5. Summary and Conclusion

5.1. Summary:

Breast cancer considered as highest diagnosed cancer in women. However, classical therapy is still under practice but a group of patients does not respond to these therapies and most of the patients show severe side effects and relapse, and resistance to chemotherapy. Hence, it is important to understand the alternative mechanisms and sign targeted therapies to overcome these problems in breast cancer patients. The tumor microenvironment of a solid tumor is considered the most complicated environment. Activation of an immune response against the cancer cell and immune systems selectively cell kill the tumorous cells became the central part of immune therapy. Interferon plays a critical role in the anti-tumor activity as interferon α and β upregulate the MHC I, II and enhance antigen presentation of tumor cells, and enhance the potential of anti-tumor response. DNA sensor cGAS activate the STING pathway during viral infection but exploitation of this pathway in breast cancer is not well understood. In the current study, we studied the differential regulation and intactness of the cGAS-STING pathway in ER/PR positive and negative breast cancer cell lines and their role in regulation during DNA damage conditions. Further, we also investigated the exploitation of the cGAS-STING pathway as an anti-cancer therapy in breast cancer.

5.1.1. Expression of cGAS and STING in breast cancer cell lines and patient tissue

- STING expression is significantly upregulated in ER (Estrogen receptor) negative breast cancer cell lines in favour of their growth and metastasis. The expression of cGAS is ubiquitous in all breast cancer cell lines selected in the study.
- Data (TIMER) from patients also support that ER expression is negatively correlated with STING expression. While cGAS expression remains ubiquitously present in all breast cancer types in patients' expression data.
- STING expression is significantly upregulated in tumorous tissue of triple-negative breast cancer cells as compared to non-cancerous tissue.

5.1.2. Role of cGAS and STING during DNA damage conditions

- DNA damage induces NF-κB activation in STING-positive triple-negative breast cancer cells as compared to STING non-expressive cells

- STING-mediated NF-κB activation induces IL-6 expression in triple-negative breast cancer cells in DNA damage conditions
- The knockdown of STING sensitizes breast cancer cells to genotoxic stress and inhibits clonogenicity
- STING-mediated IL-6 induction enhances PD-L1 expression during DNA damage in triple-negative breast cancer cells
- STAT3 inhibitor, HJC0152and doxorubicin act synergistically in breast cancer cells to inhibit cell death
- STING expression positively correlates with IL6 and PD-L1 expression and high STING expression in chemotherapy shows poor survival

  Thus, we conclude that the expression of STING shows pro-survival activity during DNA-damaging conditions. DNA damage activates NF-κB mediated IL-6 production and activates IL-6 STAT3. IL-6 also upregulated PD-L1 during DNA damage in STING-expressing and escape from CD8 T cell-mediated immune response. STAT3 inhibitor with DNA damaging agent shows synergy in anti-cancer therapy. In triple-negative breast cancer cell lines

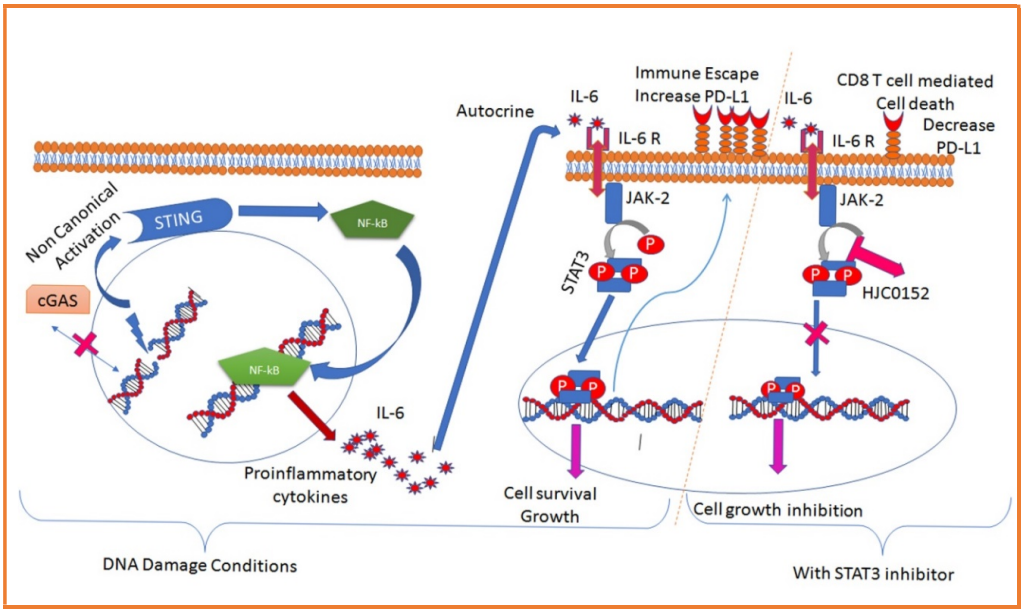

Figure 24 DNA damage induces STING-mediated IL-6-STAT3 survival pathway

During DNA damage STING activates NF-κB independent of cGAS which further induces the production of IL-6. IL-6 binds to its receptor IL-6R and enhances the phosphorylation of STAT3. The *activation of the STAT3 pathway show cell survival and upregulation of PD-L1-mediated immune escape. Combinatorial treatment of STAT3 inhibitor as HJC0152 with doxorubicin sensitizes the treatment via pSTAT3 blocking and down-regulation of PDL-1 and activates immunogenic cell death.*

5.1.3 Role of cGAS STING pathway in anti-cancer therapy

- c-di AMP induces STING-mediated IFN pathway in breast cancer cell lines
- c-di-AMP binds to STING
- c-di- AMP activates cell death in ER/PR negative breast cancer cell lines
- STING is essential for c-di-AMP-induced cell death
- IRF-3 is indispensable for c-di-AMP-induced apoptosis in ER-negative breast cancer cells
- c-di-AMP induces IRF-3 translocation to mitochondria and induces the mitochondrial-mediated intrinsic pathway of apoptosis

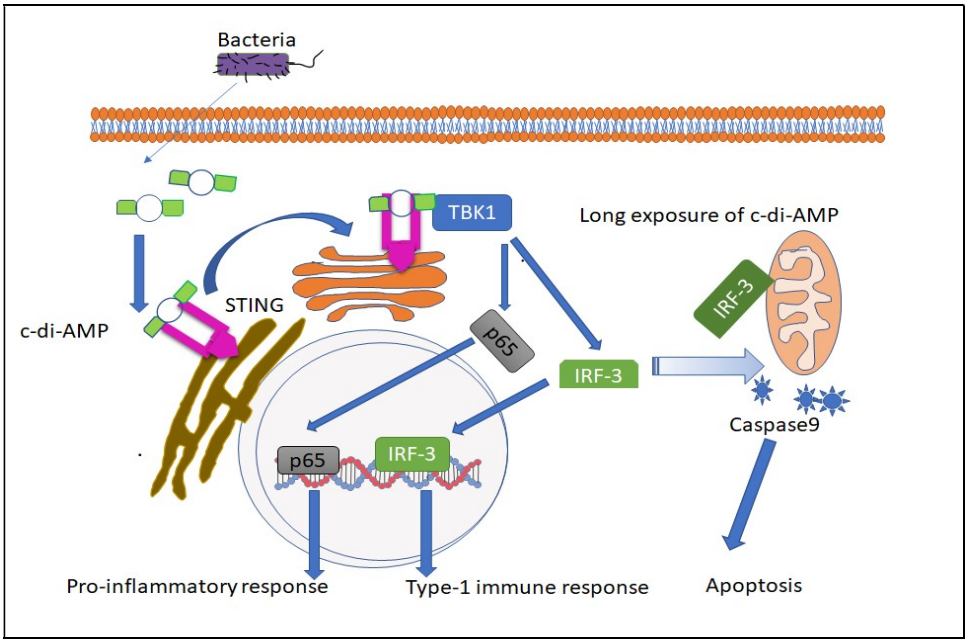

Figure 25 Analog of cGAMP, c-di-AMP induces cell death in STING expressing breast cancer cell lines

c-di-AMP derived from bacteria can bind and activate STING and downstream produce type-1 immune response and proinflammatory cytokines. Long-term treatment of c-di-AMP induces IRF-3 translocation to mitochondria and induces caspsae9-mediated apoptosis pathway.

5.1.4. Role of cGAS STING pathway in anti-cancer therapy

Different metal ion act as co-factor for many enzymes, here we investigated Mn^{2+} as a critical metal ion that activates the cGAS-STING pathway in breast cancer cells. The exploitation of Mn^{2+} mediated anticancer therapy the major findings concluded below

- $MnCl_2$ activates STING-mediated IFN response
- cGAS and STING are indispensable for $MnCl_2$-mediated IFN activation in breast cancer cells
- $MnCl_2$ sensitizes breast cancer cells to paclitaxel-induced cell death
- A combination of $MnCl_2$ and Paclitaxel enhanced immunogenic cell death of breast cancer cells.

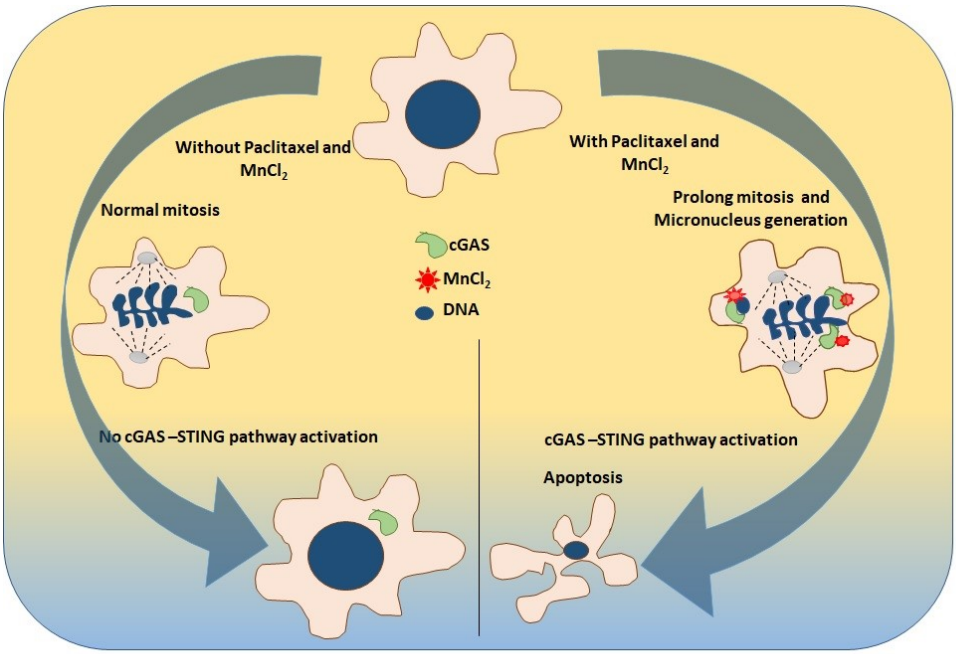

Figure 26 Combination treatment of paclitaxel and $MnCl_2$ enhances anti-tumor response via cGAS-STING pathway

During normal mitosis, cGAS remains inactive but during treatment of paclitaxel extends the mitosis time and generates a micronucleus that is sensed by cGAS. Activated cGAS with supplementary of $MnCl_2$ incudes profound activation of cGAS STING pathway. Exaggerated activation of the cGAS-STING pathway induces a synergistic effect of MnCl2 with paclitaxel during anti-cancer treatment.

5.2. Conclusion:

Triple-negative breast cancer is aggressive and metastatic breast cancer type and shows immune evasion, drug resistance, relapse, and poor survival. Anti-cancer therapy like ionizing radiation and chemotherapeutic drug majorly induces DNA damage hence, alteration in DNA damage repair and downstream pathways may contribute to tumor cell survival. DNA damage during chemotherapy is sensed by cyclic GMP-AMP synthase(cGAS)-stimulator of interferon genes (STING), which determines the anti-tumor immune response by modulating the expression of programmed cell death ligand-1 (PD-L1), immune suppressor, in the tumor microenvironment. Triple-negative breast cancer cells are cGAS-STING positive and modulation of this pathway during DNA damage response for survival and immune escape mechanism is not well understood. Here we demonstrate that doxorubicin-mediated DNA damage induces STING mediated NF-κB activation in triple-negative as compared to ER/PR-positive breast cancer cells. STING-mediated NF-κB induces the expression of IL-6 in triple-negative breast cancer cells and activates pSTAT3, which enhances cell survival and PD-L1 expression. Doxorubicin and STAT3 inhibitor act synergistically and inhibit cell survival and clonogenicity in triple-negative breast cancer cells. Knockdown of STING in triple-negative breast cancer cells enhances CD8-mediated immune cell death of breast cancer cells. The combinatorial treatment of triple-negative breast cells with doxorubicin and STAT3 inhibitor reduces PD-L1 expression and activates immune cell-mediated cancer cell death. Further STING and IL-6 levels show a positive correlation in breast cancer patients and poor survival outcomes. The study here strongly suggests that STING-mediated activation of NF-κB enhances IL-6-mediated STAT3 in triple-negative breast cancer cells which induces cell survival and immune-suppressive mechanism.

Further immune adaptor protein like STING/MITA regulate innate immune response and plays a critical role in inflammation in the tumor microenvironment and regulation of metastasis including breast cancer. Chromosomal instability in highly metastatic cells releases fragmented chromosomal parts in the cytoplasm, hence the activation of STING via an increased level of cyclic dinucleotides (CDNs) synthesized by cGMP-AMP synthase (cGAS). Cyclic dinucleotides 2' 3'-cGAMP and its analog can potentially activate STING-mediated pathways leading to nuclear translocation of p65 and IRF-3 and transcription of inflammatory genes. The differential modulation of the STING pathway via 2' 3'-cGAMP and its analog and its implication in breast tumorigenesis is still not well explored. In the current study, we demonstrated that c-di-AMP can activate type-1 IFN response in ER-negative breast cancer cell lines which correlates with STING expression. c-di-AMP binds to STING and activates downstream IFN pathways in STING-positive cells. Prolonged treatment of c-di-AMP induces cell death in STING-positive metastatic cells mediated by IRF-3. c-di-AMP induces IRF-3 translocation to mitochondria and initiates Caspase-9 mediated cell death and inhibits clonogenicity of triple-negative breast cancer cells. This study suggests that c-di-AMP can activate and modulates the STING pathway to induce mitochondrial-mediated apoptosis in estrogen-receptor-negative breast cancer cells.

We also demonstrated that the cGAS can be directly activated via Mn^{2+} and that generates STING-mediated anti-tumor response. Mn specifically activated cGAS and STING pathway in triple-negative breast cancer cells which are STING positive and showed no activity in MCF-7 ER/PR positive cells. The combination of the Mn^{2+} and paclitaxel also activated the immunogenic of triple-negative breast cancer cells.

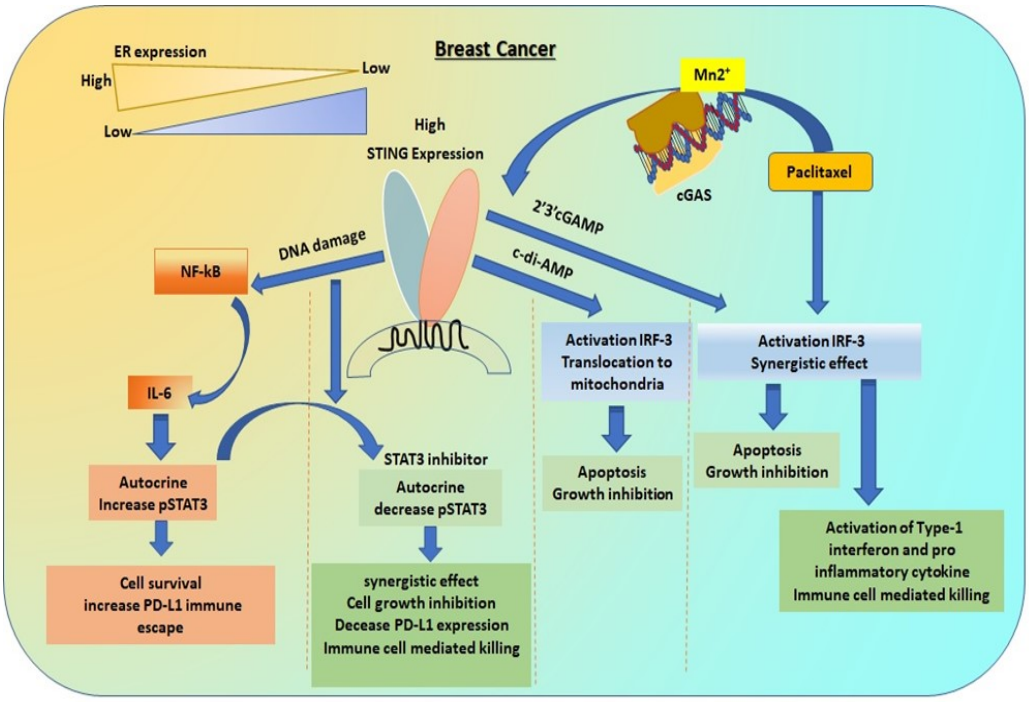

Figure 27 Conclusion

5.3. Limitation of the study

The study provided some interesting leads for the possible development of a combinatorial therapeutic regimen for breast cancer however there are some limitations which have been summarized below:

- The expression of the cGAS-STING study was performed only on a limited number of numbers of cell lines and patients due to the limited availability of samples. The study should be further extended on in a panel of cell lines and a larger cohort of breast cancer patients.
- The patients can further be classified based on ER, PR, and HER2 status and its correlation with cGAS and STING should be further explored. This correlation may help devise personalized therapy for patients.
- Data generated and conclusions have been drawn from cell lines *in vitro* activity during DNA damage conditions. Genotoxic agents and a combination with different STAT3 inhibitor should be also confirmed *in vivo* model of breast cancer.

- c-di-AMP is hydrophilic and exhibits less cell permeability; hence higher dosage is required for an effective outcome. It will be interesting to generate nanoparticles or liposomes for the delivery of c-di-AMP to tumor cells for better efficacy.
- Mn^{2+} and Paclitaxel-formulated liposomes should be generated and tested *in vivo* may have more therapeutic importance.

5.4. Future perspective

The study here answered some of the interesting questions related to the cGAS-STING pathway in breast cancer cell lines providing leads to further investigate the therapeutic potential of this pathway using combinatorial therapies:

1. The role of cGAS both STING dependent/independent should be further investigated in breast cancer and its implication in the regulation of inflammation and breast tumor cell proliferation.
2. The study here shows that the STING upregulates IL-6 STAT3 and promotes breast cancer survival during DNA damage conditions. It will be interesting to extend these findings to '*in vivo m*odels' systems and breast cancer organoids derived from breast cancer patients.
3. Inhibition of IL-6 STAT3, using IL-6 receptor using Tocilizumab, downstream JAK2 inhibitor as ruxolitinib, and different STAT3 inhibitors like niclosamide, HJC0152 in combination with STING agonist should be tested in the different breast cancer cell types (ER/PR positive and ER/PR negative).
4. STING agonist as c-di-AMP had lower cell permeability and is prone to ENPP1 mediated degradation hence chemical modification of structure is required to generate moiety which is ENPP1 resistant.
5. Several STING agonists have been generated by all-natural dinucleotides (CDNs) that have limited cell permeability and hence synthetic molecules are required.
6. Apoptosis threshold can be also decreased by developing a combination of the STING agonist, chemotherapeutic drugs, and Bcl-/Bcl-Xl mimetic.
7. Intravenous and subcutaneous treatment leads to adverse effects like cytokine storm. So, liposome or antibody-drug conjugate that specifically deliver STING agonist in tumor cell only should be generated to avoid cytokine storm during cGAS-STING therapy.