ABSTRACT

Background: Natural killer (NK) cells are highly effective cytotoxic lymphocytes that exhibit rapid and direct responses to combat transformed and cancer cells. Deficiencies and functional alterations in NK cells have been associated with an increased risk of developing malignancies. The metabolic characteristics of NK cells play a decisive role in their functionality, and any alterations in their metabolism can have a profound impact on their immune responses. The hostile and harsh circumstances in tumor microenvironment (TME) present a formidable challenge for the metabolism of tumor-infiltrating NK cells. These conditions can disrupt the metabolic processes of NK cells, ultimately resulting in immunosuppression. The imbalance in nutrients and metabolites levels is one of the hallmarks of TME that can contribute to the disruption in the immunometabolic processes ending in dysfunction of tumor-infiltrating immune cells. 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 have been established, its influence on NK cells remains unexplored.

Aim: Our present study aimed to investigate 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.

Methods: The NK92 cell line was grown in mono-culture and co-culture system with MDA-MB-231, a breast cancer cell line, supplemented with different folate and succinate conditions. We examined the alteration in the NK92 cell activation status by assessing the expression of CD56 and IFNγ, as well as the expression of *GZMB*, and *PRF1*genes. Additionally, we examined the impacts of some key genes in folate metabolic pathway including; *RFC*, *DHFR*, and *MTHFR*, and key genes in mTOR signaling pathway including; *HIF1α* and *SREBP1*. Moreover, considering the importance of regulation for the functionality

of NK cells, we analyzed the levels of cytokines and chemokines in the supernatant of the co-culture system under different folate and succinate treatments.

Results: our findings revealed several important insights,

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 the levels of CD56 and pro-inflammatory cytokine IFNγ ($p < 0.05$, $p < 0.0001$) and the percentage of IFNγ+ cells were observed in the high concentration (0.2 mM) of 5-MTHFA cultures ($p < 0.05$). Further, *GZMB* gene expression was down-regulated in 5-MTHFA cultures ($p < 0.005$), indicating lower cytotoxic potential of NK92 cells.

In the co-culture system with the breast cancer cell line MDAMB231, NK92 cells maintained the highest CD56 levels in a high concentration (0.2 mM) of FA ($p < 0.0001$), while folate deficiency decreased CD56 levels ($p < 0.0001$). IFNγ levels and the percentage of IFNγ+ cells decreased significantly in the FA over-supplemented (0.2 mM) and folate-deficient cultures ($p < 0.0001$). The highest levels of IFNγ were observed in the high concentration (0.2 mM) of 5-MTHFA cultures ($p < 0.0001$). NK92 cell viability in the co-culture system decreased in the control FA condition (0.02 mM) and folate-deficient condition compared to the NK92 viability in monoculture, suggesting a possible sequestration of folate by cancer cells.

The HIF1α levels increased in mono-cultured NK92 cells with high folate doses and folate deficient condition ($p < 0.0001$) but decreased in co-cultured NK92 cells ($p < 0.0001$), suggesting possible effects of folate availability on NK cell activities mediated by the alterations in HIF1α expression.

The expression of mTOR pathway-related genes in mono-cultured NK92 cells was also affected by folate levels. Excessive folate (0.2 mM) treatments resulted in down-regulation of the gene expression levels of *Raptor*, *Rictor*, *RPS6KB1*, *RPS6KB2* and *AMPKα1* ($p < 0.05$) while high FA dose resulted in *SREBP1* upregulation in mono-cultured NK92 cells ($p < 0.001$), suggesting possible impacts on NK cell proliferation, metabolic regulation and functionality.

IL8 and CXCL9 (MIG) levels in the supernatant of co-culture system were significantly lower in high folate doses and folate-deficient condition ($p < 0.0001$) while CCL2 (MCP1) levels were higher in these conditions compared to the control folate treatment ($p < 0.001$).

This creates a hostile microenvironment for NK cells to exert their cytotoxic activities against tumor cells.

Succinate treatments of mono-cultured NK92 cells led to a reduction in CD56 and IFNγ levels ($p < 0.0001$ and $p < 0.005$, respectively), as well as a decrease in the percentage of CD56+ and IFNγ+ cells ($p < 0.001$ and $p < 0.0001$, respectively). Further, supplementing the culture medium with high succinate concentrations (500 μM) increased the HIF1α levels more in co-cultured NK92 cells compared to mono-cultured cells ($p < 0.0001$). 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.

Expression of *MTHFR* and *RFC* genes were down-regulated ($p < 0.0001$) in succinate treatments, while *DHFR* gene expression was up-regulated at high succinate concentrations; 100 μM and 500 μM, ($p < 0.05$ and $p < 0.005$, respectively), suggesting an influence of succinate on the folate metabolic pathway. Further, 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 ($p < 0.0001$). This suggests possible impacts on NK cell proliferation, metabolic regulation and functionality.

Moreover, supplementing the co-culture system with 50 μM of succinate in high folate or folate-deficient conditions resulted in decreased CD56 and IFNγ levels ($p < 0.0001$), but increased HIF1α levels as well as increased concentrations of the cytokines; IL10, IL6, and IL8 and the chemokines; CCL2 (MCP1) and CXCL9 (MIG) ($p < 0.0001$). 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, in turn, might influence the NK cell anti-cancer activities.

Conclusion: our research has unveiled new understanding about how folate and succinate may affect the activation and metabolic regulation of NK cells within the TME. These insights shed light on the intricate interplay between these metabolites and their influence on NK cell function and metabolism within the TME, contributing to a broader comprehension of the regulatory mechanisms at play in cancer immunity.