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Calreticulin as a Molecular Adjuvant in Cancer Immunotherapy

Calreticulin jako molekulární adjuvans v nádorové imunoterapii

Diploma thesis

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Declaration

I hereby declare that my thesis represents my own original research work. Wherever the contribution of others is involved, every effort is made to indicate this clearly including reference to the literature. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Prague, 2025 Bc. Ilina Liudmila

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Abstract

Intratumoral immunotherapy is a promising strategy in cancer treatment aimed at overcoming the immunosuppressive tumor microenvironment and triggering a local immune activation, which can further lead to systemic anti-tumor responses. Calreticulin, a key mediator released during immunogenic cell death, has been proposed as a promising target for cancer immunotherapy due to its immunostimulatory features and ability to enhance phagocytosis of tumor cells. This diploma thesis investigates the role of calreticulin as an adjuvant in intratumoral immunotherapy, administered alone or in combination with TLR agonists. Using two different murine tumor models, the poorly immunogenic B16-F10 melanoma and the immunogenic MC38 colon carcinoma, we evaluated the therapeutic efficacy of intratumoral CRT administration. While calreticulin alone did not show significant therapeutic effect in either model, the combination of CRT with TLR agonists led to notable therapeutic benefit for MC38-beraing mice. The combination therapy resulted in prolonged survival, reduced tumor growth, and a trend toward increased infiltration of dendritic and CD8+ T cells in the tumor microenvironment. Furthermore, treated mice exhibited markers of a systemic, durable, and tumor-specific adaptive immune response. These findings suggest the potential of calreticulin as an effective adjuvant combined with other immunostimulatory agents in intratumoral immunotherapy.

Key words: calreticulin, intratumoral immunotherapy, adjuvant, immunogenic cell death, TLR agonists, tumor microenvironment

Abstrakt

Intratumorální imunoterapie představuje slibnou strategii protinádorové léčby, jejímž cílem je překonat imunosupresivní prostředí nádoru a indukovat lokální aktivaci imunitního systému, s potenciálem navodit i systémovou protinádorovou odpověď. Calreticulin, protein uvolňovaný během imunogenní buněčné smrti, je v současnosti studován jako cíl po protinádorovou imunoterapii díky svým imunostimulačním vlastnostem a schopnosti podpořit fagocytózu nádorových buněk. Tato diplomová práce se zaměřuje na hodnocení účinnosti calretikulinu jako adjuvans v rámci intratumorální imunoterapie, a to jak při samostatném podání, tak v kombinaci s agonisty TLR. Během experimentů byly použity dva myší nádorové modely: málo imunogenní melanom B16-F10 a více imunogenní kolorektálním karcinom MC38. Výsledky ukázaly, že samotný calretikulin nevykazuje signifikantní terapeutický efekt v žádném z těchto modelů. Naproti tomu jeho kombinace s TLR agonisty vedla k prodloužení celkového přežití, zpomalení růstu nádoru a u myší s MC38 nádory k trendu zvýšené infiltrace dendritickými buňkami a CD8+ T lymfocyty v nádorovém mikroprostředí. Taky byly pozorovány známky systémové, dlouhodobé a nádorově specifické adaptivní imunitní odpovědi. Získané výsledky naznačují, že calretikulin má potenciál sloužit jako efektivní adjuvans v kombinaci s jinými imunostimulačními látkami v kontextu intratumorální imunoterapie.

Klíčova slova: calretikulin, intratumorální imunoterapie, adjuvans, imunogenní buněčná smrt, TLR agonisté, nádorové mikroprostředí

Content

List of abbreviations

ACT	Adoptive Cell Transfer
ADCC	Antibody-Dependent Cellular Cytotoxicity
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ARG1	Arginase 1
ATP	Adenosine Triphosphate
CAF	Cancer-Associated Fibroblast
CAR	Chimeric Antigen Receptor
CCL	C-C Chemokine Ligand
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CDC	Complement-Dependent Cytotoxicity
cDC	Conventional Dendritic Cells
CpG	Cytosine-Phosphate-Guanine
CRT	Calreticulin
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
DAF	Decay-Accelerating Factor
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
ERp57	Endoplasmic Reticulum Protein 57
FACS	Fluorescence-Activated Cell Sorting
FasL	Fas Ligand
FBS	Fetal Bovine Serum

Foxp3	Forked Box P3
GFP	Green Fluorescent Protein
GPI	Glycosylphosphatidylinositol
HLA	Human Lymphocyte Antigen
HMGB1	High-Mobility Group Box 1
i.t.	Intratumoral
iAEs	immune-related adverse events
ICD	Immunogenic Cell Death
ICI	Immune Checkpoint Inhibitor
IDO	Indolamine-2,3-Dioxygenase
IFN	Interferon
IL	Interleukin
KDEL	Lys-Asp-Glu-Leu Retention Sequence
LAG-3	Lymphocyte-Activation Gene 3
LPS	Lipopolysaccharide
LRP1	Low Density Lipoprotein Receptor-Related Protein 1
LTA	Lipoteichoic Acid
mAb	Monoclonal antibodies
MAPK	Mitogen-Activated Protein Kinase
MBL	Mannose-Binding Lectin
MDA5	Melanoma Differentiation-Associated Protein 5
MDSC	Myeloid-Derived Suppressor Cell
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MyD88	Myeloid Differentiation Primary Response 88 Protein
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK	Natural Killer cell
NO	Nitric Oxide
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PD-1	Programmed Cell Death-1

pDC	Plasmacytoid Dendritic Cells
PD-L1/2	Programmed Cell Death Ligand-1/2
PERK	PKR-like endoplasmic reticulum kinase
PLC	Peptide-Loading Complex
Poly(I:C)	Polyinosinic:Polycytidylic Acid
PRR	Pattern Recognition Receptor
R-848	Resiquimod
rCRT	Recombinant Calreticulin
RFP	Red Fluorescent Protein
RIG-I	Retinoic Acid-Inducible Gene I
ROS	Reactive Oxygen Species
s.c.	Subcutaneous
SEM	Standard Error of The Mean
TA	Toll-Like Receptor Agonists
TAA	Tumor-Associated Antigen
TAE	Tris-acetate-EDTA buffer
TAM	Tumor-Associated Macrophage
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper cell
TLR	Toll-Like Receptor
TLS	Tertiary Lymphoid Structure
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
Treg	Regulatory T cell

1. Introduction

According to the latest data from the GLOBOCAN database, about 20.0 million new cancer cases were diagnosed worldwide in 2022, and approximately 9.7 million deaths from the disease were observed. Statistics indicate that around 1 in 5 people, regardless of sex, will develop cancer at some point in their lives; among them, 1 in 9 men and 1 in 12 women will die from it. Additionally, over than 35 million new cancer diagnoses are expected by the year 2050 based on the population aging and growth trends (Bray *et al.*, 2024).

Despite extensive research over several decades, effective cancer treatment remains problematic in many cases, especially when diagnosed at later stages or when tumors develop resistance to existing standard therapies. Therefore, there is still a need to improve current treatment methods, search for new targets, and develop novel therapeutic strategies to enhance therapy effectiveness, minimize side effects, and improve patient survival and quality of life. The intratumoral immunotherapy has emerged as a promising strategy to overcome the immunosuppressive tumor microenvironment and minimize systemic side effects by the direct injection of immunostimulatory agents into the tumor site.

Among the molecules involved in cancer immunotherapy, calreticulin has gained increasing attention as a potential modulator of anti-tumor immunity. It is a multifunctional protein primarily localized in the endoplasmic reticulum, but in response to certain types of chemotherapy or radiotherapy, it can translocate to the cell surface or be secreted into the extracellular milieu. The exposure of CRT is a key marker of immunogenic cell death, thereby promoting phagocytosis of tumor cells and effective anti-tumor immune response. These findings make it a valuable target for strategies aimed at enhancing the immunogenicity of tumors.

In this thesis, we focus on investigating the potential of calreticulin as an immunostimulatory agent for intratumoral immunotherapy, particularly its role in modulating immune response within the tumor microenvironment.

2. Literature review

2.1 Immune surveillance

One of the main functions of the immune system is to identify and destroy malignant cells before they become tumors. The development of effective anti-tumor immune responses depends on the coordinated activity of both the innate and adaptive immune systems.

2.1.1. Innate immune response

2.1.1.1. Natural Killers

Natural Killer (NK) cells play a crucial role in controlling tumor development. Unlike T cells, they recognize tumor cells in an antigen-independent manner. They detect abnormal cells through the coordination of activating and inhibitory signals from their surface receptors. In the context of cancer immunology, NK cells can kill malignant cells via the 'missing-self' mechanism. NK activation is normally suppressed through the binding of their inhibitory receptors to MHC class I molecules on the surface of healthy cells. However, many cancer cells downregulate MHC I expression to avoid recognition by cytotoxic CD8+ T cells. This loss of MHC I removes inhibitory signals to NK cells, triggering their activation. NK cells directly destroy these abnormal tumor cells mainly through releasing cytotoxic granules containing perforin and granzymes**.** Perforin makes pores in the plasma membrane of the target cell, allowing granzymes to enter and initiate a caspase-dependent apoptotic cascade. Additionally, NK cells can also provide cell destruction via other mechanisms, including Fas-FasL or TRAIL pathways and antibody-dependent cellular cytotoxicity (Coënon *et al.*, 2024). Moreover, upon activation, NK cells release pro-inflammatory cytokines like IFN-γ and TNF-α, as well as chemokines that enhance antigen presentation and recruit other immune cells to the tumor site, strengthening the anti-tumor response (Vitale *et al.*, 2005; Böttcher *et al.*, 2018). NK cells have also been shown to limit metastasis formation through the destruction of circulating tumor cells (Ichise *et al.*, 2022; Vyas *et al.*, 2023).

2.1.1.2. Macrophages

Macrophages are a heterogeneous and plastic population of immune cells that infiltrate tumors. Within the TME, they can polarize into two main types in response to local signals and cytokines: classically activated M1 macrophages and alternatively activated M2 phenotype. However, growing evidence from single-cell RNA sequencing suggests that macrophage classification is actually more complex, and macrophages in the tumor microenvironment often exhibit mixed or intermediate phenotypes (Müller *et al.*, 2017; Song *et al.*, 2020).

The M1 phenotype is considered pro-inflammatory and is typically polarized in response to TLR stimulation and cytokines such as IFN-γ and TNF. M1 macrophages secrete high levels of pro-inflammatory cytokines, such as IL-1, IL-12, TNF-α, produce ROS and NO, have enhanced antigen-presenting capacity, and exhibit higher expression of costimulatory molecules. This phenotype supports anti-tumor immunity by promoting the activation of cytotoxic T cells; it also inhibits tumor growth and metastasis (Yuan *et al.*, 2017).

2.1.1.3. Dendritic cells

Dendritic cells are key mediators between the innate and adaptive immune systems. Generally, three main populations of DCs have been identified: conventional DCs, which are divided into two cDC1/cDC2 subtypes, and plasmacytoid DCs (pDCs). cDC1s are commonly thought to specialize in the presentation of cell-associated antigens and cross-presentation of exogenous antigens on MHC I, leading to CD8+ T cell activation. In contrast, cDC2s are more efficient in antigen presentation via MHC class II, promoting activation of CD4+ helper T cells (Wculek *et al.*, 2019).

Dendritic cells also promote anti-tumor immune response by producing pro-inflammatory cytokines such as IL-12 and TNF. IL-12 plays a critical role in enhancing the cytolytic activity of NK and CD8+ cells and stimulating production of IFN-γ (Zhang *et al.*, 2008; Vacaflores *et al.*, 2016). In CD4+ T cells, IL-12 activates the STAT4 signalling pathway, which induces the transcription factor T-bet, promoting the differentiation of Th1 cells and stimulating IFN-γ production (Thieu *et al.*, 2008). This cytokine environment also supports the polarization of macrophages toward the M1 phenotype (Yu *et al.*, 2016). However, IL-12 function can be

inhibited by IL-10 within the immunosuppressive TME, leading to a weakened anti-tumor response (Ruffell *et al.*, 2014). Additionally, DCs produce CXCL9 and CXCL10 chemokines, which promote the recruitment of CD8+ T cells into the TME (Spranger *et al.*, 2017).

pDCs are known to produce huge amounts of type I and type III IFNs upon activation, playing a key role in antiviral immunity (Scheuplein *et al.*, 2015). However, in tumor immunity, pDC can play a dual role depending on the immunological context. Type I IFNs show notable anti-tumor effects by inhibiting tumor cell proliferation, inducing transcription of pro-apoptosis genes in malignant cells, and enhancing antigen presentation. However, defective and weak type I IFN signalling may support tumor progression by upregulating PD-L1 on cancer cells and secretion of immunosuppressive cytokines (Holicek et al., 2024). The immunosuppressive TME, especially the presence of immunosuppressive cytokines, inhibits IFN-I production in pDC and promotes their shift toward a tolerogenic phenotype (Han *et al.*, 2018; Terra *et al.*, 2018). Additionally, hypoxic conditions within the TME also support tolerogenic properties of pDC (Fan *et al.*, 2022).

2.1.2. Adoptive immune response

The adaptive immune system plays a crucial role in identifying and eliminating tumor cells in an antigen-specific way by recognizing tumor-associated antigens (TAAs) that differ from those found in healthy cells. The appearance of these abnormal proteins is a consequence of various genetic mutations that occur and accumulate during tumorigenesis (Smith *et al.*, 2019).

A conceptual framework that describes the step-by-step process through which the immune system recognizes and eliminates cancer cells is known as the cancer-immunity cycle and was first proposed by Chen and Mellman in 2013. This model highlights the dynamic interactions between tumor cells and the immune system (Chen and Mellman, 2013).

The anti-tumor immune response begins when the newly formed TAAs are released from stressed or dying cells and captured by APCs, primary dendritic cells. Upon stimulation by DAMPs and pro-inflammatory signals, DCs undergo maturation and migrate to the draining lymph nodes, where they efficiently present processed antigens on MHC molecules to naïve T cells. This antigen presentation, combined with the necessary co-stimulatory signals and cytokine stimuli, leads to the activation and clonal expansion of tumor-specific effector T cells. Once activated in the lymph nodes, these effector T cells circulate through the bloodstream and

infiltrate the tumor. Within the TME, the activated CTLs recognize cancer cells displaying the specific tumor antigens presented on MHC class I molecules, leading to targeted tumor cell killing. The tumor cell death results in the release and accumulation of additional TAAs and immunostimulatory signals, which can further recruit and activate other immune cells, thereby amplifying the immune response against cancer (Chen and Mellman, 2013).

2.1.2.1. T cells

Cytotoxic CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), play a central role in adaptive immune responses against tumor. A high infiltration of CD8+ T cells in tumors corelates with better prognosis and overall survival in patients with various types of cancer (Fluxá *et al.*, 2018; Xu *et al.*, 2019; Oshi *et al.*, 2020). These activated effector cells directly destroy tumor cells by releasing cytotoxic granules, the same mechanism that NK cells use. Additionally, CD8+ T cells express FasL, which binds to its receptors on target cells. This interaction activates death domains and initiates a caspase cascade that also results in programmed cell death. Additionally, CD4+ helper T cells, particularly the Th1 subset, provide essential support in anti-tumor immunity. Th1 cells secrete key pro-inflammatory cytokines, such as IFN-γ and IL-2, which help establish and amplify robust anti-tumor immune response. IFN-γ enhances tumor antigen recognition by upregulating MHC class I and II molecules on APCs. IL-2 is a critical growth factor that supports the proliferation, survival, and cytotoxic function of CD8+ T cells and NK cells (Ahmed *et al.*, 2023).

2.1.2.2. B cells

Different phenotypic subsets of B cells, representing various stages of their development, are present within the tumor microenvironment and are often found in association with organized lymphoid structures known as tertiary lymphoid structures (TLSs) (Chen *et al.*, 2020; Xia *et al.*, 2023). Mature TLSs contain germinal centers, where B cells can undergo selective activation, clonal expansion, somatic hypermutation, and class switch recombination. Following maturation, B cells differentiate into plasma cells, which secrete high-affinity tumor-specific IgG antibodies (Meylan *et al.*, 2022). These antibodies bind to tumor cells and can induce NK cell-mediated ADCC or antibody-mediated phagocytosis of tumor cells by macrophages (Mazor *et al.*, 2022).

B cells can also serve as APCs and present tumor antigen to CD4+ TILs, supporting T cell-mediated immune responses (Bruno *et al.*, 2017; Jiang *et al.*, 2019). Additionally, B cells also secrete a variety of cytokines and chemokines, including IFN-γ, TNF-IL-12, GM-CSF, and CXCL8, which help recruit and activate other immune cells, thereby supporting the anti-tumor response (Montfort *et al.*, 2016; Garaud *et al.*, 2019).

2.2. Tumor escape mechanisms

Even though the immune system employs a wide range of mechanisms designed to detect and eliminate malignant cells, tumors are able to develop various immunosuppressive strategies to escape immune surveillance, allowing them to grow and spread without being destroyed. The mechanisms that allow tumors to evade the immune system are complex and involve changes not only in the tumor cells but also in the surrounding environment.

2.2.1. Loss of antigen

One of the important mechanisms by which tumors escape immune surveillance is by reducing their immunogenicity. Tumor cells can change or lose the expression of TAAs, which are crucial for recognition by the adaptive immune system. Loss of antigenicity can occur as a result of numerous mechanisms, including genomic mutations, alternative splicing, or epigenetic modifications. These changes make tumor cells less recognizable to the immune system, especially for activated effector CD8+ T cells. In the context of CAR-T cell immunotherapy, this mechanism of evasion can lead to therapeutic resistance and disease relapse, limiting the long-term efficacy of the treatment (Cortés-López *et al.*, 2022; Fioretti *et al.*, 2023).

2.2.2. Loss of antigen presentation

Another common strategy by which tumors evade immune detection is the downregulation of MHC class I molecules to avoid effective antigen presentation and recognition by effector CD8+ T cells (Masuda *et al.*, 2007; Kaneko *et al.*, 2011; Ferns *et al.*, 2016). Some tumors lose a functional β2-microglobulin, which is an important component of the MHC class I complex. This loss leads to the disruption of the proper assembly and expression of MHC I on the cell surface (Del Campo *et al.*, 2014).

As mentioned above, when tumors lack MHC class I molecules, they are typically recognized and eliminated by NK cells as ‘missing-self’. However, many tumor cells evade this response by upregulating non-classical MHC I molecules, such as HLA-G, HLA-E, and HLA-F, which interact with inhibitory receptors on NK cells, thereby suppressing NK cell activation and cytotoxicity (Ferns *et al.*, 2016; Ormandjieva, 2022). Additionally, some tumors evade NK cell recognition by proteolytic cleavage and releasing a soluble form of MICA, a stress-induced

antigen on the cell surface that normally activates NK through the NKG2D activating receptor. These soluble ligands bind to NKG2D, leading to its downregulation on NK cells and impairing their cytotoxicity (Kim *et al.*, 2024).

2.2.3. Induction of tolerance

In the context of tumor immune evasion, many solid tumors frequently overexpress inhibitory molecules, which in physiological conditions act as immune regulators that prevent excessive inflammation. The best characterised molecules relevant to tumor immune escape are PD-1/PD-L1 and CTLA-4. PD-1 is primarily found on activated T cells. When PD-1 binds to PD-L1 on tumor cells, it triggers signalling pathways that inhibit T cell activation and proliferation. CTLA-4 competes with the costimulatory receptor CD28 for binding to CD80/CD86 on APCs. Because CTLA-4 binds to CD80/86 with higher affinity, it blocks the necessary costimulatory signal, leading to T cell anergy. However, immune checkpoint molecules are not limited to these; other inhibitory molecules, such as LAG-3 and TIM-3, are also known to contribute to T cell exhaustion and immunosuppression in the tumor microenvironment (Rezazadeh-Gavgani *et al.*, 2025). The therapeutic blockade of these pathways using immune checkpoint inhibitors (ICIs) has demonstrated significant clinical impact on various types of tumors (Kazandjian *et al.*, 2016; Larkin *et al.*, 2019; Finn *et al.*, 2020).

2.2.4. Tumor microenvironment

In addition to direct mechanisms of immunosuppression by tumor cells themselves, a crucial role in the regulation of cancer progression and metastasis plays the tumor microenvironment (TME). It is now well known that tumors are not only composed of cancerous cells but also include various immune cells, such as dendritic cells, tumor-associated macrophages, granulocytes, NK cells, T and B lymphocytes; non-immune cells, such as endothelial and stromal cells, and cancer-associated fibroblasts (CAFs); blood and lymphatic vessels; and extracellular matrix components. Different secreted molecules, such as cytokines, chemokines, growth factors, and proteases, are also present in the TME (Zhang *et al.*, 2020; de Visser and Joyce, 2023). All these elements together create a heterogeneous dynamic tumor stroma that can either support or inhibit cancer progression, depending on the composition and interplay between its components (Mellman *et al.*, 2023).

2.2.4.1. Regulatory T cells

Regulatory T cells (Tregs) are an immunosuppressive subset of CD4+ T cells, characterized by high expression of CD25, also known as the IL-2 receptor alpha chain, and the transcription factor FOXP3, playing a crucial role in maintaining peripheral tolerance (Hori, Nomura and Sakaguchi, 2003).

Tumors actively recruit Tregs by secreting chemokines, such as CCL22, CCL17, and CCL5, which bind to CCR4 and CCR5, respectively, on Tregs (Pere *et al.*, 2011; De Oliveira *et al.*, 2017). High infiltration of Tregs in tumors is associated with poor prognosis in various cancer types (Liotta *et al.*, 2011; O'Callaghan *et al.*, 2015; Stenström, Hedenfalk and Hagerling, 2021). Tregs suppress effector immune reactions through multiple mechanisms. They secrete immunosuppressive cytokines, especially IL-10, IL-35, and TGF-β, which inhibit the activity of cytotoxic T cells and NK cells. Immune checkpoint molecules, including CTLA-4 and LAG-3, are also expressed on activated Tregs. Additionally, Tregs mediate immunosuppression through metabolic modulation. Due to their high expression of CD25, Tregs consume large amounts of IL-2, limiting its availability for the proliferation of surrounding effector T cells. Surface ectoenzymes CD39 and CD73 on Treg convert extracellular ATP into adenosine, which binds to the A2A receptor and suppresses effector T cells. Indoleamine 2, 3-dioxygenase (IDO) metabolizes the essential amino acid tryptophan, leading to T cell starvation and exhaustion (Ohue and Nishikawa, 2019).

2.2.4.2. Regulatory B cells

Like T cells, B cells also include a regulatory subset known as regulatory B cells. Bregs represent a relatively small population of B cells with immunosuppressive functions, producing immunosuppressive cytokines, such as TGF-β, IL-10, and IL-35 (Garaud *et al.*, 2019; Mirlekar *et al.*, 2020). Bregs also express immune checkpoint molecules, including PD-L1, which further contributes to T cell exhaustion (Wu *et al.*, 2020). It has also been demonstrated, that Bregs can contribute to CD4+ T cell differentiation into Tregs in cervical cancer (Yang *et al.*, 2024).

2.2.4.3. Tumor-associated macrophages (TAMs)

As mentioned before, macrophages can polarize into two main types depending on surrounding stimuli. M2 macrophages exhibit an immunosuppressive phenotype and are more abundant in tumors than M1. Hypoxia and cytokines, such as IL-6, IL-8, IL-10, and TGF-β, within the TME drive macrophage polarization toward the M2 phenotype (Huber *et al.*, 2016; Li *et al.*, 2019). In turn, M2 macrophages secrete additional immunosuppressive cytokines and factors that create a positive feedback loop within the TME, thereby promoting more macrophages to polarize to the M2 and recruiting other immunosuppressive cells (Fu *et al.*, 2019). TAMs have also been reported to support metastasis by producing various soluble factors, particularly IL-6. IL-6 has been shown to activate the epithelial-mesenchymal transition (EMT) in tumor cells, a process where cells lose their adhesion and acquire mesenchymal features, including increased migration and invasion (Zhong *et al.*, 2020; Chen *et al.*, 2022).

2.2.4.4. Myeloid-derived suppressor cells (MDSCs)

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells with immunosuppressive properties. Under normal physiological conditions, myeloid progenitors differentiate into mature cells, such as monocytes, DCs, and granulocytes. However, under pathological conditions, like chronic inflammation or tumor progression, this differentiation process is disrupted, leading to the accumulation of MDSCs. MDSCs are broadly classified into two major subsets based on their origin and surface markers: monocytic MDSCs (M-MDSCs) and granulocytic, also known as polymorphonuclear MDSCs (PMN-MDSCs) (Cassetta *et al.*, 2019). Both subsets suppress T cell activity through several mechanisms, including the production of high levels of immunosuppressive cytokines, ROS, and NO. Additionally, MDSCs express arginase-1 (ARG1), an enzyme that depletes arginine and cysteine from the tumor microenvironment. Furthermore, IDO expression is also upregulated in MDSCs. This depletion of essential amino acids impairs effector T cell proliferation and activation (Yang *et al.*, 2020).

2.2.4.5. Extracellular Matrix

The extracellular matrix (ECM) is a complex and dynamic network of proteins, glycoproteins, and polysaccharides that provides structural and signalling support to surrounding cells. Within the tumor, the ECM becomes highly dysregulated. The main driver of ECM remodeling are cancer-associated fibroblasts (CAFs), which produce large amounts of ECM components, especially collagens. This excessive collagen deposition increases ECM stiffness, creating a dense physical barrier that limits immune cell infiltration and promotes tumor progression. CAFs also produce enzymes such as lysyl oxidase and matrix metalloproteinases, which catalyze collagen cross-linking and ECM remodeling. These structural changes promote tumor cell invasion and facilitate metastasis (Desai *et al.*, 2025). Additionally, CAFs also secrete immunosuppressive cytokines, thereby supporting tumor progression (Cho *et al.*, 2018; Garcia Garcia *et al.*, 2022).

CAFs are mostly originate from tissue-resident fibroblast in response to different signals present within TME including chronic oxidative stress or TGF-β secreted from tumor cells (Toullec *et al.*, 2010; Yoon *et al.*, 2021). Moreover, other cell types, such as endothelial cells, mesenchymal stem cells can also be transformed to CAFs (Yeon *et al.*, 2018; Miyazaki *et al.*, 2020).

2.3. Cancer immunotherapy

Immunotherapy is one of the types of cancer treatment that aims to restore and enhance the immune system's natural ability to fight cancer by targeting different points in the cancer-immunity interaction. Ongoing research is focused on a deeper understanding of tumor immunology, discovering new potential immunotherapy targets, and optimizing combinations of existing treatment approaches to improve outcomes for patients with various cancer types.

2.3.1. Intratumoral therapies

Systemic immunotherapies have been extensively studied over the years, and various types have demonstrated effectiveness in targeting and eliminating cancer cells. However, they can also affect healthy tissue leading to unwanted side effects. Some immunotherapy drugs, especially immune checkpoint inhibitors, can trigger immune-related adverse events (iAEs) because of overactivation of the immune system. These iAEs can range from mild symptoms, such as skin rash, fatigue, and nausea, to severe autoimmune reactions that may affect various organs (Ascierto *et al.*, 2017; D'Angelo *et al.*, 2018). Additionally, systemic immunotherapy does not always effectively reach the tumor site. Physical barriers such as dense stroma or poor vascularization can also limit the effectiveness of cancer therapy (Gao *et al.*, 2019; Li *et al.*, 2021).

These limitations can potentially be overcome by using intratumoral immunotherapies that involve the direct injection of immunomodulatory drugs into the solid tumors. The goal is to locally affect the immunosuppressed TME that is difficult to reach with a systemic therapy. It could be a promising strategy that allows the use of a higher local dose of drugs with lower systemic toxicity compared to conventional systemic administration (Margolin *et al.*, 2018). Importantly, intratumoral administration has also been shown to induce systemic anti-tumor immune responses, including improved reduction of non-injected distant metastases (Zanker *et al.*, 2020; Dang *et al.*, 2021; Osorio *et al.*, 2024).

2.3.1.1. Intratumoral therapies under investigation

Several intratumoral immunotherapy strategies have been studied in preclinical models. Some of them have also progressed into early phase clinical trials, where they have demonstrated

promising efficacy as monotherapy or in combination with other immunotherapies. Selected examples are discussed below.

2.3.1.1.1. Monoclonal Antibodies

Monoclonal antibodies (mAb), an important group of targeted drug therapies, are successfully used in cancer treatment. Therapeutic mAbs are designed to recognize specific antigens with high affinity, often overexpressed within the tumor microenvironment. By directly binding to these molecular targets, mAbs can block critical signalling pathways important for tumor growth. They can also activate immune responses like ADCC, where NK cells destroy tagged cancer cells, and CDC, which leads to direct tumor cell lysis through complement activation (Zahavi and Weiner, 2020). Monoclonal antibodies can also be conjugated with a cytotoxic drug designed to deliver cytotoxic agents directly to cancer cells (Maiorano *et al.*, 2023).

Traditionally, mAbs are administered systemically, but they are also being investigated as an intratumoral therapy. For instance, a recently published phase I study examined intratumoral administration of an anti-CD40 agonistic antibody (2141-V11). The trial enrolled 12 patients with various solid tumors metastatic to skin. The treatment was well-tolerated, with only mild adverse events and no dose-limiting toxicities reported. Among the treated patients, anti-tumor response could be evaluated in 10 patients: two of them achieved complete responses, six had stable disease, and two showed disease progression. Importantly, tumor size reduction was observed not only in injected tumors but also in non-injected skin metastases, suggesting induction of local and systemic robust immune activation. Post-treatment analysis of tumor biopsies revealed that injected tumors displayed increased infiltration of CD8+ T cells and B cells, but not CD4+ cells (Osorio *et al.*, 2024).

2.3.1.1.2. Checkpoint Inhibitors

One of the most widely used types of monoclonal antibodies for cancer immunotherapy are immune checkpoint inhibitors, which target molecules such as CTLA-4 and PD-1 to enhance T cell-mediated anti-tumor responses. Although their investigation as intratumoral therapy remains limited, a phase I clinical trial investigated the local intracerebral administration of CTLA-4 (ipilimumab) and PD-1 (nivolumab) immune checkpoint blocking mAb in

combination with additional systemic administration of nivolumab in patients with recurrent glioblastoma. The therapy showed to be safe and better overall survival than what is typically observed in recurrent glioblastoma. These findings support further investigation of this local immunotherapy approach in brain tumors (Duerinck *et al.*, 2021).

2.3.1.1.3. Adoptive Cell Therapies

Adoptive cell transfer involves the isolation, *ex vivo* modification, and reinfusion of autologous or allogeneic effector immune cells with tumor-killing potential, APCs, or a combination of both. The goal of ACT is to increase the quantity and functionality of tumor-specific immune cells to overcome the immunosuppressive features of the TME and achieve durable anti-tumor responses (Du *et al.*, 2023).

Ilixadencel is an experimental immunotherapy using intratumoral injections of allogeneic pro-inflammatory DCs after *in vitro* activation with polyI:C, R848 and IFN-γ. In preclinical mouse models, ilixadencel has been shown to induce a pro-inflammatory tumor microenvironment, promoting activation and migration of host bystander DCs to draining lymph nodes, and enhancing the recruitment of CD8+ T cells and NK cells to the tumor site (Fotaki *et al.*, 2018).

Ilixadencel has also been evaluated in clinical settings. In a randomized phase II study involving patients with newly diagnosed metastatic renal cell carcinoma (mRCC), ilixadencel was administered i.t. and followed by sunitinib monotherapy. Sunitinib, a tyrosine kinase inhibitor that targets the VEGF receptor and other kinases related to tumor progression, is a common medical treatment for mRCC. The drug combination did not result in improved OS compared to treatment with sunitinib alone. However, this combination therapy was associated with a nearly two-fold higher rate of confirmed responses, including confirmed complete responses, while no CR has been reported in the sunitinib alone (Lindskog *et al.*, 2022).

2.3.1.1.4. CAR T cells

CAR (Chimeric Antigen Receptor) T cell therapy is another type of adoptive cell transfer that involves genetically engineering T cells to target tumor-specific antigens and kill tumor cells in a non-MHC-dependent manner. This method is successfully used in the treatment of hematological malignancies (Fowler *et al.*, 2021; Bouchkouj *et al.*, 2022). CAR T cell therapy

is not yet approved for solid tumors; however, several clinical trials are currently investigating its safety and effectiveness.

For example, a first-in-human pilot study tested the safety and feasibility of a repetitive local administration of CAR CTL cells targeted the high-affinity IL-13 receptor α2 in 3 patients with recurrent glioblastoma (GBM). IL-13Rα2 has been shown to be overexpressed in more than 50% of GBM, but not in the healthy brain tissue. The results showed an acceptable safety profile and evidence of transient antitumor activity (Brown *et al.*, 2015). Based on these initial findings, a recent phase I clinical trial examined autologous IL13Rα2 CAR T cells in combination with IL-2 in six patients with GBM. The CAR T cells were infused into the MRI-defined tumor site. The treatment again was well-tolerated with mild transient adverse events. Radiographic evidence of increased tumor necrosis was observed in two patients. Two other participants underwent craniotomy because of a lack of imaging-based responses to treatment and symptomatic progression. Histological analysis revealed confluent coagulative necrosis localized near the infusion site. Later, the pathological samples from these individuals showed CD8+ T cell infiltration, with a higher density near the injection site than in the distal tumor regions. Although both studies demonstrated biological activity of intratumoral CAR T cell therapy, no objective improvement in survival or clinical response was noted (Brown *et al.*, 2022).

2.3.1.1.5. TLR agonists

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) that play a crucial role in the initiation of innate immune response. TLRs can recognize specific conserved PAMPs from invading microorganisms as well as DAMPs released by damaged or dying host cells. In humans, 10 TLRs are known. Each TLR binds a specific type of ligand, allowing the immune system to detect and respond to a wide range of pathogens (Li and Wu, 2021). Currently, several TLR agonists are being studied for their potential in cancer therapy.

For example, the intratumoral administration of G100, a synthetic TLR4 agonist, has been tested in patients with Merkel cell carcinoma. The treatment was well-tolerated without additional toxicity. The results showed increased infiltration of CD8+/CD4+ T cells and upregulation of immune-related genes in the injected tumors (Bhatia *et al.*, 2019). Additionally, in a later phase I/II clinical trial involving patients with follicular lymphoma, the intratumoral

administration of G100 in combination with ICI anti-PD-1 (pembrolizumab) resulted in a 33.3% overall response rate and led to tumor regression in 72.2% of patients (Halwani *et al.*, 2022).

Another class of TLR agonists being explored in cancer immunotherapy are CpG oligodeoxynucleotides, which activate TLR9. It has been shown that the intratumoral administration of CpG can significantly enhance the anti-tumor effect of low-dose local radiation therapy in a murine model of soft tissue sarcoma. This combination therapy induced a stronger immune response compared to CpG or radiation therapy alone (Su *et al.*, 2024).

A murine model of single intratumoral administration of TLR7/8 agonist 3M-052 significantly reduced mammary tumor growth, induced a T cell-inflamed TME, and reduced metastatic spread to the lungs. Notably, tumor-specific T cells were also identified in lungs of 3M-052-treated mice, suggesting the induction of systemic anti-tumor immunity (Zanker *et al.*, 2020).

2.3.1.1.6. MBTA therapy

MBTA is an abbreviation for a therapeutic mixture designed to stimulate a strong local immune response within the tumor through the intratumoral application. It consists of three TLR agonists (resiquimod (R-848), poly-inosinic–polycytidylic acid (poly(I:C)), and lipoteichoic acid (LTA)), a phagocytosis-stimulating agent (mannan-BAM), and an anti-CD40 antibody (Caisová *et al.*, 2018; Uher, Caisova, *et al.*, 2021).

Mannan is a branched polysaccharide composed of mannose units present in the cell walls of fungi and some bacteria. Mannan is recognized by mannose-binding lectin, leading to the activation of the lectin complement pathway. Mannan can also be recognized by the mannan receptor on the surface of APCs. In MBTA, mannan is attached to a biocompatible anchor for the membrane (BAM), which allows the complex to bind to the surface of the tumor cell and promote tumor cell phagocytosis (Waldmannová *et al.*, 2016).

R-848 is recognized by TLR7/8 in human cells and TLR7 in mice. It triggers a MyD88-dependent signalling pathway that leads to the activation of NF-κB transcription factor and the production of Th1-type proinflammatory cytokines such as IFN-α, IL-1β, and IL-6 (Zhou and Sun, 2015).

Poly(I:C) is a synthetic analogue of double-stranded RNA, which activates immune system through TLR3, MDA5, and RIG-I. It mimics viral infection and triggers the production of induction of type I interferons, which enhance CD8 + T cell responses, activate NK cells, and support DC maturation (Cheng and Xu, 2010).

Lipoteichoic acid is a component of the cell wall of gram-positive bacteria, which activates TLR2, leading to pro-inflammatory cytokine production (Seo, Michalek and Nahm, 2008).

CD40 is a member of the TNF-receptor superfamily and is primarily expressed on APCs, including DCs, B cells, and macrophages. Its ligand is mainly found on activated T cells. Activation of CD40 promotes maturation of DCs by upregulation of MHC and costimulatory CD80/CD86 molecules, and IL-12 production (Bullock, 2022).

Together, these components establish a pro-inflammatory microenvironment within the TME, triggering the activation of both innate and adaptive immune responses in murine models (Uher, Huynh, *et al.*, 2021). MBTA therapy effectively reduced injected tumors and significantly enhanced the overall survival of treated mice across various cancer types (Caisová *et al.*, 2018; Uher, Caisova, *et al.*, 2021). Notably, the therapeutic effects were also observed in non-injected distal metastases, indicating the induction of systemic anti-tumor immune response. Furthermore, MBTA therapy supported long-term immunological memory, as all rescued mice showed no evidence of tumor growth after the second challenge with the same cancer cell type, highlighting the potential of MBTA to induce durable tumor-specific immunity (Medina *et al.*, 2020).

Thus, the intratumoral administration of TLR agonists combined with phagocytosis-stimulating agents represents a promising therapeutic strategy for inducing a strong and sustained anti-tumor immune response, leading to tumor regression and improved survival. This work is based on a similar concept to MBTA therapy.

2.4. Calreticulin

2.4.1. Structure and physiological functions of CRT

Calreticulin (CRT) is a multifunctional calcium-binding chaperone that plays a critical role in various physiological and pathological processes. CRT is predominantly located in the lumen of the endoplasmic reticulum, where it regulates intracellular calcium homeostasis. Additionally, CRT is an important lectin-binding chaperone protein. Together with calnexin, it assists in the proper folding of newly synthesized glycoproteins passing through the ER (Michalak *et al.*, 2009).

Calreticulin consists of a N-terminal signal peptide and three structural and functional domains: a globular lectin-like N-terminal domain, which binds misfolded proteins and is involved in quality control of newly synthesized proteins; a central proline-rich P-domain that binds calcium ions with high affinity but low capacity and also participates in CRT chaperone activity; and an acidic C-terminal domain, which binds calcium with low affinity but high capacity and serves as a calcium reservoir. The C-terminal domain also contains a KDEL amino acid retention sequence responsible for retaining CRT within the ER (Michalak, 2024).

2.4.2. Role of calreticulin in cancer immunity

2.4.2.1. Role in antigen-presentation

Calreticulin is a part of the peptide-loading complex (PLC), responsible for the proper assembly of MHC class I molecules within the ER. Together with other proteins such as tapasin, ERp57, and TAP (the transporter associated with antigen processing), it stabilizes the complex and ensures the proper loading of high-affinity peptide antigens, which are derived from degraded intracellular proteins, onto MHC class I molecules (Blees *et al.*, 2017).

2.4.2.2. Immunogenic cell death

CRT is considered a hallmark of immunological cell death (ICD), a form of regulated cell death that has been observed in tumors after exposure to certain chemotherapeutic agents or radiation therapy. In contrast to apoptosis, where dying cells are typically cleared without triggering an

immune response, ICD is characterized by the release of certain DAMPs leading to immune system activation that can help target and eliminate cancer cells. In addition to calreticulin, the best-known DAMPs released during ICD include ERP57, HMGB1, ATP, and heat shock proteins. The combination of these signals triggers the immune response against cancer (Galluzzi *et al.*, 2023).

Under cellular stress conditions, especially in response to ICD-inducing agents, calreticulin is translocated from the ER to the cell surface or released into the extracellular space (Obeid et al., 2007; Panaretakis et al., 2009; Garg et al., 2012; Osman et al., 2017; Abdullah et al., 2022).

The exact mechanism of CRT exposure on the cell surface is not fully understood. It is believed that cellular stress signals initiate CRT translocation by PERK activation and subsequent phosphorylation of eIF2α and caspase-8 activation. These processes have been shown to be required for the pre-apoptotic CRT exposure. CRT is thought to be translocated in complex with ERp57. The CRT-ERp57 complex is transported through the secretory pathway, involving ER-to-Golgi trafficking and vesicular transport. Finally, vesicles with CRT-ERp57 complex are exocytosed through SNARE-mediated fusion with the plasma membrane (Panaretakis *et al.*, 2009b; Liu *et al.*, 2019). However, a recent study has shown that some anticancer drugs, such as oxaliplatin and 5-fluorouracil, promote CRT translocation not through the ER stress induction but through another mechanism, presumably involving activation of the p53 signalling pathway (Naito *et al.*, 2024).

Calreticulin is also believed to be essential for determining the immunogenicity of tumor cells (Obeid *et al.*, 2007). Mice vaccinated with cancer cells that had been pretreated *in vitro* with ICD inducers showed strong resistance to new tumor growth of the same cancer type. However, this anti-tumor effect was significantly reduced when the ecto-CRT expression was suppressed, resulting in the loss of the vaccine's ability to activate an effective anti-cancer immune response (Garg *et al.*, 2015).

2.4.2.3. Role in phagocytosis

The surface-exposed calreticulin acts as an “eat me” signal and plays a crucial role in the recognition and uptake of dying cancer cells by professional phagocytes. Once CRT is present on the cell surface, it can be recognized by the LRP1/CD91 receptor on antigen-presenting cells. It leads to direct engulfment of dying tumor cells by phagocytes (Gardai *et al.*, 2005).

Early studies highlight the importance of calreticulin translocation to the cell surface in reaction against tumors, demonstrating that CRT blocking or knockdown in cancer cells significantly decreases phagocytosis efficiency and inhibits immunogenic cell death reaction. Importantly, these functions can be restored by adding a recombinant protein (Obeid *et al.*, 2007; Garg *et al.*, 2015; Matsusaka *et al.*, 2022).

However, it is believed that tumor cells are able to counterbalance the effect of CRT exposure by upregulation of CD47. This protein binds to signal regulatory protein alpha (SIRPα) on the surface of APCs and serves as a "don't eat me' signal for them. This mechanism allows cancer cells to avoid engulfment and destruction by the immune system and promotes tumor survival and growth. Therefore, the balance between surface CRT and CD47 determines how effectively dying cells are eliminated (Chao *et al.*, 2010). More recently, the novel mechanism by which tumor cells can avoid CRT exposure was reported. It has been shown that tumor cells can upregulate stanniocalcin-1 (STC1), and its upregulation is associated with poor overall survival. Investigations of the immunosuppressive mechanisms of STC1 revealed that it directly interacts with CRT, leading to its retention in the cytoplasm, impaired phagocytosis of cancer cells, and a weak T-cell immune response (Lin *et al.*, 2021).

Interestingly, it has also been revealed that activated macrophages can secrete calreticulin into the extracellular space to label target cells for subsequent removal through macrophage-mediated programmed cell removal (Feng *et al.*, 2015, 2018). A similar mechanism has been observed in microglia, which release CRT in response to bacterial LPS stimulation. In this context, CRT acts as an opsonin and promotes clearance of bacteria by microglia (Cockram, Puigdellívol and Brown, 2019; Reid *et al.*, 2022). Additionally, CRT chemoattracts microglia, even in nanomolar concentrations, and induces production of proinflammatory cytokines such as TNF-α, IL-6 and IL-1β. CCL2 chemokine secretion was also triggered by CRT. Although the exact mechanism of CRT-induced microglial activation remains unclear, it does not appear to involve LRP1, as its inhibition has no effect on cytokine secretion (Reid *et al.*, 2022).

2.4.2.4. Immune cell activation

Findings regarding the ability of calreticulin to activate different cell types are controversial. On the one hand, it has been reported that CRT alone does not promote DC maturation or activation and requires additional signals, such as those released by dying tumor cells (Obeid

et al., 2007). On the other hand, more recent studies have demonstrated that CRT can also act as a direct immunostimulatory signal for APCs, leading to their activation and cytokine production, supporting anti-tumor response. For instance, a recombinant CRT/39-272 fragment has been reported to induce maturation of DCs after 24 h *in vitro* incubation, resulting in increased expression of co-stimulatory molecules and pro-inflammatory cytokine secretion (Li et al., 2015; 2016).

These findings were further supported by blocking CRT activity with anti-CRT antibody, which led to lower expression of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β *in vitro*. In an *in vivo* mouse model of acute lung injury (ALI), intraperitoneal administration of the anti-CRT antibody significantly suppressed inflammation. The treated mice showed lower levels of pro-inflammatory cytokines and reduced infiltration of neutrophils and T lymphocytes within the injured lung tissue compared to untreated controls (Jiang *et al.*, 2020).

Although the exact mechanism by which CRT activates immune cells remains unclear, evidence suggests that CRT may signal through the TLR4/CD14 pathway. This is supported by findings that DCs derived from TLR4 mutant C3H/HeJ mice and CD14 knock-out C57BL/6 mice showed significantly reduced cytokine responses after stimulation with rCRT/39-272, compared to wild-type controls. Moreover, rCRT/39-272 treatment induced increased expression of phosphorylated Akt observed 60 minutes after stimulation, suggesting that the PI3K/Akt pathway is involved in the CRT-induced activation of DCs (Li et al., 2015). Another study proposed that extracellular rCRT activates macrophages through the MAPK-NFκB signalling pathway, increasing TNF-α and IL-6 expression in macrophages (Duo *et al.*, 2014).

A more recent study also showed that expression of CRT on the tumor cell surface was positively correlated with the infiltration of mature DCs in tumor tissues *in vivo* in mouse models of non-small cell lung cancer (NSCLC). Furthermore, this study highlighted that membrane CRT on tumor cell NSCLC can interact with TLR4 triggering TLR4-MyD88 signalling. It leads to secretion of TNF-α and CCL19, which are involved in DC recruitment and maturation. Notably, the levels of TNF-α and CCL19 were significantly decreased in TLR4 knockdown cells (Chen et al., 2021).

Calreticulin also positively correlated with enhanced NK cytotoxicity in acute myeloid leukemia when exposed on malignant blasts. Treatment of purified NK cells with rCRT alone did not affect their ability to release cytotoxic granules or secrete IFN-γ. However, rCRT added to PBMCs significantly increased NK cells activity upon stimulation. This suggests that rCRT

enhances NK cell function indirectly by promoting the activity of other immune cells. Specifically, rCRT promoted maturation of CD11cCD14^{high} monocytes, increasing their expression of IL-15Rα, a key molecule for presenting IL-15 to NK cells and promoting their activation (Truxova *et al.*, 2019). Moreover, a more recent study demonstrated that CRT expressed on the surface of cells undergoing ER-stress can directly activate NK cell cytotoxicity through the interaction with NKp46 receptor (Sen Santara *et al.*, 2023).

2.4.2.5. Prognosis factor in cancer

CRT has been reported to be overexpressed in various types of malignancies compared to their corresponding healthy tissues. In some cancer types, CRT expression levels in tumor tissue are correlated to better prognosis and clinical outcomes in patients.

Calreticulin has been shown to be increased on malignant blasts in AML patients even before chemotherapy. High levels of ecto-CRT were shown to positively correlate with increased frequency of circulating tumor-specific CD4+ and CD8+ cells in the peripheral blood, compared to patients with lower CRT expression (Fucikova, Truxova, *et al.*, 2016).

A positive correlation between CRT expression on tumor cell membranes and mature DC infiltration has been observed in NSCLC (Fucikova et al., 2016; Chen et al., 2021). Patients with high CRT expression showed significantly higher densities of mature DCs, but no significant difference in CD8+ T cell infiltration was noted between CRT-high and CRT-low cohorts. The CRT-high/DC-high patients demonstrated the best prognosis, while CRT-low/DC-low patients had the highest risk of death. Similar trends were seen with CRT expression and CD8+ T cell density. Interestingly, CRT-low patients at stage I had a worse survival prognosis than CRT-high patients at advanced stages (Fucikova et al., 2016). Additionally, in advanced NSCLC patients treated with ICI combination therapy, elevated plasma CRT levels correlated with better progression-free and overall survival, serving as a marker of the efficacy of therapy (Tsutsumi *et al.*, 2023).

However, despite the strong evidence supporting the anti-tumor properties of CRT, several studies have reported that high levels of CRT expression in tumor tissues are associated with poor clinical prognosis in patients with many cancer types.

For instance, CRT was found to be highly expressed in nasopharyngeal carcinoma (NPC) tissues compared to healthy controls. This elevated expression was positively correlated with

advanced clinical stage and metastasis. The underlying mechanism might be associated with activation of the Stat3 signalling pathway. This study showed that knockdown of CRT in NPC CNE2 cells led to decreased Smad3 phosphorylation and inhibited migration and invasion ability of the cells *in vitro.* Blocked CRT expression also contributed to the inhibition of EMT by downregulating invasion-related proteins such as vimentin and increasing levels of E-cadherin (Ye *et al.*, 2020). Additionally, CRT expression in NPC tumor cells can also activate the Stat3 signalling pathway, thereby promoting cell proliferation, migration, and invasion (Han *et al.*, 2019).

A similar trend was observed in gastric cancer (GC), where both protein and mRNA levels of CRT were significantly upregulated in tumor tissues and associated with poor prognosis in patients. Stable CRT overexpression in GC cell lines enhanced their migratory capabilities and led to increased expression of EMT-related markers such as Snail and ZEB1, as well as cell adhesion molecules such as MMP2, fibronectin, and integrin β1. In contrast, CRT knockdown had opposite effects, decreasing these protein expression levels and increasing epithelial markers like E-cadherin and ZO-1 (Wang *et al.*, 2022).

The role of CRT in promoting metastasis and tumor progression through induction of EMT has also been demonstrated in other types of cancer, where this process has been shown to be mediated by various distinct signalling pathways (Sheng *et al.*, 2017; Zheng *et al.*, 2020).

Taken together, calreticulin exhibits multiple mechanisms by which it can promote phagocytosis of tumor cells and activate pro-inflammatory responses, suggesting its potential to serve as an effective adjuvant in cancer immunotherapy. However, CRT could also contribute to tumor progression, but this function could depend on the immune context within the TME. Therefore, in this thesis we investigated the effects of CRT either alone or in combination with other anti-tumor agents as part of intratumoral immunotherapy approach.

3. Aims

This thesis investigates the hypothesis that extracellular calreticulin can function as an effective adjuvant in intratumoral immunotherapy by enhancing immune activation and promoting effective anti-tumor responses. To test this, the study aimed to:

- Engineer tumor cell lines with stable expression of GPI-anchored CRT on their surface using a plasmid construct and subsequently evaluate their ability to promote phagocytosis *in vitro* and induce anti-tumor response *in vivo*, compared to control cells lacking this expression.

- Evaluate whether recombinant CRT, either alone or in combination with TLR agonists, is able to enhance tumor control and improve survival when administered as an intratumoral therapy *in vivo*.

- Compare the immunological and therapeutic impact of CRT-based treatment in two murine cancer models with different immunogenicity.

4. Materials & Methods

4.1. Cells cultivation

Murine melanoma B16-F10 (CYT-305157, Cytion) and colorectal carcinoma MC38 (CYT-305223, Cytilon) cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS and 5% streptomycin/penicillin (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2.

4.2. Cell preparation

Cells were harvested at 70-90% confluence growth, the culture medium was removed, and the cells were washed with sterile PBS. To detach adherent cells, a trypsinization solution (0.02% trypsin and 0.25% EDTA) was added, and the cells were placed in an incubator at 37°C for 5 minutes. The trypsinization reaction was then stopped by adding DMEM supplemented with 10% FBS. The cells were stained with trypan blue for viability assessment and counting in a Bürker chamber.

4.3. Plasmids

The following plasmids were used in experiments:

- pTagRFP-H2B (FP368, Evrogen)
- pEGFP-N1 (37375, AddGene)
- pV5-CALR-DAF1 plasmid was generated and isolated by the Laboratory of Mitochondrial Physiology at the Institute of Physiology of the ASCR (plasmid map and nucleotide sequence are shown in the supplementary section).

4.4. Lipofectamine 3000 transfection

Lipofectamine™ 3000 Transfection Reagent (Invitrogen) was used.

One day before transfection, cells were seeded on a 6-well plate, so they covered approximately 70-80% of the well area. The transfection reaction was performed in serum-free and antibiotic-

free growth medium following a commercial protocol. Four hours after transfection, serum-free medium was replaced with complete culture medium supplemented with 5% FBS. The transfected cells were incubated for 24 hours at 37°C in a humidified incubator with 5% CO_2 and subsequently collected for transfection efficiency analysis.

4.5. Electroporation

The serum-free and antibiotic-free Opti-MEM (Gibco) medium without any supplements was used as an electroporation buffer.

1×10^6 cells were mixed with 10 µg plasmid DNA in electroporation buffer to a final volume of 100 µl. The mixture was gently mixed with a pipette, and the entire volume was then transferred into a pre-chilled electroporation cuvette. Electroporation was performed using the NEPA21 electroporator with the following parameters:

- The poring pulse was set to 200 V, with a 3 ms pulse length, 50 ms pulse interval, 2 pulses, positive polarity, and 10% decay rate.
- Subsequently, the transfer pulse was applied at 20 V, with a 50 ms pulse length, 50 ms pulse interval, 5 pulses, alternating polarity, and 40% decay rate.

After electroporation, the cells were immediately transferred to a culture tube with pre-warmed complete growth medium. The cells were incubated for 24 hours at 37°C in a humidified incubator with 5% CO_2 and subsequently collected for transfection efficiency analysis.

4.6. Agarose gel electrophoresis

0.8% agarose gel electrophoresis was used to evaluate the integrity of plasmid DNA.

Agarose powder was dissolved in 1× TAE buffer by heating the mixture in a microwave for approximately 1 min with regular swirling until the agarose was completely dissolved. The flask was then cooled by swirling until it was comfortable to touch, and ethidium bromide was added to a concentration of 0.5 µg/ml for DNA visualization. The solution was poured into a gel tray with a comb and allowed to solidify at room temperature.

After the gel solidified, it was placed in an electrophoresis chamber and filled with 1× TAE buffer to cover the gel surface. Plasmid DNA samples were mixed with 6× loading dye and

transferred into a well in the gel. Gel electrophoresis was performed at a constant voltage of 100 V for 45-60 minutes.

4.7. Mice and tumor transplantation

Female C57BL/6 mice, 6-8 weeks old, obtained from AnLab s.r.o., were subcutaneously injected with 1×10^6 B16-F10 or 1×10^6 MC38 cells in 0.1 ml of serum-free DMEM into the previously shaved right flank.

Mice were maintained under specific pathogen-free conditions in individually ventilated cages with free access to food and water under a 12-hour light/dark cycle.

4.8. Treatment

Materials used for the intratumoral treatment mixture:

- RS09 – LPS peptide mimic (HY-D1056, MedChemExpress)
- R-848 – Resiquimod (HY-13740, MedChemExpress)
- Poly(I:C) – Polyinosinic-polycytidylic acid (HY-135748, MedChemExpress)
- Mouse recombinant calreticulin protein (HY-P72112, MedChemExpress)

Seven days after tumor cell transplantation, mice were randomly divided into 4 groups of 8, and the therapy was started (day 0). The treatment plan included six injections administered intratumorally at six-day intervals. The volume of each dose of the mixtures was 50 µl/mouse. The control group was treated with the same volume of saline.

The therapeutic mixtures used for immunotherapies:

- **CRT + TLR agonists**: 0.5 mg/ml R-848, 0.5 mg/ml poly(I:C), 0.5 mg/ml RS09, 10 µg/dose of a recombinant CRT dissolved in saline.

- **TLR agonists only**: 0.5 mg/ml R-848, 0.5 mg/ml poly(I:C), 0.5 mg/ml RS09 dissolved in saline.

- **CRT only:** 10 µg of a recombinant CRT dissolved in saline.

4.9. Tumor measurement

Tumor sizes were measured with a caliper every two or three days from day 7 after tumor cell inoculation. Tumor volumes were calculated using the following formula:

$$V = 0.5 \times \text{length} \times \text{width}^2$$

Mice were sacrificed when tumor volume exceeded 2500 mm^3 or signs of severe suffering were evident.

4.10. Serum and tissue collection

Mice were first anesthetized with an intraperitoneal injection of ketamine-xylazine solution. The abdominal cavity was carefully opened, and blood was collected from the inferior vena cava. The collected blood was transferred into collection tubes and allowed to clot at room temperature. Then the blood samples were centrifuged at 3500 rpm for 10 minutes to separate the serum. The sera were carefully collected and stored at -20°C for further ELISA analysis.

Tumors were also harvested. The tissues were homogenized using mechanical cell homogenizers and filtered through 70 μm cell strainers (BD Falcon) to obtain single-cell suspensions. Cells were then resuspended and stored in Streck Cell Preservative (Streck Inc.) for subsequent flow cytometry analysis.

4.11. Flow cytometry

4.11.1. Plasmid expression

To evaluate the expression of transfected genes, flow cytometry was performed on cells after plasmid delivery via either Lipofectamine-based transfection or electroporation. For the detection of surface-expressed CRT, cells were stained with a Cy5-conjugated anti-calreticulin antibody (bs-0062R-Cy5, Bioss). For plasmids encoding RFP or GFP, intrinsic fluorescence was measured without the need for antibody staining. Untransfected cells were used as negative controls. The analysis was performed on the NovoCyte Penteon flow cytometer and were analyzed using NovoExpress software.

4.11.2. Analysis of leukocyte population

The collected tissue suspensions were centrifuged to remove the preservative solution (1500 rpm, 5 min, 4°C). The supernatant was discarded, and the cell pellet was washed twice with cold FACS buffer (PBS supplemented with 2% FBS) and centrifuged (1500 rpm, 5 min, 4°C). Before staining, suspensions were filtered through a 70 µm filter (BD Falcon).

The cells were seeded into 96-well U-bottom plates and then stained with 20 µl of the prepared mixture of the following monoclonal antibodies:

- ABflo® 488 anti-mouse CD3 (A27161, ABclonal Technology)
- ABflo® 594 anti-mouse CD45 (A23709, ABclonal Technology)
- Alexa Fluor® 700 anti-mouse I-A/I-E Antibody (M5/114.15.2, BioLegend)
- APC/Cyanine7 Rabbit anti-Mouse CD11b (A26935, ABclonal Technology)
- Brilliant Violet 605™ anti-mouse/human CD45R/B220 (RA3-6B2, BioLegend)
- PE anti-mouse CD11c (N418, BioLegend)
- PE/Cyanine7 anti-mouse CD4 (A27150, ABclonal Technology)
- PerCP anti-mouse CD8a (A27335, ABclonal Technology)

The cells were incubated on ice in the dark for 30 minutes. Subsequently, the cells were twice washed with cold FACS buffer and centrifuged (1500 rpm, 5 min, 4°C). The pellet was resuspended in 100 µl of FACS buffer and analyzed. The analysis was performed on the NovoCyte Penteon flow cytometer. The data were gated and analyzed using FlowJo 10.6 software.

4.12. ELISA

Serum IFN-γ and IL-10 concentrations were assessed using enzyme-linked immunosorbent assay (ELISA). Cytokine levels were measured using the following commercial kits:

- Mouse IFN-γ ELISA Kit (RK00019-96, ABclonal Technology)
- Mouse IL-10 ELISA Kit (RK00016-96, ABclonal Technology)

All procedures were carried out according to the manufacturer's protocols. Absorbance was read at 450 nm using a microplate reader, and cytokine concentrations were determined by comparison to a standard curve. The sensitivity of detection was based on the lower limit indicated by each kit.

4.13. Statistical analysis

All statistical analysis and graphs were done in RStudio. The statistical significance level was set at $p < 0.05$ for all tests. Error bars correspond to the SEM.

Two-tailed Student's t-test was used to assess the significance of differences between two groups. One-way ANOVA with Tukey's multiple comparison post hoc test was used to evaluate differences between multiple groups.

The survival analysis was performed using the “survival” R package. Kaplan-Meier curves were generated to visually represent cumulative survival trends over time. Statistical significance of differences in survival times between groups was evaluated using the Mantel-Cox log-rank test.

5. Results

5.1. *In vitro* experiments.

Initially, we aimed to generate tumor cells expressing CRT on their surface. To achieve this, a plasmid was designed encoding CRT without the KDEL retention sequence and with the addition of a DAF (decay-accelerating factor) GPI-anchor (Legler et al., 2005).

Cells were transfected using two different techniques: lipid-based transfection reagent (Lipofectamine 3000) and electroporation. Flow cytometry was subsequently performed to determine whether CRT was expressed on the cell surface (Fig. 1 and Fig. 2). Unfortunately, no surface expression of CRT was detected in either method.

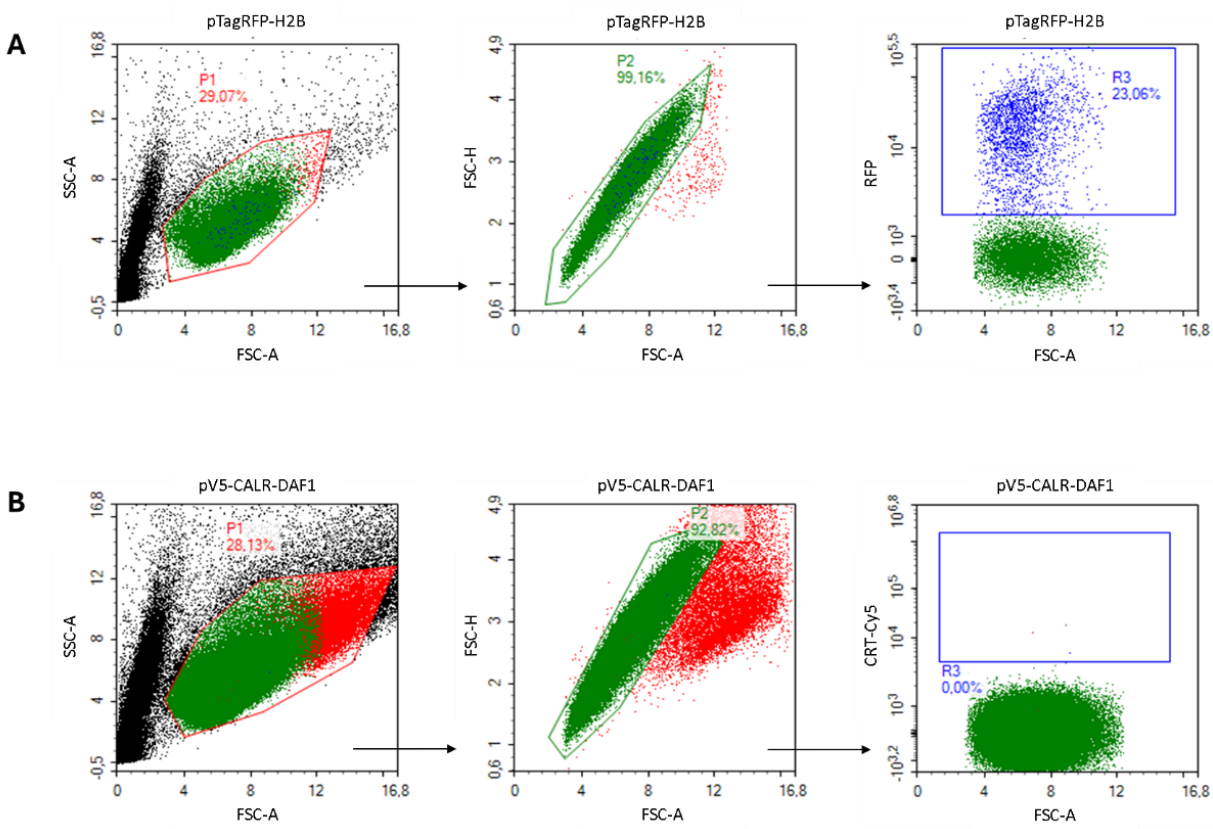


Figure 1. *Expression of plasmids in B16-F10 cells after transfection using Lipofectamine 3000. A – cells transfected with a control plasmid expressing RFP, B – cells transfected with our plasmid.*

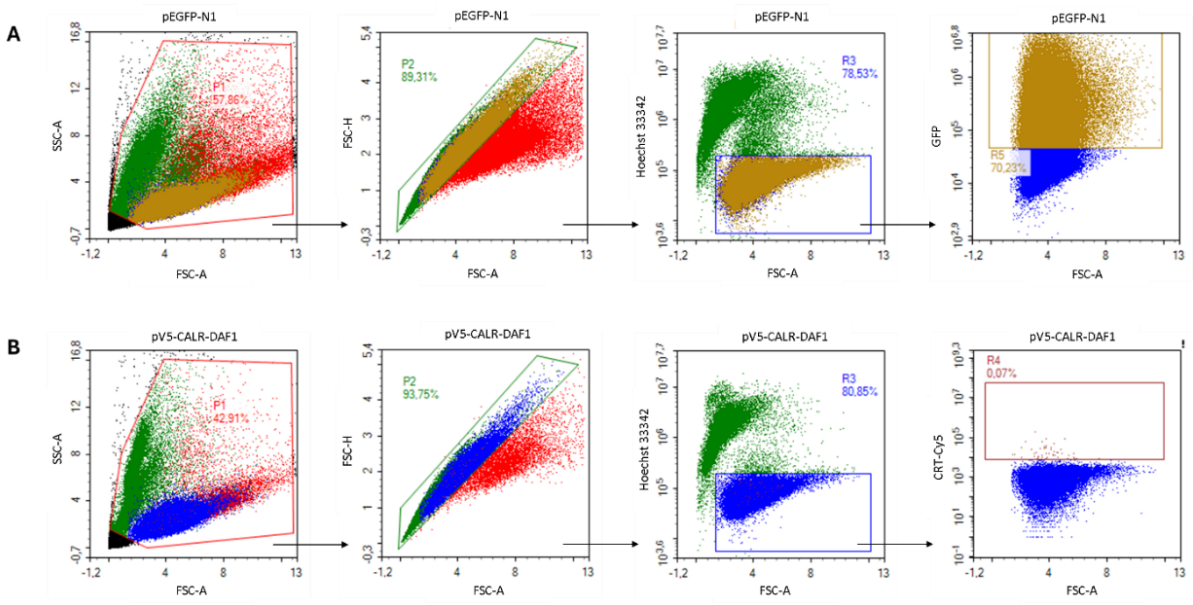


Figure 2. *Expression of plasmids in cells after electroporation.*
A – cells transfected with a control plasmid expressing GFP, B – cells transfected with our plasmid.

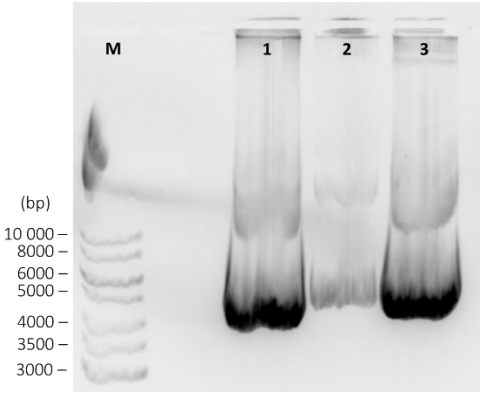


Figure 3. *0.8% agarose gel electrophoresis of the pV5-CALR-DAF1 plasmid.*
M – DNA marker; 1,2,3 – lines represent the same plasmid from different aliquots.

After several unsuccessful attempts to insert plasmids, the integrity of plasmid DNA was confirmed by electrophoresis on 0.8% agarose gel (Fig. 3). The analysis showed that the plasmid DNA was not degraded.

Because of time constraints, we decided to stop this line of research within this diploma thesis and focus on *in vivo* studies.

5.2. *In vivo* experiments.

The next aim of this thesis was to investigate the therapeutic efficacy of recombinant calreticulin alone or in combination with TLR agonists as intratumoral immunotherapy strategy. Four treatment mixtures were used: control (treated with saline), CRT (treated with recombinant calreticulin only), TA (treated with TLR agonists mix), and CRT+TA (treated with a combination of recombinant calreticulin and TLR agonists mix). The therapeutic scheme is illustrated below (Fig. 4).

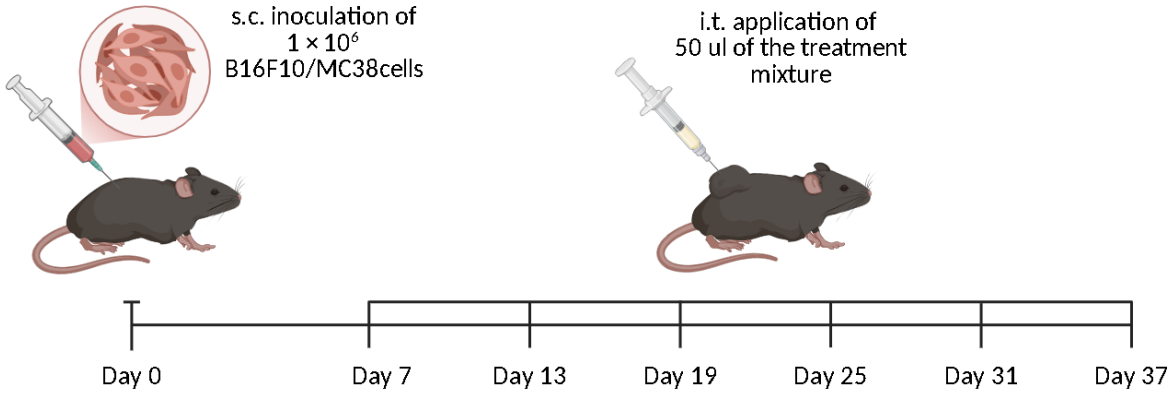


Figure 4. *Treatment scheme. C57BL/6 mice were subcutaneously injected with 1 × 10^6 B16-F10 or MC38 cells on day 0. The therapy was initiated on day 7 after tumor inoculation. The mice received six intratumoral injections at six-day intervals. The picture was created in BioRender.*

5.2.1. B16-F10 murine model

Firstly, we examined our intratumoral therapies in the B16-F10 melanoma murine model. The effects of the treatments on tumor growth over time are shown in Figure 5. No significant difference was observed between the CRT-treated group and the control. In contrast, treatment with TLR agonists, as well as the combination of TA and CRT, demonstrated a significant therapeutic effect, leading to a reduction in tumor sizes compared to the control group. A slight reduction in tumor growth was noted in the CRT+TA group compared to the TA group, but this difference did not reach statistical significance.

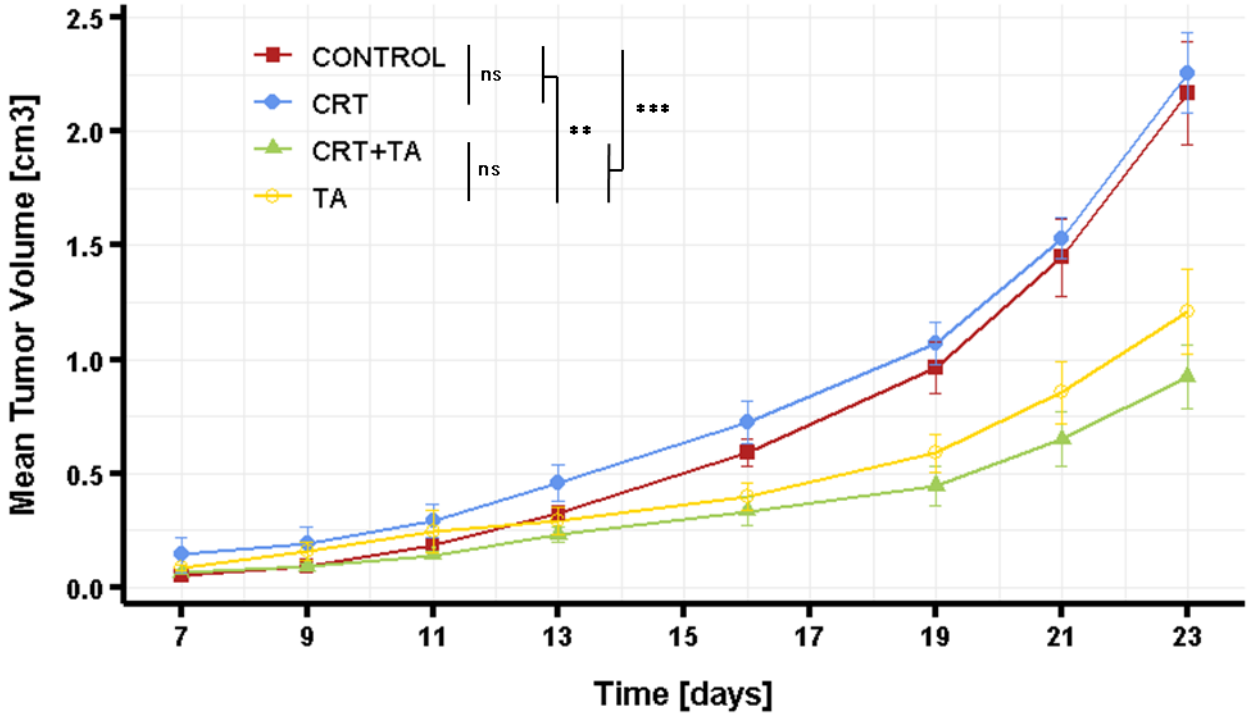


Figure 5. *B16-F10 murine model. The curves represent mean tumor growth over time for each treatment group: control (shown in red), CRT (shown in blue), TA (shown in yellow), CRT+TA (shown in green). Statistical analysis was performed by one-way ANOVA with post-hoc Tukey's multiple comparison test at day 23, n= 8-10 mice/group. (ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)*

In the survival analysis experiment, we observed similar trends in treatment outcomes. Specifically, mice treated with TA and TA+CRT lived significantly longer than the control and CRT-treated groups. However, there were no significant differences in survival between the CRT and control groups, as well as between TA and TA+CRT groups (Fig. 6).

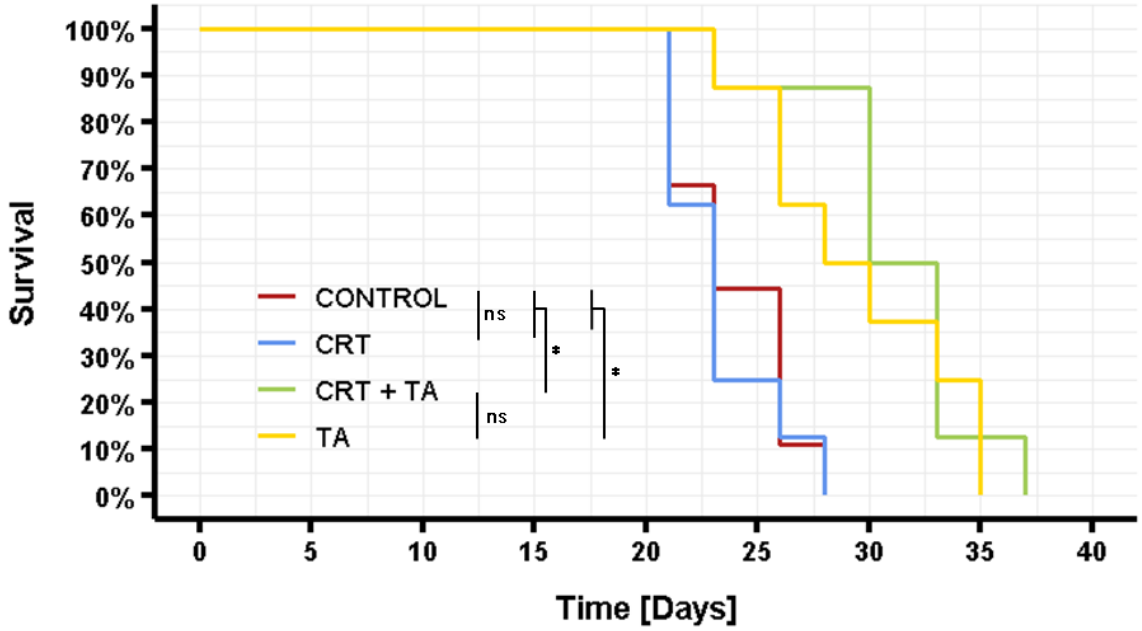


Figure 6. *B16-F10 murine model. Kaplan-Meier survival curves show an overall survival over time across four treatment groups: control (shown in red), CRT (shown in blue), TA (shown in yellow), CRT+TA (shown in green). Statistical differences were evaluated using the log-rank test, n = 8-10 mice/group. (ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)*

5.2.2. MC38 murine model

5.2.2.1. Tumor growth and mice survival analysis

Since the therapy did not give positive results in the melanoma model, we chose the murine colorectal carcinoma MC38 cells as a more immunogenic model for further study of the immunotherapeutic efficacy of our therapeutic mixtures. The treatment conditions were the same as in the previous experiment.

In the MC38 colorectal carcinoma model, as in the B16-F10 tumors, treatment with calreticulin alone did not improve tumor growth or overall survival, showing no significant difference compared to the control group. In contrast, treatment with TLR agonists led to a significant reduction in tumor sizes. Moreover, the addition of CRT to TA treatment significantly prolonged overall survival compared to TA alone. The tumors were eliminated in 3 of 16 experimental animals in the CRT+TA group in two independent experiments.

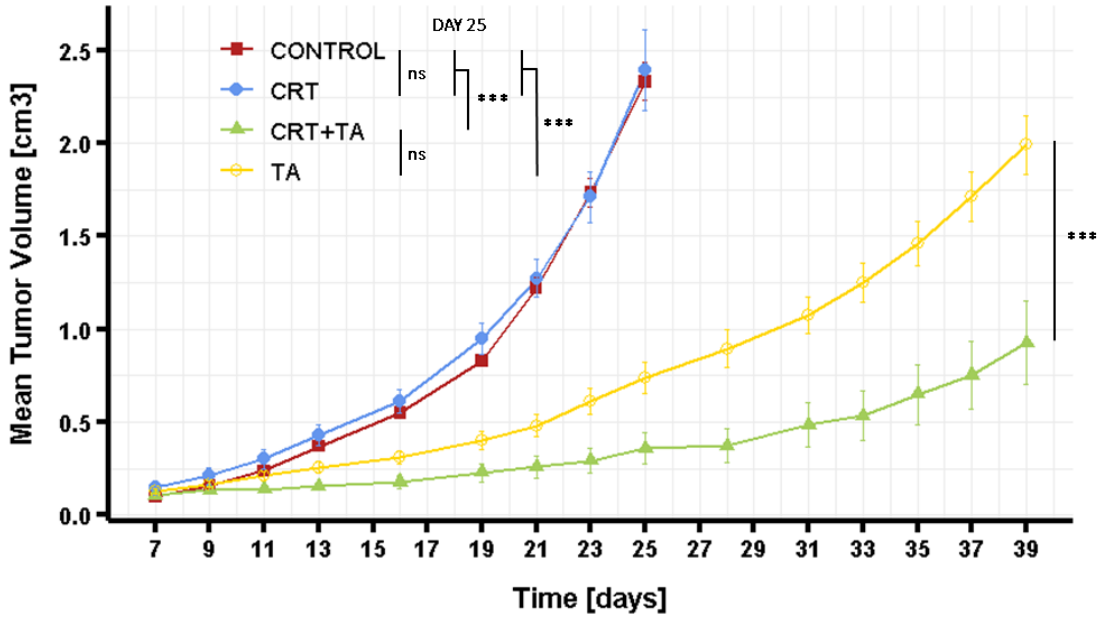


Figure 7. *MC38 murine model. The curves represent mean tumor growth over time for each treatment group: control (showed in red), CRT (shown in blue), TA (shown in yellow), CRT+TA (shown in green). Statistical analysis was performed using one-way ANOVA with post-hoc Tukey's multiple comparison test to compare all groups on day 25. Subsequently, Student's two-tailed t-test was used for pairwise comparisons between two surviving groups on day 39. The experiment was independently repeated twice, n = 8-10 mice/group. (ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)*

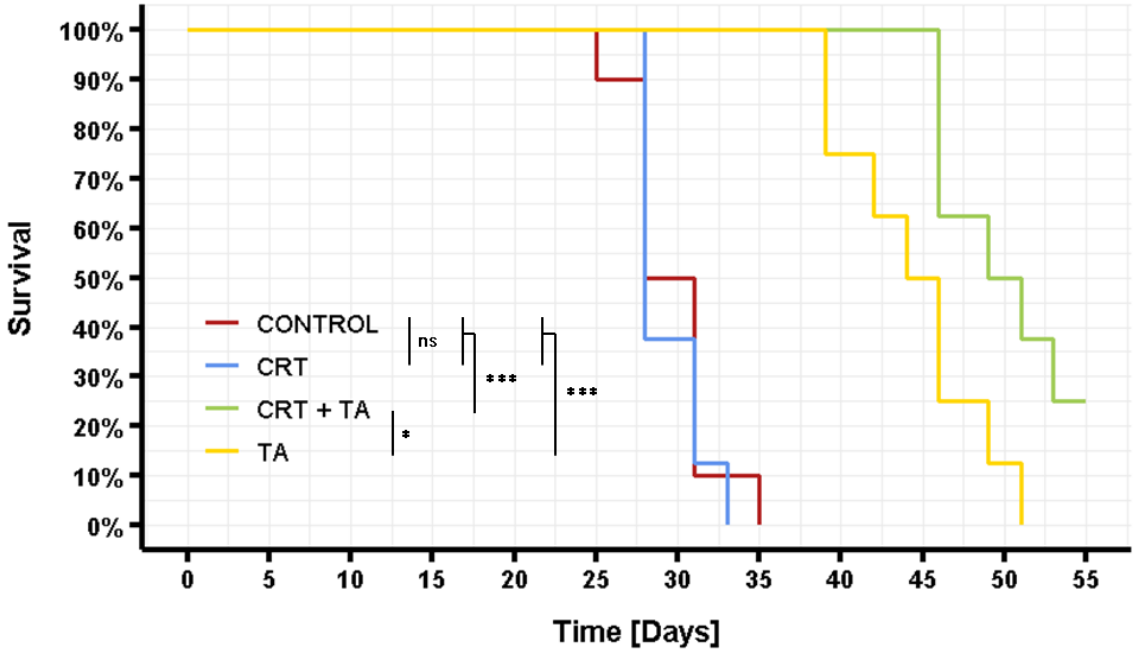
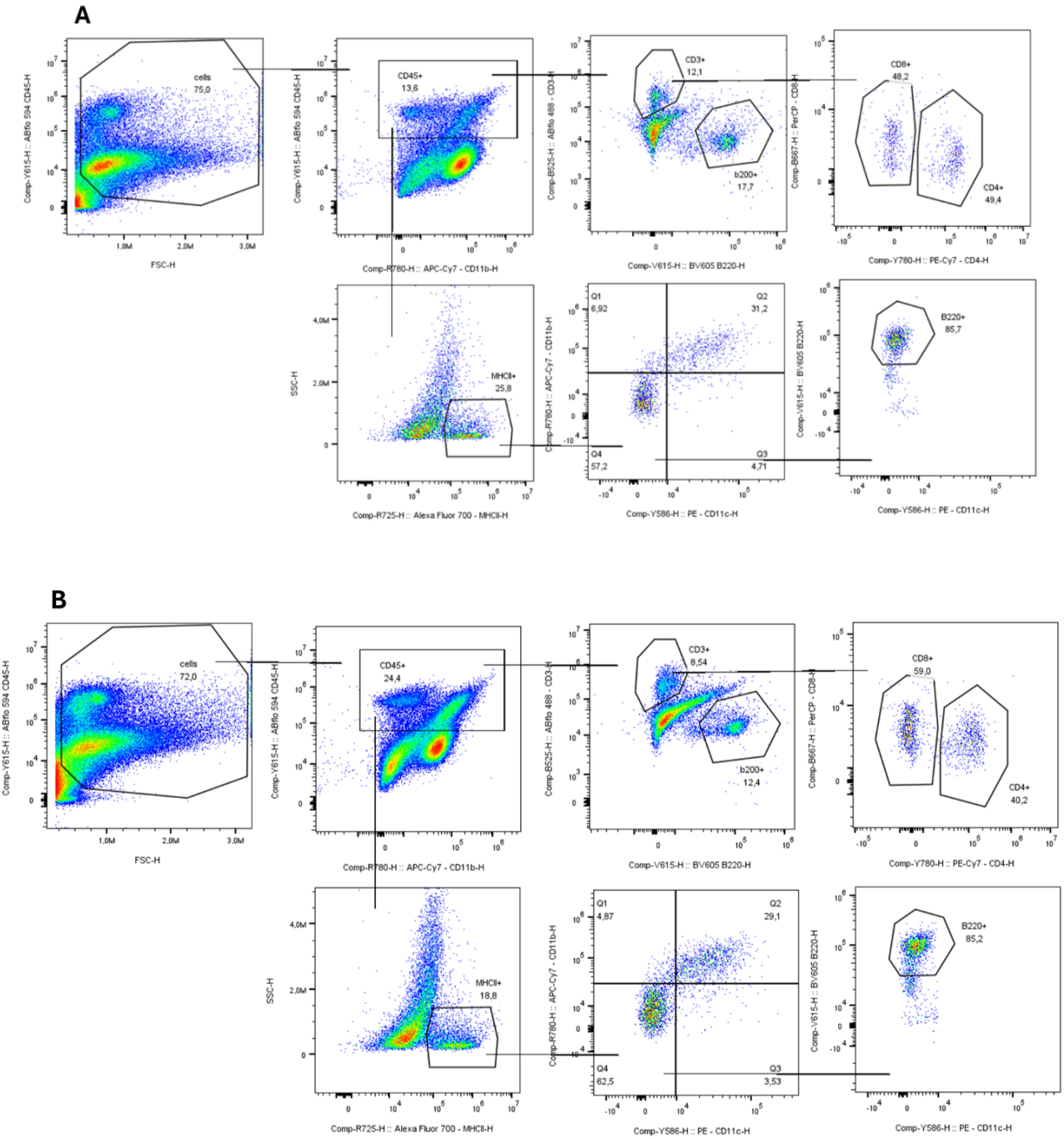


Figure 8*. MC38 colorectal carcinoma model. Kaplan-Meier survival curves show a comparison of overall survival over time between four treatment groups: control (shown in red), CRT (showed in blue), TA (shown in yellow), CRT+TA (shown in green). Statistical differences were evaluated using the log-rank test, n= 8-10 mice/group. (ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)*

5.2.2.2. Analysis of tumor-infiltrating lymphocytes

By day 25, tumors in the control and CRT groups had become too large to continue the treatment, so mice were euthanized. To enable comparative analysis across all experimental groups, four mice from the TA and TA+CRT groups were also sacrificed at the same time point. Tumors were collected for analysis of tumor-infiltrating lymphocytes by flow cytometry. The representative gating strategies applied in this experiment and obtained results are presented below (Fig. 8 and Fig. 9 respectively).



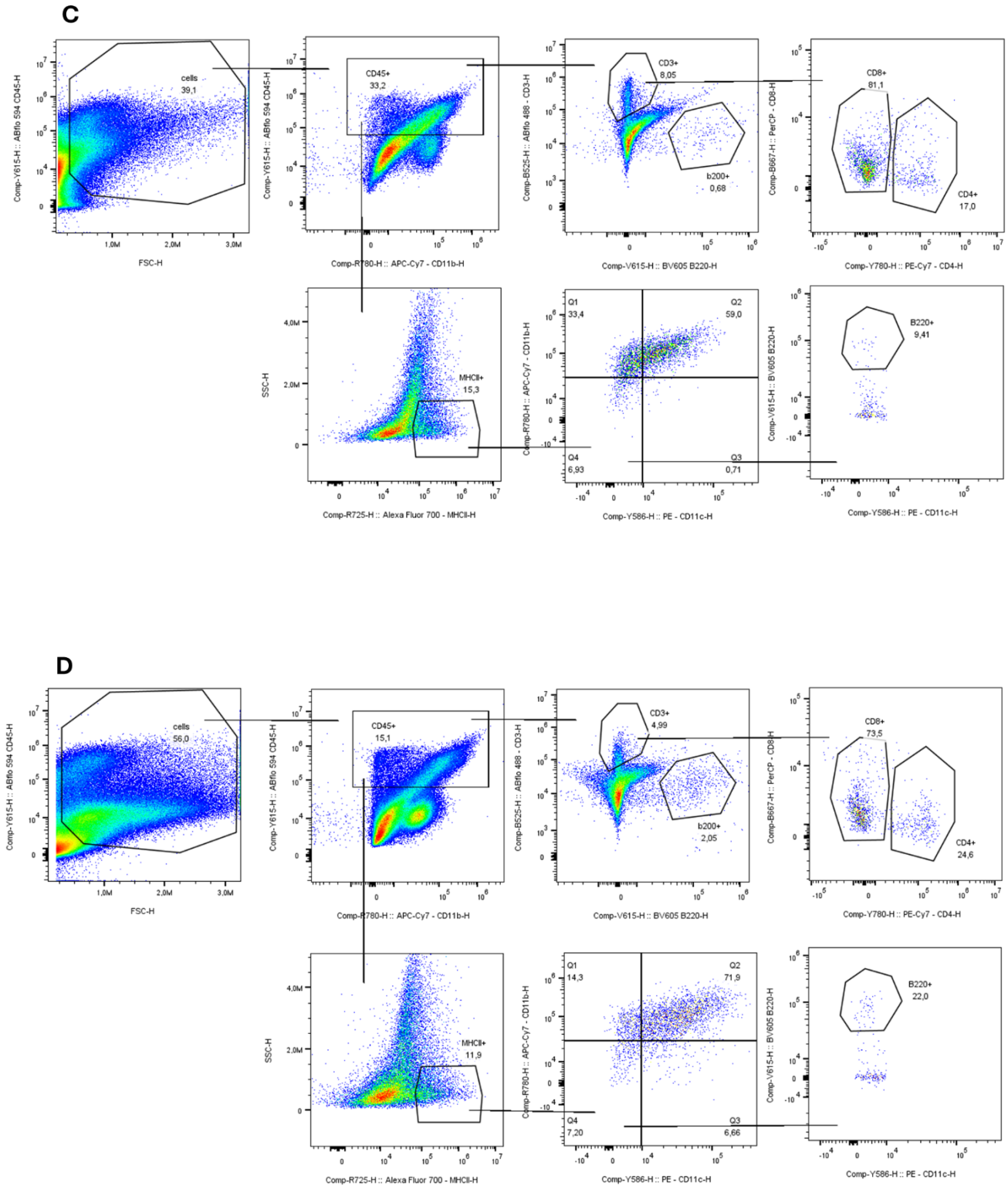


***Figure 9**. Representative gating strategies from MC38 tumors after treatment are shown for each experimental group: A – control, B – CRT, C – TA, D – CRT+TA.*

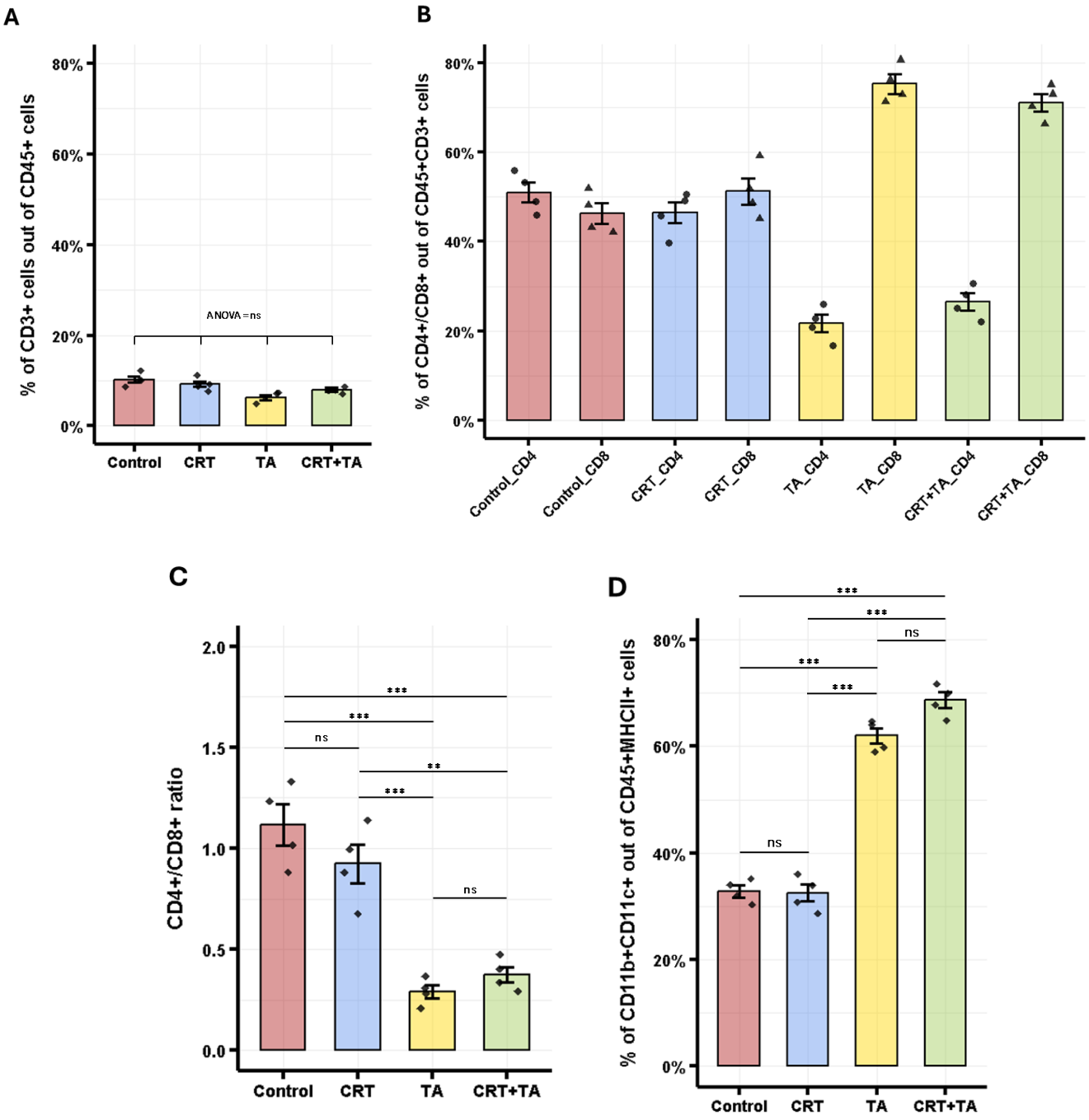


Figure 10. *Flow cytometry analysis of TILs. A – overall T cell infiltration, B – percentages of CD4+ and CD8+ T cells among tumor-infiltrating T cells, C – CD4+/CD8+ T cell ratio, D – CD11b+/CD11b+ cells among CD45+MHCII+ tumor-infiltrating APCs. Individual data points are shown. Statistical analysis was performed using one-way ANOVA with post-hoc Tukey's multiple comparison test (ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)*

Flow cytometric analysis of tumor-infiltrating lymphocytes revealed no differences in overall T cell infiltration (CD45+CD3+ cells) between all groups, suggesting that neither treatment significantly altered the total number of tumor-infiltrating T cells compared to the control group. However, tumors from the TA and CRT+TA groups exhibited a significantly lower CD4+/CD8+ ratio than those in the control and CRT groups. This indicates a marked shift toward CD8+ T cell dominance. In the both control and CRT-treated groups, CD4+ and CD8+ T cells were expressed at similar levels, suggesting a balanced T cell profile.

Additionally, we focused on antigen-presenting cells. Among MHC+ APC cells, the control and CRT groups showed higher proportion of CD11b-CD11c-, the majority of which were B220+, suggesting greater infiltration of B cells. In contrast, in the TA and CRT+TA groups, we observed a shift toward a higher proportion of CD11b+CD11c- and CD11b+CD11c+ cells, corresponding to monocytes, macrophages, MDSCs, and dendritic cells, respectively. Notably, the CRT+TA group showed the highest proportion of DCs, although this increase did not significantly differ from the TA-treated group.

5.2.2.3. Analysis of systemic cytokine response

Next, in order to assess the systemic cytokine responses induced by the therapeutic mixtures, we also measured plasma levels of IFN-γ and IL-10 using ELISA. The results revealed that the control and CRT groups exhibited minimal IFN-γ levels (around 20 pg/ml) with no statistical differences between them. In contrast, the mice treated with TA exhibited a significant increase in IFN-γ levels, reaching around 787 pg/ml. Interestingly, we observed that the CRT+TA group showed the highest levels of IFN-γ, approximately 936 pg/ml, which were significantly higher than all other groups. In contrast, IL-10 levels were below the detection limit in all treatment groups. These results suggest that the TA in combination with CRT can synergistically enhance systemic Th1-type immune response, as evidenced by elevated IFN-γ levels, without inducing compensatory immunosuppressive IL-10.

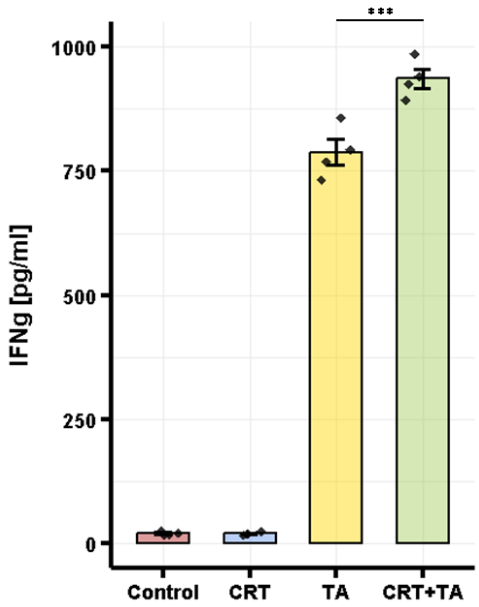


Figure 11. *IFN-γ concentrations in mice sera across treatment groups. Individual data points are shown. Statistical analysis was performed using one-way ANOVA with post-hoc Tukey's multiple comparison test (ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)*

5.2.2.4. Mice rechallenge

The remaining mice in the TA and CRT+TA groups completed the full course of therapy. In this experiment, as well as in the previous separate survival study, only mice from the CRT+TA group exhibited complete tumor regression (2 out of 8 in the current study, and 1 out of 8 in the survival experiment). To assess the development of immunological memory, these mice were rechallenged by s.c. injection of the same number of MC38 tumor cells as in the initial inoculation. None of the cured mice developed new tumors, suggesting the establishment of a durable and tumor-specific immune response.

6. Discussion

This thesis is focused on the investigation of calreticulin as a potential adjuvant in cancer immunotherapy. The primary aim was to explore an immunotherapeutic strategy designed to mimic the natural release of damage-associated molecular patterns, as occurs during immunogenic cell death. ICD is a form of regulated cell death that stimulates the immune response against cancer by releasing immunostimulatory signals. One of the key hallmarks of ICD is the translocation of CRT to the surface of tumor cells. The surface-exposure CRT acts as an "eat me" signal for macrophages, promoting tumor cell phagocytosis(Galluzzi *et al.*, 2023). We hypothesized that mimicking this process through an immunotherapeutic strategy could lead to increased phagocytosis of tumor cells and activation of the host immune response against cancer.

Firstly, the main goal was to generate various cancer cell lines expressing exogenous calreticulin and assess their ability to elicit cell phagocytosis *in vitro* and induce the anti-tumor immune response *in vivo* compared to corresponding control cells without stable expression of CRT on the cell surface. For this purpose, a plasmid was designed. The plasmid encodes CRT lacking the C-terminal KDEL retention sequence, which is responsible for keeping proteins localized within the ER. Loss of this signal leads to secretion of CRT into the extracellular space (Howe *et al.*, 2009). To ensure its localization to the cell surface, a DNA fragment encoding the GPI-anchor signal from the DAF protein was incorporated into the construct downstream of the CRT sequence (Legler et al., 2005). As a result, the engineered construct is supposed to produce a recombinant calreticulin anchored to the outer plasma membrane in lipid rafts through the GPI-anchor. The plasmid map and complete nucleotide sequence are shown in the supplementary section.

We attempted to transfect several cancer cell lines using a lipid-based transfection reagent Lipofectamine 3000 and electroporation. Although the integrity of the plasmid DNA was confirmed, transfection did not result in detectable CRT expression. Further analysis would be needed to identify the reasons why CRT is not expressed on the cell surface. For example, whether mRNA is expressed after transfection and whether CRT levels are increased in the cytoplasmic membrane instead of the plasma membrane. However, due to time constraints, we decided not to continue this research direction within this thesis.

Based on existing evidence that calreticulin can not only be expressed on the surface of dying tumor cells but can also be secreted into the extracellular space, thereby supporting the initiation of robust anti-tumor responses, we decided to investigate the potential use of CRT as an adjuvant in cancer intratumoral immunotherapy. As a reference approach, MBTA therapy has been chosen. MBTA is a therapeutic mixture, which contains mannan attached to BAM, a TLR agonists mix (resiquimod, poly(I:C), lipoteichoic acid) and additionally anti-CD40 antibody. This immunotherapeutic approach involves direct intratumoral injections of these agents to enhance phagocytosis of tumor cells through mannan-mediated opsonization and promote immune activation via TLR signalling and additional CD40 stimulation (Caisová *et al.*, 2018).

Mannan is a polysaccharide present on several types of bacteria, which is recognized by the immune system through mannose-binding lectin. MBL is encoded by the *MBL2* gene, and the presence of certain polymorphisms in this gene can reduce serum concentration of MBL and alter its functional activity, increasing susceptibility to different infections (Baioumy *et al.*, 2021; Sena *et al.*, 2022; Leman *et al.*, 2025). As a result, individuals with these genetic variations may not respond effectively to MBTA therapy. We hypothesized that CRT could be an alternative phagocytosis-stimulating agent suitable for a wider population. Therefore, we aimed to assess whether soluble CRT in a therapeutic mixture with TLR agonists could enhance immune activation and promote a more effective anti-tumor response. The treatment schedule has also been chosen based on the experiences from Dr. Zenka's laboratory. Recently, a study showing optimization of numbers of intratumoral injections of MBTA therapy was published. The results showed that the group treated with the 6x1 treatment schedule (six treatment cycles with one injection during each cycle) achieved the best survival outcomes (Uher *et al.*, 2025).

In this study, we used two cell types: murine melanoma (B16-F10) and murine colorectal carcinoma (MC38). Four treatment mixtures were used: control (treated with saline), CRT (treated with recombinant calreticulin only), TA (treated with TLR agonists mix), and CRT+TA (treated with a combination of recombinant calreticulin and TLR agonists mix). We observed a robust therapeutic response to immunotherapy with TA and CRT+TA in mice bearing MC38, whereas B16-F10-bearing mice showed a much lower response to these therapies under the same treatment conditions. Although the exact reasons remain unclear, we can hypothesize that various factors may likely contribute to the low immunogenicity of the B16-F10 melanoma model.

In our study, even the administration of TA alone had only a slight effect on tumor growth regression and survival outcomes in the B16-F10 model. These results are in contrasts to

findings from other studies that used different TLR agonists as i.t. immunotherapy in B16-F10 models and observed a stronger therapeutic response (Caisová *et al.*, 2018; Lee *et al.*, 2022). These differences may be caused by different numbers of administered cells. In our experiment, we used a higher initial cell dose compared to the studies that reported reduced tumor growth and much longer survival times, also leading to complete tumor regression compared to controls. Therefore, it may have resulted in more aggressive cancer progression from the beginning, which could partly explain the reduced therapeutic effect observed in our study. In addition, differences in the therapeutic plan and specific composition of TA in the therapeutic mixtures could also contribute to differences in efficacy.

A possible reason why the MC38 model responded to our therapy while B16-F10 did not is that the tumor microenvironment of B16-F10 tumors may be less immunogenic, thereby reducing the efficacy of immunotherapy. Based on the immunological profiles in the TME, tumors can be classified as immunologically "hot" or "cold," reflecting their response to immunotherapy. Hot tumors are typically immunologically active and characterized by high infiltration of immune effector cells, particularly CD8+ cytotoxic T lymphocytes. In contrast, immunologically cold tumors often exhibit an immunosuppressive environment that lacks immune effector cells (Wu *et al.*, 2024).

There is limited literature that investigates both B16-F10 and MC38 models at the same time in the context of immunotherapy. However, a study analyzing of the TME of six commonly used tumor models revealed significant differences in immunogenicity between MC38 and B16-F10 tumors. B16-F10 was characterized by lower infiltration of CD45+ lymphocytes, whereas MC38 showed higher immune cell infiltration, especially higher levels of CD8+ T cells. In athymic nude mice, MC38 tumors grew significantly faster than in wild-type mice, highlighting their high immunogenicity and strong connection with adaptive immune mechanisms, especially T cell-mediated responses. In contrast, B16-F10 showed similar tumor growth in both nude and wild-type mice, suggesting lower immunogenicity. Interestingly, MC38 also exhibited higher levels of Tregs than B16-F10. It could also reflect an active immune microenvironment, where the presence of activated CD8+ T cells triggered compensatory immunosuppressive mechanisms, by which tumors attempted to evade immune destruction (Carretta *et al.*, 2023).

Another study that used both models characterized B16-F10 tumors as less responsive to immunotherapy with ICIs. While both B16-F10 and MC38 tumors showed a significant increase in CD8+ T cell infiltration, the overall therapeutic response in B16-F10 tumors was

lower. The study suggested that this differential response may be related to different metabolic profiles between these two tumor types. Moreover, necrotic cell death induced as a result of lymphocyte-mediated anti-tumor response was more notable in MC38 tumors than in B16-F10. Additionally, ICI treatment led to improved blood perfusion in MC38 tumors, whereas only minimal changes were observed in B16-F10 tumors (Saida *et al.*, 2021). Together, these findings support the idea that the B16-F10 model is less sensitive to immunotherapy than the MC38 model.

To evaluate the underlying mechanisms driving anti-tumor response to the therapies in the MC38 model, we performed analysis of tumor-infiltrated lymphocytes. The tumor samples were collected on day 25, as most tumors in the control and CRT groups had reached excessively large sizes before the therapy was completed. In order to perform comparative analysis between all groups, some mice from the TA and TA+CRT groups were also sacrificed, tumors were harvested for flow cytometry analysis, and blood samples were collected for ELISA analysis. The remaining mice from the TA and CRT+TA groups completed the entire therapy course.

Regarding T cell populations, no significant differences were observed in the overall percentage of T cells among all groups. However, both TA and CRT+TA therapeutic mixtures induced a significant shift toward CD8+ T cell predominance. This finding reflects an enhanced anti-tumor immune response and may partially explain the improved therapeutic efficacy of these treatments. In contrast, in both control and CRT groups, CD4+ and CD8+ T cells were expressed at similar levels, suggesting a balanced T cell profile. The finding that the distribution of T cell subsets in the CRT group remained similar to the control group suggests that CRT alone does not significantly affect the composition of intratumoral T cell subtypes.

Next, we investigated the population of dendritic cells within the tumor microenvironment, based on the hypothesis that calreticulin exposure may influence DC infiltration and activation. In this study, we defined DCs as CD11c+CD11b+MHCII+ cells. However, it is important to note that this gating strategy may not fully distinguish DCs from macrophages, as these markers could be shared. Therefore, additional markers such as F4/80 would be needed for more detailed and accurate separation of the cell populations. We observed a trend toward a higher proportion of DCs among MHCII+ APCs in tumors treated with the combination therapy compared to TA alone, although the difference did not reach statistical significance. This observation suggests that the combination of TA with CRT may preferentially promote dendritic cell infiltration and subsequently more efficient antigen presentation to effector T cells.

The results obtained from the flow cytometry analysis are in line with observed tumor growth patterns. On day 25, when tumors were harvested, both TA and CRT+TA showed significantly smaller tumor volumes compared to the control and CRT-treated groups. At this time point, most mice in the control and CRT groups had developed excessively large tumors and therefore were sacrificed. The smallest mean tumor size was noted in the CRT+TA-treated group. However, this difference was not statistically significant compared to the TA-only group. After the completion of treatment, differences in tumor sizes between the TA and CRT+TA groups reached statistical significance, primarily due to the presence of rescued mice. In two independent experiments, complete tumor regression was observed only in the CRT-treated group, with 3 out of 16 mice achieving full remission. Upon rechallenging these mice with MC38 cells, no tumor growth was detected, indicating the development of long-term protective immunity.

We also investigated the differences in cytokine profiles among the treated mice. The obtained results provide important insights into the immune-modulatory effects of the tested treatments. Notably, mice receiving the combination therapy showed markedly increased levels of plasma IFN-γ, approximately 936 pg/ml, which was significantly higher than levels observed in the TA group (~787 pg/ml). This cytokine was almost absent in the CRT and control groups.

The significantly higher levels of IFN-γ in the CRT+TA group compared to the TA-only group may be explained by synergy of several factors. In this group, we also observed a noticeable trend toward a higher proportion of DCs. Moreover, previous studies have reported that recombinant CRT fragment can stimulate DCs to produce increased levels of IL-12 (Li et al., 2015; Gong et al., 2022). This cytokine is well known for its ability to stimulate CD8+ T cells to produce IFN-g (Wilson, Matthews and Yap, 2008; Vacaflores *et al.*, 2016). Consistent with this mechanism, we also observed a significant shift toward CD8+ T cells in tumors treated with CRT+TA, as well as with TA alone. Together, these findings support the hypothesis that CRT enhances the antitumor immune response through activation of DCs and subsequent stimulation of CTL. Additionally, it has been reported that splenocytes from mice which were s.c. immunized with OVAp-CRT showed increased IFN-*γ* production, significantly higher compared to mice immunized with OVAp alone (Gong *et al.*, 2022). This finding is also in line with our results.

In contrast, we observed that IL-10 levels remained below the detection limit across all treatment groups, indicating a lack of systemic immunosuppression. Its absence further shows

that CRT+TA immunization creates a favorable immune environment for antitumor activity without triggering regulatory suppressive mechanisms.

Overall, the trends observed in our results are in line with the known immunogenic properties of calreticulin as a DAMP and highlight its ability to enhance the efficacy of tumor immunotherapy when combined with TLR agonists. Treatment with CRT alone did not show any significant differences compared to control, suggesting that the additional stimulation signals are needed. The robust systemic cytokine response in the CRT+TA group and the presence of rescued mice that remained tumor-free even after rechallenging only in this group add further support for its potential as an effective strategy for inducing durable and potent anti-tumor immunity. However, the interpretation of these results is highly limited by several factors. Most notably, immune cell infiltration and systemic cytokine responses were analyzed only in the first single experiment with a small sample size, which significantly limits the statistical power of the obtained results. To confirm and strengthen these findings, future studies with larger sample sizes and additional replicates are necessary. Additionally, expanding the flow cytometry panel to include markers for other immune cell types such as macrophages, NK cells, and Tregs would also be beneficial for a broader understanding of the immunomodulatory effects of CRT-based therapies.

7. Conclusion

This thesis is focused on calreticulin-based immunotherapy, aiming to explore its potential role as adjuvant in modulating the anti-tumor immune response via intratumoral administration. We observed no effect on tumor growth, immune response, or overall survival, when CRT was used alone. However, the combination of CRT with a TLR agonist showed a synergistic effect, resulting in enhanced anti-tumor immunity and improved treatment outcomes.

Unfortunately, it is not yet possible to make a definitive conclusion from the current data. To get more meaningful understanding, further studies with are needed. However, based on the obtained results from *in vivo* experiments, flow cytometry analysis, and existing literature, we can suggest that calreticulin has potential as an additional component in cancer intratumoral immunotherapy, with the ability to support a more effective immune response against tumors.

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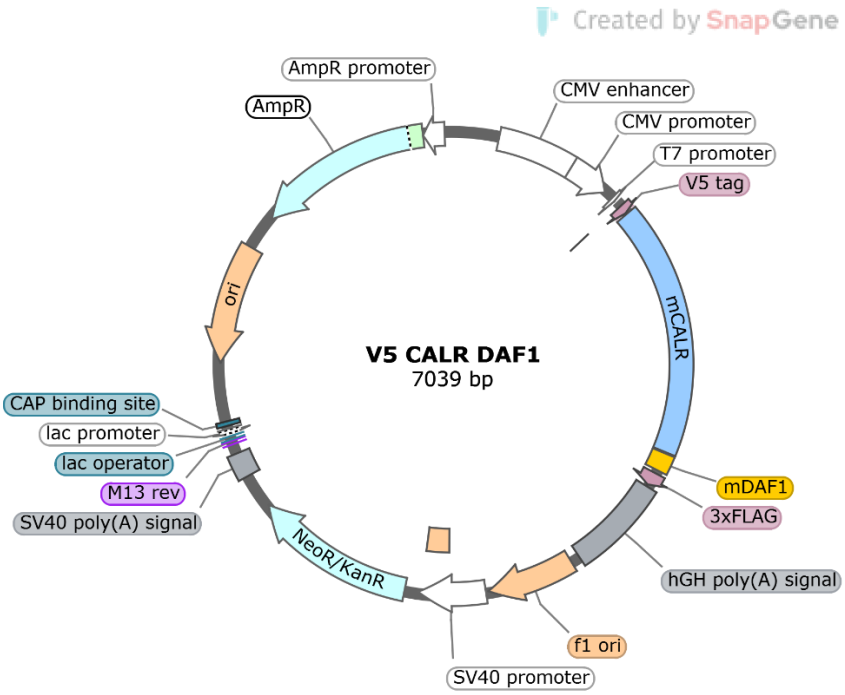
Supplementary

V5-mCALR-DAF1 plasmid map



V5-mCALR-DAF1 plasmid sequence

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