# Chapter V

# *Discussion*

## 5. Discussion:

Numerous studies have provided evidence of an association between the availability and metabolism of folate and the proper functioning of the immune system in relation to infections, autoimmune diseases, and neurodegenerative disorders [59, 62, 279, 280]. This association highlights the potential impact of folate on immune responses. Immune response is widely recognized as a hallmark of cancer development and progression [323], thereby it would be reasonable to anticipate a role for folate in the development and progression of cancer. However, there is conflicting evidence regarding the impact of folate on cancer risk and malignancy progression, which can vary depending on factors such as the dosage and exposure timing to folate. Additionally, it can be attributed to the type of epidemiological study conducted, the assessment of dietary or circulating folate levels, and the type of cancer under investigation [310, 324-326].

The vital role of natural killer (NK) cells in immune defense against pathogens and surveillance of tumor cells attracts our attention to study how folate could affect the function and regulation of NK cell metabolism. Both epidemiological studies [65, 66, 68] and experimental studies using animal models [62, 64, 69, 310] or *in vitro* [67] have indicated a correlation between folate intake and levels and the function and cytotoxicity of NK cells. These studies suggest that impaired folate metabolism may contribute to the impairment of NK cell function. This underscores the importance of understanding the relationship between folate metabolism and NK cell immune function and metabolic regulation, particularly in the context of cancer and malignancy.

Therefore, our study aimed to explore how folate levels affect the activation and metabolic regulation of the NK92 cell line, with a specific focus on the mTOR signaling pathway, within the tumor microenvironment (TME). To achieve this, we conducted experiments using two forms of folate: folic acid (FA) and 5-methyltetrahydrofolate (5-MTHFA). FA is commonly found in supplements, fortified food, and cell culture media, while 5-MTHFA is the predominant form of folate in dietary sources and peripheral blood [327].

We cultured the NK92 cell line under logarithmically increasing concentrations (0.02mM, 0.2mM, and 2 mM) of FA and 5-MTHFA, as well as a folate-free medium to simulate folate deficiency. In our study, the folate concentration measured in the complete folic acid-free medium, after adding 10% fetal bovine serum (FBS) and 10% horse serum (HS), was 0.1 nmol/L.

Before proceeding with further experiments, we assessed the effects of folate form and concentration on the viability of NK92 cells. We observed that a concentration of 2 mM folate had detrimental effects on NK92 cells, particularly in the 5-MTHFA cultures, as the cells exhibited cluster disaggregation after 72 hours. However, compared to the 5-MTHFA form, the FA form at a concentration of 2 mM appeared to be less lethal or toxic to NK92 cells. This finding is consistent with a previous study conducted on primary lymphocyte cultures, which reported a higher tendency for apoptosis in 5-MTHFA cultures compared to FA cultures [328]. In this study, the researchers found that the frequency of micronucleated binucleated cells (MNed BNCs) and DNA damage were higher in 5-MTHFA cultures, and it was positively correlated with the higher apoptosis in 5-MTHFA cultures. A plausible explanation for this effect could lie in folate transportation. Folate can be transported to cells mainly by the universal reduced folate carrier (RFC), which has a high affinity for 5-MTHF but is lower for FA [329], and by folate receptors (FRs), which are limited to specific cell types [330] and have a higher affinity for both forms than RFC [331]. In our study, *FRa* (*FOLR1*) was generally not expressed by NK92 cells; however, *RFC* gene expression was down-regulated in high folate treatments. Thus, it is possible that NK92 cells down-regulate the RFC in the milieu with high folate concentrations in a trial of cells to avoid the toxic impact of high folate doses. Alternatively, when 5-MTHFA is the dominant available form of folate in cell culture media, it may disrupt one-carbon metabolism, which is necessary for cellular homeostasis and proliferation. High levels of 5-MTHF could inhibit the methionine synthase (MS), leading to the inhibition of methylation reactions by S-adenosylhomocysteine (SAH) and the accumulation of homocysteine. Further, high levels of 5-MTHF may interfere with the metabolism of FA, reducing the levels of active tetrahydrofolate (THF) and impacting purine and thymidylate synthesis, which are necessary for DNA synthesis and repair [332].

Our findings showed that a high dose of FA (0.2 mM) increased *DHFR* gene expression. This result aligns with the findings of a clinical trial study conducted on healthy individuals, where they observed higher *DHFR* mRNA expression following a 90-day folic acid intervention. Furthermore, in line with our findings, the researchers also observed a significant reduction in the number of NK cells following the folic acid intervention [65]. Some researchers suggested that a high dose of FA leads to inhibiting the DHFR enzyme, accumulation of UMFA, and depletion of the active form of folate, tetrahydrofolate (THF), the enzymatic co-factor in one-carbon

metabolism [333]. This can disturb the one-carbon metabolism and other related metabolic pathways, including the methylation cycle [332].

Our findings from mono-cultured NK92 cells revealed that folate levels affected the expression of CD56 and IFNγ and the percentage of positive cells throughout 72 hours.

We observed that the 5-MTHFA form of both concentrations (0.02 mM and 0.2 mM) had the most pronounced effect on increasing CD56 levels compared to other folate treatments. We also found that the highest levels of pro-inflammatory cytokine IFNγ and the percentage of IFNγ+ cells were observed in the 0.2 mM concentration of 5-MTHFA form in mono-cultured NK92 cells after 72hrs. Further, gene expression analysis showed that IFNγ transcript levels were higher in 0.2mM FA and folate-deficient conditions compared to the control in mono-cultured NK92 cells. Our observations imply that folate levels influence NK92 cell cytotoxicity and can shift the cell population towards $CD56^{bright}$ NK cells which are less cytotoxic and more inclined to secrete cytokines [30]. Our results align with previous studies, which reported an association between high FA intake and reduced NK cell cytotoxic responses [65, 66].

We assume that excessive folate levels may modulate NK cell function through methylation alteration. Indeed, a line of evidence has suggested that methylation alterations in NK cell function-related genes can influence NK cell cytotoxicity. For instance, researchers found that demethylation treatment for the human NK-92MI cell line and human polyclonal NK cells with a methyltransferase inhibitor (5-azacytidine) modulated KIRs expression and altered NK cell cytolytic activity by over-expression of inhibitory KIRs and impaired granzyme B (GzmB) and perforin (Prf1) release [334, 335]. This is consistent with our finding that *GZMB* gene expression was down-regulated in the media containing a high dose (0.2 mM) of 5-MTHFA. Furthermore, it was found that the expression of activating receptors implicated in cytotoxicity against tumor cells such as NKG2D is correlated with the demethylation of the CpG promoter and the acetylation of histone H3K9 in human NK cells [336]. Hence, high doses of folate may induce alterations in DNA methylation and stability, which in turn could reduce NK cell cytotoxicity through dysregulation in the expression of activating and inhibitory NK cell receptors.

In our co-culture system with the breast cancer cell line MDAMB231, a high concentration of FA (0.2 mM) helped maintain higher CD56 levels in NK92 cells, while CD56 levels decreased in folate-deficient (NoF) conditions. Further, the NK92 cell population with the presence of the cancer cells in high doses and deficient folate conditions maintained a high percentage of CD56+

cells (more than 97%) while it decreased in the NK92 cells in the control folate treatment (about 95%). In contrast, IFNγ levels in co-cultured NK92 cells and the percentage of IFNγ+ cells decreased significantly in FA-over supplemented and folate-deficient groups. However, IFNγ levels in the co-cultured NK92 cells and the percentage of IFNγ+ cells maintain the highest levels at the high dose of 5-MTHFA among the other folate treatments. Cancer cells are broadly reported as voracious in consuming and sequestering folate to fulfill their elevating demands for nucleic acid and protein synthesis, which are needed in their rapid proliferation and aggressiveness [337]. Therefore, cancer cells increase the expression of folate receptor alpha (FRα), which has a higher affinity to bind folate than other folate transporters. Cancer cells seem to be more efficient in dealing with a low concentration of folate than NK cells by upregulating the expression of unidirectional FRα and downregulating bidirectional folate transporter RFC [338].

In our study, the viability of NK92 cells co-cultured with the breast cancer cells decreased in the control FA condition (about 85%) compared with the NK92 cell viability in the mono-culture condition (more than 90%). Further, the NK92 cell viability in NoF media decreased to 80% in the co-culture system, while it was comparable to the control FA media (0.02 mM) in the mono-culture condition. The decrease in viability of NK92 cells co-cultured with cancer cells in contrast to NK92 cells alone suggests a possible sequestration of folate by cancer cells. Moreover, the expression of CD56 and IFNγ in the mono-cultured NK92 cells in the folate-deficient condition (NoF) was comparable with the control FA treatment (0.02 mM); however, the co-cultured NK92 cells in the NoF condition remarkably decreased their expression of CD56 and IFNγ. This might be due to the cormorant consumption of the available folate in the co-culture microenvironment by the cancer cells, hence creating a drastic folate deficiency in this co-culture system. Consequently, this might lead to the occurrence of methylation alterations in excess folate dose and folate deficiency. Further, rewiring of cancer cell metabolism in these folate treatments as well as onco-factors that could be secreted in the microenvironment by the cancer cells [339, 340] could also explain the decreased levels of IFNγ and CD56 in co-cultured NK92 cells in our study.

The hypoxia-inducible factor, HIF1α, expression levels were increased in mono-cultured NK92 cells at both the protein and transcript levels in the presence of high doses of both folate forms and folate-deficient conditions. However, in co-cultured NK92 cells, HIF1α protein levels were lower compared to the control group. It is worth noting that the control group exhibited comparable HIF1α levels in mono- and co-cultured NK92 cells.

To the best of our knowledge, there is no previous study exploring HIF1α expression in relation to folate status in NK cells. However, it has been found that folic acid suppresses the inflammatory responses in a dose-dependent manner in monocyte THP-1 cells under hypoxic conditions via down-regulation of HIF1α expression [341]. Besides, it has been reported that HIF1α inhibition or deficiency in NK cells unleashes potent NK cell anti-tumor effector function and, in turn, reduces tumor growth [342]. In cytotoxic T cells, a suppressive effect of HIF1α has been reported by modulating the production of pro-inflammatory cytokines such as IFNγ [343]. Likewise, it was found that HIF1α-deleted T cells had enhanced IFNγ release upon T cell receptor activation compared to the wild type [344]. Altogether, this suggests that high folate doses in TME may help increase the anti-cancer activity of NK cells through HIF1α down-regulation. This is consistent with our data, where HIF1α levels were the lowest concomitantly with the highest levels of IFNγ in the NK92 cells co-cultured with breast cancer cells in high dose of 5-MTHFA among all other treatments.

Furthermore, in our study, co-cultured NK92 cells in folate-deficient condition had an increased percentage of CD56+ cells with lower levels of CD56 and lower IFNγ levels with higher levels of HIF1α compared to high folate dose groups, suggesting a possible shift within the NK92 cell population towards $CD56^{dim}$ NK cells, which are characterized to have more cytotoxic activity than that of $CD56^{bright}$ cells [30]. However, our data on transcript expression for cytolytic proteins *GZMB* and *PRF1* does not support the increased cytolytic potential of mono-cultured NK92 cells in folate-deficient conditions where HIF1α levels were higher compared to the control group. Further protein analysis is needed for NK cell cytolytic activity in co-culture settings.

Comparison of differences in HIF1α levels at different concentrations of folate between mono-cultured and co-cultured NK92 cells with breast cancer cells suggests an interplay of tumor cell-mediated factors in influencing HIF1α levels.

A possible mechanism for the effect of folate status on HIF1α expression is that high folate concentration or deficiency might disturb the balance in one-carbon metabolism, leading to altered DNA and histone methylation patterns. This may lead to altered gene expression of the *HIF1α* gene or the genes involved in HIF1α regulation or stability [345, 346]. Hence, epigenetic modifications including DNA or histone methylation influenced by folate status may play a role in the regulation of *HIF1α* gene expression by hindering the methylation process. For instance, it was reported in a study on the immature hematopoietic cell line HMC-1, which exhibits low levels of

HIF1A expression compared to other cell lines, that *HIF1α* gene expression in this cell line is suppressed by a process dependent on DNA methylation [347].

The interaction between IL-2 and HIF1α in human NK cells has been suggested by a recent study, in which researchers found that increasing the expression of HIF1α is dependent on the presence and concentration of IL-2 under hypoxic conditions [348].

In our study, exogenous IL-2 was essential and critical for the proliferation and survival of NK92 cells. Therefore, we added not only to monoculture conditions but also to the co-culture system. The concentration used in our study was, 10 IU/mL, the minimum recommended amount to maintain and support NK92 cell growth.

Our study was conducted under normoxic conditions and thus the elevation of HIF1α in mono-cultured NK92 cells in high doses of folate and deviated folate conditions, suggesting a pseudo-hypoxic effect for high folate treatments, mainly in high doses of 5-MTHFA form, more than in deficient condition. However, the decreasing levels of HIF1α in co-cultured NK92 cells in the high doses of folate and deviated folate conditions might be explained by the fact that the utilized concentration of exogenous IL-2, 10 IU/mL, was not enough in the co-culture system to elevate HIF1α in high doses and deficient folate treatments. This explanation for the suggested effect of folate status on HIF1α expression can be supported by our finding, in which we found no notable changes in the levels of HIF1a between mono- and co-cultured NK92 cells in the control folate treatments (0.02 mM).

The balance of cytokines and chemokines in the TME can have critical effects on the function and recruitment of immune cells generally and NK cells especially. In our study, we analyzed the cytokines and chemokines profiles in the supernatant of the co-culture system and found that IL8 and CXCL9 (MIG) were significantly lower, while CCL2 (MCP1) was higher in high folate doses and folate-deficient groups than that in the control folate treatment. IL8 (CXCL8) and CXCL9 (MIG) have been reported to play a role in attracting, recruiting, and migrating NK cells to sites of inflammation, including tumors [349-351]. Therefore, in our scenario, low levels of IL8 (CXCL8) and CXCL9 (MIG) can lead to a reduction in the chemotaxis and infiltration of NK cells into TME. Besides, high levels of CCL2/MCP1 in TME are associated with the recruitment of suppressor immune cells, such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), which can hinder the anti-tumor activities of NK cells [352].

Altogether, high folate doses and folate-deficient conditions in TME might create a hostile microenvironment for NK cells to exert their cytotoxic activities against tumor cells.

The mTOR pathway plays a vital role in regulating the metabolism and function of NK cells by promoting NK cell proliferation and enhancing their capacity to produce cytokines. mTOR pathway inhibition can impair the function of NK cells and diminish their ability to combat tumors effectively [24, 26, 143]. In our study, the expression of mTOR pathway-related genes in mono-cultured NK92 cells was also affected by folate levels. We observed that excessive folate treatments resulted in downregulation of the gene expression levels of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2*, and *AMPKα1* while a high FA dose led to *SREBP1* upregulation. In folate deficient condition (NoF), *mTOR* gene expression was significantly downregulated compared to all other folate treatments.

However, protein analysis using ELISA did not reveal statistically significant differences in SREBP1 levels or phosphorylated proteins within the mTOR pathway between the folate treatment groups. Concomitantly, we also observed downregulation of IFNγ expression in high FA treatment and NoF condition. Although these decreases did not reach the significance in mono-culture NK92 cells, it was significantly decreased in co-culture NK92 cells. As we didn't analyze the expression of mTOR pathway genes in co-cultured NK92 cells, further investigation is needed to understand the functional consequences.

Raptor and Rictor, as components of mTORC1 and mTORC2, respectively, have distinct functions in NK cells. Raptor-mediated mTORC1 activation is involved in promoting NK cell proliferation, cytokine production, and cytotoxic activity. Rictor-dependent mTORC2 signaling is important for NK cell development, survival, and maintenance of their effector functions. The down-regulation of Raptor and Rictor in high folate dose and downregulation of mTOR in folate-deficient condition (NoF) may lead to decrease NK cell proliferation [179], which is consistence with our findings in high folate concentrations, where the viable NK92 cell counts were deceased.

Ribosomal protein S6 kinases, RPS6KB1 and RPS6KB2, are downstream effectors of mTOR signaling, involved in regulating protein synthesis, cell growth, and metabolism [354]. SREBP (sterol regulatory element-binding protein) is implicated in cytokine-induced metabolic reprogramming of NK cells. SREBP supports glycolysis and OxPhos by regulating key metabolic enzymes of the CMS and influences the cytotoxic responses of NK cells [29].

To the best of our knowledge, the mTOR pathway concerning folate metabolism has not been studied in immune cells. However, mTOR is reported as a sensor of folate availability in trophoblast and brain cells [354-359]. Furthermore, the MTHFD2 enzyme in cancer folate metabolism has been shown to have a non-metabolic role in which IFNγ stimulates it through the AKT–mTORC1 pathway resulting in increased cMYC stability and PD-L1 transcription [360]. Likewise, the mTORC1 signaling pathway in breast cancer cells (MCF-7) has been connected to the FA levels which are observed to be involved in the regulation of metabolic, apoptotic, and malignancy pathways [361].

Our findings provide insights into the impact of folate levels on mTOR pathway-related gene expression in NK cells. However, further research is needed to fully understand the functional consequences and interactions between folate and the mTOR pathway in NK cell biology, particularly in the context of cancer.

In the other line of our research study, we investigated the potential mechanisms underlying the effect of succinate on NK92 cell activation and metabolism. Succinate is an important intermediate in the tricarboxylic acid (TCA) cycle that has been reported to be involved in tumorigenesis [193]. Investigating succinate as a metabolic signaling molecule may enhance our understanding of metabolic regulation in the cancer-immunity cycle. While the role of succinate in immune cells such as macrophages [48, 49, 271], dendritic cells [50, 51], and T cells [47, 362, 363] has been established, its role in NK cells has not been explored yet.

Therefore, in this study, we also aimed to assess the effect of succinate on the activation status and mTOR pathway of the NK92 cell line in the TME by investigating its influences on NK92 cells in mono- and co-culture systems with the breast cancer cell line.

In terms of succinate treatments, different concentrations of succinate were selected based on normal and pathological levels. Normal succinate levels in human plasma range between 2 and 20 μM [364], while in serum, it is between 2 and 3 μM [365]. Higher succinate levels have been detected in obese individuals ($101.72 \pm 9.37$ μM) compared to lean individuals ($78.24 \pm 4.4$ μM) [366]. In cell culture models, comparable levels of succinate ranging from 0.25mM to 0.57mM have been measured in the conditioned media of different types of cancer cells, while immune cells such as macrophages showed lower levels of 0.07mM [367]. Further, succinate concentrations in the blood of lung cancer patients ($0.53 \pm 0.038$ mM) were significantly

higher than in healthy subjects [368]. Based on these studies, concentrations of 50, 100, and 500 µM of succinate were selected to study the effects of succinate on the activation status and mTOR pathway in NK92 cells.

We noticed that the viability of NK92 cells significantly decreased after 72 hours in all succinate treatments, regardless of the dose, compared to the non-succinate control group.

Besides, we observed a reduction in CD56 and IFNγ levels in mono-cultured succinate-treated NK92 cells. The percentage of cells positive for CD56 and IFNγ also decreased. In contrast, in co-cultured NK92 cells, CD56 levels increased with succinate treatments and increased IFNγ levels were noticed in high concentrations of succinate (500 µM). Additionally, we found that the gene expression of *GZMB* (granzyme B) increased in mono-cultured NK92 cells, particularly with 100µM succinate treatment. These findings resemble previous observations in T cells exposed to succinate (0.5-10 mM) in terms of reduced CD4+ and CD8+ T cell frequencies expressing IFNγ and decreased IFNγ secretion [47].

However, unlike our results in NK92 cells, succinate exposure did not affect T-cell viability, proliferation, or activation. Suppression of IFNγ was observed in T cells cultured in a conditioned medium from succinate dehydrogenase complex subunit B (SDHB)-deficient immortalized mouse chromaffin cells mimicking the TME. The expression of GZMB in T cells was not altered by succinate exposure in those experiments. In those experiments, succinate-mediated inhibition of T cell cytokine secretion was associated with impaired glucose oxidation, decreased mitochondrial membrane potential, and suppressed ATP synthesis. Restoring the TCA cycle flux reversed the succinate-induced suppression, indicating its involvement as a key mechanism [47].

We observed that succinate treatments increased HIF1α expression levels in both mono- and co-cultured NK92 cells after 72 hours. In mono-cultured NK92 cells, high concentrations of succinate (100 µM and 500 µM) led to a significant six-fold increase in *HIF1α* gene expression. This suggests a pseudo-hypoxic effect of succinate on NK cells similar to what has been observed in macrophages [272]. Elevated HIF1α expression in NK cells has been reported to diminish their anti-tumor activity [342]. HIF1α has been also reported to have a suppressive effect on T cells by modulating the production of pro-inflammatory cytokines including IFNγ. Studies have shown that HIF1α-deleted T cells exhibited enhanced IFNγ releasing upon T cell receptor activation when

compared to the wild type. Thus, promotes T cell proliferation and production of pro-inflammatory cytokines such as IFNγ [343, 344].

In our study, 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 [30]. This is supported by the increased percentage of IFNγ-secreting NK92 cells in the co-culture system of our study.

In contrast to our finding in NK92 cells, T cells treated with succinate did not show an increase in HIF1α levels, despite elevated intracellular succinate levels [47]. On the other hand, similar to our findings, macrophages were reported to adopt HIF1α up-regulation in response to succinate elevation [369]. As we have mentioned in the discussion of folate status effect, the interaction between exogenous IL-2 and HIF1α in human NK cells has been suggested by a recent study, in which researchers found that increasing the expression of HIF1α is dependent on the presence and concentration of IL-2 under hypoxic conditions [348].

Our study was conducted under normoxic conditions, and thus the elevation of HIF1α in mono-cultured NK92 cells in succinate treatments, suggests a pseudo-hypoxic effect of succinate. Succinate supplementation was reported to result in promoting the accumulation of intracellular succinate, thus creating a state of pseudo-hypoxia by activating HIF1α signaling in cancer cells [370, 371].

Moreover, we noticed that supplementation with a high succinate concentration (500 μM) increased the HIF1α levels in co-cultured NK92 cells more than those in mono-cultured NK92 cells. This might be because of the elevated levels of succinate secreted by the cancer cells in our co-culture system [367, 368].

Activated NK cells have been shown to possess a tendency to prevent succinate accumulation by rewiring the TCA cycle towards CMS, promoting OxPhos and generating the necessary energy molecules [29]. This observation makes us suggests that NK cells may not be equipped to handle the high levels of succinate found in pathological conditions, including cancer. The accumulation of succinate in such scenarios could potentially lead to severe complications in NK cell physiology and functionality.

Our gene expression analysis showed that succinate treatments affected gene expression related to folate metabolism. *MTHFR* and *RFC* gene expression was downregulated, while *DHFR* gene expression increased at succinate concentrations (100 μM and 500 μM). These data suggest a regulatory role of succinate in folate metabolism. High levels of succinate are often associated with mitochondrial dysfunction or metabolic imbalance and can lead to alteration in one-carbon metabolism. This disruption, we suggest, can influence the availability of folate and other cofactors involved in one-carbon metabolism [372, 373]. The relationship between succinate and folate metabolism requires further research. Additionally, succinate can affect the mTOR pathway [374, 375] which also can modulate folate and one-carbon metabolism [341, 354].

In our study, we observed that succinate added to the culture medium of mono-cultured NK92 cells impacted the expression of genes associated with the mTOR pathway. Specifically, we found a decrease in the gene expression levels of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2*, *AMPKα1*, *SREBP1*, and *ATF3* in succinate-treated NK92 cells. The down-regulation of SREBP1 has been shown to decrease NK cell cytotoxicity [29], which could potentially explain the decrease in IFNγ and CD56 observed in mono-cultured NK92 cells treated with succinate in our study.

Further investigation was conducted in our study to explore the interplay between folate levels and exogenous succinate treatment in the co-cultured system. Specifically, we examined how the availability of folate and the presence of a low concentration of supplemented succinate (50 μM) may impact the activation status of NK92 cells in a co-culture system with MDAMB231 cancer cells. Additionally, we measured the concentrations of cytokines and chemokines secreted in the supernatant of this simulated TME to further elucidate the impact of folate status with succinate supplementation on the modulation of NK92 cell immune response.

We found that adding 50μM succinate in high folate or folate-deficient conditions to the co-culture system reduced CD56 and IFNγ levels, while increasing HIF1α levels in co-cultured NK92 cells compared to the control condition. However, we noticed that the levels of CD56 in the presence of exogenous succinate were lower than that in conditions without succinate, irrespective of folate levels. IFNγ levels in the presence of succinate decreased only in high 5-MTHFA concentration and increased in folate-deficient condition. In contrast, the HIF1α levels in high folate doses or folate-deficient conditions with succinate were sharply higher than those in the same folate conditions without succinate supplementation.

Furthermore, our results revealed that IL10, IL6, and IL8 concentrations as well as the chemokines CCL2 (MCP1) and CXCL9 (MIG) concentrations were considerably elevated in cultures with 50μM of succinate under both excessive and deficient folate conditions. Additionally, we observed that adding 50μM succinate to the co-culture system with control folate treatment (0.02mM FA) increased IL8 and IL6 concentrations in the supernatant. However, the concentrations of CXCL10 (IP10) and CCL5 (RANTES) did not show remarkable changes in folate groups between the conditions of with and without succinate addition.

Secretion of IL6 and IL8 in TME was reported to impair NK cell activity and function through the activation of STAT3 signaling pathway [376]. The gene display of IL10-induced NK cells showed an enhancement in mRNA levels of cell activation and cytotoxicity-related genes [377]. Elevation of IL10 secreted by tumor cells can inhibit the cytotoxic activity of NK cells and the production of IFNγ [378]. The conflicting findings regarding the role of IL10 in cancer may be attributed to the intricate regulation of its function, which is influenced by a delicate balance of cytokines and other factors in TME [379]. The combination of secreted cytokines in TME could regulate the anti-tumor activity of NK cells [380].

The monocyte chemotactic protein-1 chemokine (MCP1; CCL2) increased significantly under excessive and deficient folate conditions in the co-culture system supplemented with 50 μM of succinate in our study. CCL2 (MCP1) can be produced by different types of cells including NK cells [381], epithelial and endothelial cells, and it serves as a regulator for migration and infiltration of not only monocytes and memory T lymphocytes, but also NK cells into tumor and inflammation sites [382]. Over-expression of CCL2 (MCP1) in breast cancers is correlated with induced invasion and metastasis [383-387]. CCL2 (MCP1) expression by breast cancer cells is strongly associated with the presence of tumor-associated macrophages (TAMs), and CCL2 with increased VEGF expressions have been found to correlate with TAM infiltration, angiogenesis, and poorer survival rates in breast cancer [388-390]. Altogether, in our findings, increased CCL2 (MCP1) concentration in excessive and devoted folate conditions with 50 μM of succinate was combined with a significant decrease in the percentage of IFNγ+ NK cells and CD56 levels and associated with highly significant elevation in HIF1α levels in co-cultured NK92 cells which suggest inhibition in the NK92 cytotoxicity by interplay between folate and succinate in TME. Further, elevating HIF1α of mono- and co-cultured NK92 in excess and deprivation of folate in the presence of succinate suggests 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.

Overall, our results shed light on a possible complex interplay between succinate treatments, folate levels, and modulation of NK cell activation status. The observed changes in protein expression and cytokine/chemokine concentrations provide valuable insights into the potential mechanisms underlying immune regulation of NK cell anti-tumor activity in the co-culture system.
Further investigations are needed to delineate the specific pathways involved, elucidate the underlying mechanisms and explore the potential implications of these findings in the context of NK cell immune response modulation and cancer.

In conclusion, our results demonstrated the potential way of folate and succinate in modulating NK immune cell responses through regulation of the metabolic signaling pathway (mTOR) and cytokine and chemokine profiles. As the field of NK cell metabolism continues to advance, further research is warranted to unravel the precise mechanisms and clinical applications of such metabolites (folate and succinate).