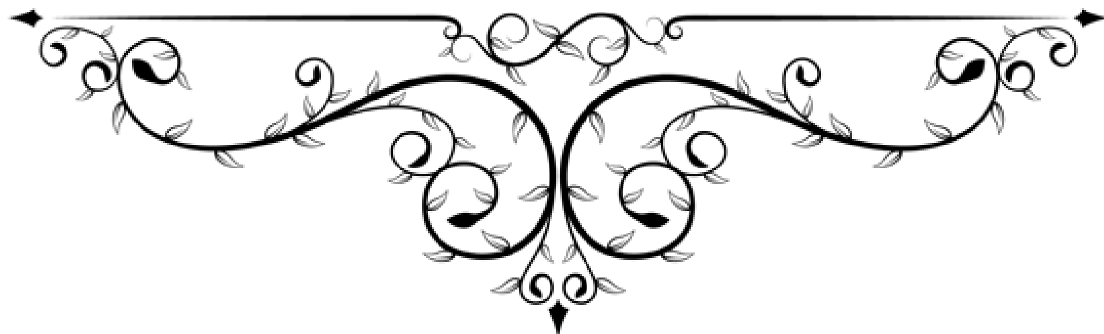
Chapter I

Introduction



1. Introduction:

Immunometabolism, which refers to the interaction between immune cell responses and metabolic processes, plays a crucial role in determining the differentiation and functional capabilities of immune cells. Any alterations in the metabolic characteristics of immune cells can significantly impact their functionality [1, 2].

Among the most effective immune cells in fighting cancer are natural killer (NK) cells, a type of innate cytotoxic lymphocytes. These cells exhibit rapid and direct killing responses against viral-infected cells and cancer cells without the need for prior priming. Additionally, NK cells play vital roles in directing and cooperating with adaptive immunity [3-5]. For instance, NK cells can regulate and shape T-cell responses [4, 5], and they can promote the recruitment of conventional type-1 dendritic cells (cDCs), which are critical for anti-tumor immunity, into solid tumors by releasing CC-chemokine ligand-5 (CCL5), XC-chemokine ligand-1 (XCL1) and XCL2 [6]. Furthermore, tumor-infiltrating NK cells regulate tumor-promoting inflammation in vivo through functional modification of neutrophils by a mechanism dependent on IFNγ and C-X-C motif chemokine receptor-3 (CXCR3) [7].

In terms of functionality, NK cells share similarities with cytotoxic CD8+ T lymphocytes, as they can directly induce cytotoxic responses and produce pro-inflammatory cytokines [4, 8]. The activation of NK cells is controlled by a wide array of signals received through a combination of activating and inhibitory receptors, as well as stress-induced ligand receptors, which detect changes in the expression patterns of their ligands on the surface of various cancer cells [3, 9, 10]. Inhibitory receptors on NK cells recognize self-proteins and transmit signals that maintain tolerance to normal cells [10-12]. When activating receptors on NK cells engage ligands expressed by tumors, and an appropriate number of inhibitory receptors are not co-engaged, the NK cells become activated and initiate the killing of target cells by secreting cytotoxic granules containing apoptosis-inducing granzymes and perforin [10].

NK cells can also eradicate target cells through tumor necrosis factor (TNF) and Fas ligand, which serve as apoptosis-inducing signals [11-13]. NK cells can recognize viral-infected or malignant cells that up-regulate stress-induced activating ligands or down-regulate MHC class-I molecules. Consequently, malignant transformed cells often down-regulate MHC class-I

molecules to avoid recognition by CD8+ cytotoxic T cells, while simultaneously inducing the NK cells activation through a missing self-recognition mechanism [10]. However, certain types of cancer cells overexpress MHC class-I molecules, which can lead to the dysfunction of NK cell activation through interactions with KIR receptors, thereby contributing to the evasion of cancer cells from the anti-tumor activities of NK cells [14, 15]. NK cells also express cellular adhesion molecules and multiple cytokine receptors (e.g., receptors for IL-12, IL-15, and IL-18) [12]. Furthermore, NK cells express FcγRIIIa (CD16s) receptors, which recognize the constant region (Fc) of specific IgG antibodies bound to target tumor cells. This recognition enables NK cells to kill target cells through a process known as antibody-dependent cellular cytotoxicity (ADCC), which has proven to be an effective method in NK cell-based immunotherapy for cancer [10, 16].

Deficiencies and functional alterations in NK cells have been associated with an increased risk of developing malignancies [17-21]. Like other immune cells, the fate and immune functions of NK cells are also influenced by their metabolism [22, 23]. Resting NK cells have low basal metabolic rates of glycolysis and oxidative phosphorylation (OxPhos), which are important for preserving NK cell effector functions (such as IFNγ production). However, NK cell stimulation for a defined period, impacts metabolic changes in NK cells [25, 27]. Furthermore, the metabolic reprogramming of NK cells is likely to depend on the type of activation stimulus (receptors or cytokines). While cytokine-stimulated NK cells can produce IFN-γ independent of glycolysis or OxPhos, activating-receptor-stimulated NK cells require OxPhos increase [26]. However, receptor stimulation is more sensitive to metabolic inhibition than cytokine stimulation [25, 26].

Furthermore, impairment of glucose metabolism and disruption of the mammalian target of rapamycin (mTOR) signaling pathway lead to diminished cytotoxic activity in NK cells [24, 26]. mTORC1 is critical for regulating glucose uptake and glycolysis, as well as controlling the production of granzyme B and perforin in activated NK cells [26]. Upon activation, NK cells increase their glucose uptake and flux through aerobic glycolysis, supported by increased expression of glycolytic enzymes and nutrient transporters. Additionally, activated NK cells enhance their OxPhos rates, accompanied by an increase in mitochondrial mass [27-29].

Human NK cells can be classified into two subsets; $CD56^{bright}$ and $CD56^{dim}$, distinguished by their CD56 expression levels. The $CD56^{bright}$ cells exhibit lower cytotoxic activity than the $CD56^{dim}$ cells, albeit they are much more efficient in cytokines production (e.g. IFNγ, TNFa, IL-10). On the other hand, the $CD56^{dim}$ cells have higher cytolytic activity to target cells and can embody more perforin and granzymes than $CD56^{bright}$ cells [30]. These two NK cell subsets also differ in their metabolic responses to stimulation. Illustratively, $CD56^{bright}$ NK cells exhibit stronger metabolic responses to cytokines stimulation (e.g IL-2 and (IL12+ IL15)) than CD56dim NK cells, resulting in the upregulation of nutrient receptors like; glucose transporter GLUT1, amino acid transporters (SLC1A5, SLC7A5, and CD98), and transferrin receptor CD71 to a greater degree in $CD56^{bright}$ NK cells than in $CD56^{dim}$ NK cells [31, 32]. Additionally, cytokine-activated $CD56^{bright}$ cells demonstrate greater activation of mTORC1 and higher rates of glucose uptake compared to $CD56^{dim}$ cells, indicating higher metabolic requirements for $CD56^{bright}$ cells [31]. Tissue-resident NK cells also increase the expression of nutrient transporters after stimulation, although to a lesser extent than blood NK cells [33].

The role of immunometabolism in regulating NK cell function and its implications in cancer immunotherapy has gained research attention [3, 10]. The understanding of NK cell metabolism is less comprehensive compared to T cells and macrophages, and the influence of the tumor microenvironment (TME) on NK cell metabolism remains largely unknown [34-36]. The harsh metabolic competition in the TME, characterized by hypoxia, acidosis, and nutrient deprivation, can disrupt the metabolism of tumor-infiltrating immune cells, leading to immune suppression [35, 37-41]. Furthermore, the accumulation of metabolites in the TME can shape the phenotypes and regulate the responses of immune cells [42, 43].

The tricarboxylic acid (TCA) cycle provides intermediate metabolites that are utilized in critical biosynthetic processes in the cells [44]. However, the mutations or inhibition of the TCA enzymes that can be found in cancers might lead to the accumulation of TCA metabolites, which can critically shape the immunological functions in the TME [45]. One key metabolites of TCA cycle is “Succinate” which has been implicated in tumorigenesis. The accumulation of succinate in the TME has been detected as a key signaling factor regulating the metabolic machinery and functionality of tumor-infiltrated immune cells [46], including; T cells [47, 362, 363] macrophages [48,49, 271], and dendritic cells [50,51].

The effects of succinate on NK cell activity and metabolism are yet to be explored [52-55]. However, an interesting discovery made by Assmann et al. regarding the metabolic behavior of IL2- and IL12-activated NK cells showed that these NK cells primarily utilize glucose through aerobic glycolysis, converting it to pyruvate and then lactates in the cytosol. However, when pyruvate enters the mitochondria, it is predominantly metabolized through citrate-malate shuttle (CMS), rather than entering the conventional TCA cycle for OxPhos and ATP production. The CMS relies solely on glucose as its fuel source. In this process, citrate is exported from the mitochondria to the cytosol in exchange for malate, via the mitochondrial citrate carrier (CIC). These findings highlight the importance of the CMS in activated NK cells for driving OxPhos and ATP production [29].

Besides, nutrients availability has also been reported to impact immunometabolism and various immune functions [56, 57]. One of the most important nutrients for proliferating cells, including tumor cells and immune cells is “Folate”, an essential group of water-soluble B9 vitamin derivatives [58, 59]. Folate cycle occupies the centre of a highly compact interconnected network, known as folate-mediated one-carbon metabolism (FOCM) which regulates various metabolic pathways and biological processes in normal and tumor cells [60]. Folate bioavailability and metabolism have been correlated with modifying the immunological functionality [61, 62] and have been considered a risk factor for malignancies [63].
In terms of NK cells, some epidemiological, *in vivo* and *in vitro* experimental studies have suggested an association between intake and levels of folate, and the function and cytotoxicity of NK cells. These findings suggest that impaired folate metabolism may contribute to the impairment of NK cell function [62, 64-69]. However, the molecular crosstalk between folate metabolism and the incriminated pathways involved in NK cell activation in the TME needs further investigation.

Therefore, in our present study, we pertain to examine the effect of folate and succinate on NK92 cell line activation status and HIF1a expression both in mono-culture and co-culture conditions. Additionally, within the mono-cultured NK92 cells, we tested the effect of folate and succinate on the expression of key genes in mTOR signaling pathway. Furthermore, we investigated the effect of folate status and succinate supplementation on the secreted cytokines and chemokines profile within the co-cullture system.

The Objectives of our study were as follows:

1. Study the effect of folate and succinate concentrations on phenotype, activation status and expression of key metabolic and functional genes of NK-92 cell line in mono-culture setting.

2. Study the effect of folate and succinate levels on the activation status of NK-92 cells and secreted cytokines/chemokines profile in a tumor mimetic 2D co-culture system.