**Title of the Thesis:**

# Anticancer Effects of AK4 in Macrophages and T-Lymphocytes

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## Table of Contents

1. ABSTRACT ..... 4
2. INTRODUCTION ..... 5
2.1 Relevance of the topic ..... 5
2.2 Aim of study ..... 6
2.3 Research problem ..... 6
3. LITERATURE REVIEW ..... 6
3.1 Immunotherapy and CAR-T cell therapy ..... 6
3.2 Limitations of CAR-T and overview of CAR-M ..... 8
3.3 Macrophages in the tumor microenvironment ..... 11
3.4 Classification of macrophages and polarization ..... 12
3.5 Adenylate Kinase ..... 13
3.6 AK4 in Macrophages, role in inflammation ..... 14
3.7 Role of Overexpression of AK4 ..... 14
4. AIMS AND RESEARCH PROBLEMS ..... 16
4.1 Main aim ..... 16
4.2 Research problem ..... 16
5. HYPOTHESIS ..... 16
6. MATERIAL AND METHODS ..... 16
6.1 Study Design ..... 16
6.2 Description of Methods ..... 17
6.3 Lentivirus packaging and cell transduction ..... 18
6.4 Western blot ..... 19
6.5 RT-PCR ..... 20
6.6 cDNA synthesis ..... 20
6.7 Flow cytometry ..... 21
7. RESULTS ..... 22
7.1 Confirmation of overexpression of AK4 by Western blot and qRT-PCR ..... 22
7.2 Cytometry measurements of ROS and mitohondrial activity ..... 25
8. DISCUSSION ..... 26
8.1 The Key Factors Driving M2 Polarization in Macrophages of THP-1 cells ..... 27

**8.2 Metabolic rearrangement caused by overexpression of AK4 ........ 28**
**8.3 Suppression of pro-inflammatory signaling pathways (NF-kB, STAT1) ........ 28**
**9. CONCLUSIONS ........ 29**
**10. REFERENCES ........ 30**

## ABSTRACT

Despite multiple therapies, including chemotherapy and immunotherapy, cancer-related mortality is still extremely high. Resistance to treatment and relapse are the two most common reasons (Yang et al., 2022). Immunotherapy is a promising method in cancer therapy. There are new forms of immunotherapy, such as CAR-T cell therapy, have use patient cells, taken, isolated, and genetically modified. The genetic modification allows T-cells to identify cancer cells and destroy them in the body (Niu et al., 2024). The new forms of immunotherapy have a proven track record in the treatment of cancer disease, most notably hematological malignancies. For example, in leukemias and B-cell lymphomas, CAR-T cell therapy has demonstrated a tremendous response (Maude et al., 2018). However, the application of this method of therapy is fraught with challenges against solid tumors due to their heterogeneity and immunosuppressive properties within the tumor microenvironment (Wang et al., 2023).

The macrophage is an immune cell, and its main function is to eliminate an organism's defenses against a variety of infections by acting via phagocytosis (Chin et al., 2021). Macrophages are polarized into two categories, M1 are pro-inflammatory and activated by interferon-gamma, which stimulate the release of cytokines and inflammation (Wujak et al., 2021), M2 is an anti-inflammatory macrophage activated by IL-2, and restores tissue as a major function. Multiple studies have examined tumor-associated macrophages (TAM), the greatest population of macrophages represents our cancer microenvironment, and contributes in two ways to tumor progression and metastasis by secreting growth factors (VEGR, EGF, etc.) and secreting immunosuppression cytokines - TGF-B (Wang & Joyce, 2010). Also, tumor-associated macrophages will express the ligand as the receptor to bind with the PD-1 receptor (programmed cell death receptor) as a means to induce T-cell exhaustion.

The overexpression of AK4 affects the functional activity of macrophages, especially the M1 pro-inflammatory activity. The data supports that AK4 increased more in the M1 type compared to the M2 type of macrophage. Thus, overexpression of AK4 is related to an inflammatory response (Chin et al., 2021). Therefore, in this regard, we view AK4 not only as a regulator of metabolism and inflammation, but also potential therapeutic target for developing better CAR-M therapies for the immunotherapy of solid tumors.

## 1. INTRODUCTION

The therapeutic goal for solid tumors is to eliminate tumor cells and further prevent relapse. The therapy of most solid tumors consists of local therapy, including surgery and radiotherapy, and systemic treatment: chemotherapy and immunotherapy. Conversely, for cancer that has metastasized, the main goal is to palliate symptoms and prolong the patient's life.

Some researchers found that the morbidity of cancer patients can be improved with chemotherapy and immunotherapy. Unfortunately, this high morbidity is attributed to morbidity rate - solid tumors retain their high rate of morbidity, which is due to solid tumors developing resistance to chemotherapy and immunotherapy (Yang et al., 2022). To try and improve the morbidity ratio in cancer patients with solid tumors, scientists have discovered a novel targeted therapy, Engineered adoptive T-cell therapies (E-ACT), an immunotherapy where patients' cells are collected, and then genetically modified. There are two types of E-ACT therapy: chimeric antigen receptor (CAR) T-cell and T-cell receptor (TCR).

Despite the interventions through newly created therapies, such as CAR-T cells and E-TCR, the tumor microenvironment, as well as the toxic effects, are large impediments for therapies to be successful in addressing solid tumors. Many studies have found that the main role that promotes cancer progression and relapse seems to be cells, specifically, tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), as well as myeloid-derived suppressor cells (MDSCs), and early exhaustion of T cells (Niu et al., 2024).

Macrophages play a huge role in the immune response because the macrophage is an immune cell, and the main role of macrophages is to protect organisms against different types of infections by phagocytosis (Chin et al., 2021). Macrophages are polarized into two types referred to as M1 and M2. M1 type of macrophage is a pro-inflammatory macrophage that is activated by interferon-gamma, leading to the release of cytokines and inducing inflammation (Wujak et al., 2021). M2 type is considered an anti-inflammatory macrophage, which becomes activated by IL-2 and whose main function is to repair tissue.

Adenylate kinase 4 (AK4) is a nucleoside monophosphate kinase that is located in the mitochondrial matrix. AK4 in macrophages promotes cell survival and growth by catalyzing the transfer of the phosphate group from ATP to AMP, producing two molecules of ADP (Jan et al., 2017). Thus, AK4 maintains the energy balance in the mitochondria, which allows for the growth of tumors and supports the tumor-associated macrophage. AK4

can be a target of anti-tumor drugs, as well as a marker of metastasis in cancers (Wujak et al., 2021).

Overexpression of AK4 enhances M1 macrophage polarization induced by interferon-γ (IFN-γ) and lipopolysaccharide (LPS). Additionally, overexpression of AK4 in macrophages will change their metabolism and contribute to an enhanced anticancer effect.

This master's thesis will examine the role of adenylate kinase 4 in macrophages and assess the hypothesis that overexpression of AK4 influences the polarization of macrophages, which has the potential for aiding in the treatment of solid tumors.

# 3. LITERATURE REVIEW

## 3.1 Immunotherapy and CAR-T cell therapy.

CAR-T cell therapy is a genetically modified T-cell expressing receptors on the surface of the cell that recognize, target, and destroy cancer by locating tumor antigens. CAR-T cells have been successfully applied to hematological malignancies. In 2017, the FDA approved CAR-T cell therapy for the resistant form of acute leukemia and relapse of lymphoma (Chen et al., 2023). The first two CAR-T protocols that gained successful approval in this period were Kymriah (tisagenlecleucel) and Yescarta (axicabtagene ciloleucel). Chen et al. (2023) reported that CAR-T was approved for patients with relapsed/refractory ALL2 and DLBCL3 in patients older than 25 years old at this time chronologically, to drug approvals (Chen et al., 2023). Recently, the FDA also approved another CAR-T: brexucabtagene autoleucel (Tecartus) in 2020 for patients with refractory/relapsed mantle cell lymphoma (Yang et al., 2022).

The structure of CAR-T cells has four sections: (1) extracellular domain that harboring a single chain variable fragments (scFv), (2) an extracellular spacer causing a space between the extracellular domain and tumor surface, (3) a transmembrane domain that helped anchor the CAR- T cell to the patients T lymphocytes, and, (4) an intracellular signaling domain containing CD3zeta (CD3) and a co-stimulatory domain indispensable to activate T cells (Yang et al, 2022) (Figure 1).

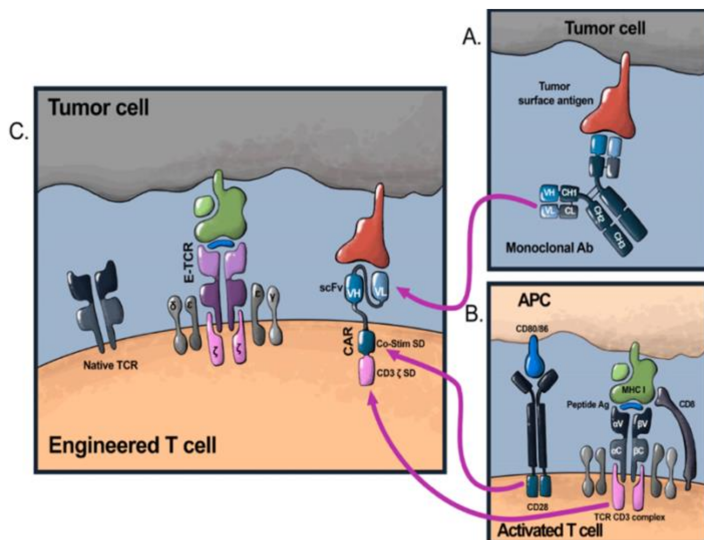



**Figure 1. Structure and mechanism of CAR-T cell therapy.**
A. Binding of monoclonal antibodies (mAbs) with tumor antigens by heavy chains and light chains.
B. T-cell binds antigen-presenting cell (macrophage). The second signal that is needed to activate the T-cell is the co-stimulatory signal, CD28. C. The right side is the second generation of CAR-T, the left side is E-TCR (Yang et al., 2022).

Though CAR-T cell therapy produces a strong response in hematological malignancies, in solid tumors, this type of therapy has produced a more elusive anti-tumor response as a result of biochemical and physiological barriers. Furthermore, tumors can be categorized into “hot” and “cold” tumors based on their tumor microenvironments and immunological characteristics (Wang et al., 2023). A “hot” tumor has a large amount of immune cells, especially T-cells, and these tumors have a lot of immune cells, which makes the tumor visible to the immune system and has a good response for the treatment of immunotherapy. On the contrary, having an immune response of the immunological characteristics typically has a low inflammatory signature with little to no immune cells that can recognize this type of cancer (Wang et al., 2023). Hence, a “cold” tumor has a low response to chemotherapy.

In addition, the immunological feature of a tumor is regulated by the tumor microenvironment and has immune checkpoints (Rojas-Quintero et al., 2024). The tumor microenvironment has tumor cells, immune cells, extracellular matrix, fibroblasts, blood vessels, and stromal cells that promote the growth of the tumor and support it. Immune cells include macrophages, T lymphocytes, dendritic (DC) cells, natural killer (NK) cells, as well as myeloid-derived suppressor cells (MDSC) (Wang et al., 2023). The tumor microenvironment (TME) of “cold” tumors has a low rate of immune cells (T lymphocytes and macrophages) due to the activation of immune checkpoints and many other immunosuppressor factors. In comparison, the TME of a “hot” tumor will reveal a high level of T lymphocytes and macrophages, with activation of the immune response CD28/B7,

CD40/CD40L. An immune checkpoint like PD1 (Programmed Death Receptor), as well as cytotoxic T-lymphocyte-associated protein-4 (CTLA4), is another significant barrier to CAR-T cells' resistance against solid tumors. In cancer, immune checkpoints serve as a means of circumventing recognition from the immune system. Such as its inhibitory receptor role with T-cells, one such as PD-1 and CTLA4, the ligand of molecules expressed by tumors, that work in immune escape (Wang et al., 2023).

### 3.2 Limitations of CAR-T and overview of CAR-M

Considering the hostile reaction of CAR-T cell therapy to solid tumors is mainly attributed to heterogeneity, poor trafficking, and unfavorable tumor microenvironmental characteristics, some studies have found improved survival rates shown through the implementation of CAR-T cell immunotherapy in breast cancer. Being one of the most diagnosed conditions globally, the number of new cases is rising by 2.3 million cases a year.

Yang et al. (2022) showed multiple pre-clinical studies that identified approximately 22 CAR-T cell constructs with 12 antigen targets in breast cancer patients. The most prevalent target as a tyrosine kinase receptor (RTK), HER2 in breast cancer. However, only 3 of 5 of the HER2-CAR constructs have continued with clinical studies (NCT04650451, NCT03740256, NCT03696030, and NCT04430595). Another target is cell surface proteins, mesothelin, mucin1 (MUC1), and Epithelial Cell Adhesion Molecule (EpCAM). Pre-clinical trials only found 6 of 12 cell surface protein targets, have advanced to the clinical study (Yang et al., 2022).

Although several clinical studies demonstrated promising results in treating breast cancer patients with CAR-T cell therapy, problems still exist, similar to solid cancers discussed in this review. Firstly, there is the overall immunosuppressive microenvironment of breast cancer tumors. Secondly, chemotactic cytokine receptor mismatches between CAR-T cells and chemotactic cytokines in tumors. Thirdly, we are witnessing antigen expression variability on the tumor's surface (Yang et al., 2022).

To overcome the CAR-T cell resistance to solid tumors with heterogeneity, scientists decided to alter the assembly of CAR-T so it could recognize many of the tumor antigens. This assembly reduces the risk of solid tumor escape, particularly glioblastoma (Zhang et al., 2022). The scientist also altered the extracellular domain of CAR-T, resulting in the creation of two single-chain variable fragments - bi-specific T-cell engager (BiTE) targeting EGRF and CD3 (Figure 2).

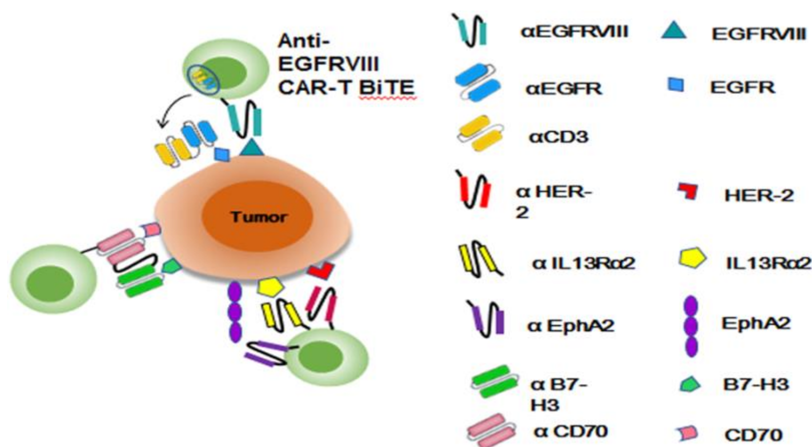



**Figure 2.** Engineering CAR-T cells, BiTe to thwart tumor escape. CAR-T cell therapy in solid tumors displayed improved outcomes by knockout of CD5 (Zhang et al., 2022).

Researchers are using an additional method designed to mitigate tumor escape after CAR-T cell therapy in solid tumors. Using CRISPR/Cas9 to delete CD5, Patel et al. (2024) at the Perelman School of Medicine took a prostate cell line targeted at HER2+. The breast cancer cells have been knocked out of CD5 using multiple guide RNA targeting CD5 on day 0 before activation of the anti-CD3/CD28 bead and further lentiviral transduction (Figure 3). By this method, the CAR-T cells become less exhausted, have better persistence, and demonstrate robust anti-tumor activity.

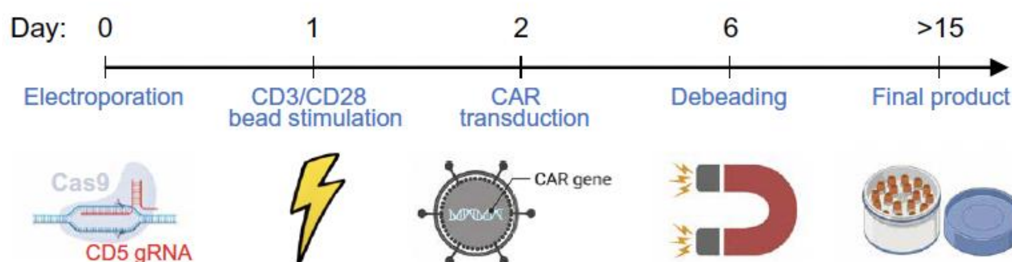



**Figure 3. Patel et al. (2024). CD5 knockout CAR-T expansion protocol.**

Recognizing the genuine limitations of CAR-T cell therapy in treating solid tumors, scientists are presenting the compelling promise of CAR-M therapy as a replacement for CAR-T cell therapy. CAR-M can infiltrate tumors with the use of the intracellular domain CD147 (Figure 4), an important marker for enhanced upregulation of MMS (matrix metalloproteinases) that can degrade the extracellular matrix, allowing for the infiltration of immune cells into the tumor (Chen et al., 2024). In addition, CAR-M utilizes different signaling pathways; for example, MerTK (tyrosine-protein kinase Mer), Megf10 (multiple EGF-like-domains protein 10), and TLR (Toll-like receptor) that induce phagocytosis,

enhancing an antitumor response. CAR-M can also facilitate the recruitment and activation of other immune cells like T lymphocytes (Chen et al., 2024) (Figure 4).

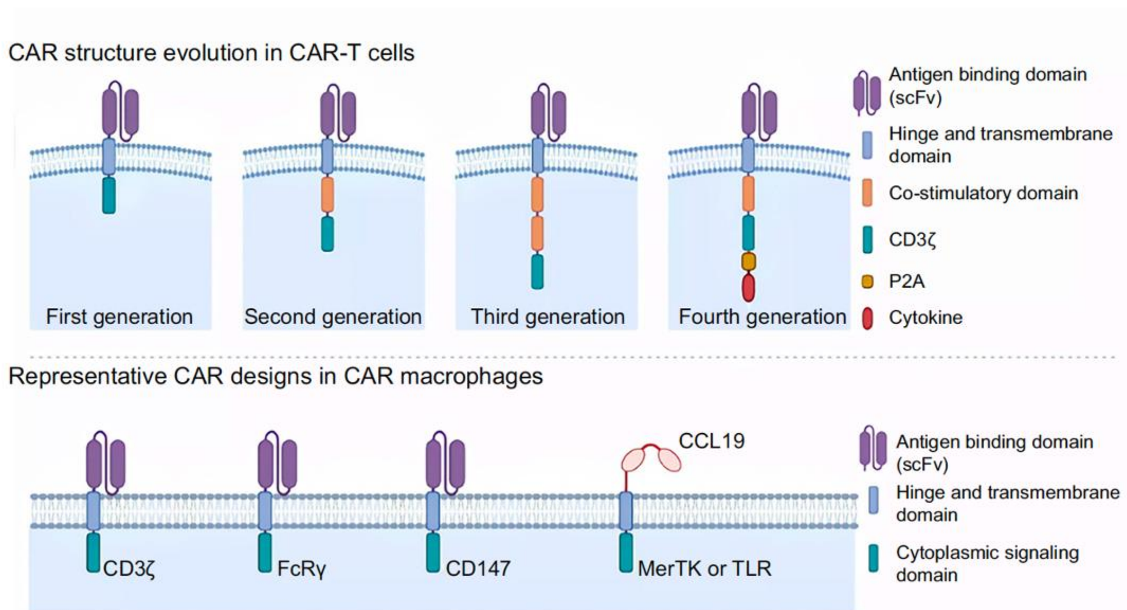



**Figure 4. Constructs of CAR-T and CAR-M therapy with targets.**

Additionally, CAR-M has a different signaling pathway, such as MerTK (tyrosine-protein kinase Mer), Megf10 (multiple EGF-like-domains protein 10), and TLR (Toll-like receptor), inducing phagocytosis, modulating the tumor microenvironment, and activating a complete signal transduction pathway resulting in an enhanced antitumor response. Furthermore, CAR-M can recruit other immune cells, including T lymphocytes, presenting tumor antigens with further activation of T lymphocytes (Chen et al., 2024) (Figure 5).

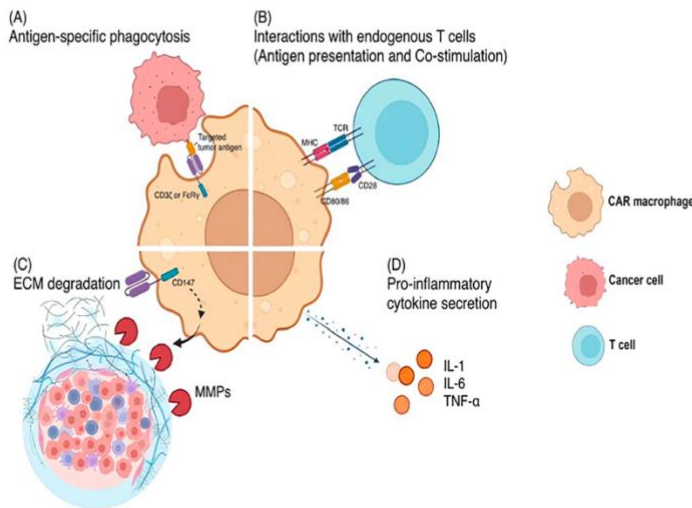



**Figure 5. CAR-M antitumor mechanism.**

CAR-M is a promising immunotherapy for solid tumors, providing advantages in comparison to CAR-T cell therapy. For the primary difference, CAR-M has a different antitumor mechanism, including phagocytosis from general immune cells and secreting TNF-α. A second point is that macrophages have a greater infiltration than other immune cells. Consequently, CAR-M can infiltrate the largest tumors. Furthermore, natural macrophages are inhibited by immunosuppressive molecules such as CD47 (which tumours secrete), which polarises macrophages to M2, which is anti-inflammatory. Conversely, CAR-Macrophages can overcome this obstacle and polarise macrophages M1, which are pro-inflammatory. Finally, macrophages can penetrate the tumor more easily and remain longer than T lymphocytes. Thus, this provides CAR-M factors that give CAR-M advantages over CAR-T (Chen et al., 2024). Certainly, there are many hurdles, such as the challenge of finding the right tumor-associated antigen and issues with tumor escape.

### 3.3 Macrophages in the tumor microenvironment

The macrophage is an immune cell, and the major function of macrophages is to protect and defend the organism against different types of infections by the phagocytic pathway (Chin et al., 2021). Macrophages are differentiated into two different phenotypes (M1 and M2). M1 macrophage is a pro-inflammatory macrophage that is activated by interferon-gamma, resulting in the production of cytokines and inflammation (Wujak et al., 2021). M2 is an anti-inflammatory macrophage that is activated by IL-2 and is primarily involved with tissue repair. There have been several studies that are focused on tumor-associated macrophages (TAM), which are part of the cancer microenvironment that

contribute to the growth of a tumor and metastasis through the release of growth factors (VEGR, EGF) and immunosuppressive cytokines (TGF-B) (Wang & Joyce, 2010). The other function of TAM is to express ligands that bind to programmed death receptor (PD-1), leading to depletion of T-cells.

### 3.4 Classification of macrophages and polarization

Macrophages have the ability to develop into either M1 or M2 types of macrophages in response to cytokines. The M1 type is a pro-inflammatory macrophage, mostly associated with inflammation and an antimicrobial role. The M1 and M2 types of macrophages utilize distinct metabolic pathways. M1-type macrophages associated with inflammation are highly capable of taking up glucose through HIF-1α. The amount of glucose leads to increased aerobic glycolysis, leading to ATP and lactate. This establishes a pathway to produce energy that supports cells in inflammation and supports immune cell activation in the change of oxidative phosphorylation to aerobic glycolysis that results in decreased pro-inflammatory interleukin IL-10 and increased pro-inflammatory IL-1β. In addition, M1 macrophages generate reactive oxygen species such as superoxide and hydrogen peroxide that are important for killing microbes and cancer cells (Nakamizo & Kabashima, 2024). M2 macrophages are anti-inflammatory and use a different metabolic pathway than M1 macrophages – oxidative phosphorylation, associated with more healing tissue.

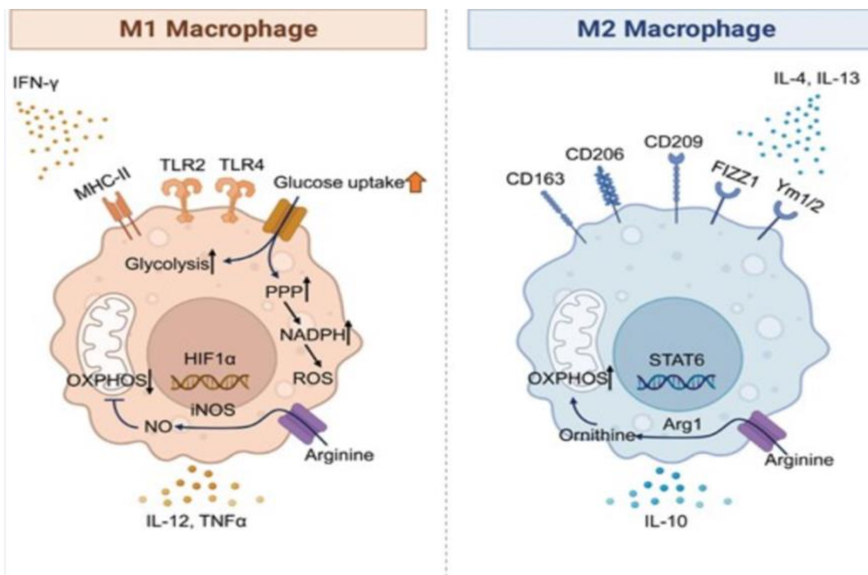



**Figure 6. Macrophage polarization.** This figure depicts the polarization and metabolic shifts in macrophages. M1 macrophages, activated by IFN-γ (Interferon gamma) utilize glucose metabolism.

Therefore, this metabolic reprogramming caused by Macrophages and M1 macrophages leads to ROS production. In contrast, M2 macrophages, activated by the cytokines IL-4 (interleukin-4) and IL-13 (interleukin-13), are associated with oxidative metabolism.

Mitochondria have an important role in the regulation of macrophages. The formation of ROS is one of the essential functions of mitochondria. M1 macrophage uses IFN-γ and LPS and indicates pro-inflammatory activation characterized by high rates of glycolysis, together with the production of nitric oxide (NO) and ROS, secretion of pro-inflammatory cytokines such as TNF-α, IL-6, and ILβ. M2 macrophage has high levels of mitochondrial rates of activity with low production of ROS, which is linked to the metabolism of ROS macrophages as well as the differentiation for polarization of macrophages. The inhibition of ROS is correlated to the differentiation of macrophages to the M2 type, which is seen in chronic inflammation and cancer environments. In consequence, ROS continues to be targeted in polarization in inflammatory disease and cancer (O'Neill & Artyomov, 2019).

**3.5 Adenylate kinase 4: mechanism**

Adenylate kinases (AK) are a class of nucleotides that regulate the energy environment in cells. AK 4 is a type of AK family, contributes to the growth and survival of cancer cells in the process of transporting the phosphate group in ADP/ATP (Chin et al., 2021).

Adenylate kinase 4 (AK4) is located in the mitochondrial matrix. AK4 is encoded by the AK4 gene and is expressed in the brain, liver, heart, kidney, and stomach. In addition, AK4 is implicated in resistance to anti-tumor agents and is one of the biomarkers of metastasis in cancers (Wujak et al., 2021). In macrophages, AK4 contributes to the survival and growth of cells by catalyzing the phosphorylation of the phosphate group, transferring from ATP to AMP, forming 2 ADPs (Jan et al., 2017).

AK4 has not been thoroughly examined, but it is known that AK4 is a biomarker of an aggressive cancer, including HER-2 positive breast cancer, and therefore, the detected AK4 could be a definitive marker that is an indicator of breast cancer progression ( Zhang et al., 2019). The data we presented demonstrates that AK4 depletion causes a decline in proliferation and invasion of MDA-MB-231 cells, but the depletion of AK4 decreased metastasis of HER-2 breast cancer in vivo (Zhang et al., 2019). In addition, AK4 could be a target facet in breast cancer therapy.

### 3.6 AK4 in Macrophage, role in inflammation

Adenylate kinase 4 (AK4) controls the ATP, ADP, and AMP concentrations in cells by converting either ATP or AMP into ADP. Given the influence on ATP/ADP, AK4 is modulating mitochondrial function, which is critical to macrophage inflammation. In addition, AK4 also controls transcriptional factor Hif-1a (Hypoxia-inducible factor 1-alfa) by influencing ATP or ADP/AMP concentration, which activates M1 polarization and regulates inflammatory genes. Therefore, AK4 is controlling the differentiation of macrophages by either activating or suppressing Hif-1a signaling with significance to ATP levels. Furthermore, AK4 regulates reactive oxygen species (ROS), activating NF-kB (transcriptional factor of Hif-1a). Consequently, AK4 is positively regulating factors associated with inflammatory genes (B, IL-1β, IL-6, and TNF-α) produced by the M1 macrophage type (Chin et al., 2021). However, Chin et al. (2021) reported that AK4 modulated the magnitude of inflammation, but not the polarization of M1/M2 type.

### 3.7 Role of Overexpression of AK4

The overexpression of AK4 affects the functional activity of macrophages, especially the M1 pro-inflammatory activity. The data supports that AK4 increased more in the M1 type compared to the M2 type of macrophage. Thus, overexpression of AK4 is related to an inflammatory response (Chin et al., 2021).

AK4 maintains cellular nucleotide homeostasis, regulating ATP and reactive oxygen species (ROS). Knock-out (KO) of AK4 increases ATP and decreases ROS, subsequently reducing antimicrobial response and glycolysis (Chin et al., 2021). AK4 also regulates inflammatory gene expression (Il1b, Il6, Tnfa, Nos2, Nox2, Hifa) through the inhibition of AMPK and a Hif-1a positive feedback loop. AK4 could change cellular homeostasis by overexpression, but not regulate the polarization of macrophages. AK4 is a key target regulating the immune response. AK4 had effects for inflammation and cancer disease specifically; AK4 overexpression increased activities of macrophages to promote inflammation, but did not alter their polarization; AK4 is changing macrophage metabolism anyways by altering mitochondrial potential, increasing glycolysis, and ROS that enhance the inflammatory response (Chin et.al., 2021).

**Conclusion:**

This literature review has described some new therapies and barriers to relapse and death in patients diagnosed with breast cancer. The newest therapy is CAR-T cell therapy for

hematological cancers. Recently, these techniques have been applied to solid cancers, but there are still many barriers to these new therapies: (1) heterogeneity of cancer and also tumor microenvironment; and (2) especially in the context of cold tumors (Salas-Benito et.al., 2023). "Cold" tumors were described by a low inflammatory signature: low immune cells, or no immune cells, immune cells not being able to recognize this cancer (Wang et al 2023). This tumor had a low response to chemotherapy. Cold tumors, also express soluble growth factors (VEGR, EGF), and soluble immunosuppressive cytokines (ex, TGF-B) that increase cancer growth and metastasis (Wang & Joyce, 2010). The authors altered the extracellular domain of the CAR-T cells in such a way as to lessen the immunosuppressive immunity of solid tumors in breast cancer, engineered two single-chain variable fragments - bi-specific T-cell engager (BiTE) directed against EGRF and CD3 (Zhang et al., 2022). Another method is to knock out CD5, which also decreases T-cell exhaustion in T-cells but promotes T-cell persistence, so the antitumor effects are seen to be substantial (Miaw & Chin, 2023).

CAR-M is another novel method of immunotherapy for solid tumours that has unique advantages over CAR-T cells, as CAR-M has a different antitumor mechanism, as it relies on phagocytosis and TNF-α secretion. Further, CAR-M is also known to infiltrate better than any other immune cell, so CAR-M can infiltrate the largest, if not most effective, modality in the solid tumor treatment toolbox.

Since the functional role of AK4 is performing the necessary role of maintain mitochondrial function, overexpression of AK4 (Adenylate Kinase 4) may provide beneficial potential regulation of macrophage metabolism and inflammatory states. AK4 is a mitochondrial enzyme involved in the redistribution of adenyl nucleotides, with the function of maintaining energy homeostasis. During inflammatory states, AK4 can be overexpressed, which causes a greater decrease in mitochondrial membrane potential and mitochondrial ROS, which then requires a metabolic shift towards the classical glycolysis activity associated with M1-polarized macrophages.

That overexpression can also come with activation of inflammatory signaling pathways (eg, NF-κB) and transcriptional factors that increase production of cytokines such as IL-1β, IL-6, and TNF-α.

Therefore, AK4 does not enhance ATP synthesis (although it does provide some contribution to the redistribution of ATP) but rather is associated with an overall decreased mitochondrial activity, ultimately driving proinflammatory activation. Given the development of CAR-M (chimeric antigen receptor macrophages) technologies - the new

frontier of cellular immunotherapy, where macrophages are programmed to recognize specific tumor antigens - the relevance of AK4 is especially significant. Given that CAR-M efficacy relies on macrophages migrating to the tumor, maintaining their M1 phenotype, and being able to produce at least some cytokines, tuning levels of AK4 could be a crucial link for producing more effective therapeutic anti-tumor cells. Additionally, expanding levels of AK4, we can also add another paradigm in stabilizing the pro-inflammatory, tumor-tacking state of CAR-M, which is again especially important in an immune-suppressive tumor microenvironment. Therefore, in this regard, we view AK4 not only as a regulator of metabolism and inflammation, but also potential therapeutic target for developing better CAR-M therapies for the immunotherapy of solid tumors.

## 4. AIMS AND RESEARCH QUESTIONS

### 4.1 Main aim

Use THP-1 macrophage polarization assays to characterize how AK4 overexpression will change the polarization towards M1 with LPS and IFN-γ stimulation, and then the anti-tumor effect of THP-1 macrophage as CAR-M therapy in solid tumours.

### 4.2. Research question

Generate lentiviral transduced THP-1 cells with AC4 overexpression confirmed with western blot and RT-PCR. Differentiate THP-1 macrophages (PMA). Stimulate with LPS/IFN-γ. Measure M1 genes with qPCR.
Utilize probes linked with fluorescence to measure ROS and other mitochondrial functions.

## 5. HYPOTHESIS

The overexpression of AK4 for THP-1 cells will lead to M1 polarization in the macrophages and increase its phagocytic properties as well as increase its anti-tumor properties.

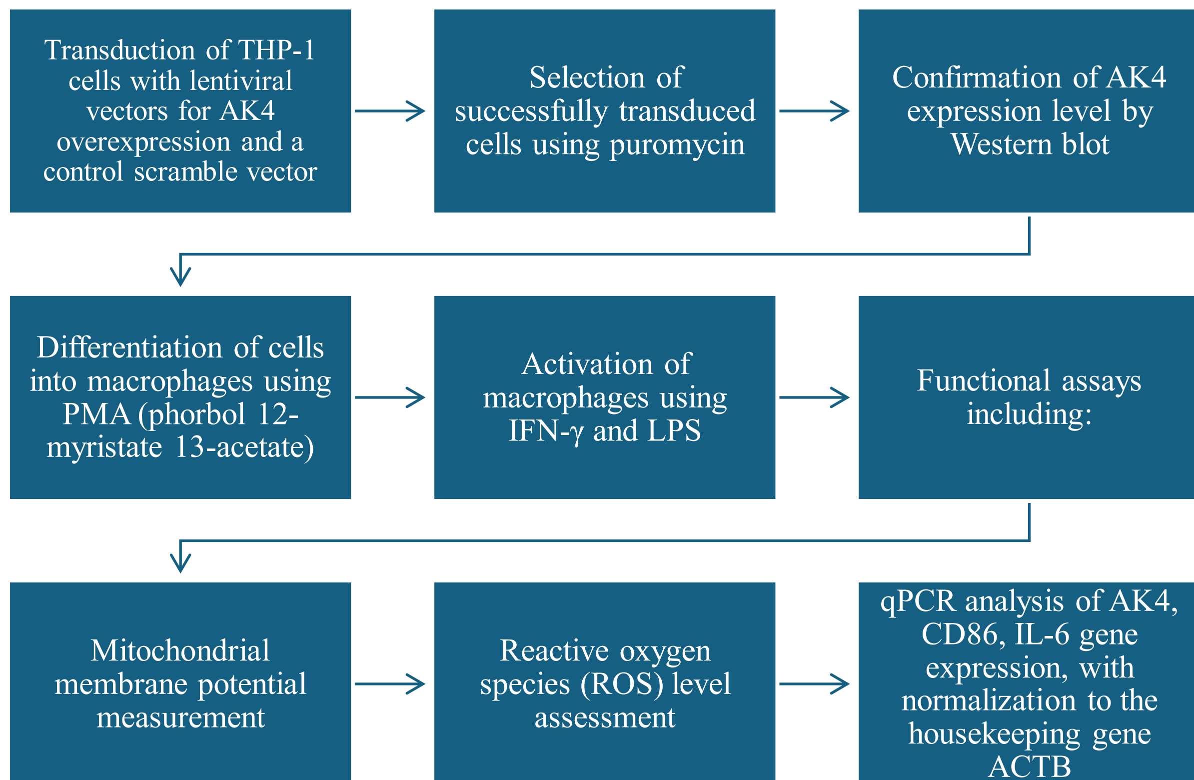
## 6. MATERIALS AND METHODS

### 6.1 Study Design

The purpose of this study was to characterize the functional influence of AK4 gene overexpression on the THP-1 monocytic cell line in regards to mitochondrial activity and the phenotypic polarization of macrophages.
This rationale led to the development of an experimental design that included two different groups of cells:

**Scramble - THP-1 cells transfected with a non-specific vector**

**OE AK4 - THP-1 cells with overexpression of the AK4 gene**




### 6.2 Description of Methods

- **The lentiviral plasmid, AK4_overexpression,** was used to develop the AK4 gene overexpression construct. The plasmid will enable stable expression of the AK4 gene in cells; both dividing and non-dividing. The vector is a CMV promoter vector the CMV promoter is a human cytomegalovirus (CMV) virulent promoter that has high activity in eukaryotic cells. The CMV promoter drives transcription of the gene in host cells.
- **CMV promoter:** This virulent promoter of human cytomegalovirus (CMV) has high activity in eukaryotic cells, which drives transcription of a polycistronic mRNA transcript that includes the AK4 gene and puromycin resistance gene (PuroR) (Radhakrishnan et al., 2008).
- **Kozak sequence:** The optimal sequence for the initiation of translation in eukaryotic cells to initiate AK4 protein synthesis. AK4 ORF (exogenous gene): The AK4 gene is adenylate kinase 4, which is a mitochondrial enzyme involved in regulating ATP levels and cellular energy metabolism.

- **P2A sequence:** A specific short sequence of amino acids (~18-22 amino acids) that features a ribosomal skipping mechanism that allows for two independent proteins (AK4 and PuroR) to be synthesized from a single mRNA transcript. This technology allows for both proteins to be synthesized in a high-efficiency manner, not requiring one to have additional promoters or other elements such as IRES (de Lima & Lanza, 2021).
- **PuroR (puromycin resistance gene):** A gene that ensures the selection of transduced cells that effectively express both AK4 and PuroR, while only those cells that successfully accept the construct can survive when puromycin is added to the nutrient medium.
- **PRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element):** An element that enhances the stability and expression of mRNA, contributing to an increase in the productivity of protein synthesis in the cell.
- **LTR (Long Terminal Repeat) sequences:** Long terminal repeats that are necessary for effective integration of lentiviral RNA into the genome of the host cell. The use of the modified ΔU3 region in the 3' LTR guarantees a decrease in promoter activity after integration, minimizing the possible effect on the expression of neighboring genes.

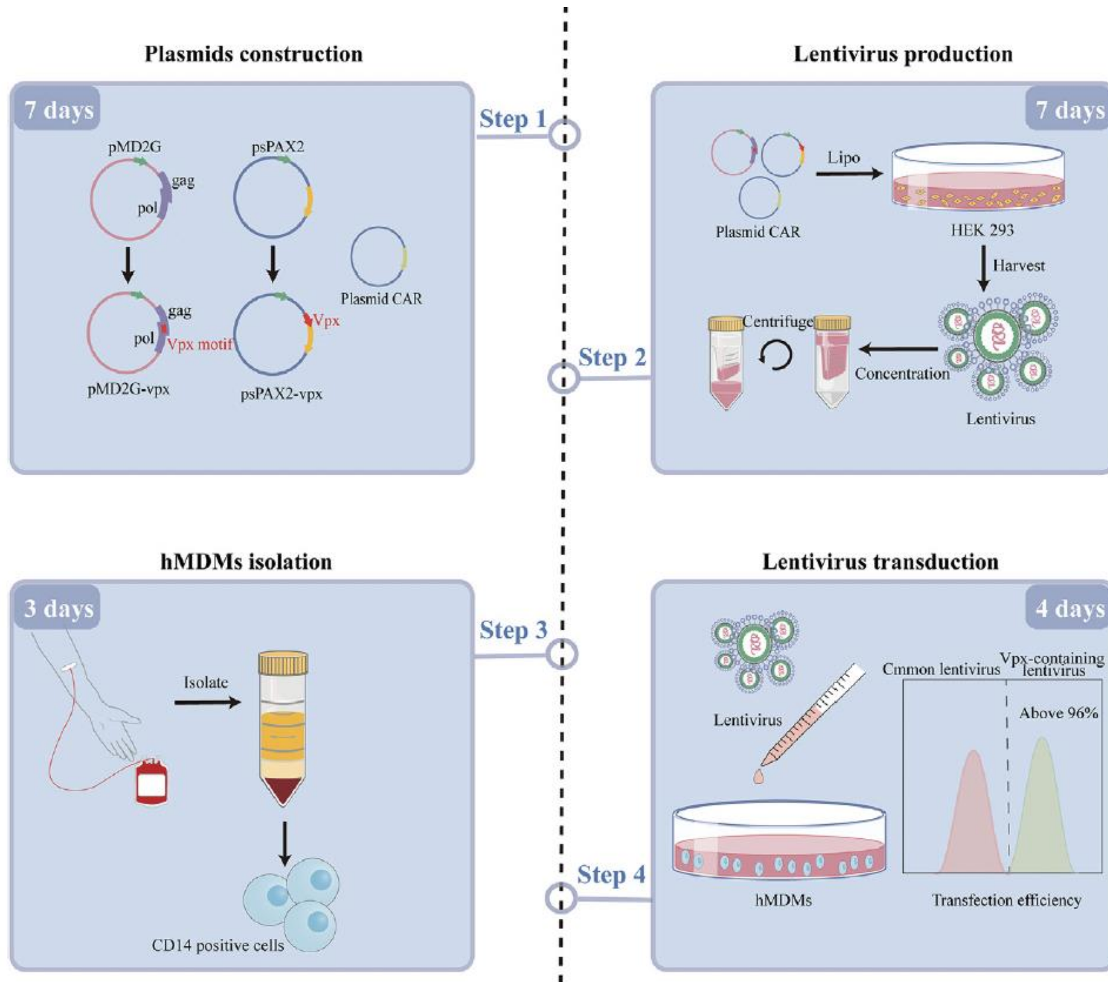
Ampicillin Resistance (AmpR) and Ori (Origin of replication): The ampicillin resistance gene, as well as the beginning of replication in bacteria, which allows for plasmid amplification in Escherichia coli (strain DH5a) for the subsequent production of a sufficient amount of high-quality plasmid DNA.

### 6.3 Lentivirus packaging and cell transduction

The lentiviral vector AK4_overexpression was used to package viral particles in HEK293T cells using pMD2 packaging plasmids pMD2.G и psPAX2.

The lipid reagent Lipofectamine 2000 (Thermo Fisher) was used for cell transfection. 48-72 hours after transfection, a supernatant containing viral particles was collected, filtered, and used to transduce target cells such as THP-1 cell lines. After transduction, the cells were subjected to selection using puromycin selection (1-2 µg/mL) for 5-7 days. Cells that successfully integrated the construct expressing both AK4 and PuroR survived, which allowed their further use for the analysis of cellular function and metabolism (Gao et al., 2024) (Figure 8).

**Figure 8. A protocol for engineering lentiviruses (Gao et al., 2024).**




### 6.4 Western blot

**Preparation of protein samples.** The cells were cultured until 80-90% confluence was achieved. For protein extraction, cells were washed with phosphate-salt buffer (PBS) and lysed in a RIPA buffer (Thermo Fisher Scientific, USA) containing protease inhibitors (cOmplete™, Roche, Germany) and phosphatases (PhosSTOP™, Roche, Germany). The samples were incubated on ice for 30 minutes with periodic shaking, then centrifuged at 13,000 rpm for 15 minutes at 4 °C.

**Electrophoresis and membrane transfer.** A 10-12% polyacrylamide SDS-PAGE gel was used to separate the proteins. 20-30 micrograms of protein were loaded into wells, and electrophoresis was performed at 100-120 V. After electrophoresis, the proteins were transferred to a PVDF membrane (Immobilon-P, Millipore, USA) by wet transfer at 100 V for 90 minutes at 4 °C.

**Blocking and incubation with antibodies.** The membranes were blocked in a 5% skimmed milk solution in TBST (Tris-buffered saline with 0.1% Tween-20) for 1 hour at room temperature. The membranes were then incubated with primary antibodies against AK4, β-tubulin (1:1000) at 4 °C overnight. The next day, the membranes were washed 3 times for 10 minutes in TBS and incubated with secondary HRP-conjugated antibodies (1:5000) for 1 hour at room temperature.

**Detection.** Proteins were visualized using an ECL chemiluminescent substrate (Thermo Fisher Scientific) and documented using a gel documentation system (ChemiDoc™, Bio-Rad). Quantitative analysis. The intensity of the signals was analyzed using the ImageJ program. The expression of a housekeeping protein (β-tubulin) was used for normalization.

## 6.5 RT-PCR

**Isolation of total RNA**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The cells were lysed with an RLT buffer containing β-mercaptoethanol, then the lysate was passed through a column to remove DNA and other contaminants. The RNA was eluted in 50 µl of nuclease-free water.

**Assessment of RNA concentration and quality.**

The concentration and purity of RNA were evaluated using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific).

## 6.6 cDNA synthesis

cDNA synthesis was performed using the MMLV RT kit (Evrogen, Russia) based on AMV reverse transcriptase, according to the manufacturer's instructions. For each reaction, 1 microgram of total RNA and an oligo(dT) primer were used to specifically initiate synthesis from poly-A mRNA tails.

The reaction mixture (with a total volume of 20 µl) included:

1 μg of total RNA, 1 μl of oligo(dT) primer (0.5 μg/μl), 1 μl of dNTP mix (10 mM of each nucleotide), 4 μl of 5× RT buffer, 1 μl of MMLV reverse transcriptase (200 units), nuclease-free water — up to 20 μl.

The reaction was incubated according to the following program: 70 °C — 5 minutes (primer annealing), 4 °C — cooling and adding the remaining components, 42 °C — 60 minutes (reverse transcription), 70 °C — 10 minutes (enzyme inactivation). The resulting cDNA was stored at -20 °C before being used in qPCR.

**Real-time PCR (qPCR)**

Amplification was performed on the QuantStudio™ 5 Real-Time PCR system (Applied Biosystems) using PowerTrack SYBR Green Master Mix for qPCR (2X) (Thermo Fisher Scientific).

Each reaction volume of 10 μl included: 5 μL of Master Mix 2x, 1 μL of direct primer (10 microns), 1μL of reverse primer (10 microns), 1 μL of cDNA, 2 μl of nuclease-free water.

The amplification program: 95 °C — 10 minutes (initialization), 40 cycles: 95 °C - 15 seconds, 60 °C — 30 seconds. After PCR was completed, the melting curve was analyzed to confirm the specificity of amplification.

**Data analysis.**

The relative expression level was analyzed by the ΔΔCt method, with normalization to ACTB as a housekeeping gene. Each reaction was performed in two technical repetitions. Biological repeats were at least three. Statistical processing was performed in the GraphPad Prism program, and the data are presented as the mean ± standard deviation (SD).

**6.7 Flow cytometry**

Four groups of THP-1-associated macrophages were used in the experiment:

Scramble (control) — macrophages induced by a scrambled sequence.

AK4 OE are macrophages with AK4 ovp expression (AK4-overexpressing).

Scramble + IFNy/TNFa are cytokine—activated control macrophages.

AK4 OE + IFNy/TNFa — AK4-overexpressing macrophages after cytokine activation.

Activation was performed using IFN-γ (20 ng/ml) and TNFα (20 ng/ml) for 24 hours before staining.

Staining of mitochondria and reactive oxygen species (ROS) Mitochondrial Staining (MitoTracker™ Red CMXRos) MitoTracker™ Red CMXRos (Thermo Fisher Scientific), a membrane potential-sensitive dye, was used to evaluate the mitochondrial mass and potential of the mitochondrial membrane. The cells were resuspended in a preheated (37 °C)

serum-free medium containing MitoTracker™ Red CMXRos at a final concentration of 100 nM. Incubation was carried out for 30 minutes at 37 °C in a $CO_2$ incubator. After incubation, the cells were washed with 1× PBS, centrifuged at 300×g for 5 minutes, and resuspended in PBS for subsequent analysis.
ROS Staining (CellROX® Deep Red Reagent) CellROX® Deep Red Reagent (Thermo Fisher Scientific) was used to detect intracellular reactive oxygen species (ROS). The cells were incubated with CellROX® at a final concentration of 5 microns for 30 minutes at 37 °C in a $CO_2$ incubator. The cells were then washed with PBS, centrifuged, and resuspended in PBS. Important: The stained cells were protected from light at all stages after the addition of the dye.

**Flow cytometry.**

The stained cells were analyzed on a BD FACSCanto II flow cytometer. Lasers and filters used: MitoTracker™ Red — 561 nm excitation, 610/20 emission, CellROX® Deep Red — excitation 640 nm, emission 660/20. At least 10,000 events were recorded for each sample. The exclusion of double cells and debris was carried out using the FSC/FSC and SSC parameters.

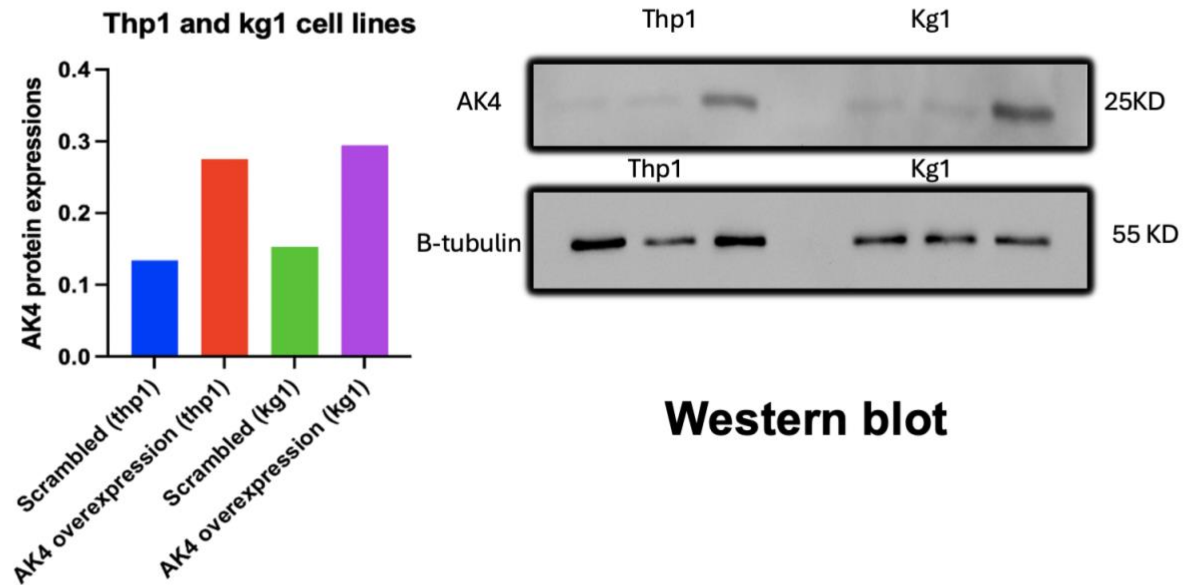
**Data processing.**

The fluorescence intensity was analyzed. The comparison between the groups was based on the average fluorescence value (MFI) for each dye. The control (unstained) cells were used to set the background threshold.

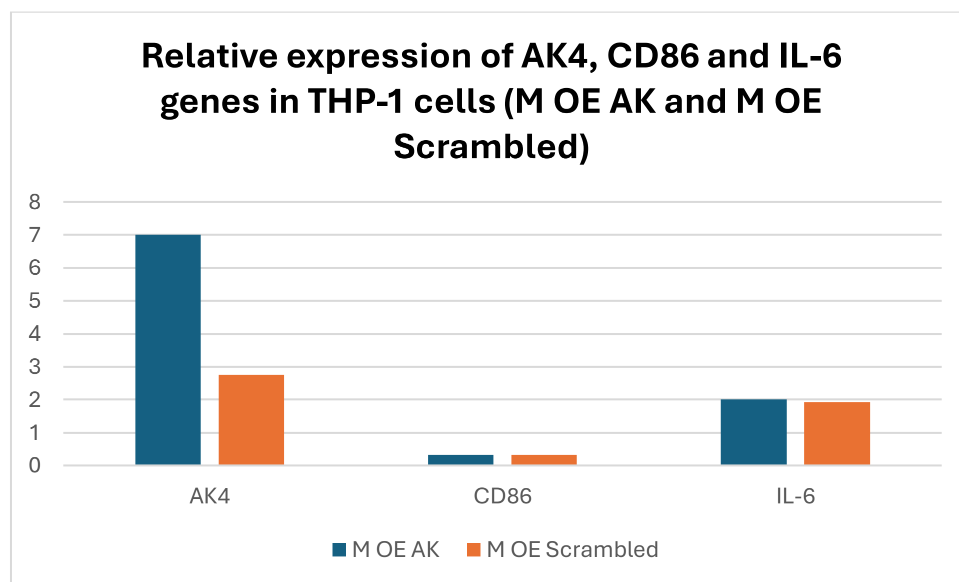
## 7. RESULTS

### 7.1 Confirmation of overexpression of AK4 by Western blot and qRT-PCR.

Western blotting was performed to evaluate the expression of AK4 protein in differentiated THP-1 macrophages after transfection with AK4 and Scramble control. The images show that the cells with overexpression of AK4 have an increased signal compared to the control. The appearance of an additional band corresponding to the AK4 molecule confirms successful transfection and pronounced AK4 expression. In the Scramble and WT control cells, the signal was significantly weaker, indicating the absence or minimal expression of AK4.




**RT-PCR.**

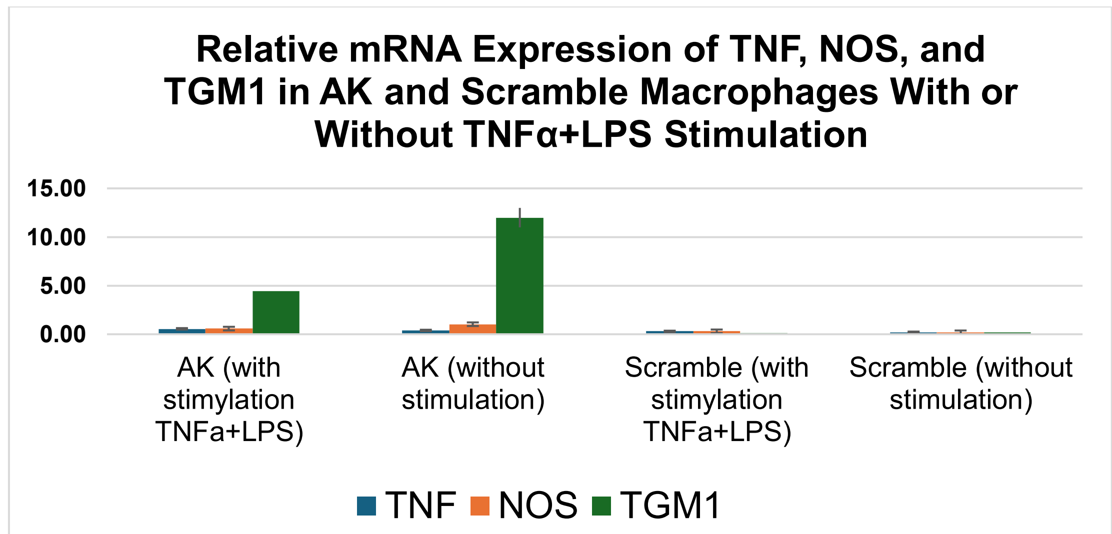



The results were analyzed using RQ ± SD. The RQ (Relative Quantification) method shows a change in gene expression in the AK4 overexpression group (OE AK) compared with the control group (Scrambled) in THP-1 associated macrophage. Each group was compared by RQ values for each group based on their standard deviation (SD).

**AK4 expressions:** AK4 expression in OE AK (RQ = 7.00 ± 1.49) is significantly higher than in OE Scrambled (RQ = 2.76 ± 0.63), by 2.54 times (range 1.63–3.99). This confirms that overexpression of AK4 (EE AK) successfully increases the expression of this gene, as it was discussed earlier, and highlights its role in metabolic processes.

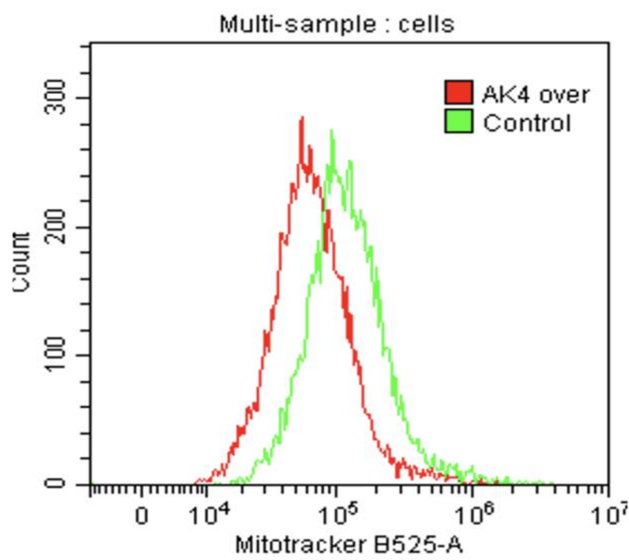
**CD86:** CD86 expression is the same in both groups (RQ = 0.33 ± 0.05). This confirms that there is no effect of AK4 overexpression on CD86-related immune pathways.

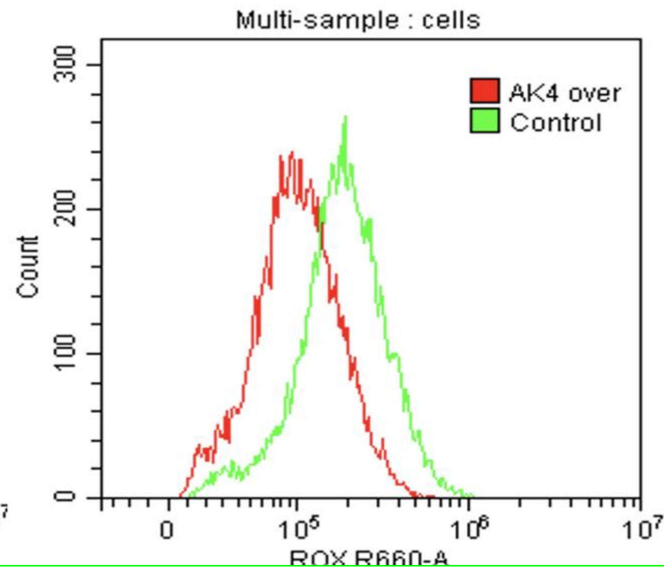
**IL-6:** The expression of IL-6 in 1M OE AK (RQ = 2.00 ± 1.20) and OE Scrambled (RQ = 1.92 ± 1.16) is almost identical (an increase of only 4%, range 0.26–4.21), analysis confirms that AK4 overexpression has no significant effect on this pro-inflammatory cytokine.




During the analysis of gene expression by quantitative PCR, it was revealed that the transcriptional activity of the TGM1 gene significantly increases in cells with overexpression of AK4 (AK4-over), especially in conditions without TNFa and LPS stimulation, reaching a more than 12-fold increase compared with the control (Scramble). When stimulated by inflammatory agents, the level of TGM1 in these cells decreased by about three times, but remained significantly higher than in the control groups. At the same time, TGM1 expression remained at a low level in Scramble cells, regardless of the stimulation conditions. The expression of the pro-inflammatory TNF and NOS genes did not show significant differences between the groups, indicating that AK4 probably does not have a pronounced effect on the classical inflammatory response, but may participate in alternative programs of macrophage activation and tissue remodeling mediated by TGM1.

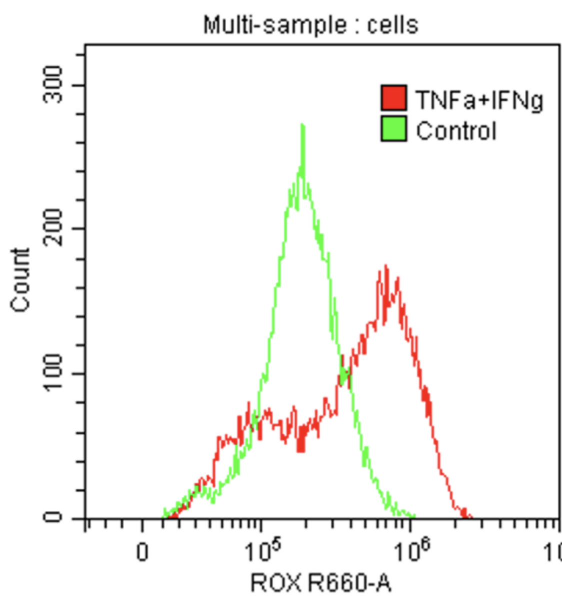
## 7.2 Cytometry measurements of ROS and mitochondrial activity

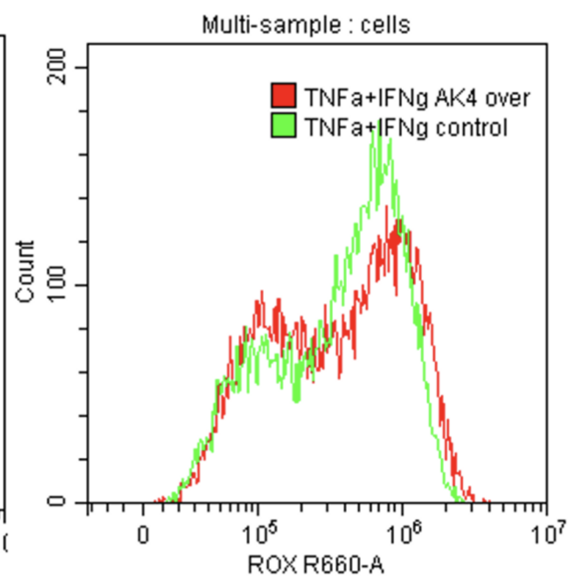



Mitochondrial potential in macrophages with high AK4 expression is lower than in the control sample.

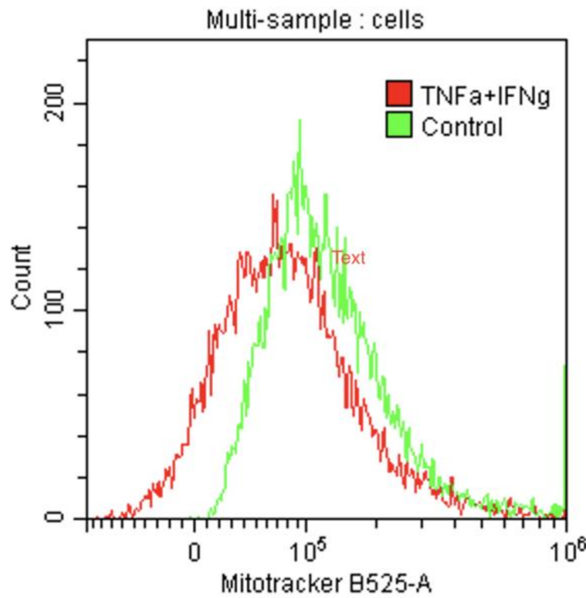
ROS levels in macrophages with high AK4 expressions were lower than in control sample



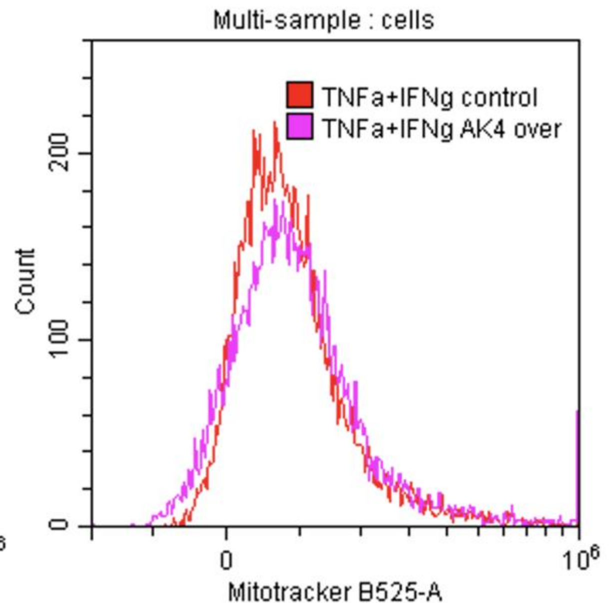




Cytokine cocktail TNFa+IFNg increases ROS levels in macrophages

The TNFa+IFNg cytokine cocktail eliminates the differences in ROS between macrophages with overexpression of AK4 and controls, increasing ROS to a similar level.




TNFa+IFNg cytokines cocktail lowers mitochondrial potential in macrophages.




TNFa+IFNg cytolines cocktail lowers the mitochondrial potential in macrophages, regardless the level of AK4.

## 8. DISCUSSION

Based on the article by Chin et al. (2021), Adenylate kinase 4 is an enzyme that catalyzes the transfer of the phosphate group in adenine nucleotides, converting ATP and AMP to ADP, maintaining cellular homeostasis, and regulating the Hifa1 (Hypoxia-inducible factor 1-alfa) by concentration of ATP/ADP. As a result, Hifa1 induces polarization and enhances inflammatory genes. Moreover, AK4 controls ROS (reactive oxygen species) and activation of NF-kb. Thus, overexpression of AK4 increases mitochondrial activity, making mitochondria more active and producing more ROS, differentiating macrophages into the M1 subtype, and increasing the anticancer effect.

Decreased mitochondrial potential: AK4, localized in the mitochondria, presumably reduces the efficiency of the mitochondrial respiratory chain, thereby reducing the membrane potential. This may indicate a decrease in the activity of mitochondrial metabolism and suppression of mitochondrial function. Understanding the level of active oxygen composition (ROS): A decrease in the mitochondrial potential reduces the production of ros, since the main source is the "leakage" of electrons at a high potential of the respiratory chain. A decrease in ROS levels can lead to suppression of redox signaling pathways and less activation of the inflammatory phenotype of macrophages (a shift towards alternative activation, the M2 phenotype).

However, according results of my master's thesis, it is revealed that overexpression of AK4 decreases mitochondrial potential. AK4, localized in the mitochondria, presumably reduces the efficiency of the mitochondrial respiratory chain, thereby reducing the membrane potential. This may indicate a decrease in the activity of mitochondrial metabolism and suppression of mitochondrial function. A decrease in the mitochondrial potential reduces the production of ROS, since the main source is the "leakage" of electrons at a high potential of the electron transport chain. A decrease in ROS levels can lead to suppression of reduction-oxidation signaling pathways and less activation of the inflammatory phenotype of macrophages (a shift towards alternative activation, the M2 phenotype).

**8.1 The Key Factors Driving M2 Polarization in Macrophages of Thp-1 cells**

Leukemic cells, such as THP-1, have increased glycolysis activity, but Mitochondrial function is impaired due to mutations or changes in OXPHOS-related gene expression.

THP-1 is a cell line derived from monocytes of a patient with acute monoblastic leukemia (AML-M5). Leukemic cells, including THP-1, often exhibit altered metabolism similar to the Warburg effect, where even in the presence of oxygen they prefer glycolysis over OXPHOS to rapidly produce the energy needed for proliferation. However, after differentiation into macrophages (e.g. by PMA), Thp-1 may exhibit metabolic plasticity, but their leukemic origin affects metabolic and signaling pathways, which may alter their response to stimuli such as IFN+LPS (Genin et al., 2015).

Leukemic cells such as THP-1 have increased basic glycolysis activity, but their mitochondrial function is often impaired due to mutations or changes in OXPHOS-related gene expression. Overexpression of AK4 as a mitochondrial enzyme could "correct" or enhance mitochondrial activity by increasing OXPHOS. This corresponds to the metabolic profile of M2 macrophages, which rely on OXPHOS and fatty acid oxidation (FAO) rather than glycolysis like M1 (Genin et al., 2015).

The first graph shows a high expression of AK4 (~7 in M OE AK), which indicates an increase in mitochondrial function. The low expression of M1 markers (CD86: 0.3, IL-6: 1.5) confirms the absence of the glycolytic shift expected from IFN+LPS.

M2 macrophages depend on OXPHOS, which is consistent with the effect AK4, which probably "switched" Thp-1 metabolism towards OXPHOS, enhancing M2 polarization.

### 8.2 Metabolic rearrangement caused by overexpression of AK4

AK4 (adenylate kinase 4) is a mitochondrial enzyme that catalyzes the reversible phosphate group transfer reaction between adenylic nucleotides (2ADP ↔ ATP + AMP), maintaining energy homeostasis in mitochondria. Macrophages exhibit metabolic plasticity: M1 macrophages rely on glycolysis to rapidly produce energy necessary for pro-inflammatory functions, whereas M2 macrophages use oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) to support reparative and anti-inflammatory processes (Kelly & O'Neill, 2015).

Overexpression of AK4 probably enhances mitochondrial activity, increasing the effectiveness of OXPHOS. This creates a metabolic profile favorable for M2 polarization, even when stimulated by IFN-γ and LPS, which normally induce glycolysis and the M1 phenotype. AK4 can increase the AMP/ATP ratio, which activates AMP-activated protein kinase (AMPK), a key regulator of metabolism. AMPK suppresses glycolysis and enhances OXPHOS, directing macrophages to the M2 phenotype (Kelly & O'Neill, 2015).

The results show a high expression of AK4 in Macrophage OE AK (~7 relative to THP-1), which confirms successful transfection. The low expression of M1 markers (CD86: 0.3, IL-6: 1.5) indicates the absence of the glycolytic shift characteristic of M1.

M2 macrophages depend on OXPHOS and FAO (Fatty Acid Oxidation), and AMPK activation contributes to this metabolic profile by suppressing M1 polarization (Kelly & O'Neill, 2015). AK4 overexpression probably enhances OXPHOS, which promotes M2 polarization, even when stimulated by IFN+LPS. The article also describes the role of AMPK in suppressing glycolysis, which may be related to the effect of AK4.

### 8.3 Suppression of pro-inflammatory signaling pathways (NF-kB, STAT1)

IFN-γ and LPS induce M1 polarization through activation of the NF-kB and STAT1 signaling pathways. IFN-γ binds to the IFNg-R receptor, activating JAK-STAT1, which leads to the transcription of pro-inflammatory genes (for example, iNOS, IL-6, TNF). LPS, interacting with TLR4, activates NF-kB through MyD88 and TRIF, enhancing the expression of M1 markers. However, overexpression of AK4 appears to suppress these pathways.

AK4, enhancing mitochondrial function, can reduce the production of reactive oxygen species (ROS) in mitochondria. ROS play a key role in activating NF-kB, enhancing the inflammatory response. The decrease in ROS caused by AK4 can inhibit NF-kB,

preventing M1 polarization. In addition, AMPK activated by AK4 can suppress STAT1 through cross-current with anti-inflammatory pathways such as STAT6 (Liu et al., 2014).

The results shows low expression of CD86 (0.3 in M OE AK) and IL-6 (1.5), and the low expression of TNF and iNOS (<1), even when stimulated by IFN+LPS. This indicates suppression of pro-inflammatory pathways.

Decrease in ROS and inhibition of NF-kB promote the transition of macrophages to the M2 phenotype, which may be related to the effect of AK4 (Liu et al., 2014).

## 9. Conclusion

Functional significance of high AK4 expression in macrophages: High levels of AK4 may play a regulatory role by keeping macrophages in a less activated state, reducing the inflammatory response by suppressing ROS formation. Macrophages acquire an anti-inflammatory, M2-like phenotype that promotes tissue regeneration and suppresses excessive inflammation. Reducing ROS levels also reduces bactericidal activity, potentially making cells less effective at fighting infections, but at the same time helping to protect tissues from damage caused by oxidative stress. Thus, the biological role of AK4 in macrophages, according to the presented data, consists mainly in controlling mitochondrial activity and reducing oxidative stress, which leads to the regulation of the inflammatory status of the cell and a shift in the immune phenotype towards a less inflammatory state.

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