5. Summary and Conclusion

Chapter 5

5.1 Summary:

Breast cancer tumor microenvironment is complex and consists of different cell population and inflammatory cytokines and chemokines that drive tumorigenic potential of these cells. TNF-α plays a critical role in breast cancer cell proliferation, correlates with higher tumor grade and metastasis. However, the inflammation mediated metabolic adaption of different breast cancer cell types in not yet understood. In the current study, we investigated the role of TNF-α in differentially modulating the mitochondrial proteins, functions and metabolism in genotypically different breast cancer cell lines ER/PR +ve (MCF-7) and ER/PR -ve (MDA-MB-231). Further, we analysed the role of LYRM7, an assembly factor for mitochondrial complex I/III and studied its role in breast cancer invasion and metastasis. The study also explored an overlap between two inflammatory axis TNF-NF-κB and ATX-LPA in breast cancer metastasis.

5.1.1 TNF-α mediated differential regulation of mitochondrial proteins in ER/PR +ve and ER/PR -ve breast cancer cell lines

- TNF-α induces differential expression of proteins regulating mitochondrial function in breast cancer subtypes: MCF-7 (ER/PR +ve) and MDA-MB-231 (ER/PR –ve) cells
- TNF-α showed differential expression of proteins of OXPHOS complexes in breast cancer subtypes: MCF-7 (ER/PR +ve) and MDA-MB-231 (ER/PR –ve) cells
- TNF-α significantly decreased mitochondrial and cellular ATP levels, complex I and complex III activities and enhanced ROS in MDA-MB-231 cells compared to MCF-7 that gives growth and metastasis advantage.
- TIMER database also showed that TNF-α is negatively correlated with mitochondrial complex proteins in Basal breast cancer patients compared to Luminal.

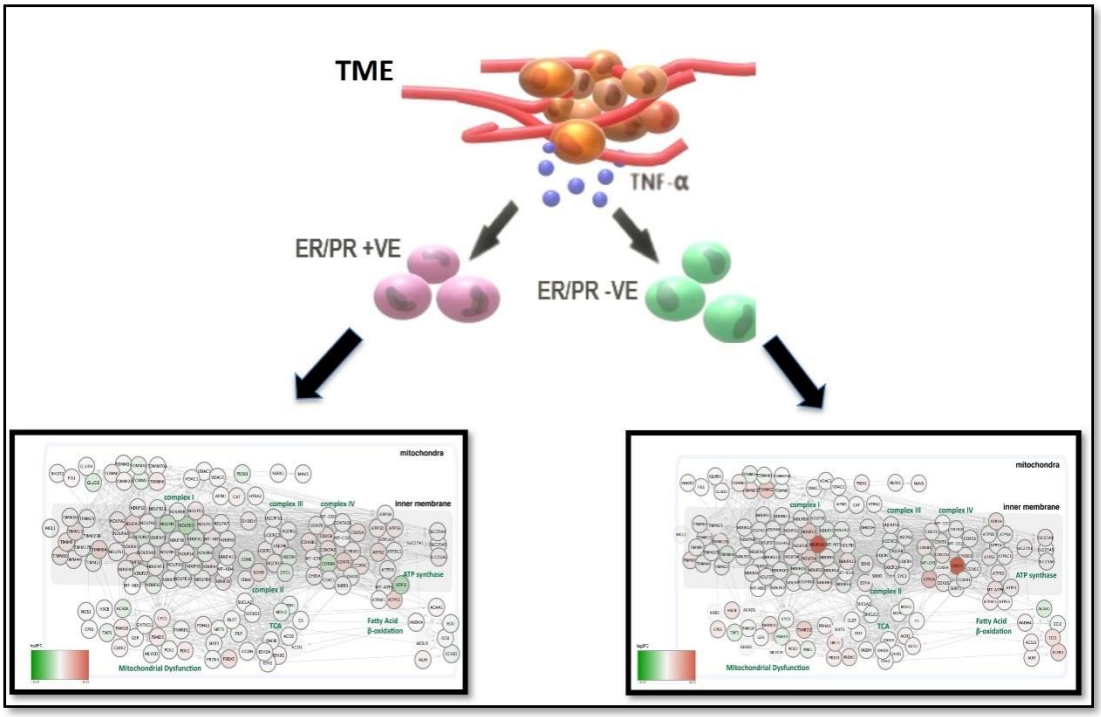

Figure 29: Role of TNF-α in differentially modulating mitochondrial proteins

TNF-α differentially modulates the expression of mitochondrial proteins ER/PR +ve and ER/PR -ve breast cancer cells

5.1.2 TNF-α differentially alters metabolic pathways in ER/PR +ve and ER/PR-ve breast cancer cells

- ER/PR (-ve) MDA-MB-231 cells also showed enhanced glycolysis pathway and pentose phosphate pathway that are essential for nucleotide biosynthesis for cell proliferation
- TNF-α treated ER/PR (-ve) MDA-MB-231 cells shows decreased levels of SDH and accumulation of succinate as compared to ER/PR (+ve) MCF-7 cells
- TNF-α treated ER/PR (-ve) MDA-MB-231 cells shows accumulation of pyruvate and aspartate that gave cells growth and metastatic advantages compared to MCF-7

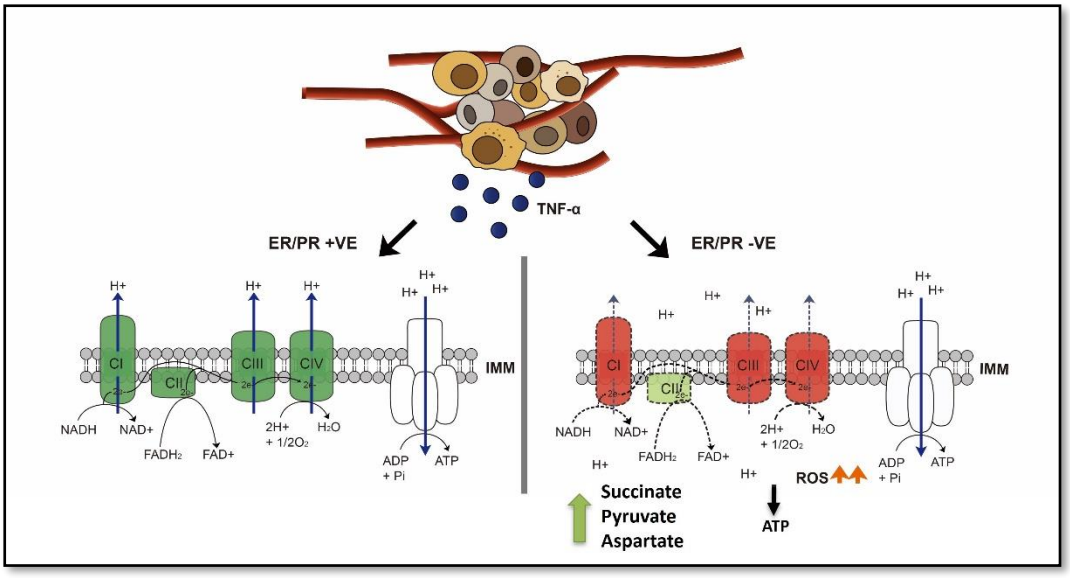

Figure 30: TNF-α modulates the mitochondrial functions and metabolite levels in breast cancer cells

TNF-α induces decrease in mitochondrial complex activities in ER/PR -ve breast cancer cells as compared to ER/PR +ve cells. TNF-α modulates glycolysis and TCA pathway along with accumulation of metabolites like succinate, pyruvate and aspartate. The defects in mitochondrial complex activity induce generation of ROS and reduces mitochondrial ATP levels in MDA-MB-231 cells as compared to MCF-7

5.1.3 TNF-α induced NF-κB regulates mitochondrial protein LYRM7

- NF-κB subunits RELA, RELB, REL, NFKB1 and NFKB2 are differentially expressed in subtypes of breast cancer patients
- KM plot survival analysis showed that higher expression of NFKB1 was associated with increased overall survival in Luminal A subtype
- High RELA expression was associated with a distinct increase in overall survival in the basal subtype of breast cancer patients
- p65 knockdown decreases clonogenic ability in presence of TNF-α in MDA-MB-231 cells
- TIMER and GEPIA2 databases show that TNF-α positively correlates with RELA and NFKB1 expression in breast cancer patients
- TNF-α-induced NF-κB pathway regulates the LYRM7 expression in MDA-MB-231 cells and inhibition by curcumin and p65 knockdown showed decreased LYRM7 expression

- Infliximab targeting TNF-α levels reduced LYRM7 expression in 4T1/BALB/c mice model
- LYRM7 regulates super complex assembly, ATP, mitochondrial membrane potential and ROS under TNF-α induced inflammatory conditions in MDA-MB-231 cells as compared to MCF-7 cells
- LYRM7 regulates breast cancer cell migration and invasion under inflammatory condition in MDA-MB-231 cells
- LYRM7 expression was reduced in TNBC cells and patients compared to other subtypes

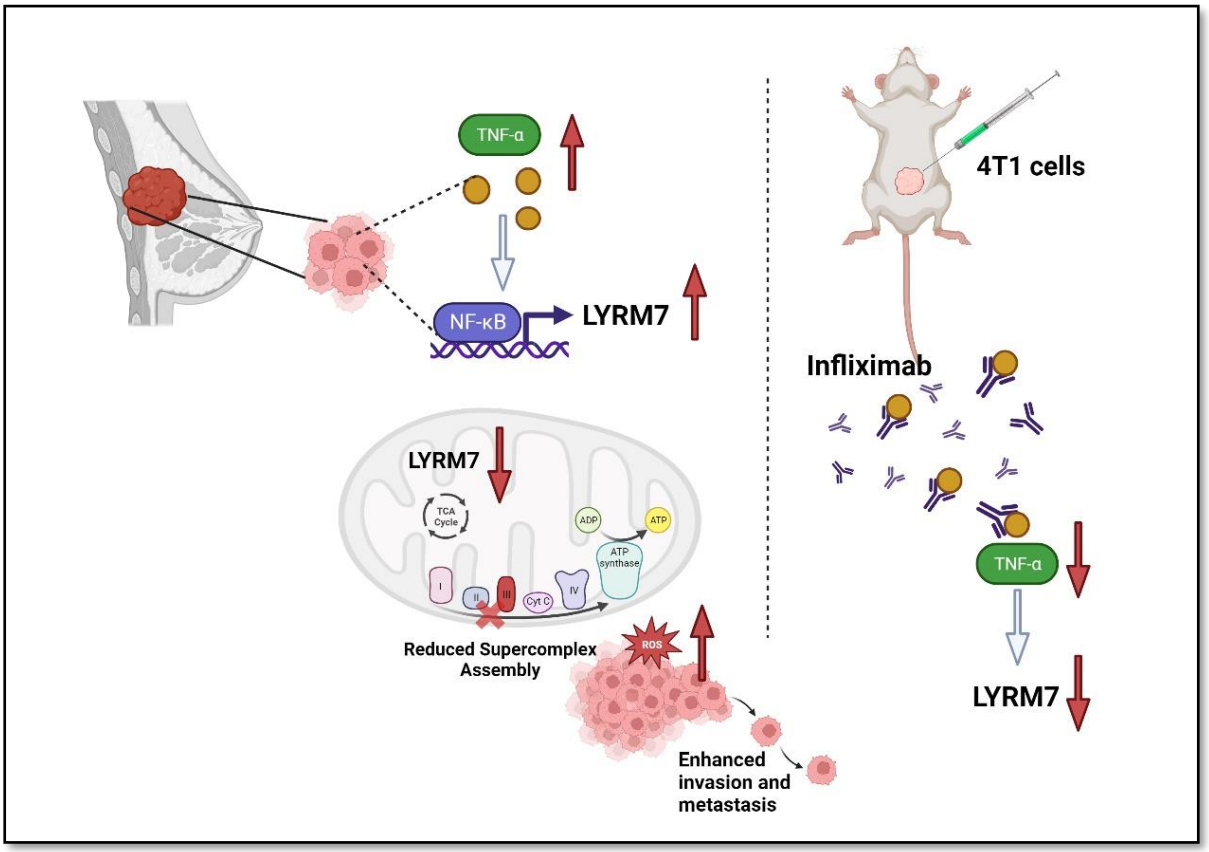

Figure 31: TNF-α induced NF-κB regulates LYRM7 expression in breast cancer

TNF-α activates NF-κB pathway by nuclear translocation of p65. Inhibition of NF-κB reduces LYRM7 expression that further decreases mitochondrial super complex assembly. Reduced LYRM7 expression generates ROS and reduced mitochondrial membrane potential. Reduced LYRM7 expression increases migration and invasion potentials of breast cancer cells and is associated with ER/PR -ve, metastatic tumor and TNBC patients. Infliximab reduces TNF-α levels in 4T1 induced tumor in BALB/c mice and this decreases LYRM7 in primary tumor

5.1.4 Role of TNF-α in regulating Autotaxin in breast cancer

- Infliximab reduces tumor growth and metastasis of 4T1 cells to lungs in BALB/c mice model
- Infliximab reduces inflammatory genes like TNF-α, RELA, IL-6 and IL-18 and NF-κB activation in primary tumor
- Infliximab regulates autotaxin expression and enzyme activity in primary tumor
- IOA, an inhibitor for autotaxin reduces RELA expression in primary tumor
- NF-κB inhibition decreases TNF-α induced autotaxin in 4T1 cells
- TNF-α and LPA regulates colony size in 4T1 cells

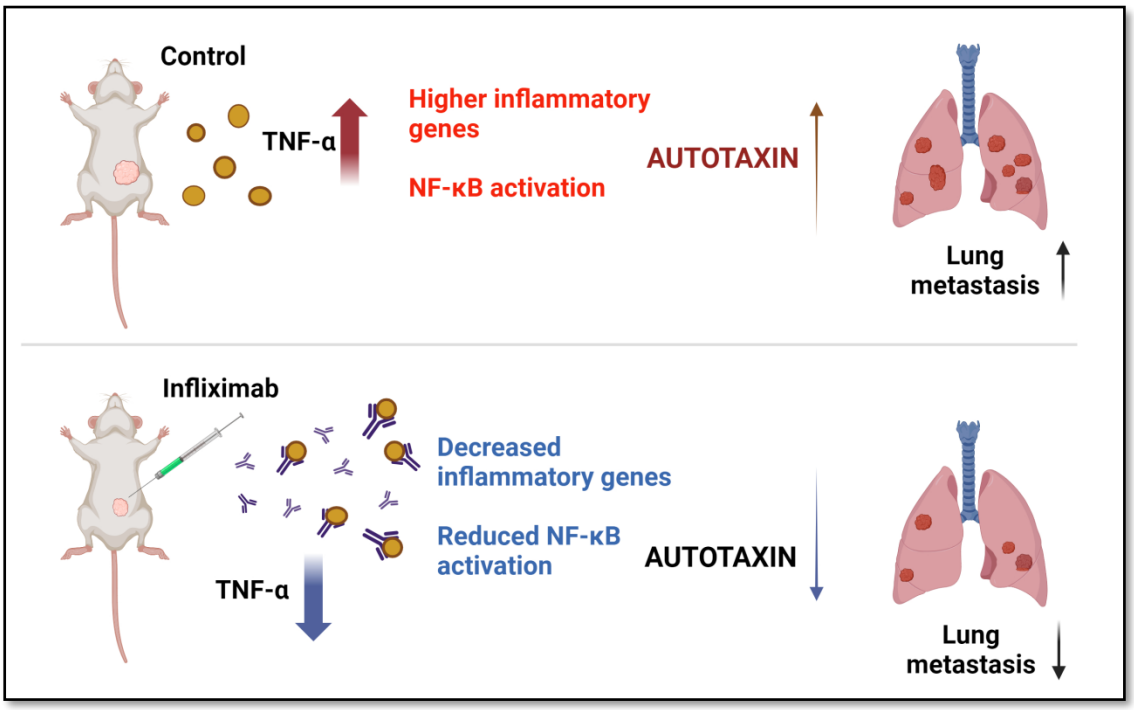

Figure 32: Infliximab decreases TNF-α induced NF-κB activation and autotaxin expression that regulates breast cancer metastasis

Infliximab decreased inflammatory genes and NF-κB activation in 4T1 induced BALB/c breast cancer mice model. Infliximab also decreased breast cancer metastasis to lungs as compared to control. Infliximab decreased autotaxin levels in primary tumor which further corelates with TNF-α induced autotaxin levels in 4T1 cells

5.2 Conclusion:

Breast cancer is a heterogeneous disease that shows a higher degree of complexity. Breast cancer subtypes have distinguished characteristics and hence a well-designed approach is necessary to achieve better treatment responses. ER/PR +ve and ER/PR -ve breast cancer cells

have different molecular patterns and metastatic and invasion potentials that have laid foundations for therapeutics. ER/PR -ve or TNBC have limited therapeutic targets and shows high immune invasion and recurrence rates. Inflammation and metabolism are two major determinants in the TME that drive the tumor cell survival and dictate changes underlying resistance to therapies. TNF-induced NF-κB pathway regulate multiple cellular processes in the cancer cells. Mitochondria has emerged a platform for immune-metabolic changes thus act as critical platform integrating inflammation in TME due to various cell type for metabolic adaption in tumor cells which may differentially effect tumor progression in different breast cancer subtypes. Inflammation induced mitochondrial adaptation in different breast cancer cell types and breast cancer cells survival is not well understood. Here we demonstrated that TNF-α modulates expression of mitochondrial proteins in ER/PR +ve and ER/PR -ve breast cancer cells. TNF-α modulates metabolism differentially in ER/PR +ve and ER/PR -ve breast cancer cells by modulating the levels of critical assembly factors and subunits involved in mitochondrial respiratory chain super complexes. TNF-α modulated metabolic reprogramming and shows accumulation of metabolites like pyruvate, aspartate and succinate that favours survival and proliferation of more aggressive ER/PR -ve breast cancer cells. Further, large scale database of breast cancer patient showed TNF-α negatively correlates with major subunits of mitochondrial complexes in Basal subtype compared to luminal breast cancer patients. The study here strongly suggests that TNF-α induced metabolic pathways and mitochondrial protein expression in ER/PR +ve and ER/PR -ve breast cancer cells induced cell migration and clonogenicity and are novel targets for therapeutics to prevent progression in aggressive breast cancers.

Further, changes in mitochondrial proteins and mitochondrial complex activities contribute to generation of ROS which is a major driver for metastasis in breast cancer cells. The study identified LYRM7, an assembly factor for mitochondrial complex III that modulates breast cancer invasion and migration under inflammatory conditions. TNF-induced NF-κB regulate the expression of LYRM7 in MDA-MB-231 breast cancer cells. The study further identifies TNF-α induced LYRM7 in regulating the breast cancer cell migration and invasion potentials by modulating mitochondrial OXPHOS capacity. The expression of LYRM7 decreased in TNBC patients as compared to other subtypes. Infliximab, a monoclonal antibody against TNF-α showed reduced LYRM7 gene expression as compared to control in breast cancer mice model. Thus, the study indicates that TNF-α induced NF-κB is a critical regulator of LYRM7,

a major factor for modulating mitochondrial functions under inflammatory conditions, which determines growth and survival of breast cancer cells.

Infliximab that reduces TNF-α in the breast cancer mice model showed reduced tumor growth and metastasis to lungs specifically. TNF-α induced NF-κB can regulate the expression of autotaxin which play a role in clonogenicity of breast cancer cells. This work provides an increased understanding of how to regulate the ATX-LPA-inflammatory cycle and prevent it from becoming maladaptive and pro-metastatic. This concept could be developed as a novel treatment for improving the outcomes for breast cancer patients by using infliximab to decrease the effects of TNF-α together with ATX-LPA inhibitors.

The study suggest that TNF-induced pathway can be targeted using Infliximab and other developing biologics or developing combinatorial therapy targeting the OXPHOS and metabolic pathway acting synergistically in presence inflammatory milieu of breast cancers.

5.3 Limitations of the study: The study provided some interesting targets of inflammation, metabolism, and mitochondria for therapeutic regimen for breast cancer however there are several limitations of the study which have been summarized below:

1. TNF-α modulated mitochondrial proteins were identified in breast cancer cell lines; however, their validations in mice models and patients derived tumors will further strengthen the hypothesis.
2. The data generated and conclusions drawn were from two cell lines. More cell lines can be further explored to generalize the results obtained. Primary cells developed from different breast cancer subtypes should be used to validate the findings.
3. Many other proteins were identified in the proteome data however only few proteins have been validated. It is important and interesting to validate and analyze the differential role of identified proteins in metabolic adaptations.
4. The study lacks ChIP-seq data validating NF-κB binding in the promoter region of upregulated target genes.
5. Orthotopic syngeneic mouse model should be used to further validate the effects of identified mitochondrial proteins in regulating metastasis
6. Effects of mitochondria inhibitors can be important in understanding importance of specific mitochondrial complexes on breast cancer cell survival, migration and clone

forming ability and in vivo model should be sued. Further breast cancer organoid model could be used to understand the differential metabolic adaptations.

7. Infliximab has been associated with side effects in breast cancer patients. Hence developing combinatorial therapy minimising the side effects should be evaluated for targeting TNF-α in TME

5.4. Future perspective

The study here investigated inflammation induced changes related to the TNF-α induced NF-κB pathway and its regulation of mitochondrial proteins in breast cancer cell lines providing leads to further investigate the therapeutic potential:

1. The role of TNF-α induced inflammation and changes to mitochondrial dynamics and its implication in regulating breast cancer metastasis should be investigated.
2. The study showed that TNF-α reduces OXPHOS assembly and activity of mitochondrial complexes breast cancer cell lines. It will be interesting to study its effects in cell lines derived from different subtypes of breast cancer patients
3. Inhibition or enhancing the activity of mitochondrial complex using inhibitors, antioxidants or compounds that can modulate the complex activity should be tested in different breast cancer models
4. Different anti-TNF antibodies or drugs in combination with other anti-inflammatory drugs can be tested in breast cancer models to study its potential as therapeutics.
5. Infliximab in combination with ATX inhibitors like IOA can be tested in understanding inflammation in TME and its effects on breast cancer metastasis
6. TNF-α induced NF-κB pathway regulates basal homeostasis and is important in cell survival, proliferation and apoptosis. Therefore, a well-designed anti-TNF approach that can specifically target TME should be developed and studied in breast cancer