical research.

6.4 Future Directions

Future studies are also needed to explore how we can exploit the MSC and CSC interaction to target the CSCs during therapies. Several studies have been reported on cross-talk between MSCs and cancer cells in the context of angiogenesis, cancer cell proliferation, invasion and metastasis (Cuiffo BG *et al*., 2012, Jing Y *et al*., 2022, Liu S *et al*., 2011, Mishra PJ *et al*., 2009). However, targeting CSC-MSC interaction for a therapeutic approach is not yet successful. Recently, the present lab has shown that BCG can induce TSD phenotype in CSC population of SCC-25 cell line and then cause bystander apoptosis (Bhuyan S *et al*., 2022). This invitro and invivo finding suggest that the CSCs activating TSD based niche defense mechanism may have

therapeutic potential. Thus, future studies may be conducted in HNSCC subjects whether BCG can target the TSD phenotype.

The model of stem cell niche defense could be utilised to develop biomarker for early detection of disease recurrence. The findings of the association of c-MSC having intracellular *Mtb* with TB recurrence has great implication in the field of TB management. Even with the preliminary data, our findings on rpoC and rpoB mutations in c-MSC *Mtb* isolates potentially offer valuable insights into future recurrence with drug resistance phenotype of *Mtb*. Importantly, it is reported that 18% MDR TB cases are previously treated drug sensitive TB cases (Sharma N *et al*., 2020, Shivekar SS *et al*., 2020). In this context, our findings of stem cell niche in MDR-*Mtb* evolution in a TB patient cohort from north east region may provide a novel approach for early diagnosis of MDR TB and TB recurrence.

Similarly, the findings of TSD+ CTCs and ASC+ c-MSCs from HNSCC recurrence subjects suggest potential role of TSD and ASC based defense mechanism in cancer recurrence. The CTC in HNSCC has been extensively studied as prognostic marker and for personalized treatment strategy (Tinhofer I *et al*., 2018, Deng Z *et al*., 2022). However, CTC detection still remained as a challenging task due to its transient nature as they undergo epithelial-to-mesenchymal transition (EMT) to acquire a CSC like phenotype (St John, M. A. *et al.* 2009, Kupferman, M. E. *et al.* 2010). In this context, our method of isolating the EpCAM population followed by ABCG2 population from the EpCAM+CTCs and the TSD characterisation of these EpCAM+/ABCG2+ CTC may provide an efficient method of CTC detection. Additionally, our findings of expansion of circulating MSCs in the HNSCC recurrence subjects may provide a novel insight in the field of significance of MSC in tumor progression and metastasis. Future study in a large population may identify ASC+MSCs to complement TSD+ CTC as a clinically relevant biomarker to predict metastasis and recurrence.

6.5 Conclusion

As hypothesized, the study has demonstrated that *Mtb* and CSCs may hijack the stem cell niche defense mechanism for recurrence following therapy. Using a mice model of Mtb dormancy in BM-MSC, this study demonstrated that during MHV-infection, the Mtb harboring MSCs in the lung reprogram into ASCs to protect the niche from infection-induced

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inflammation. The intracellular *Mtb* exploits this ASC reprogramming for intracellular replication followed by extracellular release leading to PTB reactivation. Importantly, we speculated that the BM-MSC harboring d*Mtb* may migrate to the lung during inflammation and induce reactivation. Indeed, from the mice with d*Mtb* present only in the BM, we could obtain circulatory MSCs harboring *Mtb* following MHV-1 infection, indicating that the source of these *Mtb* harboring MSCs are BM stem cell niche. Further, we validated this finding in clinical subjects. We isolated viable *Mtb* from c-MSCs of TB subjects, and interestingly these subjects showed recurrence post 8-15 months of successful anti TB treatment. The reprogrammed c-MSCs and intracellular *Mtb* were obtained from the recurrent TB cases. Similar to TB, c-MSCs having ASC phenotype were also isolated from subject with cancer subjects suffering from HNSCCs. In an in vitro assay, CSCs of cisplatin-treated SCC-25 cells reprogrammed into the TSD phenotype characterized by invasive, and inflammatory stemness phenotype. Notably, the conditioned media of the TSD phenotype activated stem cell niche defense mechanism by reprogramming the MSCs into niche modulatory ASCs. These ASCs are immunosuppressive, thus may promote tumor growth in the niche. Further, we could isolate TSD+CTCs and c-ASCs from HNSCC subjects under cisplatin treatment. This study revealed that higher number of c-ASCs may be associated with higher potential of HNSCC recurrence. Future study and larger sample size is required to validate the role of ASCs in disease recurrence and treatment failure. Moreover, this study revealed a fundamental and underlying mechanism of disease progression; ASC based niche defense (Figure 38). Therefore, this ASC based niche defense has significance in diagnostic, therapeutics and translational research, specially in the field of infectious diseases and cancer.

Moreover, this study has demonstrated a novel insight into stem cell niche defense mechanisms in disease progression, treatment failure, and recurrence. The stem cell niche defense mechanism may lead to the development of diagnostics for early detection of TB reactivation, MDR evolution, and HNSCC recurrence. It has also presented a unique way of conducting long-term clinical investigations in a rural population using an indigenous knowledge-based network system (Das B *et al.*, 2020). Therefore, this study has significant implications in the field of public health development by contributing in TB and cancer management, especially in rural populations.

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Abbreviation

ABC	ATP-Binding Cassette
ABCG2	ATP-Binding Cassette Subfamily G Member 2
AFB	Acid Fast Bacilli
AKT	AK Strain Transforming
ALDH	Aldehyde Dehydrogenase
ANOVA	Analysis of Variation
ARI	Acute Respiratory Infection
ASC	Altruistic Stem Cell
ATCC	American Type culture collection
BCG	Bacillus Calmette-Guerin
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BM-MSC	Bone Marrow- Mesenchymal Stem Cell
BMP	Bone Morphogenic Protein
BSC II	Biosaftey Cabinet Class Ii
BSO	Buthionine Sulphoximine
CAF	Cancer-Associated Fibroblast
CBNAAT	Cartridge-Based Nucleic Acid Amplification Test
CBPAR	Community Based Participatory Action Research
CCL2	C-C motif chemokine ligand 2
CD	Cluster Of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony Forming Units
CHC	Community Health Centre
CM	Conditioned Media
c-MSC	Circulatory MSC
COPD	Chronic Obstructive Pulmonary Disease
CRP	C- Reactive Protein

CSC	Cancer Stem Cells
CTC	Circulating Tumor Cells
CXCL 8	C-X-C Motif Chemokine Ligand 8
CXCR 2	C-X-C Motif Chemokine Receptor 2
DMEM	Dulbecco's Modified Eagle Medium
dMtb	Dormant *Mycobacterium tuberculosis*
DNA	Deoxyribonucleic Acid
DST	Drug Sensitivity Test
DTC	Disseminated Tumor Cells
EAI	East African Indian
EC	Endothelial Cells
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immune Sorbent Assay
ELDA	Extreme Dilution Limiting assay
EMT	Epithelial-To-Mesenchymal Transition
EpCAM	Epithelial Cellular Adhesion Molecule
EPTB	Extra Pulmonary Tuberculosis
ERK	Extracellular Signal Regulated-Kinase
ESC	Embryonic Stem Cell
ESR	Erythrocyte Sedimentation Rate
FBS	Fetal Bovine Serum
FCR	Fear Of Cancer Recurrence
FGD	Focused Group Discussion
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
GCL	γ-glutamylcysteine synthetase
GSH	Glutathione
HEPES	4-(2-Hydroxyethyl) Piperazine-1-Ethanesulfonic Acid, N-(2-Hydroxyethyl) Piperazine-N′-(2-Ethanesulfonic Acid)

hESC	Human Embryonic Stem Cells
HGF	Hepatocyte Growth Factor
HIF1α	Hypoxia Inducible Factor 1 Alpha
HMGB 1	High Mobility Group Box 1
HNC	Head And Neck Cancer
HNSCC	Head And Neck Squamous Cell Carcinoma
HOS	Osteosarcoma
HPV	Human Papillomavirus
HRP	Horseradish Peroxidase
HRQOL	Health Related Quality Of Life
HSC	Hematopoietic Stem Cells
IAEC	Institutional Animal Ethics Comittee
IBSC	Institutional Biosafety Comittee
ICM	Injured Conditioned Media
IFN	Interferon
IFI6	Interferon-alpha inducible protein 6
IKIN	Indigenous Kamarupa Information Network
IKS	Indigenous Knowledge System
IL	Interleukin
INH	Isoniazid
ISG	IFN- Stimulating Genes
JAK	Janus Kinase
JUT	Jiva Upakara Tantra
JUV	Jiva Upakarvada
KTC	Kavikrishna Telemedicine Care
LJ	Lowenstein-Jensen
LTBI	Latent TB Infection
MAPK	Mitogen-Activated Protein Kinase
MDM 2	Murine Double Minute 2

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MDR	Multi Drug Resistance
MDSC	Myeloid-Derived Suppressor Cell
MET	Mesenchymal Epithelial Transition
MHC	Major Histocompatibility Complex
MHV-1	Murine Hepatitis Virus-1
miRNA	Micro Ribonucleic Acid
ML	*Mycobacterium Leprae*
MNC	Mononuclear Cell
MSC	Mesenchymal Stem Cells
Mtb	*Mycobacterium Tuberculosis*
mTOR	Mammalian Target Of Rapamycin
NAC	N-Acetylcysteine
NE	North East
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer Cells
NTEP	National Tuberculosis Elimination Program
NTM	Nontuberculous Mycobacteria
OC	Ovarian Cancer
OCT 4	Octamer-Binding Transcription Factor 4
PBMC	Peripheral Blood Mononuclear Cell
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffer Saline
PCR	Polymerase Chain Reaction
PET-CT	Positron Emission Tomography-Computed Tomography Scan
PFU	Plaque Forming Unit
PGE2	Prostaglandin E2
PHC	Primary Health Center
PI3K	Phosphoinositide 3-Kinase
PIGF	Placental Growth Factor

PMAIP1	Phorbol-12-myristate-13-acetate-induced Protein 1
PPTBL	Post-Primary Tuberculosis Of The Lungs
PTB	Pulmonary Tuberculosis
PTPRD	Protein Tyrosine Phosphate Receptor Type D
PTPR	Protein Tyrosine Phosphatase Receptor Type T
PTRR	Protein Tyrosine Phosphatase Receptor
qPCR	Quantitative Polymerase Chain Reaction
RAS	Renin-Angiotensin System
RBC	Red Blood Cell
RH-4	Rhabdomyosarcoma
RIF	Rifampicin
R-MSC	Reprogrammed Mesenchymal Stem Cell
RNA	Ribonucleic Acid
RNTCP	Revised National Tb Control Program
rpoB	Ribonucleic Acid Polymerase B
rpoC	Ribonucleic Acid Polymerase C
RRDR	Rifampicin Resistance Determining Region
SACS	Self-Assessed Collaboration Skills
SARS-COV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SCF	Stem cell factor
SC	Sub-Centers
SCC-25	Squamous Cell carcinoma 25
SDF1	Stromal Cell-Derived Factor 1
SEM	Standard Error of Mean
SiRNA	Small Interfering RNA
SNPTB	Smear Negative Pulmonary Tuberculosis
SNV	Short Nucleotide Variant
SOP	Standard Operating Procedure

SP	Side Population
SPm	Migratory Side Population
STAT3	Signal Transducer and Activator of Transcription 3
TAM	Tumor Associated Macrophage
TB	Tuberculosis
TCC	Tertiary Cancer Center
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
TPO	Thrombopoietin
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Treg	Regulatory T Cells
U-MSC	Untreated Mesenchymal stem cell
TSD	Tumor Stemness Defense
WB	Western Blot
VEGF	Vascular Endothelial Growth Factor
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively-Drug Resistant

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