Chapter VI

Summary & Conclusion

6.1. Summary:

Immunometabolism, the intricate relationship between immune cell responses and metabolic processes, is crucial for determining the differentiation and functionality of immune cells. Natural killer (NK) cells are highly effective immune cells in combating cancer and viral infections. The metabolic characteristics of NK cells play a decisive role in their functionality, and any alterations in metabolism can have a profound impact on their immune responses.

While the importance of immunometabolism in the context of cancer has been well established in T cells and macrophages, its comprehensive understanding in NK cells lags behind.

The tumor microenvironment (TME), marked by its hostile and harsh conditions including hypoxia, and acidosis, substantial nutrients and metabolites imbalances, presents a formidable challenge for the metabolism of tumor-infiltrating immune cells. These conditions can disrupt the metabolic processes within these immune cells, ultimately resulting in immunosuppression.

This further emphasizes the critical importance of unraveling the immunometabolic mechanisms in NK cells, specifically when situated within the TME.

Folate metabolism, a vital element of one-carbon metabolism, has been linked to NK cell function and cytotoxicity. Impaired or disrupted folate metabolism could potentially contribute to NK cell dysfunction. Nevertheless, the intricate interplay between folate metabolism and pathways governing NK cell activation within the TME remains a subject that necessitates further exploration. Likewise, the accumulation of metabolites within the TME, such as succinate, holds the potential to influence immune cell characteristics and responses. While the effect of succinate on immune cells including macrophages, dendritic cells, and T cells has been established, its influence on NK cells remained unexplored. However, the cross-talk between folate metabolism, succinate buildup and immunometabolic signaling pathways involved in NK cell activation in the TME remains an area that has not been highlighted in the research.

Given this, our present study aimed to shed light on the possible effects of folate availability and exogenous succinate supplementation on the activation status and metabolic regulation of NK cells, with a particular emphasis on the mTOR signaling pathway, in the context of cancer and the TME (Figure 54).

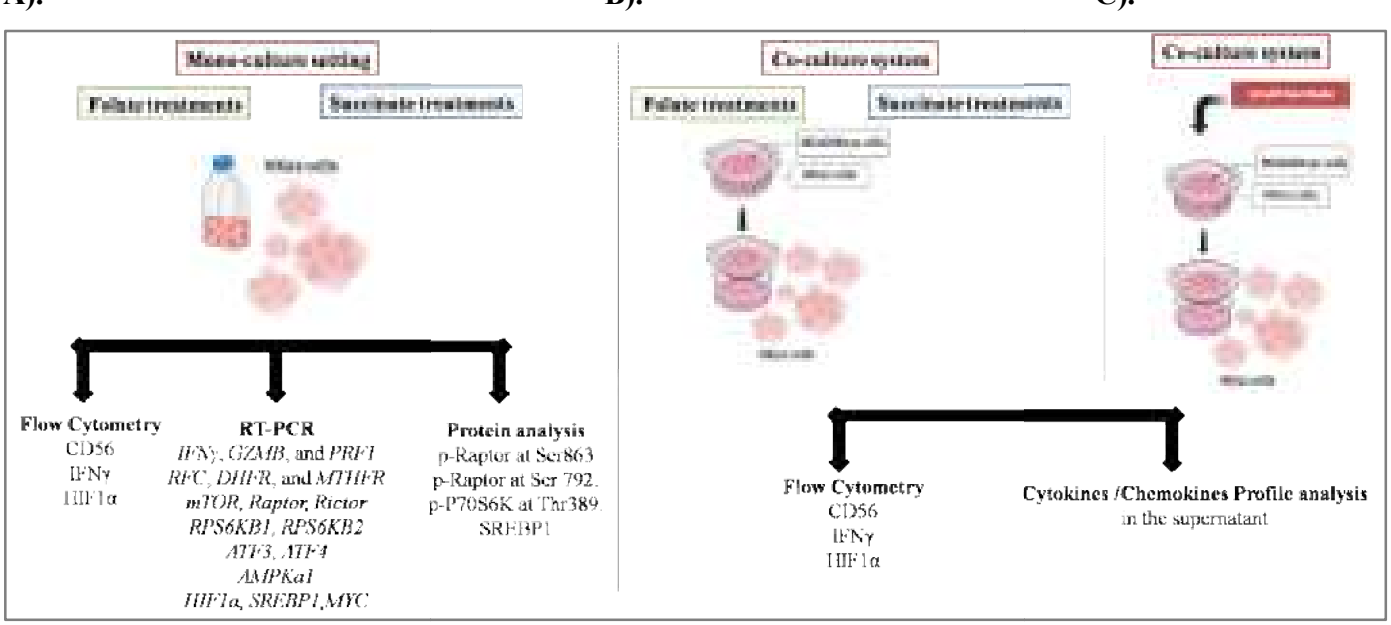

Figure (54): Schematic diagram illustrates the methodology used to investigate the potential impacts of folate availability and exogenous succinate supplementation on NK92 cells.

Firstly, NK92 cell line was recruited to be grown within mono- and co-culture systems supplemented with different folate forms and concentrations. Two forms of folate, folic acid (FA) and 5-methyltetrahydrofolic acid (5-MTHFA), were used in the experiments. Secondly, NK92 cells were cultured within mono- and co-culture systems supplemented with different succinate concentrations. Lastly, to understand preliminary the interplay between folate availability and succinate accrual in the co-culture system, we supplemented the co-culture media with 50µM of succinate in different folate concentrations. For both, we examined the alteration in expression of CD56 and IFNγ as markers for the activation status of NK92 cells. Further, we examined their impacts on transcriptional factor, HIF1α, in mono- and co-culture conditions with MDAMB-231 breast cancer cells. In addition, in mono-cultured NK92 cells, particularly, gene expression analysis was conducted to examine the NK cell functional genes (*IFNγ*, *GZMB*, and *PRF1*) and some key genes in folate metabolic pathway (*RFC*, *DHFR*, and *MTHFR*) and key genes in mTOR signaling pathway (*mTOR, Raptor, Rictor, RPS6KB1, RPS6KB2*, *ATF3, ATF4*, *AMPKa1*, *HIF1α, SREBP1* and *MYC*). Moreover, considering the importance of cytokines and chemokines regulation for the functionality of NK92 cells, we surveyed their concentrations in the supernatant of the co-culture system under different folate and succinate treatments.

The following section outlines the significant findings of our present study, which illuminate novel aspects of the intricate relationship between these metabolites (folate and succinate), and NK cell function and metabolism within TME. Additionally, the figures (55, 56, 57, 58 and 59) represent graphical models illustrating the key findings of our study (created with BioRender.com).

- **Folate effects on NK92 cells:**

1- Folate availability affected the expression of CD56 and IFNγ, as well as the percentage of positive cells in mono-cultured NK92 cells after 72hrs. Among all the folate treatments examined, the 5-MTHFA cultures exhibited the most pronounced effect in increasing CD56 levels and pro-inflammatory cytokine IFNγ and the percentage of IFNγ+ cells was observed in the high concentration (0.2 mM) of 5-MTHFA cultures, the *GZMB* gene expression was down-regulated in these cultures, indicating lower cytotoxic potential of NK92 cells (Figure 55).

2- In the co-culture system with the breast cancer cell line MDAMB231, NK92 cells maintained the highest CD56 levels in a high concentration of FA (0.2 mM) while folate deficiency decreased CD56 levels. IFNγ levels and the percentage of IFNγ+ cells decreased significantly in the FA over-supplemented (0.2mM) and folate-deficient groups. The highest levels of IFNγ were observed in the 0.2 mM concentration of 5-MTHFA media. NK92 cell viability in the co-culture system decreased in the control FA condition (0.02mM) and folate-deficient condition, suggesting a possible sequestration of folate by cancer cells (Figure 56).

3- HIF1α levels increased in mono-cultured NK92 cells with high folate doses and folate deficiency, but decreased in co-cultured NK92 cells, suggesting possible effects of folate availability on NK cell activities mediated by the alterations in HIF1α expression (Figures 55 and 56).

4- The expression of mTOR pathway-related genes in mono-cultured NK92 cells was also affected by folate levels. Excessive Folate (0.2mM) treatments resulted in down-regulation of the gene expression levels of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2* and *AMPKα1* while high FA dose resulted in *SREBP1* upregulation in mono-cultured NK92 cells which tends to suggest possible impacts on NK cell proliferation, metabolic regulation and functionality (Figure 55).

5- IL8 and CXCL9 (MIG) levels in the supernatant of the co-culture system were significantly lower in high folate doses and folate-deficient conditions while CCL2 (MCP1) levels were higher in these conditions compared to the control folate treatment. This creates a hostile microenvironment for NK cells to exert their cytotoxic activities against tumor cells (Figure 56).

➢ **Succinate effects on NK92 cells:**

6- Succinate treatments of mono-cultured NK92 cells led to a reduction in CD56 and IFNγ levels, as well as a decrease in the percentage of CD56+ and IFNγ+ cells (Figure 57). Further, supplementing the culture medium with high succinate concentrations (500 µM) increased HIF1α levels more in co-cultured NK92 cells compared to mono-cultured cells. the elevated HIF1α levels in co-cultured NK92 cells in succinate treatments along with increased CD56 and IFNγ levels indicates a possible shift within the NK92 cell population towards $CD56^{bright}$ NK cells, which are known to be less cytotoxic and more inclined towards cytokine production (Figures 57 and 58).

7- Succinate treatments down-regulated *MTHFR* and *RFC* gene expression while increasing *DHFR* gene expression at high succinate concentrations (100 µM and 500 µM). This suggests an influence of succinate on the folate metabolic pathway (Figure 57).

8- Succinate addition to the culture medium of mono-cultured NK92 cells affected gene expression related to the mTOR pathway. *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2*, *AMPKα1*, *SREBP1*, and *ATF3* gene expression levels decreased in succinate-treated NK92 cells. This suggests possible impacts on NK cell proliferation, metabolic regulation and functionality (Figure 57).

9- Supplementing the co-culture system with 50 µM of succinate in high folate or folate-deficient conditions resulted in decreased CD56 and IFNγ levels, but increased HIF1α levels as well as increased the cytokines; IL10, IL6, and IL8 concentrations and the chemokines; CCL2 (MCP1) and CXCL9 (MIG) concentrations. This suggests inhibition in the NK92 cytotoxicity and a pseudo-hypoxic effect induced by an interplay mechanism between folate metabolism and succinate levels in our normoxic simulated TME that might influence the NK cell anti-cancer activities (Figure 59).

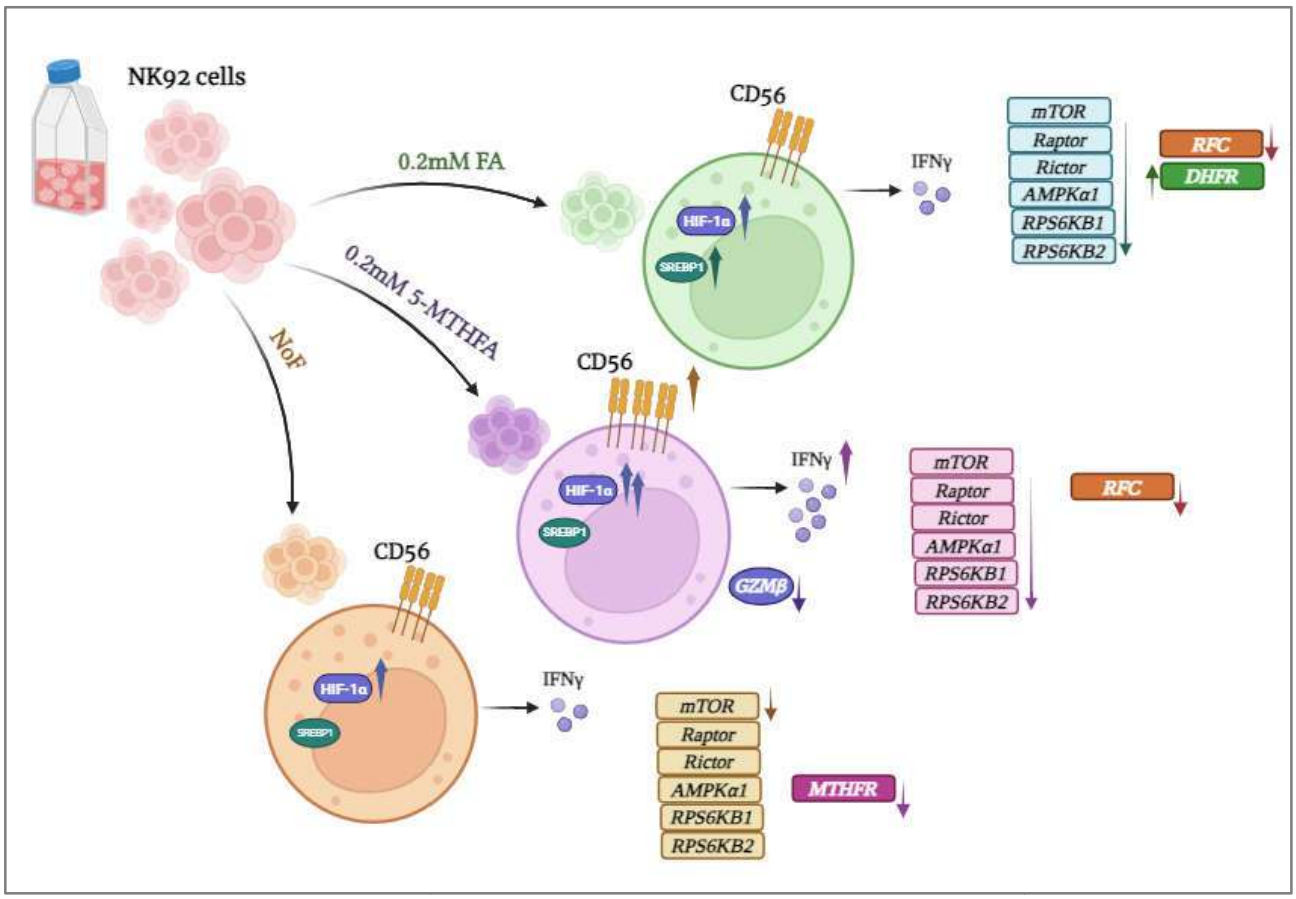

Figure (55): A graphical model represents the key findings of Folate availability on NK92 Cells in mono-culture setup after 72hrs.

Compared with the control folate treatment (0.02mM of FA), high folate concentrations (0.2mM), particularly 5-MTHFA form, negatively affected NK92 cell viability and increased the CD56 expression levels, IFNγ production, and HIF1α expression. *GZMB* gene expression was down-regulated in 5-MTHFA cultures. In the folate metabolic pathway, *RFC* gene expression was down-regulated in high folate treatments, while *DHFR* gene expression was upregulated in high folic acid (FA) dose (0.2mM). NoF (no addition folate) which simulates the folate deficiency statement resulted in down-regulation of the *MTHFR* gene expression and increased HIF1α levels. Excessive Folate (0.2mM) treatments resulted in down-regulation of the gene expression levels of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2* and *AMPKα1* while high FA dose resulted in *SREBP1* upregulation.

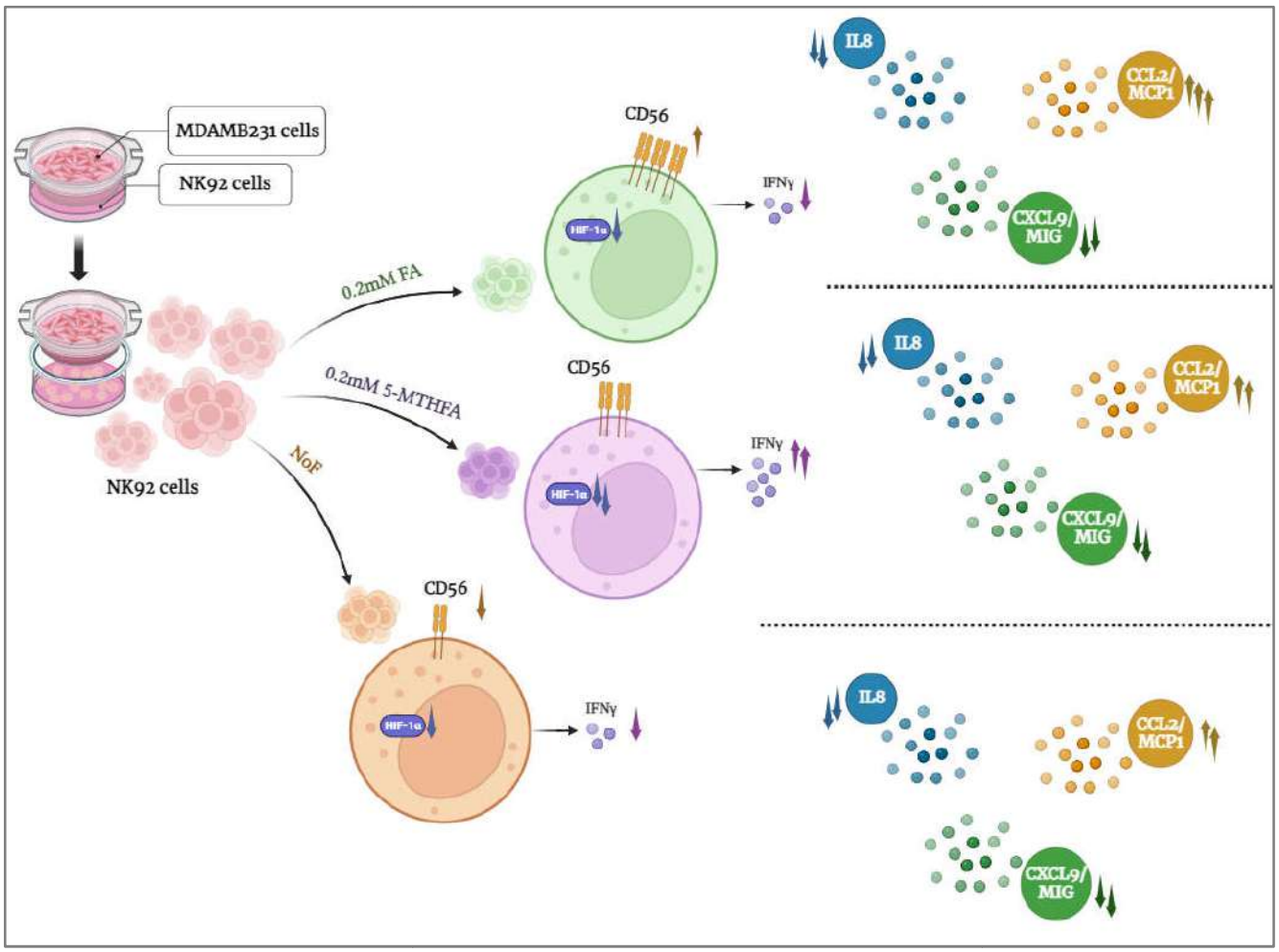

Figure (56): A graphical model represents the key findings for the effect of Folate availability on the activation status of NK92 cells and the cytokines /chemokines production in the co-culture system with the breast cancer cell line, MDAMB231.

NK92 cells maintained the highest CD56 levels in a high concentration of FA (0.2 mM) while folate deficiency decreased CD56 levels. IFNγ levels decreased significantly in the FA over-supplemented (0.2 mM) and folate-deficient groups. The highest levels of IFNγ were observed in the 0.2 mM concentration of 5-MTHFA media. HIF1α levels decreased high folate doses, particularly in 5-MTHFA, and folate-deficient condition (NoF)

IL8 and CXCL9 (MIG) were significantly lower while CCL2 (MCP1) was higher in high folate doses and folate deficient which might create a hostile microenvironment for NK cells to exert their cytotoxic activities against tumor cells.

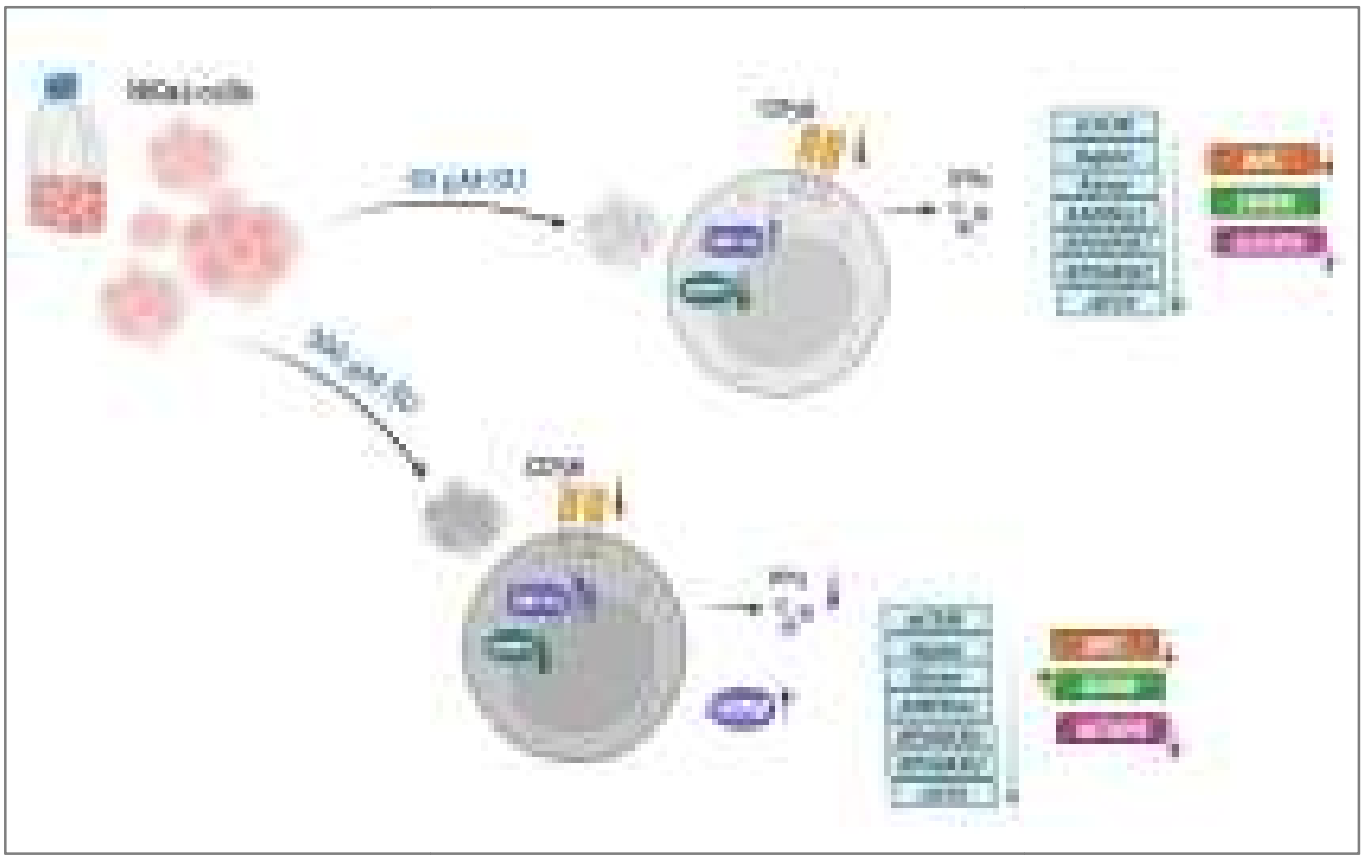

Figure (57): A graphical model represents the key findings of Succinate treatments on NK92 cells in mono-culture setup after 72hrs.

Succinate treatments led to decreasing NK92 cell viability and reduction in CD56 and IFNγ levels. *GZMB* gene expression was up-regulated in high succinate concentration (500 μM). The expression of *MTHFR* and *RFC* genes in the folate metabolic pathway was downregulated. A high succinate concentration (500 μM) resulted in *DHFR* upregulation. Succinate treatments led to decreasing the gene expression of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2*, *AMPKα1*, *SREBP1*, and *ATF3* in the mTOR pathway. HIF1α levels increased in succinate treatment, particularly with the high concentration (500 μM).

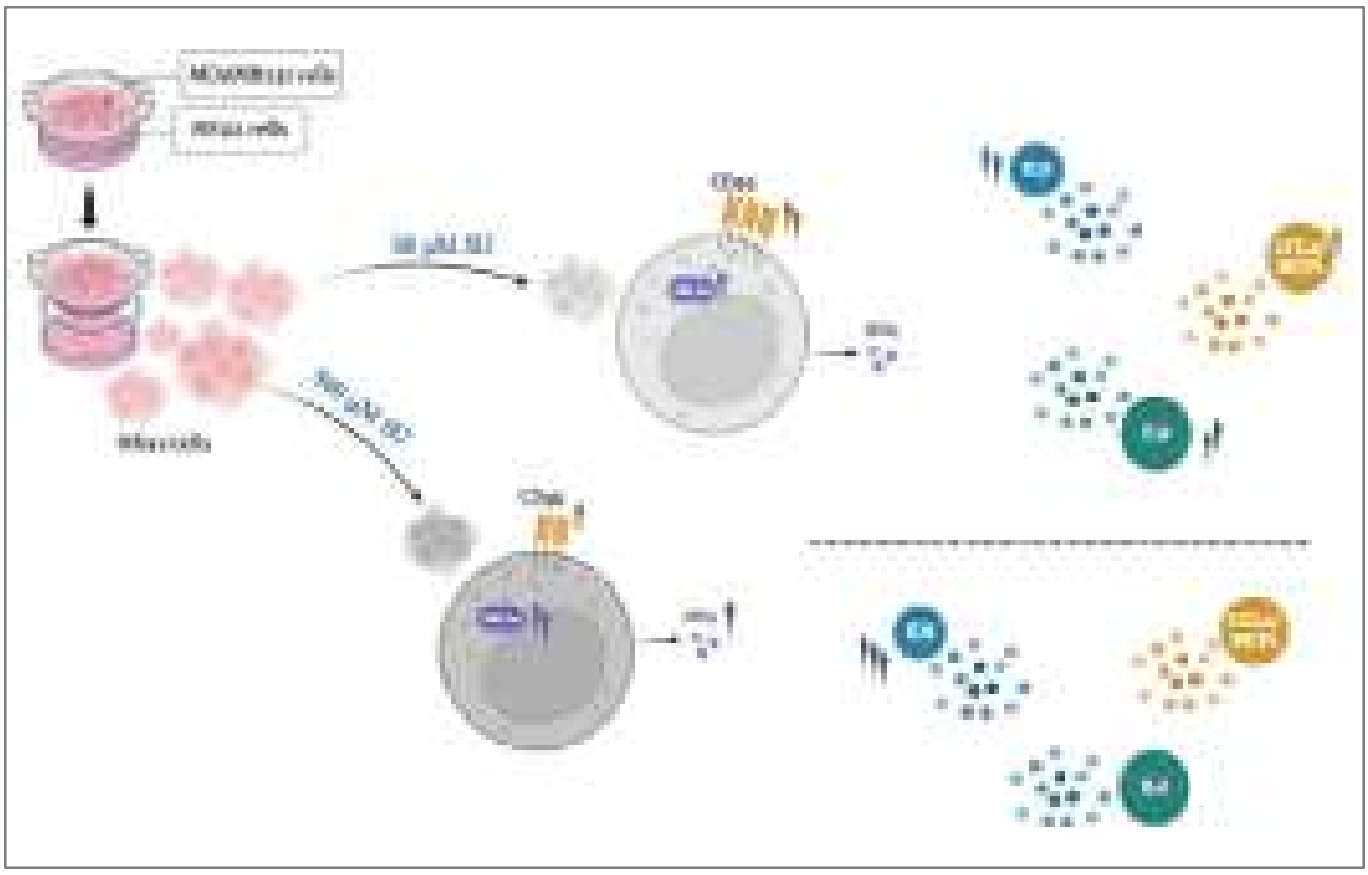

Figure (58): A graphical model represents the key findings of the effect of Succinate treatments on the activation status of NK92 cells and the cytokines /chemokines production in the co-culture system with the breast cancer cell line, MDAMB231.

CD56, IFNγ and HIF1α levels increased with succinate treatments, particularly at high concentrations (500 µM). Both succinate treatments led to increased IL8 concentration, while the concentrations of IL6 and CCL2 (MCP1) were significantly elevated in cultures treated with low succinate concentration (50µM).

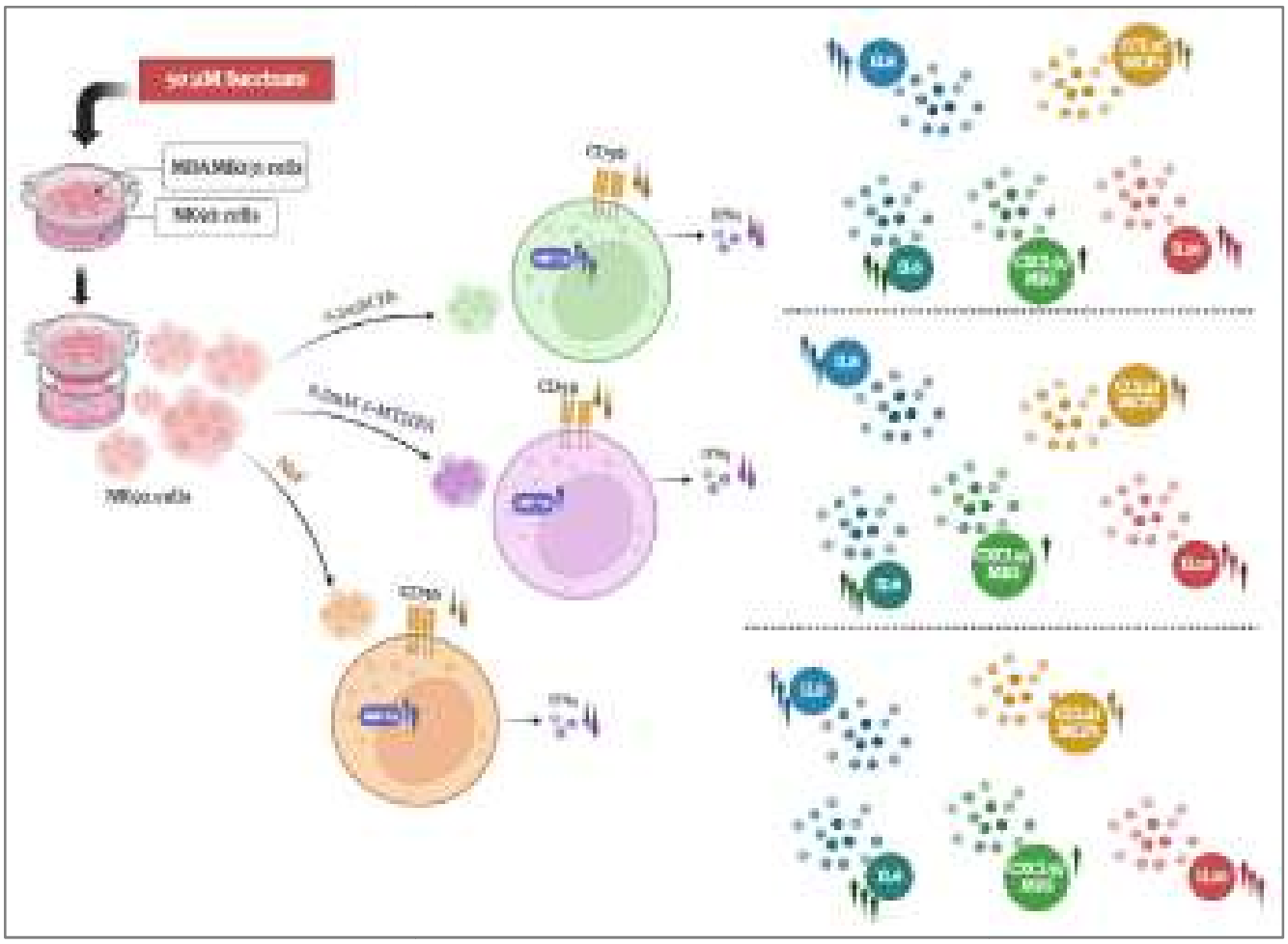

Figure (59): A graphical model represents the key findings of the influence of supplementation with 50µM of Succinate in different Folate availability on the activation status of NK92 cells and the cytokines /chemokines production in co-culture system with the breast cancer cell line, MDAMB231.

Supplementing the co-culture system with 50 µM of succinate in high folate or folate-deficient conditions resulted in decreased CD56 and IFNγ levels, but increased HIF1α levels.

The concentrations of the cytokines; IL10, IL6, and IL8, as well as the chemokines; CCL2 (MCP1) and CXCL9 (MIG) concentrations, increased in cultures with excess or deficient folate conditions compared with the control condition (0.02mM of FA). Overall, these changes in the cytokine/chemokine profile might create a hostile microenvironment for NK cells to exert their cytotoxic activities against tumor cells.

6.2. Conclusion:

The findings of our study revealed the potential effects of folate availability and succinate buildup on the activation status and metabolic regulation by mTOR pathway of NK92 cells cultured alone and within a TME mimetic co-culture system with the breast cancer cell line, MDAMB231.

These findings can be highlighted as follows;

High concentrations of folate, particularly 5-methyltetrahydrofolate (5-MTHFA) form, negatively impacted NK92 cell viability. Furthermore, folate availability influenced the expression levels of CD56, IFNγ production as well as HIF1α expression levels in the mono- and the co-culture system with MDAMB231 breast cancer cells.

NK92 cell culture supplemented with succinate exhibited decreased viability and affected CD56, IFNγ, and HIF1α levels in NK92 cells. Moreover, succinate influenced the expression of genes related to folate metabolic pathway (*RFC, DHFR, MTHFR*). Additionally, folate levels and succinate supplementation influenced the regulation of gene expression of key genes involved in the up-stream and down-stream of the mTOR pathway including; *mTOR, Raptor, Rictor, RPS6KB1, RPS6KB2, AMPKα1, and SREBP1*.

Furthermore, our findings suggest an interplay effect between folate availability and succinate buildup in the TME mimetic co-culture system, which could impact the NK cell function. We observed that succinate supplementation within different folate conditions influenced the expression levels of CD56, IFNγ, and HIF1α levels in NK92 cells and impacted the balance of cytokine and chemokine produced in the co-culture system.

While this study has shed light on a previously unexplored research domain concerning NK cell function and metabolic regulation influenced by folate and succinate levels, it is essential to acknowledge certain limitations.

The study focused on a limited set of markers and genes associated with NK cell function, activation and metabolism, and it did not delve into the underlying mechanisms through which folate and succinate exert their effects on NK cells, necessitating further research to unravel these mechanisms and investigate other crucial factors involved in the methylation cycle, mitochondrial metabolism and glycolysis.

Additionally, our findings were based on *in vitro* experiments that utilized the NK92 cell line as a model for NK cells, which may not fully represent the complexity and heterogeneity of primary

NK cells *in vivo*. The translation to *in vivo* conditions and clinical scenarios requires further investigation.

Lastly, the study primarily examined the effects of folate and succinate on NK cells within a co-culture system involving MDAMB231 breast cancer cells, limiting the generalizability to other tumor types or microenvironments.

Despite these limitations, our study provides valuable insights into the interplay between folate metabolism, succinate accumulation, and immunometabolic signaling pathway in NK cells within the TME, highlighting potential avenues for future research and investigation for therapeutic targeting in this area.