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Cytotoxicity of T cells induced by recoverin-based fusion protein against tumor cells

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Zusammenfassung:

Hintergrund: Krebs bleibt die führende Todesursache und eine erhebliche Hürde für eine erhöhte Lebenserwartung. Trotz bedeutender Fortschritte bei chirurgischen und chemoradiotherapeutischen Techniken ist ihre Wirksamkeit bei der Verlängerung des Überlebens von Krebspatienten begrenzt. Darüber hinaus wird die Behandlungseffektivität durch chirurgisches Wiederauftreten, entfernte Metastasen und die Entwicklung von Medikamentenresistenzen weiter beeinträchtigt. In den letzten Jahren hat sich die Immuntherapie als neuartige Behandlungsstrategie gezeigt, die vielversprechende Ergebnisse bei der Bewältigung dieser Herausforderungen erzielt. Unter den verschiedenen immuntherapeutischen Ansätzen haben Tumorimpfstoffe aufgrund ihrer Spezifität, Zugänglichkeit und minimalen Nebenwirkungen erhebliche Aufmerksamkeit erregt. Folglich besteht großes Interesse unter Forschern, Tumorimpfstoffe mit hoher Immunogenität zu identifizieren. Ein solcher potenzieller Kandidat ist Recoverin, ein Autoantigen, das hauptsächlich im Netzhautgewebe, insbesondere innerhalb der Blut-Retina-Barriere, vorkommt. Es wurde festgestellt, dass in verschiedenen Tumorzelltypen eine abnormale Anhäufung von Recoverin auftritt, die eine robuste Immunantwort auslöst, die sich gegen die Tumorzellen richtet. Darüber hinaus wurden hohe Expressionen des Recoverin-Proteins bei Tumorpatienten mit günstigen Prognosen in Verbindung gebracht, was auf sein Potenzial als Krebsimpfstoff hindeutet. Des Weiteren besitzt das Hitzeschockprotein 70 (HSP70) natürliche adjuvante Eigenschaften und soll die Immunogenität von Selbstantigenen verstärken.

Methoden: In unserer bisherigen Forschung haben wir gezeigt, dass die Fusion von Recoverin mit HLA-Klasse-I/II-Epitopen des HSP70-Proteins die Aktivierung von dendritischen Zellen (DCs) und CD8^{+}-T-Zellen stimuliert. In dieser Studie wollen wir das Proliferationspotenzial und die antitumorale zytotoxische Wirkung der durch das Recoverin-Fusionsprotein induzierten T-Zellen weiter untersuchen. Um die T-Zell-Proliferation zu bewerten, werden DCs, die durch das Recoverin-Fusionsprotein induziert wurden, mit CFSE-markierten T-Zellen kultiviert, und die Proliferationssignale werden mittels Durchflusszytometrie nachgewiesen. Darüber hinaus werden wir DCs, die durch das Recoverin-Fusionsprotein induziert wurden, mit T-Zellen kultivieren und diese anschließend nach einer zweiwöchigen Periode mit Y79-Zellen kultivieren. Der Prozentsatz der 7-AAD[+] Y79-Zellen, gemessen mittels Durchflusszytometrie, wird als Indikator für die durch das Recoverin-Fusionsprotein induzierte antitumorale Wirksamkeit verwendet.

Ergebnisse: Unsere Ergebnisse zeigen, dass die Kultivierung von durch das Recoverin-Fusionsprotein induzierten dendritischen Zellen (DCs) mit T-Zellen zu einem signifikanten CFSE-Signal führt, das auf eine robuste T-Zell-Proliferation hinweist. Darüber hinaus wird bei nachfolgender Kultivierung dieser T-Zellen mit Y79-Zellen ein höherer Prozentsatz von 7-AAD$^+$ Signalen, die von den Y79-Zellen stammen, nachgewiesen, was auf eine verbesserte zytotoxische Wirkung hindeutet.

Schlussfolgerung: Im Vergleich zur separaten Verabreichung von Recoverin-Peptiden oder HSP70 zeigt das Recoverin-Fusionsprotein eine überlegene Immunogenität und hebt sein Potenzial als mögeicher Krebsimpfstoff hervor.

Abstract:

Background: Cancer remains the leading cause of mortality and a significant barrier to increasing life expectancy. Despite significant advancements in surgical and chemoradiotherapy techniques, their efficacy in prolonging cancer patient survival is limited. Moreover, treatment effectiveness is further compromised by surgical relapse, distant metastasis, and the development of drug resistance. In recent years, the emergence of immunotherapy as a novel treatment strategy has shown promising results in addressing these challenges. Among the various immunotherapeutic approaches, tumor vaccines have gained considerable attention due to their specificity, accessibility, and minimal side effects. Consequently, there is great interest among researchers in identifying tumor vaccines with high immunogenicity. One such potential candidate is Recoverin, an autoantigen primarily found in the retinal tissue, specifically localized within the blood-retina barrier. Notably, abnormal accumulation of Recoverin has been observed in several tumor cell types, triggering a robust immune response that targets the tumor cells. Additionally, high expression levels of Recoverin protein in tumor patients have been associated with favorable prognoses, suggesting its potential as a cancer vaccine. Furthermore, heat shock protein 70 (HSP70) possesses natural adjuvant properties and is believed to enhance the immunogenicity of self-antigens.

Methods: In our previous research, we demonstrated that fusion of Recoverin with HLA class I and II epitopes of HSP70 protein stimulates the activation of dendritic cells (DCs) and CD8+ T cells. In this study, we aim to further investigate the proliferative capacity and anti-tumor cytotoxic effects of T cells induced by the Recoverin fusion protein. To assess T cell proliferation, DCs induced by the Recoverin fusion protein will be co-cultured with CFSE-labeled T cells, and flow cytometry will be employed to detect proliferation signals. Additionally, we co-cultured DCs induced by the Recoverin fusion protein with T cells, followed by co-culturing with Y79 cells after a two-week period. The percentage of 7-AAD+ Y79 cells, measured by flow cytometry, will be utilized as an indicator of the anti-tumor efficacy induced by the Recoverin fusion protein.

Results: Our results demonstrate that co-culturing DCs induced by the Recoverin fusion protein with T cells leads to a significant CFSE signal, indicating robust T cell proliferation. Furthermore, when these T cells are subsequently co-cultured with Y79 cells, a higher percentage of 7-AAD+ signals originating from Y79 cells is detected, suggesting an enhanced cytotoxic effect.

Conclusion: In comparison to the separate administration of Recoverin peptides or HSP70, the Recoverin fusion protein exhibits superior immunogenicity, highlighting its potential as an cancer vaccine.

List of abbreviations

%	Percentage
°C	Degree Celsius
μg	Microgram
μl	Microliter
ANOVA	Analysis of variance
RNAi	RNA Interference
TSAs	Tumor-specific antigens
BSA	Bovine serum albumin
TAAs	Tumor-associated antigens
CARs	Chimeric antigen receptors
CAR-T	Chimeric antigen receptor T cell therapy
CAR	Cancer-associated retinopathy
ACT	Adaptive cellular transfer
IL	Interleukin
IFN	Interferon
HD	High-dose
ICB	Immune checkpoint blockade
PD-1	Programmed cell death protein 1
DNA	Deoxyribonucleic acid
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
EDTA	Ethylenediaminetetraacetic acid
LAG-3	Lymphocyte-activation gene 3
FDA	Food and Drug Administration
HPV	Human papillomavirus
FP	Fusion protein
g	Gram

L	Liter
mg	Milligram
ml	Milliliter
U	Unit
pept	Peptide
PGE2	Prostaglandin E2
RNA	Ribonucleic acid
SCLC	Small cell lung cancer
HCC	Hepatocellular carcinoma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRK1	Rhodopsin kinase
GPCR	G-protein-coupled receptor
HSP	Heat shock protein
TAMs	Tumor-associated macrophages
MDSCs	Myeloid-derived suppressor cells
CTL	Cytotoxic T lymphocyte
iDC	Immature dendritic cell
mDC	Mature dendritic cell
TME	Tumor microenvironment
PVDF	Polyvinylidene fluoride
BSA	Bovine serum albumin
SD	Standard deviation
PBMC	Peripheral blood mononuclear cell
RCVRN	Recoverin
HLA	Human leukocyte antigen
TCR	T cell receptor

1. Introduction

Cancer represents a severe global health issue, imposing a substantial burden on both human life and the economy. Despite some progress achieved in traditional tumor treatment approaches, their limitations have become increasingly evident. Therefore, the pursuit of more effective therapeutic methods has become a focal point in current tumor research. As an emerging treatment approach, tumor immunotherapy has demonstrated immense potential and has recently achieved significant breakthroughs.

1.1　Global burden of cancer

Cancer stands as a primary cause of death and disease burden worldwide. According to data from the World Health Organization, millions of people are diagnosed with various types of cancer each year, and cancer-related mortality rates remain alarmingly high. This situation is particularly dire in developing countries, where inadequate medical resources and limited education contribute to an even more challenging tumor prevention and control landscape.

1.2　Traditional cancer treatment approaches and emerging therapeutic strategies

Traditional cancer treatment approaches, including surgical resection, chemotherapy, and radiation therapy, have long served as the cornerstone of cancer management [1-3]. However, these methods present certain limitations that constrain their application in specific cases [4-6]. Surgical resection may be hindered by factors such as tumor location and the overall condition of the patient, which can render complete tumor removal unfeasible, particularly when tumors are situated near critical organs or blood vessels [7]. Additionally, chemotherapy and radiation therapy often result in non-specific damage to both tumor and normal cells, leading to side effects and toxicities such as nausea, vomiting, hair loss, and immune suppression, which impose additional burdens on patients' physical and psychological well-being [8, 9]. Furthermore, certain tumors exhibit resistance to conventional treatment methods, significantly compromising treatment efficacy [10, 11]. Consequently, with the advent of biotechnology, personalized precision therapies targeting tumor cells have garnered widespread attention and have yielded exciting outcomes (**Figure 1.2**).
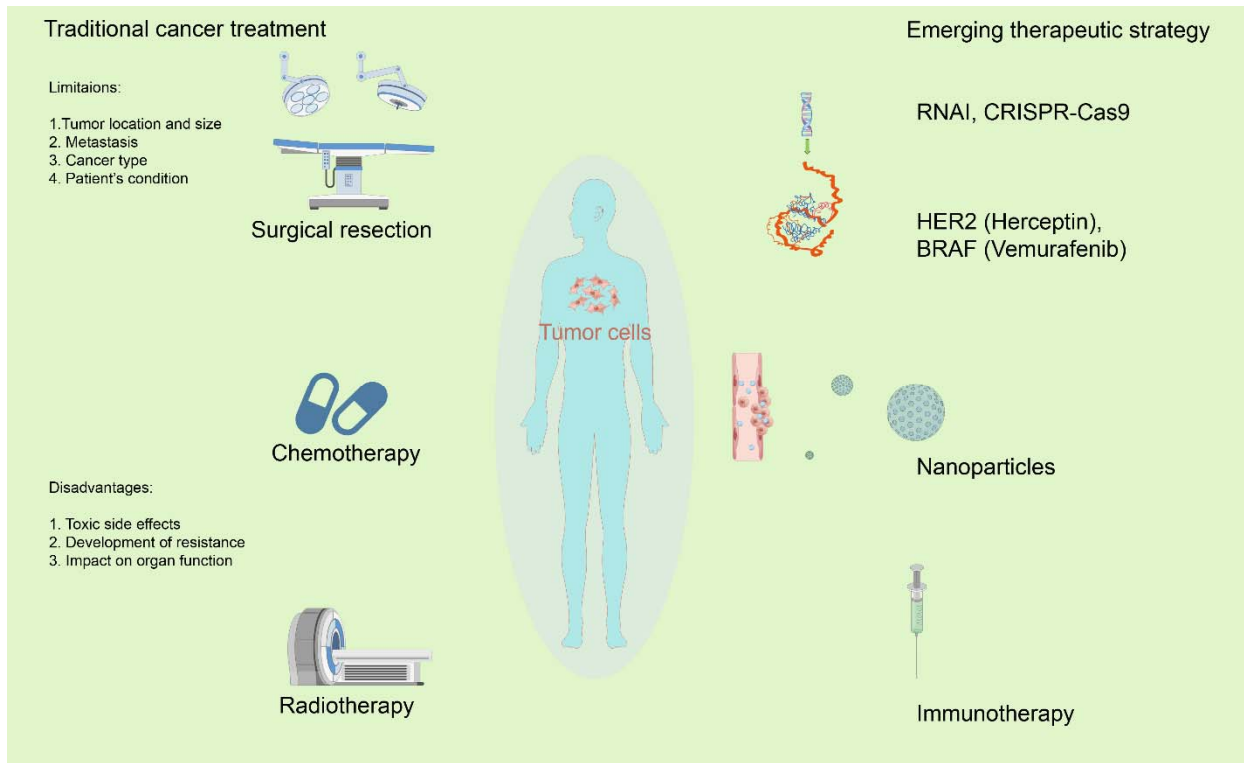
Figure 1.2: Traditional cancer treatment approaches and emerging therapeutic strategies (original art).

1. Targeted Therapy Based on Gene Mutations: Some cancers harbor specific genetic mutations that drive abnormal proliferation and survival of cancer cells [12, 13]. By identifying and targeting these specific gene mutations, targeted therapeutic drugs can effectively inhibit tumor cell growth. For instance, breast cancer patients with HER2 gene mutations can undergo treatment with trastuzumab (Herceptin), which suppresses HER2 receptor activity, thus impeding cancer cell proliferation [13, 14].

2. Targeted Therapy Based on Gene Expression: By analyzing the gene expression patterns of tumor cells, specific genes or proteins with aberrant expression can be identified [15, 16]. Drugs targeting these aberrantly expressed targets can be developed to interfere with tumor cell growth and survival [17]. For example, melanoma patients with BRAF gene mutations can receive treatment with vemurafenib, a drug that selectively inhibits mutated BRAF protein kinase activity, thereby disrupting cancer cell signaling pathways [18, 19].

3. RNA Interference (RNAi) Technology: RNAi technology offers a precise and targeted therapeutic approach by suppressing the expression of specific genes in tumor cells [20]. By employing small RNA molecules (siRNA or miRNA) that bind to target genes in tumor cells,

disease-causing genes in patients' tumor cells can be selectively silenced [21]. Researchers have developed a nanoparticle-based small RNA delivery system that encapsulates siRNA or miRNA within nanoparticles carrying specific targeting molecules [22]. This technology enables selective binding to tumor cells, interfering with the overexpression of target proteins resulting from mutations and thus inhibiting tumor cell growth and metastasis [23].

4. Gene Editing Therapy: Gene editing technologies, such as CRISPR-Cas9, have witnessed breakthrough progress [24]. By utilizing gene editing tools, critical genes in tumor cells can be precisely edited to inhibit their growth or promote cell death [25]. For instance, scientists have employed gene editing techniques to suppress the activity of the BCR-ABL gene, a key driver of chronic myeloid leukemia, in certain leukemia patients [26].

5. Nanomaterial-based Tumor Photothermal Therapy: Photothermal therapy utilizes nanomaterials to absorb and convert external light energy, generating a thermal effect that destroys tumor cells [27]. Researchers have successfully developed a variety of nanomaterials, including gold nanoparticles, carbon nanotubes, and iron oxide nanoparticles, for photothermal therapy. Upon exposure to specific wavelengths of light, these nanomaterials absorb light energy and induce a thermal effect, leading to localized heating and destruction of tumor cells, thereby achieving precise tumor treatment [28]. In summary, nanomaterials hold significant potential in personalized and precise tumor treatment. Nanoparticle-based drug delivery systems enable targeted therapy of tumor cells, while nanomaterial-based photothermal therapy allows localized destruction of tumor cells [29].

6. Metabolism-Targeted Therapy: Tumor cells exhibit distinct metabolic characteristics compared to normal cells, providing an opportunity for targeted therapy [30]. Metabolism-targeted therapy aims to interfere with the metabolic pathways of tumor cells by inhibiting key metabolic enzymes or regulating metabolic pathways, disrupting tumor cell survival and proliferation abilities [31]. This strategy relies on an in-depth understanding of tumor cell metabolic pathways and interventions tailored to the specific metabolic features of tumor cells. A typical example is lipid metabolism-targeted therapy for breast cancer. Breast cancer cells often display abnormal lipid metabolism activity, exhibiting increased reliance on lipid synthesis and utilization. Researchers leverage this characteristic to transform breast cancer cells into fat cells, achieving an anti-tumor effect [32].

7. Immunotherapy: Immunotherapy is a treatment method that harnesses the body's immune system to combat diseases. It involves activating, enhancing, or regulating immune system functions to recognize, attack, and eliminate tumor cells [33]. Mechanisms of immunotherapy

encompass the use of immune modulators, cell vaccines, antibody therapies, gene-engineered cell therapies, and more, to bolster the body's immune system defenses and clearance capabilities against diseases [34]. Immunotherapy showcases broad application prospects in the field of cancer treatment, and the remarkable results of some clinical trials have made it the most vibrant and captivating area of research currently [35, 36].

1.3   Immunotherapy strategies in cancer

1.3.1   Theoretical foundations

Immunotherapy represents a therapeutic approach that harnesses the power of the immune system to combat tumor cells by modulating and enhancing its activity. At its core, this treatment method relies on the utilization of tumor cell mutations and antigens, as well as the restoration of tumor cell recognition, to activate the immune system's response against cancer. The following key theoretical foundations underpin immunotherapy:

1. Tumor Cell Mutations: One crucial theoretical foundation of immunotherapy lies in the recognition and targeting of tumor cell mutations [37]. During the course of tumor development, genetic alterations occur within the cellular genome, resulting in discernible genetic disparities between tumor cells and their normal counterparts. These mutations give rise to novel tumor-specific antigens (TSAs) that are absent in normal cells [38]. By identifying these specific antigens, the immune system can selectively eliminate tumor cells while sparing normal cells.

2. Tumor-Associated Antigens: Another vital theoretical foundation is based on immunotherapy's ability to target tumor-associated antigens (TAAs) [39]. TAAs are characterized by their overexpression on the surface of tumor cells, with higher expression levels compared to their presence in normal cells [40]. Immunotherapy interventions stimulate the immune system to mount an immune response against TAAs, thereby facilitating the recognition and attack of tumor cells [41].

3. Restoration of Tumor Cell Recognition: Immunotherapy also encompasses strategies to restore the immune system's recognition of tumor cells. Tumor cells employ various mechanisms to evade immune surveillance, including the inhibition of T-cell activation, establishment of an immunosuppressive microenvironment, and downregulation of tumor antigen expression. Immunotherapy interventions counteract these escape mechanisms, enabling the immune system to recognize and effectively target tumor cells [42, 43]. For

instance, the inhibition of immune checkpoint molecules, such as CTLA-4 and PD-1, expressed on tumor cells enhances the cytotoxic activity of T cells against tumors [44, 45].

4. Modification and Enhancement of Immune Cells: Modifying and enhancing immune cells represent another critical theoretical foundation of immunotherapy. CAR-T cell therapy exemplifies this approach, involving the extraction of a patient's own T cells, subsequent genetic modification to express chimeric antigen receptors (CARs) specific to tumor antigens [46]. These engineered CAR-T cells possess the capability to recognize and directly attack tumor cells, while undergoing robust proliferation within the body, thereby establishing a sustained anti-tumor effect [47].

In the past decade, clinical trials exploring various theoretical foundations of immunotherapy have demonstrated immense potential in combating cancer, even resulting in complete remission for select individuals [48]. Although the current benefit of immunotherapy is limited to a minority of tumor patients, the future outlook for this treatment approach is highly promising.

1.3.2 Types of immunotherapeutic strategies

In the field of cancer treatment, a series of pioneering approaches have emerged, notably encompassing cytokine therapy, cancer vaccines, immune checkpoint inhibitors, and adaptive cellular transfer (ACT) therapy (**Figure 1.3.2**) [49]. These innovative strategies collectively underscore substantial progress within this domain, striving to incite robust anti-tumoral responses within patients and thereby providing more effective and targeted choices for managing malignancies.
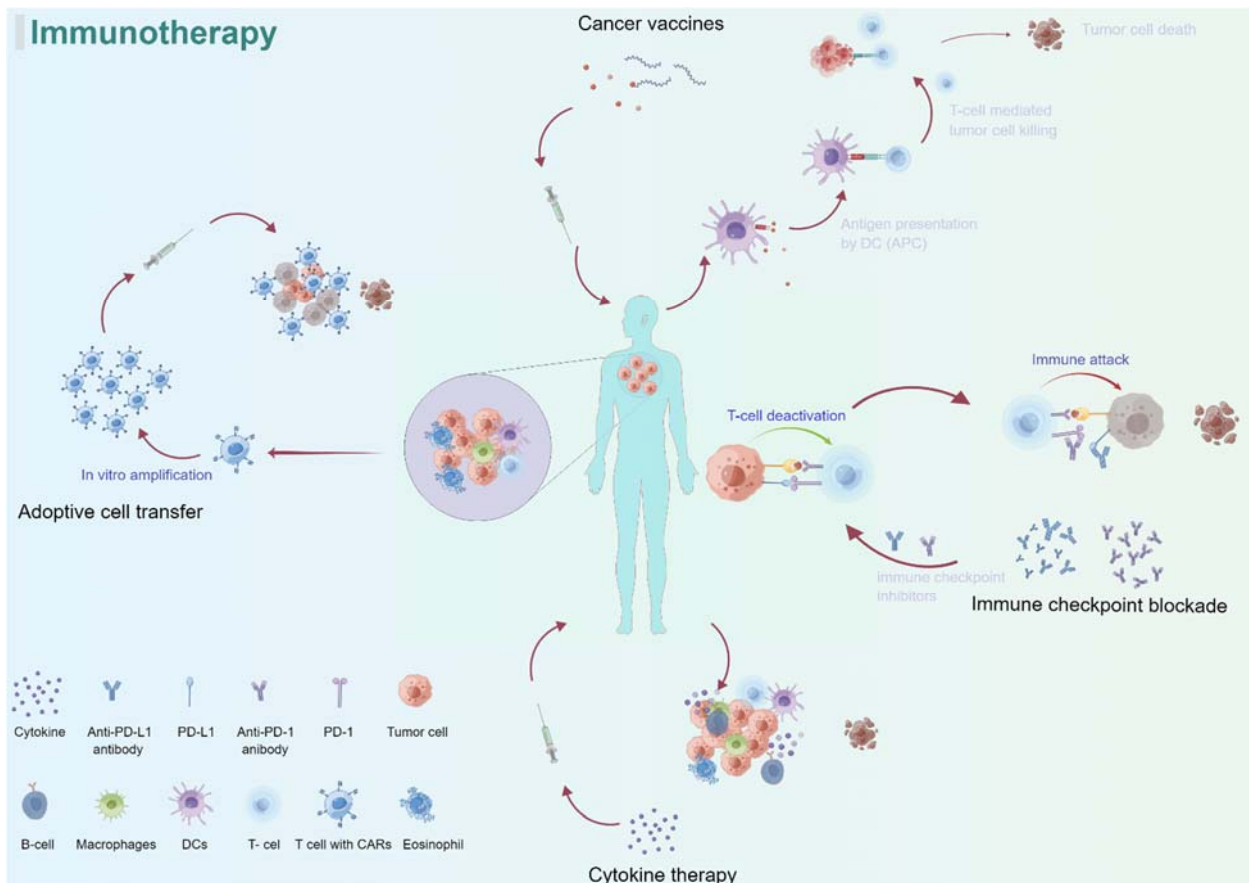
Figure 1.3.2 Four types of immunotherapeutic strategies (original art).

Cytokine therapy is primarily geared towards stimulating immunological mechanisms, orchestrating the amplification of immune cell activity to enhance the precision of targeted responses against solid tumors [50]. In contrast, immune checkpoint inhibitors offer a distinctive perspective. They intervene in the suppressive interplay between malignant and immune cells, reinstating immune cells' capacity to mount an assault against tumors, consequently enhancing therapeutic outcomes [51]. The strategy of Adoptive Cell Transfer (ACT) therapy involves the harvesting and subsequent expansion of autologous immune cells, particularly T cells. Following their activation, expansion, and restoration to vitality, these cells are reintroduced into the patient's physiological milieu, thereby enabling more finely-tuned targeted responses against tumor cells [52]. Simultaneously, cancer vaccines represent a proactive immunization approach [53]. Meticulously designed on the foundation of tumor antigens, these formulations incite tumor-specific immune reactions. The result orchestrates an immune mechanism adept at discriminating and eradicating cancerous cells, thereby achieving a precisely focused therapeutic effect.

Nevertheless, it is imperative to acknowledge that these methodologies are continuously evolving, necessitating ongoing research and refinement to maximize therapeutic efficacy while mitigating potential drawbacks. This progressive approach holds the promise of progressively enhancing the outlook for the cohort of individuals afflicted by cancer.

1.3.2.1 Cytokine therapy

Cytokines play a pivotal role in the regulation of cellular activities and are produced by various cell types. They play a critical role in the interplay and modulation of immune cells, contributing to the regulation and activation of immune responses. These multifunctional molecules are capable of activating and enhancing immune cell functions, promoting anti-tumor immune responses, as well as inhibiting tumor growth and metastasis.

Several cytokines with potent anti-tumor activities have been identified, including interleukin (IL)-2, IL-12, IL-15, interferon (IFN)-α, and IFN-γ [54-57]. These cytokines exert their effects through diverse mechanisms to enhance the response of the immune system against tumors. Firstly, cytokines can directly stimulate and augment immune cell functions. For instance, IL-2, a potent T-cell growth factor, facilitates T-cell proliferation, functional maturation, and enhances their cytotoxicity against tumor cells [58, 59]. IL-12 and IL-15 promote the activity of natural killer (NK) cells, reinforcing their ability to eliminate tumor cells [60, 61]. IFN-α and IFN-γ exert antitumor effects by suppressing tumor cell proliferation and inducing apoptosis [62, 63].

Additionally, cytokines play a significant role in modulating the tumor microenvironment, influencing immune cell infiltration and function. The tumor microenvironment comprises a complex network of cells and molecules that contribute to immune suppression. Cytokines possess the capability to modify the immunosuppressive characteristics of the tumor microenvironment, thereby increasing immune cell infiltration and activity. For example, IL-2 and IL-12 have been shown to enhance the infiltration of lymphocytes into tumors, augmenting their antitumor response [64, 65]. IFN-γ can counteract immunosuppressive factors within the tumor microenvironment, thereby reducing immune evasion. Notably, IL-7, as a cytokine therapy in cancer treatment, exhibits superior activity compared to IL-2 in inducing the expansion of tumor-specific T cells in breast cancer, highlighting its potential as an adjunctive molecule in the field of oncology [66]. Studies have demonstrated that IL-7 can restore the activity of CD8+ T cells by downregulating PD-1 expression [67]; Furthermore, in

addition to its impact on T cells, IL-7 has been shown to regulate immune responses in B cells, dendritic cells, and natural killer cells with precision [68-70]. Recently, novel cytokines have been identified as secreted immune checkpoints, demonstrating their potential in immune modulation for therapeutic interventions [71].

High-dose (HD)-IL-2 (aldesleukin) has received approval from the U.S. Food and Drug Administration (FDA) for the treatment of specific solid tumors [72]. Furthermore, cytokines can be synergistically combined with other therapeutic strategies to enhance treatment efficacy. By augmenting the recognition of tumor antigens and immune cell responses, cytokines can be integrated with tumor antigen-targeted therapies. Moreover, the combination of cytokines with immune checkpoint inhibitors can enhance anti-tumor immune responses by activating immune cells and inhibiting immunosuppressive pathways [73-75]. Several ongoing clinical trials are investigating the combined use of cytokines with adoptive cell transfer (ACT). Studies have reported that the co-administration of low-dose IL-2 and adoptive T cell therapy significantly prolongs the survival of T cells in patients with metastatic melanoma compared to those without IL-2 administration [76].

Despite the immense potential of cytokine-based anti-tumor immunotherapy, several challenges and limitations persist. Firstly, certain cytokines exhibit severe toxic side effects, which restrict their clinical utility. Secondly, the preparation and delivery techniques of cytokines still present challenges that necessitate further refinement and optimization. Moreover, the intricate nature of the tumor microenvironment hampers the efficacy of cytokine-based interventions.

1.3.2.2 Immune checkpoint blockade (ICB)

Anti-tumor immune therapy based on immune checkpoint blockade (ICB) has emerged as a promising therapeutic approach aimed at leveraging the body's immune system to attack and eliminate tumor cells [77]. Immune checkpoints encompass a group of molecules that normally maintain immune system homeostasis, preventing excessive activation and autoimmunity. However, tumor cells exploit these checkpoints to evade immune surveillance, facilitating tumor growth and dissemination. Notably, the interaction between programmed cell death protein 1 (PD-1) on activated T lymphocytes and its ligand PD-L1 on tumor cells results in the immune evasion of tumor cells by T lymphocytes [78].

The essence of ICB therapy lies in the inhibition of immune checkpoint molecules, which

serves to unleash potential anti-tumor immune responses. This therapeutic strategy predominantly employs antibodies to block immune checkpoints, including cytotoxic T lymphocyte-associated protein 4 (CTLA-4), PD-1, and PD-L1, with the objective of reinstating and reinforcing the body's immune response [79]. Immune checkpoint inhibitors have exhibited encouraging clinical outcomes across a variety of malignancies [80]. The CTLA-4 monoclonal antibody Ipilimumab was the first immune checkpoint inhibitor to gain approval from the US FDA [81], followed by PD-1 antibodies (Nivolumab and Pembrolizumab) receiving market authorization in late 2014. Two years later, Atezolizumab, the first PD-L1 antibody, obtained regulatory approval [82]. These agents have been sanctioned for the treatment of diverse solid tumors and hematologic malignancies [83-85], heralding a new era in clinical anti-tumor therapy through ICB.

The mechanisms of anti-tumor immune therapy encompass multifaceted interactions [86]. Initially, the inhibition of CTLA-4 impedes the transmission of inhibitory signals on immune cell surfaces, thereby augmenting the activation and proliferation of T cells. Subsequently, the disruption of PD-1/PD-L1 binding prevents immune escape signals between tumor cells and immune cells, enabling immune cells to identify and eliminate tumor cells. Nevertheless, certain PD-L1+ tumors do not respond to PD-L1 immune therapy, necessitating the identification of additional tumor immunogenic markers. These may include other immune checkpoint molecules expressed on tumor cells and the surrounding stromal cells, such as lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) [79, 83, 87-89]. The collective actions of these mechanisms ultimately facilitate immune system reconstitution and restoration, enabling the containment of tumor cell proliferation and dissemination.

Despite the remarkable therapeutic efficacy of ICB strategies in anti-tumor immune therapy, only a minority of patients exhibit durable responses [85, 90, 91]. One contributing factor is the capacity of tumor cells to downregulate the expression of surface proteins, thereby diminishing immune recognition and attack. Additionally, the presence of immune inhibitory factors within the tumor microenvironment can impede immune cell infiltration and function [92]. To enhance the effectiveness of anti-tumor immune therapy, ongoing research focuses on combining different immune checkpoint inhibitors [93], as well as their integration with other treatment modalities such as chemotherapy, radiation therapy, and targeted therapy [84, 94, 95]. Moreover, personalized treatment strategies represent a pivotal research avenue, leveraging the genomic and immunological characteristics of individual patients to

tailor optimal therapeutic regimens [96-98].

Further investigation into tumor immune evasion mechanisms and potential resistance mechanisms assumes paramount significance. For tumors harboring sufficient antigenicity and immunogenicity, immune therapy necessitates a dual focus on strategies that enhance tumor-specific immune responses, such as vaccines and/or T cell receptor-engineered T cells (TCR-T), as well as strategies that augment T cell-mediated cytotoxicity against tumor cells, such as chimeric antigen receptor-engineered T cells (CAR-T) [34, 99]. In summary, immunotherapy based on immune checkpoint blockade represents a forefront therapeutic approach that activates the body's immune system to combat tumor cells. Although challenges such as immune evasion, treatment resistance, and off-target effects persist [100-102], ongoing research and development hold the promise of improving the clinical outcomes for cancer patients.

1.3.2.3 Adoptive cell transfer (ACT) therapy

In recent years, the application of autologous T lymphocytes in cancer therapy has attracted significant attention, leading to numerous ongoing clinical trials [103]. T lymphocytes play a crucial role in tumor immune responses and possess potent cytotoxic capabilities. Adoptive Cell Transfer (ACT) Therapy is an immunotherapy technique aimed at targeting tumor cells by enhancing the recognition abilities of lymphocytes, particularly T cells [104]. This approach is distinct from non-specific immune stimulation and immune checkpoint blockade, as it focuses on actively directing immune cells to attack tumors, rather than merely augmenting pre-existing immune responses. In simple terms, the underlying principle of this therapy involves extracting a patient's immune cells, genetically modifying or activating them to enhance their recognition capabilities, and subsequently reintroducing these modified immune cells back into the patient's body [105]. These therapies hold promise for targeting immunologically poor tumors and inducing durable responses [106]. ACT therapy primarily encompasses two novel cellular immunotherapeutic techniques known as TCR-T and CAR-T, which have demonstrated encouraging results in treating certain malignancies [107-110].

TCR-T is a cell-based therapy that involves the infusion of genetically modified T lymphocytes into patients, enabling them to efficiently recognize tumor cells expressing specific targets. In comparison to cytotoxic chemotherapy and targeted therapy, TCR-T exhibits rapid activation and high specificity. Furthermore, TCR-T cells display greater sensitivity to antigen levels on tumor cells when compared to CAR-T cells [111, 112]

Unfortunately, despite demonstrating some efficacy in clinical trials [113], TCR-T therapy still faces challenges in achieving optimal clinical effectiveness. Current research efforts focus on identifying effective tumor antigens, cloning high-affinity TCR receptors, and optimizing TCR transduction efficiency [114-117].

Another compelling cell-based immunotherapy technique that has garnered significant interest among researchers is CAR-T, which currently stands as the only commercially available ACT therapy. CAR-T cells are T cells engineered to express receptors with specificity for tumor antigens. The key steps of CAR-T therapy involve collecting T cells from the patient's body, genetically modifying these cells to express chimeric antigen receptors (CARs), expanding the modified CAR-T cells to obtain a sufficient cell population, and finally reinfusing them into the patient's body to combat tumor cells [118]. Compared to conventional immunotherapies, CAR-T therapy offers several unique advantages. Firstly, CAR-T cells bypass the need for antigen processing and presentation mechanisms, enabling direct recognition of tumor-associated antigens and subsequent induction of cytotoxicity through T cell activation and cytokine secretion. Secondly, CAR-T cells overcome major histocompatibility complex restrictions by introducing CAR proteins, thereby avoiding immune rejection reactions [119].

CAR-T therapy has achieved significant breakthroughs in clinical trials. Currently, CAR-T therapy has been approved for the treatment of various hematologic malignancies, such as B-cell leukemia and lymphoma [110, 120]. Clinical trial results have demonstrated long-term remission in many patients treated with CAR-T therapy [121]. However, CAR-T therapy still faces certain challenges and limitations. Firstly, its efficacy remains relatively limited against solid tumors, as it primarily focuses on hematologic malignancies. Secondly, CAR-T therapy can induce severe side effects, including cytokine release syndrome and neurotoxicity [122] Additionally, the use of autologous T cells derived from patients often leads to damage to the patient's T cell compartment during the T cell acquisition process [123]. Furthermore, the high production cost and lengthy manufacturing process pose barriers to the widespread feasibility of CAR-T therapy [118].

1.3.2.4 Cancer vaccines

Cancer vaccines are grounded in the principles of immunology. The development and progression of cancer are frequently associated with immune system dysregulation, allowing tumor cells to evade immune surveillance and promote tumor growth and metastasis. The

objective of cancer vaccines is to stimulate the patient's immune system, enhancing its ability to recognize and attack tumor cells, thus suppressing tumor growth and preventing metastasis [124]. Based on their intended application and mode of action, cancer vaccines can be categorized as either prophylactic or therapeutic vaccines [125].

Prophylactic vaccines are designed to prevent cancer development in individuals who are at risk. These vaccines function by activating the immune system to generate specific immune responses that provide protection against cancer. Several prophylactic vaccines have been developed, such as the human papillomavirus (HPV) vaccine, which effectively prevents HPV-associated cancers including cervical and anal cancers [126]. Additionally, the hepatitis B vaccine is considered a prophylactic vaccine, as hepatitis B virus infection is a significant risk factor for liver cancer [127].

Therapeutic vaccines, on the other hand, are administered to individuals who have already been diagnosed with cancer, aiming to activate the immune system and elicit an immune response against cancer cells. These vaccines typically incorporate specific tumor antigens, such as whole tumor cells, tumor-associated antigens, or specific tumor-associated proteins. By introducing these antigens, the immune system is stimulated to mount an immune response specifically targeting cancer cells, thereby facilitating the therapeutic effect [128]. Notable therapeutic vaccine approaches include DNA vaccines, mRNA vaccines, peptide vaccines, and dendritic cell (DC) vaccines [53, 99].

(1) DC vaccines: Dendritic cells are a specialized type of immune cells that possess antigen-presenting capabilities. They can capture and process antigens and subsequently activate other immune cells to generate immune responses [129]. Dendritic cell vaccines involve isolating dendritic cells from the patient's body and manipulating them in the laboratory to present specific tumor antigens. Subsequently, these processed dendritic cells are reintroduced into the patient's body to activate the host immune system and elicit immune responses against cancer cells [130]. Dendritic cell vaccines are highly personalized, exhibiting robust long-term immune memory effects. For instance, Sipuleucel-T (Provenge), an autologous dendritic cell vaccine, has gained FDA approval for clinical use [131]. However, similar to CAR-T cell therapy, the preparation process of dendritic cell vaccines is complex, time-consuming, requires specialized equipment, and is associated with high costs. Furthermore, the therapeutic efficacy of dendritic cell vaccines displays variability and necessitates further optimization and research [132, 133].

(2) DNA and mRNA vaccines: These vaccine modalities involve introducing DNA or mRNA

sequences encoding specific tumor antigens into the patient's body, thereby enabling the patient's own cells to produce the desired antigens and initiate immune responses [134]. While DNA vaccines and mRNA vaccines share similar principles, they differ in terms of delivery methods and the duration of immune responses [135-137]. The advantages of these vaccines lie in their capacity for customization based on the specific cancer type and individual characteristics, thus improving treatment effectiveness. Nevertheless, further research is needed to enhance antigen expression and the strength of immune responses. Additionally, the storage and transportation of DNA and mRNA vaccines present technical challenges due to the inherent instability of these biomolecules.

(3) Peptide vaccines: Peptide vaccines are composed of specific peptide segments derived from cancer cell surfaces, capable of stimulating immune cells to mount immune responses against cancer cells [125]. Peptide vaccines are typically administered in conjunction with immune adjuvants, such as antigen-presenting cell activators, to enhance the magnitude and persistence of immune responses [138]. The design of peptide vaccines can be tailored to target specific cancer-specific antigens, thereby increasing treatment precision [139]. Compared to DNA and mRNA vaccines, peptide vaccines offer enhanced stability and simplified preparation and storage.

In contrast to cytokine therapy, cancer vaccines provide a more precise means of eliciting immune responses by targeting specific tumor antigens, thereby minimizing damage to normal cells [140]. Moreover, cancer vaccines induce long-term immune memory, enabling the immune system to mount rapid and effective responses to cancer recurrence [141]. While immune checkpoint blockade therapy (ICB) restores the immune system's ability to target tumors by blocking inhibitory signals, its efficacy varies among individuals, and some patients may exhibit limited responsiveness to ICB treatment. In contrast, cancer vaccines stimulate the patient's own immune system to generate more specific and targeted immune responses, thereby improving treatment efficacy to some extent [142]. Furthermore, cancer vaccines can be synergistically combined with other immunotherapeutic approaches, including ICB, to further enhance treatment outcomes [143, 144]. Unlike CAR-T cell therapy, which is typically restricted to specific tumor antigens, cancer vaccines can address the diversity and heterogeneity of tumors. Additionally, cancer vaccines demonstrate lower side effects and toxicity and can be more effectively personalized through vaccine administration [145]. Although cancer vaccines have made notable advancements in clinical applications, they still face challenges such as tumor heterogeneity and immune escape mechanisms, necessitating further research and exploration.

1.3.3 Antigens selection for cancer vaccines

Cancer vaccines have emerged as a novel therapeutic approach for tumor treatment, and the significance of antigen selection cannot be disregarded [146]. Antigens play a crucial role in recognizing and activating the immune system, and their selection directly impacts the specificity and efficacy of tumor vaccines. Therefore, comprehensive consideration of various factors, including tumor heterogeneity [147], antigen expression levels, immunogenicity, and immune evasion [148, 149], is essential in the antigen selection process for cancer vaccines.

The types of antigens selected for cancer vaccines can be categorized as tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) [150]. In the field of cancer immunotherapy, the development of effective vaccines is an important yet challenging task. Vaccine design necessitates careful deliberation of antigen selection and how to elicit and enhance the patient's immune response to achieve the inhibition of tumor growth and dissemination.

TSAs refer to antigens that are exclusively expressed on tumor cells and completely absent in normal cells [151]. TSAs primarily consist of mutated proteins and viral-derived proteins, endowing them with superior recognition and targeting abilities towards tumor cells [152]. This enables TSAs to precisely activate immune cells, inducing an attack specifically against tumor cells while causing minimal harm to normal tissues [153]. Consequently, TSAs are often regarded as ideal antigen choices due to their high specificity in recognizing and eliminating tumor cells [154].

In contrast to TSAs, TAAs are antigens that are overexpressed in tumor cells but expressed at low levels in normal cells [146]. TAAs encompass a common class of cancer antigens, including fetal antigens, carcinoembryonic antigens, carbohydrate antigens, tyrosinase, protein kinases, and growth factor receptors [150, 155]. Since TAAs are expressed in both tumor and normal cells, the immune system exhibits reduced specificity towards them, potentially leading to effects on normal cells [156]. Consequently, vaccine designers need to explore vaccine strategies targeting TAAs that enhance immune attack against tumor cells while minimizing immune damage to normal tissues.

The use of TSA vaccines is also constrained by the diversity of somatic cell mutations in different tumor types and their individual specificity. Studies have demonstrated a positive correlation between immune activity and tumor mutation burden (TMB) [157, 158]. Only 10%

of non-synonymous mutations in tumor cells can generate highly MHC-affine mutant peptides, and a mere 1% of these peptides can be recognized by patient T cells. Therefore, in theory, the higher the TMB, the greater the number of novel antigens that T cells in the tumor can recognize [154]. Different malignancies exhibit substantial variations in TMB. Tumors with high TMB, such as melanoma, demonstrate a higher response rate to immune therapy [159], whereas solid tumors with generally low TMB are less amenable to existing neoantigen vaccine systems. It is noteworthy that due to the stochastic nature of mutations, there is minimal overlap of TSAs among cancer patients [160, 161], significantly limiting the widespread application of TSA vaccines. In contrast, the advantage of TAA vaccines lies in their broad applicability. Since TAAs are present in various tumor types, TAA vaccines can be extensively employed against diverse malignancies [162, 163]. This broad spectrum makes TAA vaccines a universal vaccine strategy applicable to multiple tumors. However, most clinical trials targeting TAAs have failed to demonstrate long-lasting beneficial effects due to central (thymic) or peripheral tolerance mechanisms [164, 165]. Cancer-germline antigens (CGAs) represent a unique subset of TAAs that are expressed in healthy tissues but are restricted to immune-privileged sites [166, 167], such as the blood-testis barrier [168]. Consequently, CGAs possess high immunogenicity to the human immune system and evoke robust immune stimulation when exposed, while exhibiting reduced adverse effects on normal tissues compared to conventional TAA vaccines [169-171]. To date, Cancer-Testis Antigens have been extensively investigated in clinical research and have demonstrated promising clinical benefits [172-175].

Similar to the blood-testis barrier, the retinal barrier constitutes a vital physiological structure that safeguards and regulates the intra-retinal environment. Comprising multiple intricate and highly specialized cell layers, the retinal barrier forms a tightly knit physical and biochemical barrier, restricting free diffusion of substances within the retina. Within the retinal barrier, specific photoreceptor proteins are exclusively expressed locally in the eyes of non-tumor patients, and aberrant expression has been observed in certain tumor cells. These photoreceptor proteins collectively encompass the group of cancer-retina antigens, including arrestin, rhodopsin kinase, cGMP-phosphodiesterase 6 (PDE 6), etc [176]. Some studies have suggested that these aberrantly expressed cancer-retina antigens (CRAs) in tumor cells can serve as potential tumor markers and are closely associated with tumor cell homeostasis [177, 178], implying the potential of these photoreceptor proteins as targets for specific therapy, particularly as tumor vaccines. Our research team has previously reported on a retina-restricted CRA called Recoverin [179], which serves as a candidate tumor

antigen with significant potential in immunotherapy. We will elaborate on this in the subsequent section.

1.4   Recoverin in the Blood-Retinal Barries

The blood-retinal barrier serves as a crucial biological barrier, formed by the tight junctions of endothelial cells within the retinal capillaries (**Figure 1.4**）. Its primary function is to maintain the stability of the ocular environment. By restricting the unimpeded diffusion of substances, this barrier effectively safeguards the retina against the detrimental effects of exogenous agents [180]. Remarkably, the blood-retinal barrier also confers immune privilege upon the eye, thereby preventing the full recognition and immune response toward certain proteins that are intrinsic to the retina and play pivotal physiological roles [181]. The exposure of these proteins to the immune system triggers a robust immune reaction, as they are perceived as "foreign antigens" by the host [182]. Notably, among these proteins, Recoverin, a calcium-binding protein found within the photoreceptor cells, has been concurrently discovered by several research teams and identified as the primary cancer-retina antigen [179], thereby suggesting its potential value in the development of cancer vaccines.
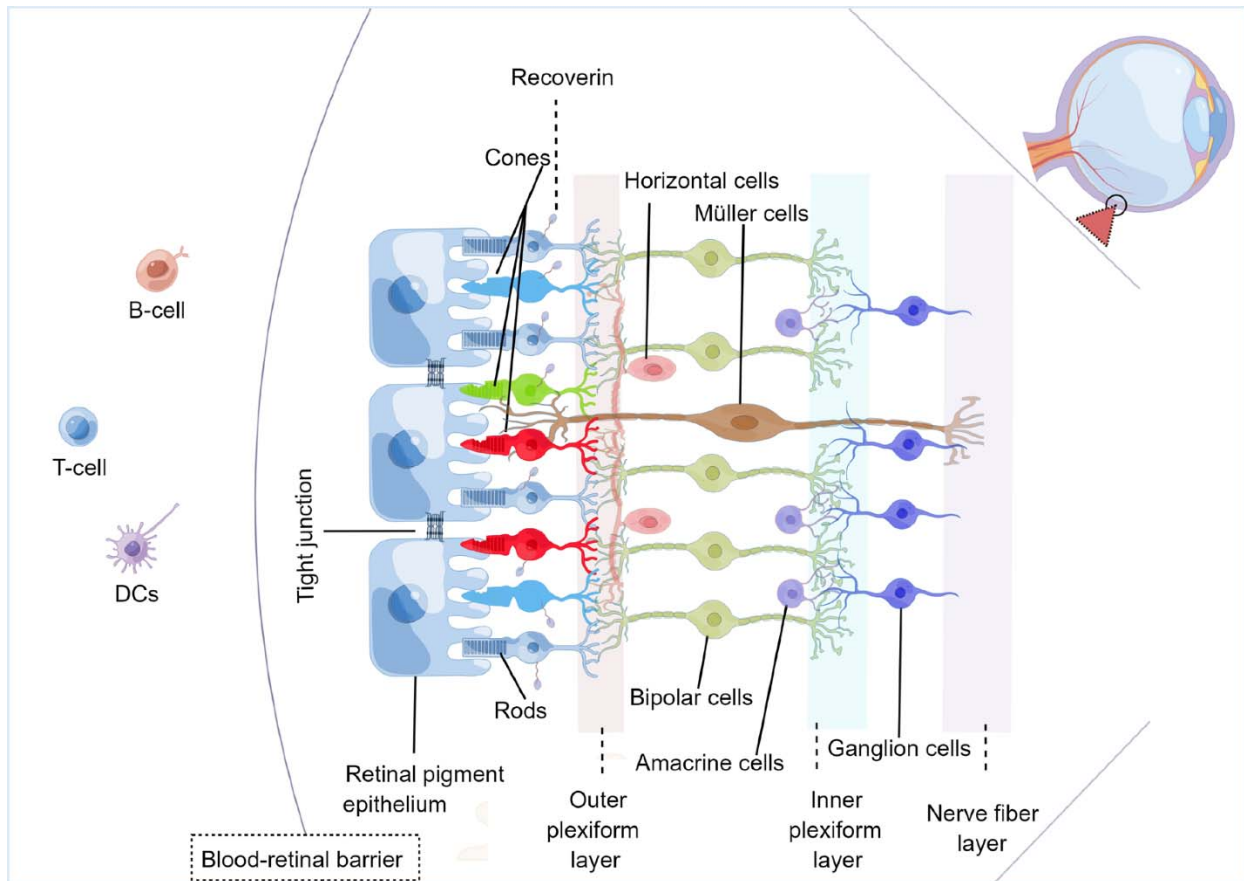
Figure 1.4 Blood-Retinal Barries (original art).

1.4.1 Physiological functions of Recoverin

Recoverin, a widely distributed protein in the retina, predominantly localizes to the outer segments of photoreceptors in vertebrate retinas. It plays a crucial physiological role in visual signal transduction by modulating the state of rhodopsin through the inhibition of rhodopsin kinase (GRK1) [183]. In retinal cells, Recoverin primarily serves as a regulator of calcium signaling, as it possesses the ability to bind calcium ions and respond to changes in intracellular calcium concentration [184]. In dark environments, the concentration of calcium ions increases in rod cells, leading to the binding of Recoverin to these ions and subsequent inhibition of GRK1, thereby preventing rhodopsin phosphorylation. Conversely, in bright light conditions, Recoverin releases calcium ions, resulting in the phosphorylation and inactivation of GRK1 on rhodopsin. This regulatory mechanism has the capacity to influence the excitatory and inhibitory inputs of retinal cells, thus modulating the transmission and processing of visual signals [185]. In both scenarios, the majority of Recoverin protein is localized in the inner segments of rods, with approximately 12% present in the outer

segments in darkness, and less than 2% remaining in the outer segments under light exposure [186]. Through this mechanism, Recoverin regulates the retina's ability to adapt to different light intensities, ensuring the normal functionality of the visual system [187]. Furthermore, the regulatory mechanism of Recoverin in cone cells is not yet fully understood, but evidence suggests that calcium-dependent regulation of GRK activity is more pronounced in cone cells compared to rod cells [188]. This implies that Recoverin may exert a more potent light adaptation regulatory function in cone cells. However, it appears that Recoverin primarily regulates the cascade of cone phototransduction under low light conditions [189].

1.4.2 Recoverin in cancers

Recent research has indicated that Recoverin may also play a potentially significant role in the field of oncology. It has been observed that the expression levels of Recoverin are markedly upregulated in various tumor tissues, particularly in retinoblastoma, breast cancer, lung cancer, and prostate cancer, among others [190-195]. A recent proteomic study has further revealed the upregulation of Recoverin protein in breast milk secretions from breast cancer patients [196]. In urologic malignancies, the combination of arrestin and Recoverin has shown promise as an effective urinary biomarker for renal cell carcinoma (AUC 0.96), although its performance in bladder cancer and prostate cancer is suboptimal (AUC 0.76; 0.7) [197, 198].

Cancer-associated retinopathy (CAR) is a rare paraneoplastic autoimmune disease characterized by painless vision loss [192]. Autoantibodies generated against cancer-associated retinal antigens cross-react with proteins located in the blood-retina barrier, ultimately leading to retinal damage [199, 200]. Immune checkpoint inhibitors (ICIs) have been widely employed in cancer therapy or clinical trials. However, cases of ICI-induced retinopathy resulting in visual impairment have been reported in patients with hepatocellular carcinoma (HCC) and small cell lung cancer (SCLC), with subsequent improvement in vision following steroid treatment [201, 202]. Notably, self-antibodies against Recoverin have been detected less frequently in the anti-retinal autoantibodies that induce CAR [203], indicating a relatively higher safety profile of Recoverin in tumor vaccines. Furthermore, CAR induced by Recoverin antibodies has shown significant improvement through intravitreal dexamethasone implantation [190, 204].

The 5-year survival rate for most patients diagnosed with SCLC is a mere 7% [205]. Interestingly, a case report described a patient with SCLC who developed CAR. Recoverin protein was detected on tumor cells, and Recoverin antibodies were present in the patient's serum. The patient experienced spontaneous regression of SCLC without receiving any anticancer treatment. Another case report described a SCLC patient who maintained long-term tumor remission and survived for 9 years after synchronous chemotherapy. Throughout this period, Recoverin antibodies remained positive [206]. Surprisingly, two additional case reports documented the survival of patients with SCLC accompanied by CAR for 15 and 17 years, respectively [207, 208]. This phenomenon has been attributed to the antitumor effect exerted by Recoverin antibodies, suggesting the potential application of Recoverin as an antigen in tumor vaccines.

The precise role of Recoverin expression in tumors remains incompletely understood, but significant progress has been made in certain studies. Recoverin, through its interaction with calcium ions, is involved in GRK-dependent cellular regulation in cancer cells [209], thereby regulating crucial processes such as proliferation, apoptosis, and migration [210-212]. Recently, a study shows that the aberrantly expressed Recoverin, upon transfection into A549 cells, could affect the vitality and drug sensitivity of A549 cells via G-protein-coupled receptor (GPCR) signaling [213].

1.5   Adjuvants

1.5.1 Definition and mechanisms of action of adjuvants

Adjuvants are auxiliary substances added to vaccines to enhance their immunogenicity. They exert their effects through several mechanisms:

1. Providing immune stimulation: Adjuvants can mimic pathogen features, activating the immune system and eliciting an immune response, thus enhancing the immunogenicity of the vaccine.

2. Improving antigen presentation: Adjuvants can modify antigen characteristics in the vaccine, facilitating recognition and presentation by immune cells, thereby improving the efficiency of immune responses.

3. Modulating immune responses: Adjuvants can regulate immune cell activation and

differentiation, promoting specific immune response types such as Th1 or Th2 responses, thereby enhancing immune effectiveness.

Cancer vaccines aim to stimulate specific immune responses that recognize and inhibit the growth and spread of tumor cells. However, standalone cancer vaccines often fail to induce sufficient immune reactions [214, 215]. To enhance the efficacy of cancer vaccines, researchers have developed adjunctive substances called vaccine adjuvants [216, 217]. The introduction of adjuvants can activate immune cells, increase antigen expression and delivery, and improve immune cell recognition and killing capacity towards antigens [218-220]. Additionally, adjuvants can recruit immune cells, facilitate immune cell interactions, and promote the formation of immune memory [221-223]. Overall, adjuvants enhance immune responses and improve the effectiveness of cancer vaccines by increasing immune cell activity and antigen immunogenicity. Among potential adjuvant choices, the heat shock protein (HSP) family, with its unique functions and biological characteristics, has emerged as a promising candidate for cancer vaccine adjuvants. Furthermore, HSPs exhibit excellent safety and biocompatibility, further supporting their feasibility as adjuvants [224]. The rapid growth and abnormal metabolism of cancer cells lead to increased stress levels, resulting in elevated expression of HSPs, providing abundant sources for extracting adjuvants for cancer vaccines [225, 226].

1.5.2 HSP family in adjuvants

Heat shock proteins (HSPs) are a highly conserved group of proteins widely present in cells. They serve various essential biological functions in organisms, including protein folding, transport, stability, degradation, and participation in signal transduction [227]. HSP proteins not only function under non-physiological conditions such as high temperature, low temperature, high salinity, and pH changes but also play significant physiological roles in normal cells [228]. Studies have demonstrated that HSPs play important protective roles in disease resistance and aging [229, 230].

The structural characteristics of HSP family proteins are closely related to their functions. HSPs consist of an ATP-binding domain and a substrate-binding domain. The ATP-binding domain of HSPs participates in ATP hydrolysis, providing the energy for their molecular chaperone function. The substrate-binding domain directly forms complexes with substrates (usually unfolded or misfolded proteins), preventing their misfolding or aggregation [231].

Notably, HSP70 was the first HSP shown to bind antigen peptides [232].

HSP70 is divided into different subtypes, each with distinct localization [233]. Extracellular HSP70, released from tumor cells through exosomes, plays a role in regulating tumor-associated immune cells within the tumor microenvironment [234]. HSP70 accumulates extensively in various types of tumors to protect tumor cells from the toxicity of chemotherapy drugs. Increased extracellular HSP70 levels after chemotherapy enhance the pro-tumor effects of tumor-associated macrophages (TAMs) by regulating TGF-β expression in breast cancer cells [235]. Inhibition of HSP70 significantly improves the efficacy of tumor chemotherapy [236]. Additionally, HSP70 is involved in a wide range of cancer occurrence and progression through dysregulation of multiple cancer-related signaling pathways [237, 238]. Studies have shown that HSP70 and insulin-like growth factor I receptor (IGF-IR) mutually regulate each other, promoting proliferation and migration of HCC cells [239]. Through the AKT-STAT3 signaling pathway, HSP70 upregulates macrophage receptor with collagenous structure (MARCO), thereby promoting macrophage phagocytosis of apoptotic tumor cells, reducing inflammatory cell infiltration, and facilitating immune evasion [209]. Furthermore, the HSP70/TLR4 signaling axis promotes the recruitment of myeloid-derived suppressor cells (MDSCs) into the tumor tissue, shaping an immunosuppressive microenvironment and leading to adaptive resistance to anti-PD-1 immunotherapy [240].

1.5.3 Application of the HSP family as cancer vaccine adjuvants

In the past few decades, HSPs have been extensively studied as antigen carriers for vaccines [241-243]. Due to their antigen-presenting functions, HSPs can enhance immune responses and are considered ideal antigen carriers [244]. HSPs form antigen-HSP complexes by binding specific antigens to their antigen-binding domains [245]. These complexes are delivered to professional antigen-presenting cells (such as dendritic cells) and presented to immune cells via the major histocompatibility complex (MHC) class I or class II pathways, activating T cells and B cells, thereby initiating specific immune responses [246]. Furthermore, some HSPs (such as HSP70) can interact with receptors on the surface of immune cells, regulating immune cell activation and function, and enhancing immune responses [247]. Clinical studies have demonstrated the potential value of HSP70 as a cancer vaccine adjuvant and have shown promising clinical outcomes [248].

In contrast to the biological functions of HSP70, HSP70-peptide complexes can induce

robust anti-tumor immune responses. In a mouse model of lung cancer metastasis, researchers observed that a vaccine combining TAA with HSP70 significantly increased the number and responsiveness of cytotoxic T lymphocytes (CTLs) and better suppressed tumor growth and metastasis compared to individual TAA vaccines. Further experiments demonstrated that HSP70 facilitated the recognition and presentation of TAAs by dendritic cells [249], a phenomenon also observed in mouse models of hepatocellular carcinoma (HCC) and ovarian cancer [250, 251]. The efficacy of such vaccines largely depends on tumor origin and the immunogenicity of antigens delivered by HSP70 [252]. Human papillomavirus (HPV) is a major risk factor for cervical cancer, and E7 is a major component of HPV that can be presented to T cells by dendritic cells. However, HPV vaccines only contribute to the prevention of cervical cancer and have no therapeutic effect. A study found that E7-HSP70 protein can exert anti-tumor effects as a therapeutic vaccine [253]. Currently, some HSP70-based vaccines have undergone clinical trials [254], and promising therapeutic effects have been demonstrated in phase 1 clinical trials [255]. As mentioned earlier, Recoverin, as a specific TAA, exhibits potent immunogenicity and has minimal side effects on normal tissues. However, as a single peptide, its ability to induce anti-tumor immune responses is limited. Therefore, combining Recoverin with HSP70 may have significant anti-tumor effects.

1.6   Research objective

Considering the abnormal expression of Recoverin in various cancers and its potent immunogenicity as a result of the blood-retina barrier, we aim to investigate the potential of a Recoverin-HSP70 fusion protein as a cancer vaccine adjuvant. Our preliminary research has observed that the Recoverin-HSP70 fusion protein can effectively induce dendritic cell maturation and activation and further activate CD8+ T cells. My study will further explore the ability of the Recoverin-HSP70 fusion protein to activate dendritic cells and induce T cell proliferation, as well as evaluate the cytotoxicity of activated T cells against Y79 tumor cells expressing Recoverin. Thus, the potential of the Recoverin-HSP70 fusion protein as an anti-tumor vaccine will be assessed.

2. Materials and methods

2.1 Materials

2.1.1 Laboratory equipment

Centrifuge	Rotina 380R, Hettich, Germany
Electronic balance	MP-3000, Waagen dienst, Germany
Flow Cytometer	LSRFortessaTM, BD Biosciences, USA
Vortex	G560E, Scientific Industries, USA
4/-20°C fridge	FKS 5000, Liebherr, Germany
-80°C fridge	Bosch, Germany
Multipette Plus	HandyStep® S, Brand, Germany
Pipettes	Transferpette® S, Brand, Germany
Pipette controller	Corning, USA
Cell counter CASY	OMNI Life Science GmbH & Co KG
Microscope	Olympus, Japan
Water bath	Julaba, Germany
37°C cell incubator	Binder, Germany
Magnetic mixer	GLW, Germany
Electronic pH meter	Chyo, Japan

2.1.2 Consumable items

0.5-10 µL L, Ep T.I.P.S.® Reloads	Eppendorf, Germany
10-200 µL L, Ep T.I.P.S.® Reloads	Eppendorf, Germany
100-1000 µL L, Ep T.I.P.S.® Reloads	Eppendorf, Germany
7.5 ml Lithium Heparin blood collection tube	S-Monovette®, Sarstedt, USA
5ml Flow Cytometry Tube (FACS tube)	FALCON®, Mexico
5 ml pipette	COSTAR®, USA
10 ml pipette	COSTAR®, USA

25 ml pipette	CELLSTAR®, USA
50 ml pipette	Nunc™ Serological Pipettes, USA

2.1.3 Chemical and reagents

Bovine serum albumin (BSA)	Biomol, Germany
Natriumacid	Morphisto, Germany
Millipore H_2O	Advantage A10, Merck, Germany
Ethylenediaminetetraacetic acid (EDTA)	E5513, SIGMA-ALORICH, USA
PBS buffer (10X)	Power BC, PanReac AppliChem, German

2.1.4 Solutions

Fluorescence-activated cell sorting (FACS) buffer	
pH	7.3
500 m L	DPBS
1 ml	Natriumacid
2.5 g	BSA

Buffer for isolation of T cells	
pH	7.2
1000 mL	DPBS
5 g	BSA
2 mM	EDTA

PBMCs Cell Culture Medium	
495 mL	1640 Medium
5 mL	plasma

Y79 Cell Culture Medium	

400 mL	DMEM
100 mL	plasma

Cell Cryopreservation System/10^7 cells	
450 µL	plasma
50 µL	DMSO

2.1.5 Antibodies

Antibody	Fluorochrome	Reactivity
Anti-CD45	BV650	Human
Anti-7AAD	PerCP Cy5.5	Human
CFSE	FITC	Human
Anti-CD3	BUV395	Human
Anti-Recoverin	-	Human
Anti-GAPDH	-	Human

2.1.6 Hardware and Software

Computer system	Windows 10, Lenovo, USA
FlowJo™ version.10	BD, USA
FACSDIVA™ SOFTWARE	BD, USA
SPSS	Version 21.0, USA
GraphPad Prism 8	Version 8.0.1, USA

2.2 Methods

2.2.1 Production of Recombinant fusion proteins - Preliminary work

As part of the preliminary investigations conducted for the present study, our international

collaborator, Dr. Alexey V. Baldin, affiliated with the Belozersky Institute of Physico-Chemical

Biology at Lomonosov Moscow State University, successfully acquired a compilation of bacterial strains of E. coli that serve as producers of recombinant fusion proteins comprising human HSP70 and two recoverin epitopes. The aforementioned strain collection encompasses two designated producers:

1. E. coli JM109/pQE80 HSP70;
2. E. coli JM109/pQE80 H2E1-HSP70-H1E1 rec.

Designations used in the name of bacterial strains, fusion proteins, plasmid vectors, etc., and their decoding are shown as follows (abbreviations):

E. coli, Escherichia coli; JM 109, Bacterial strain E. coli JM109; pQE80, Plasmid vector of QIAexpress pQE bacterial expression system; HSP70, Heat shock protein 70, Homo sapiens; H1, HLA 1 (human leukocyte antigen class I); H2, HLA 2 (human leukocyte antigen class II); E1, Epitope 1; E2, Epitope 2; rec, Recoverin.

The Recoverin protein, transcribed from the RCVRN gene (Gene ID: 5957), possesses the amino acid sequence:

MGNSKSGALSKEILEELQLNTKFSEEELCSWYQSFLKDCPTGRITQQQFQSIYAKFFPDTDP
KAYAQHVFRSFDSNLDGTLDFKEYVIALHMTTAGKTNQKLEWAFSLYDVDGNGTISKNEVLE
IVMAIFKMITPEDVKLLPDDENTPEKRAEKIWKYFGKNDDDKLTEKEFIEGTLANKEILRLIQFE
PQKVKEKMKNA. For the plasmid design based on the pQE80 system, Escherichia coli were genetically engineered to synthesize fusion proteins. Specifically, HLA II- and HLA I-specific recoverin epitopes were appended to the N- and C-terminus of HSP70, respectively. The central segment of the fusion protein consists of the amino acid sequence of the human HSP70 protein, which is encoded by the HSPA1B gene. HLA II and HLA I recoverin epitopes were chosen refer to Table 1.

Table 1. Epitopes for Recoverin.

HLA type	Name	Amino acid sequence in FASTA format	Nucleotide sequencing
HLA-A*02:01	H1E1	ALSKEILEEL	GCC CTG TCC AAG GAG ATC CTG GAG GAG CTG
HLA-DQA1*05:01	H2E1	YVIALHMTTAGKTNQ	TAC GTC ATC GCC CTG CAC ATG ACC ACC GCG GGC AAG ACC AAC CAG

HLA-DQB1*03:01	H2E1	EYVIALHMTTAGKTN	GAG TAC GTC ATC GCC CTG CAC ATG ACC ACC GCG GGC AAG ACC AAC

Note: All donors in our project showed HLA-A*02, HLA-DQA1*05, and HLA-DQB1*03 positive.

Subsequently, the plasmids encoding the fusion proteins were transfected into the Escherichia coli strain JM109 for the synthesis of the fusion proteins. Following that, Dr. Alexey V. Baldin performed the separation and purification of these fusion proteins. Considering the stimulation of endotoxins on immune cells, Dr. Alexey V. Baldin assessed the endotoxin content in the samples, and the results demonstrated that the endotoxin content in almost all fusion proteins was below 10 EU/mg. Therefore, the influence of residual endotoxins can be excluded when evaluating the immunogenicity of protein formulations [256].

Table 2. The presence of endotoxins in the preparations of isolated and purified fusion proteins consisting of HSP70 and recoverin.

#	Designation	Concentration,	Endotoxin level,	Endotoxin level
1	HSP70	0.391 mg/ml	<0.5 EU/ml	<1.27 EU/mg
2	H2E1-HSP70-H1E1	0.104 mg/ml	<0.5 EU/ml	<4.8 EU/mg

2.2.2 HLA typing for blood donors

The HLA diagnosis was performed at the Laboratory for Immunogenetics and Molecular Diagnostics of LMU Großhadern Hospital. Subsequently, a meticulous selection process identified volunteers who exhibited positive HLA types, specifically matching all three types mentioned above. These individuals were then chosen as blood donors for our project. In total, five donors participated in this study. At initially, all donors gave their consent.

2.2.3 Unveiling the expression profile of Recoverin through single-cell transcriptome analysis

Tumor Immune Single-cell Hub 2 (TISCH2) represents a scRNA-seq repository with a particular focus on the tumor microenvironment (TME) [257]. It offers comprehensive cell-

type annotation at the individual cell level, facilitating the investigation of TME across diverse cancer types. All cellular categories are meticulously annotated within TISCH2. Moreover, TISCH2 provides researchers with a rapid understanding of gene expression patterns for their genes of interest at the single-cell level. In this study, I leveraged TISCH2 to explore the expression levels of Recoverin in human retinoblastoma (dataset: RB_GSE166173) [258]. Initially, the 'NormalizeData' function within the "Seurat" package was utilized for the normalization of raw counts (UMI) in individual cells to a scale of 10,000, followed by a logarithmic transformation of the data. Simultaneously, all gene expression values were converted to transcripts per million (TPM). The quantification of gene expression within each cell was represented as log2(TPM/10+1). TPM values were divided by 10 to mitigate the effects of variable dropout rates among genes. Further, the annotation of cell clusters was conducted utilizing the marker-based annotation approach as implemented in MAESTRO. Finally, UMAP and violin plots were generated to visually portray the mRNA expression profiles of RCVRN.

2.2.4 PBMCs isolation

1. 15 ml whole blood sample was collected from each blood donor for further processing.

2. One 50 ml centrifuge tube (designated as tube A) was prepared and filled with 15 ml of DPBS (Dulbecco's Phosphate-Buffered Saline) according to the principle of equal volume dilution. Subsequently, the 15 ml whole blood sample was slowly added to tube A containing DPBS. Gentle agitation using a pipette ensured thorough mixing and uniform dilution of the blood.

3. Another clean 50 ml centrifuge tube (designated as tube B) was obtained and filled with 15 ml of Biocoll-Separating Solution, which was added in an equal volume to the blood sample. The 30 ml mixture of blood and DPBS was carefully layered onto the Biocoll-Separating Solution in tube B. It was essential to handle the tubes delicately without shaking or disturbing the separation phases to maintain their integrity.

4. Tube B was then subjected to centrifugation at a speed of 1200 rcf (relative centrifugal force) for a duration of 20 minutes, without applying the brake.

5. Following centrifugation, the uppermost layer containing serum was collected using a 15 ml centrifuge tube and transferred to the Water Bath for subsequent inactivation at 54°C for 30 minutes. This inactivation step aimed to eliminate potentially interfering components, such as inflammatory factors, present in the serum, ensuring optimal conditions for immune cell culture and preservation.

6. Subsequently, the PBMC-containing cloudy interphase was carefully transferred from tube B to a new 50 ml centrifuge tube (tube C). Special attention was given to avoid the transfer of any Biocoll-Separating Solution, as it could compromise cell viability.

7. In tube C containing the PBMC suspension, DPBS was added in a 1:5 ratio to achieve dilution of residual Biocoll-Separating Solution and serum components. The tube was then subjected to centrifugation at 500 rcf for 5 minutes, and the supernatant was discarded. This process was repeated.

8. The resulting PBMC cell pellet was resuspended in 1 ml of DPBS for further analysis. A 20 μl aliquot of the cell suspension was retrieved for cell counting using a specialized cell counter.

9. The collected PBMCs (totaling 10^7 cells) were subsequently cryopreserved and stored at a temperature of -80°C for future isolation of T cells.

2.2.5 PBMC cell cryopreservation

1. Cryopreservation medium was prepared by combining inactivated autologous serum with DMSO solution at a volumetric ratio of 9:1.

2. Cryopreservation boxes were placed at room temperature, and cryovials (1.8 ml) were meticulously labeled with pertinent information, including the time of storage, cell type, and donor identification.

3. The obtained 10^7 PBMCs were gently resuspended in 400 ml of cryopreservation medium, ensuring an even distribution, before being carefully transferred to the appropriately labeled cryovials.

4. The cryovials, now containing the PBMCs, were promptly and securely placed inside a -80°C freezer, where they underwent a controlled freezing process for long-term preservation.

2.2.6 PBMC cell revival and seeding

1. A water bath was set to maintain a constant temperature of 37°C to facilitate cell revival.

2. Retrieving the cryovials from the -80°C freezer, the cryopreserved PBMCs were quickly submerged in the water bath. Careful attention was paid to the complete thawing of the cryopreservation medium before transferring the contents into 15 ml centrifuge tubes pre-filled with 10 ml of culture medium (RPMI/1% plasma medium).

3. The subsequent centrifugation step, performed at 500 rcf for 5 minutes, effectively separated the viable cells from the cryopreservation medium.

4. Following centrifugation, the supernatant, containing residual cryopreservation medium, was discarded. The viable PBMCs were then gently resuspended in 2 ml of fresh culture medium.

5. To determine cell concentration accurately, 10 µl of the cell suspension was withdrawn for cell counting. Subsequently, the PBMCs were seeded into culture dishes, selecting appropriate dish sizes based on the cell concentration to ensure optimal growth and viability. Then cells were placed in a cell incubator at 37°C with 5% CO_2.

2.2.7 Induction of differentiation from PBMCs to immature dendritic cells

1. PBMCs were resuspended in RPMI/1% plasma medium and seeded at a density of 10^7 cells per well in 6-well plates. The plates were then placed in a cell incubator at 37°C with 5% CO_2, and the PBMCs were allowed to incubate for 90 minutes.

2. After the 90-minute incubation, the monocytes exhibited robust adhesion to the well surfaces. To remove non-adherent cells, the culture was carefully washed twice with 4 ml of serum-free PRMI1640 medium. This step ensured the retention of well-adhered monocytes, resulting in a monolayer of cells with optimal adhesion.

3. Subsequently, the serum-free PRMI1640 medium was replaced with 4 ml of RPMI/1% plasma medium supplemented with IL-4 (1000 U/ml) and GM-CSF (1000 U/ml) in each well. The cultures were then maintained in a cell incubator at 37°C with 5% CO_2 for 5 days.

4. After the 5-day incubation period, the cells were carefully examined under a microscope to assess their state and morphological changes. At this stage, the monocytes had successfully differentiated into immature dendritic cells (iDCs), which were now found suspended in the culture medium.

2.2.8 Differentiation from iDCs to mature dendritic cells (mDCs)

1. Immature dendritic cells (iDCs) were harvested and subjected to centrifugation (500 rcf, 5 minutes). The resulting supernatant was carefully discarded, and the iDC cell aggregates were subsequently re-suspended in 3 ml of RPMI/1% plasma medium supplemented with IL-4 (1000 U/ml) and GM-CSF (1000 U/ml). To ensure accurate cell enumeration, a 10 µl aliquot of the cell suspension was used for cell counting.

2. Following the cell preparation, iDCs were seeded into individual wells of a 24-well plate at a density of 2.5×10^5 cells per well. Four distinct experimental groups were established,

namely: the iDC group (iDC group), the iDC group treated with 0.4 µM Recoverin peptide/HSP70 protein/fusion protein (DC_pept/HSP70/FP group), the iDC group exposed to cytokines (DC_cyto group), and the iDC group treated with 0.4 µM Recoverin epitope/HSP70 protein/fusion protein in conjunction with cytokines (DC_pept/HSP70/FP cyto group). Subsequently, the plate was maintained in a cell incubator at 37°C with 5% CO_2 for a period of 3 hours.

3. In order to promote dendritic cell maturation, the cytokine medium mixture was prepared, consisting of RPMI/1% plasma medium supplemented with GM-CSF, IL-4, IFN-γ, TNF-α, prostaglandin E2 (PGE2), and CD40L. Specifically, the concentrations of GM-CSF and IL-4 were both set at 1000 U/ml, while IFN-γ, TNF-α, and PGE2 were maintained at 2000 U/ml, and CD40L was set at 400 ng/ml.

4. At the end of the 3-hour incubation period, the iDC_cyto and the DC_pept/HSP70/FP cyto group were added with 1 ml of the prepared cytokine medium mixture for cell resuspension. While the iDC group and the DC_pept/HSP70/FP group were added with 1 ml of RPMI/1% plasma medium containing 1000 U/ml GM-CSF and IL-4.

5. The 24-well plate housing the four experimental groups of cells was subsequently maintained in a cell incubator at 37°C with 5% CO_2 for a 24-hour incubation period. This specific duration allowed for the differentiation of iDCs into mature dendritic cells (mDCs).

2.2.9 T cells Isolation from PBMCs

Pan T Cell Isolation Kit:

1. Pan T Cell MicroBead Cocktail
2. LS column
3. MACS Separator
4. Biotin-Antibody Cocktail

Steps:

1. As previously described, PBMCs in cryopreservation were revived. Cells were washed with 10 ml DPBS and centrifuged (500 rcf, 5 minutes). The supernatant was carefully discarded, leaving only the PBMC cell pellet.

2. The PBMC cell pellet was resuspended in 40 µl of T cells Isolation Buffer. Then, 10 µl of Biotin-Antibody Cocktail from the Pan T Cell Isolation Kit was added. The cell suspension was incubated at 4°C for 5 minutes.

3. The cell suspension was retrieved from the 4°C refrigerator, and an additional 30 μl of T cells Isolation Buffer and 20 μl of Pan T Cell MicroBead Cocktail from the Pan T Cell Isolation Kit were added. The cells were incubated at 4°C for 10 minutes.

4. During this period, the MACS Separator was prepared, and the LS column was properly inserted into the MACS Separator. 3 ml of T cells Isolation Buffer was added to the LS column for column washing.

5. After 10 minutes, the cell suspension was retrieved from the 4°C refrigerator. 4 ml of T cells Isolation Buffer was added to dilute the cell suspension. Subsequently, the cell suspension was applied to the LS column, and the effluent T cell suspension was collected beneath the LS column.

6. The T cell suspension was centrifuged (500 rcf, 5 minutes), and the supernatant was discarded, then the T cell pellet was obtained.

2.2.10 Assessment of T cell proliferation induced by mDCs

1. Four groups of mDCs were prepared using the aforementioned methods: iDC, DC_pept/HSP70/FP, DC_cyto, and DC_pept/HSP70/FP cyto groups. Cell clusters from each group were collected, and 300 μl of serum-free RPMI 1640 medium was added to resuspend the mDCs. A 10 μl cell suspension was taken for cell counting.

2. T cell clusters were isolated and collected following the above-mentioned method.

3. T cell clusters were resuspended in 10 ml DPBS and then centrifuged (500 rcf, 5 minutes) to discard the supernatant. This step aimed to remove residual serum components from the cell clusters.

4. 1 ml DPBS was added to resuspend the T cells again.

5. After 15 minutes, the 15 ml centrifuge tube containing T cells was taken out, and 10 ml DPBS was added to dilute the CFSE dye. Then, centrifugation was performed (500 rcf, 5 minutes), and the supernatant was discarded to obtain the T cell clusters.

6. CFSE was diluted with DPBS and added to the T cell suspension to achieve a final CFSE concentration of 2 μM. The T cells were then transferred to a Water Bath and incubated at 37°C for 15 minutes.

7. T cell clusters were resuspended in 10 ml RPMI/1% plasma medium and then

centrifuged (500 rcf, 5 minutes). The supernatant was discarded, and the T cell clusters were obtained.

8. 1 ml serum-free RPMI 1640 medium was used to resuspend the T cells, and 10 µl of the cell suspension was taken for cell counting.

9. Following a 10:1 ratio, 5×10^5 T cells and 5×10^4 mDCs were co-cultured in a 48-well plate. Additional serum-free RPMI 1640 medium was supplemented to maintain a total volume of 1 ml per well.

10. Additionally, two extra wells were set up with only 5×10^5 T cells each. One well had the addition of PHA at a final concentration of 10 µg/ml. The total volume of the medium in each well was maintained at 1 ml.

11. The cells were then transferred to an incubator at 37°C and incubated for 7 days.

12. After 7 days of co-culturing the cells, the growth status of T cells in the six co-culture wells was observed under a microscope, and photographs were taken.

13. The cells from the six wells were separately collected, centrifuged (500 rcf, 5 minutes), and the supernatant was discarded to obtain the T cell clusters. Then, 400 µl of serum-free RPMI 1640 medium was added to resuspend the cells for each group.

14. Each group of cell suspensions was divided into two portions. One portion had 1 µl of CD3 antibody added. The cells were then incubated in the dark at room temperature for 15 minutes.

15. Flow cytometry was used to detect the proliferation signal of T cells, and Flowjo software was utilized for data analysis.

2.2.11 Assessment of cytotoxicity of T cells against Y79 induced by mDCs

1. Four groups of mature dendritic cells (mDCs) were prepared using the aforementioned methods: iDC group, DC_pept/HSP70/FP group, DC_cyto group, and DC_pept/HSP70/FP cyto group (**Figure 2.2.11**). Cell clusters from each group were collected and resuspended in 300 µl RPMI/1% plasma medium. Then, 10 µl of the cell suspension was used for cell counting.

2. T cell clusters were separated and collected following the same procedure. The T cell

clusters were resuspended in 1 ml RPMI/1% plasma medium. Next, 10 μl of the cell suspension was used for cell counting.

3. T cells (5×10^5) and mDCs (5×10^4) were co-cultured in a 48-well plate at a ratio of 10:1. RPMI/1% plasma medium was added to maintain a total volume of 1 ml per well.

4. Additionally, a separate well was set up with 5×10^5 T cells and a total volume of 1 ml.

5. The cell cultures from all five groups were then transferred to a 37°C incubator and cultured for 1 day. Simultaneously, in a clean 50 ml centrifuge tube, 20 ml of RPMI/1% plasma medium and IL-2 were added to prepare IL-2 solution (50 U/ml). Once prepared, the IL-2 solution was stored at 4°C.

6. On day 2, the 48-well plate was removed from the incubator. Carefully, 500 μl of supernatant from each co-culture well was aspirated and discarded. Subsequently, 500 μl of IL-2 solution was added to each well.

7. On day 4, day 6, day 9, day 11, and day 13, half of the culture medium was replaced with fresh medium containing IL-2 in each group.

8. On day 14, images of the five cell groups were captured under a microscope. Then, the stimulated T cells were washed and re-stimulated using mDCs again. All groups were cultured in IL-2-free medium for 24 hours in the incubator.

9. On day 15, the activated T cells from each group were collected and co-cultured with Y79 cells in a 10:1 ratio in a 96-well plate. Additionally, a separate well containing only Y79 cells with a total volume of 1 ml was set up. The cells were then transferred to the cell culture incubator for further incubation for 24 hours.

10. After 24 hours (day 16), cells from each of the six wells were collected, centrifuged (500 rcf, 5 minutes), and the supernatant was discarded to obtain cell clusters. Then, 400 μl of serum-free RPMI 1640 culture medium was added to resuspend each cell group.

11. Each cell suspension was treated with 10 μl of 7-AAD antibody dye, and then each group was divided into two portions. One portion was further stained with 1 μl of CD45 antibody dye. The cells were then incubated in the dark at room temperature for 15 minutes.

12. Cell death signals were detected using a flow cytometer, and the data were analyzed using Flowjo software.
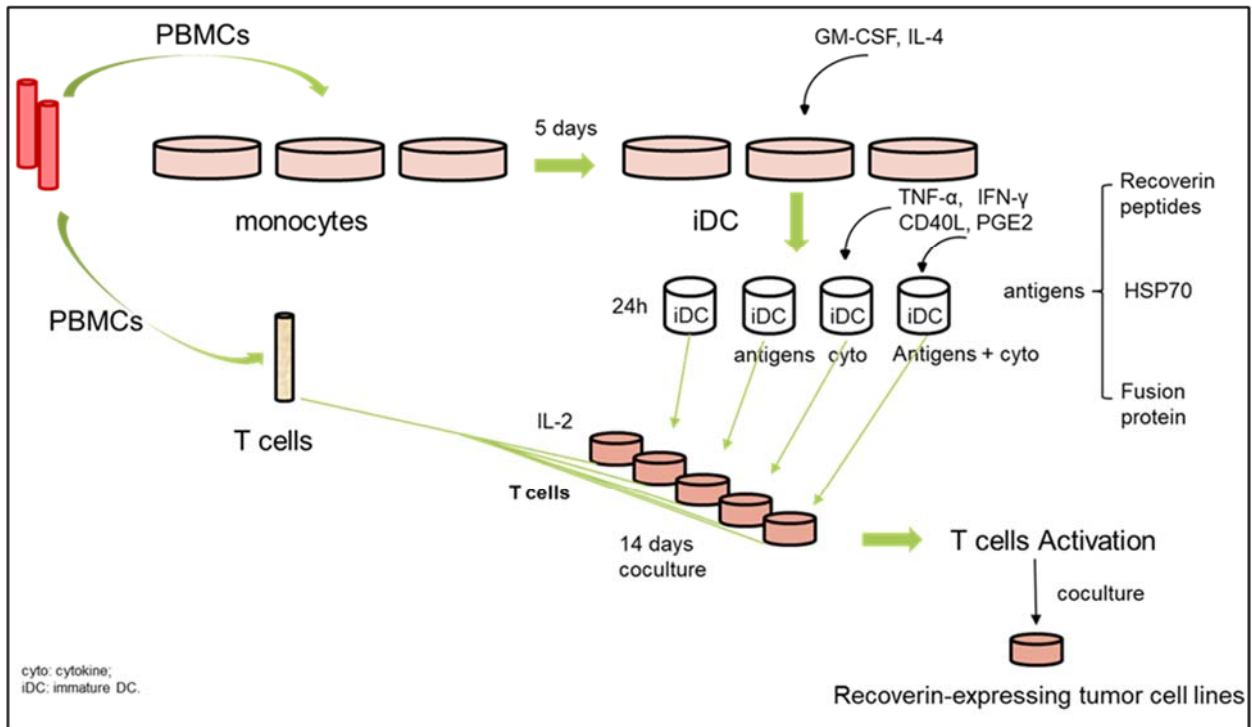
Figure 2.2.11 Work flow for the assessment of T cells cytotoxicity against Y79 induced by mDCs pulsed with antigens (original art).

2.2.12 Flow cytometry

1. Prepare the FACS buffer as described above.

2. Transfer the cells to FACS tubes and resuspend them in 200 ul of FACS buffer.

3. Add the antibody stains and incubate for 15 minutes at room temperature in the dark after vortexing.

4. After 15 minutes, proceed with cell washing by adding 2.5 ml of FACS buffer and centrifuging (500 rcf, 5 minutes).

5. Discard the supernatant from the FACS tube and resuspend the cells in 300 ul of fresh FACS buffer.

6. Perform sample analysis using a flow cytometer.

2.2.13 Western blot

Cell lysates derived from Y79 and Huh7 cell lines were meticulously prepared utilizing an IP lysis buffer enriched with a cocktail of protease inhibitors (Sigma, Lot 53002700). The

quantification of protein content was executed through the utilization of the BCA Protein Assay Kit (Pierce™, Thermo Scientific). Uniform quantities of the protein lysates were subjected to separation via SDS-PAGE and subsequent transfer onto polyvinylidene fluoride (PVDF) membranes, an essential step preceding the western blotting procedure. Impeding nonspecific binding, the membranes were subjected to blockade employing a buffer containing bovine serum albumin (BSA), succeeded by a prolonged incubation with primary antibodies; specifically, anti-Recoverin (Abcam) and anti-GAPDH (Cell Signaling), performed at a refrigerated temperature of 4°C over the course of a night. A subsequent incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (dilution: 1:3,000, Cell Signaling) ensued at ambient temperature for a duration of 1 hour. The visualization of the western blotting outcomes was achieved through the application of the western blot imaging system (ChemiDoc™, Bio-Rad).

2.2.14 Figure draw

Figdraw is an online scientific illustration platform developed by Home for Researchers. All three figures in the Introduction section were originally created by me using the Figdraw toolkit.

2.2.15 Statistical analysis

The measurement data were expressed as mean ± standard deviation (SD) of the mean. Group comparisons were conducted using Student's t test or one-way analysis of variance (ANOVA). Statistical significance was denoted as $P<0.05$.

3.   Results

3.1   HLA typing of PBMCs from blood donors

In our preliminary investigation, the HLA typing of blood donors was conducted at the Laboratory for Immunogenetics and Molecular Diagnostics, LMU Großhadern Hospital. The HLA diagnostic results allowed us to categorize the donors into two groups: HLA-A2 positive and HLA-A2 negative.

Subsequently, I conducted verification experiments on peripheral blood mononuclear cells

(PBMCs) derived from these two groups of donors using flow cytometry. The results, as illustrated in Figure 1, revealed a remarkable HLA-A2 positivity rate of 99.3% in the selected blood donors, indicating a high recognition capability of their PBMCs towards Recoverin Epitopes (**Figure 1B**). Conversely, a distinct group of donors exhibited an HLA-A2 positivity rate of 6.78% and was deemed ineligible for further experimental analysis (**Figure 1A**).

By conducting comprehensive HLA typing and verifying the PBMCs, I successfully identified suitable blood donors for our study, ensuring the validity and reliability of the subsequent experimental investigations.
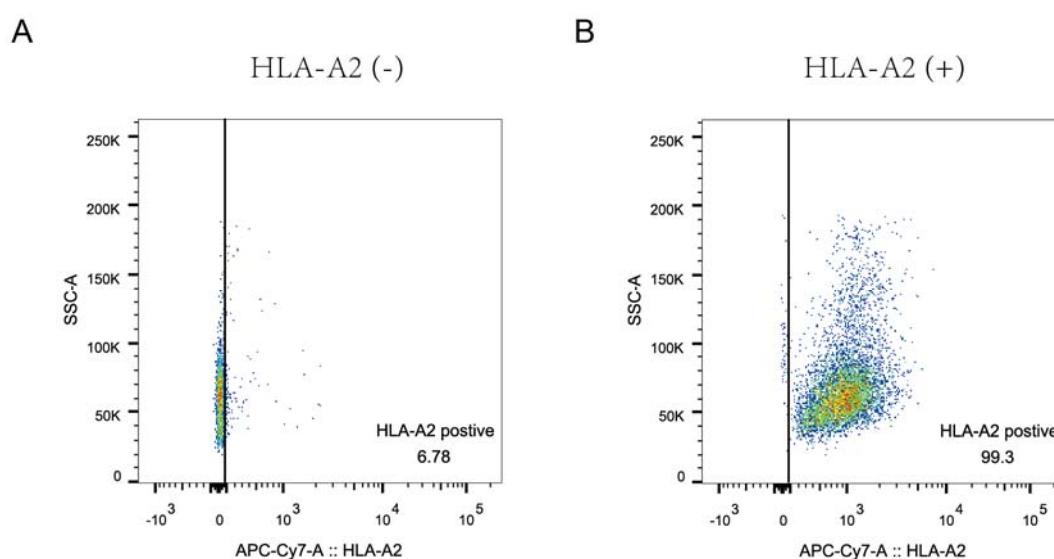
Figure 1: Flow cytometric analysis of HLA-A2 expression on PBMCs from blood donors. (**A**) HLA-A2 expression level on PBMCs from HLA-A2 negative donors. (**B**) HLA-A2 expression level on PBMCs from HLA-A2 positive donors.

3.2 DCs pulsed with Recoverin fusion protein elicits potent stimulation of T cell proliferation

In our previous research, we made a notable observation that the Recoverin fusion protein effectively activates dendritic cells (DCs), leading to a subsequent T cell activation. Given the crucial role of T cell abundance in influencing their cytotoxic effects on tumor cells, our current study aims to delve deeper into the impact of DCs pulsed with the Recoverin fusion protein on T cell proliferation. Additionally, we seek to compare this effect with T cell proliferation induced by Recoverin peptide or HSP70 alone.

3.2.1 DCs pulsed with Recoverin peptide alone fail to stimulate T cell colony formation

To initiate our investigation, we employed four distinct treatment strategies to induce the maturation and activation of DCs (**Table 3.2.1**). These strategies involved stimulating immature dendritic cells (iDCs) with three different antigens: Recoverin peptides, HSP70, and the Fusion protein. Consequently, we designated the activated DCs as iDC, DC_cyto, DC_pept/HSP70/FP, and DC_pept/HSP70/FP cyto, respectively.

Table 3.2.1 Four treatment groups for induction of immature dendritic cells (iDCs).

Group name	Treatment
iDC	IL-4 (1000 U/ml); GM- CSF (1000 U/ml)
DC cyto	IL-4 (1000 U/ml); GM- CSF (1000 U/ml); TNF-α (1000 U/ml); IFN-γ (1000 U/ml); PGE2 (1000 U/ml); CD40L (200 ng/ml)
DC-antigens	IL-4 (1000 U/ml); GM- CSF (1000 U/ml); Antigens, added with Recoverin peptides, HSP70, and Fusion protein, respectively.
DC-antigens cyto	IL-4 (1000 U/ml); GM- CSF (1000 U/ml); TNF-α (1000 U/ml); IFN-γ (1000 U/ml); PGE2 (1000 U/ml); CD40L (200 ng/ml); Antigens, added with Recoverin peptide, HSP70, and Fusion protein, respectively.

Following this, we set out to evaluate the ability of activated DCs to promote T cell proliferation. We conducted co-culturing experiments, where activated DCs were incubated with T cells from the same blood donors under serum-free conditions for a duration of seven days. To serve as controls, T cells were also cultured alone (negative control), and a well-known selective T cell mitogen, PHA, was added to the T cell culture medium (positive control). After the seventh day of co-culture, we meticulously observed and recorded T cell colony formation in various treatment groups using a microscope. This aspect of our study serves as a representation of T cell proliferation (**Figure 2A, C, E**). Subsequently, we utilized "ImageJ" software to measure the area of each T cell colony, and statistical analysis was performed using "GraphPad Prism 8" software for multiple comparisons (**Figure 2B, D, F**).

Our findings revealed that Recoverin peptide failed to induce larger T cell colony formation compared to iDC-induced T cell colonies (**Figure 2A, B**). In contrast, HSP70 and Recoverin fusion protein showed the ability to stimulate more substantial T cell colony formation (**Figure 2C, D, E, F**). These results suggest that both HSP70 and Recoverin fusion protein exhibit higher potency in stimulating T cell proliferation compared to Recoverin peptide.
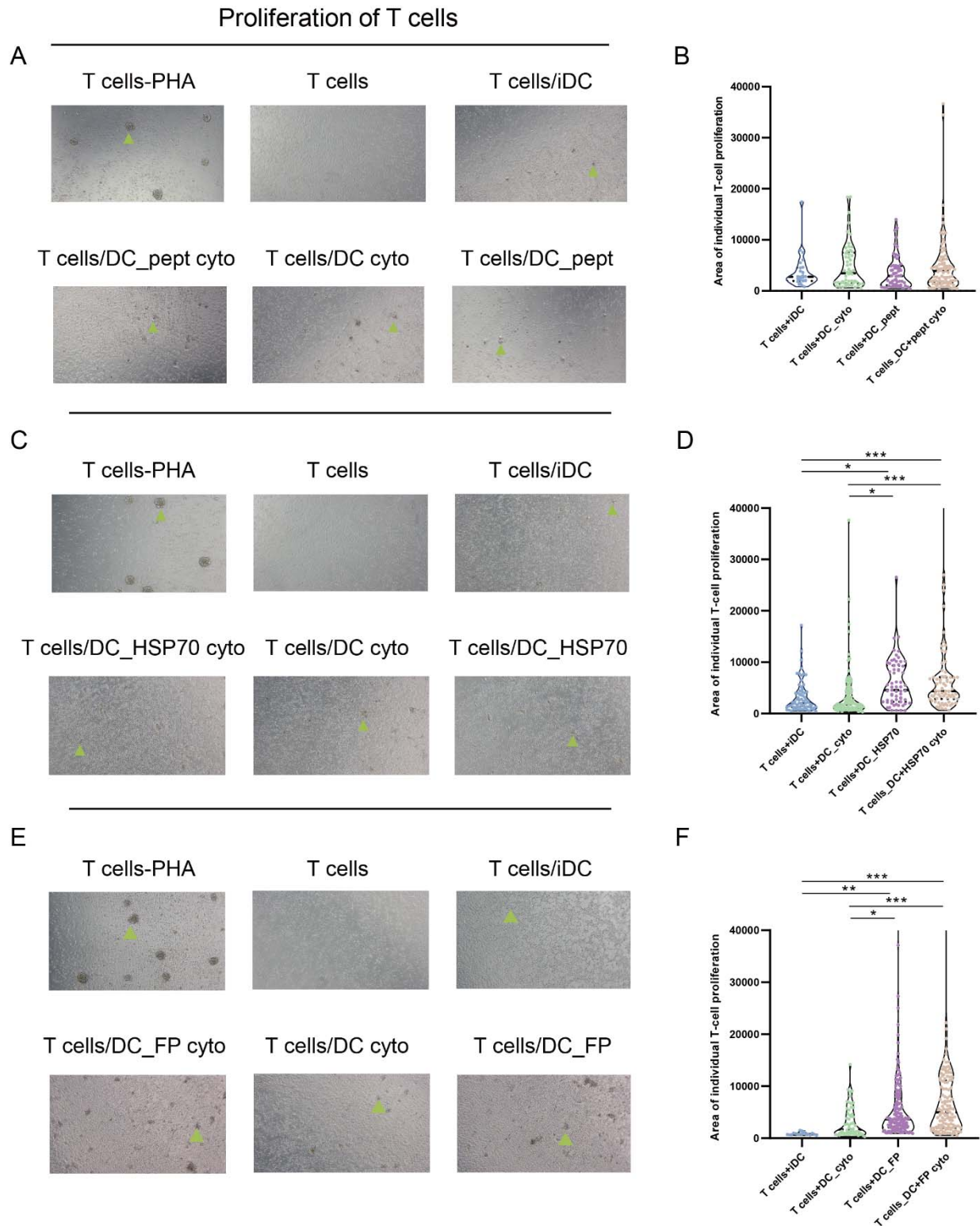
Figure 2: Evaluation of T cell colony formation induced by different treatments. (**A, B**) T cell colony formation after co-culturing with activated DCs pulsed with Recoverin peptide. (**C, D**) T cell colony formation after co-culturing with activated DCs pulsed with HSP70. (**E, F**) T cell colony formation after co-culturing with activated DCs pulsed with Recoverin fusion protein. Three independent repeated experiments were performed (* $P<0.05$, ** $P<0.01$, and

*** *P*<0.001).

3.2.2 DCs pulsed with Recoverin fusion protein stimulate strong T cell proliferation

Moving forward, we aimed to gain a more comprehensive understanding of the impact of the three different antigens on DCs' ability to promote T cell proliferation. We accomplished this by collecting CFSE-labeled T cells, as CFSE is a cell-permeable fluorescent dye commonly employed for tracking cell proliferation and division. The CFSE-labeled T cells were subjected to different treatments, and their fluorescence intensity was detected using a flow cytometer after a specified period, allowing us to precisely assess T cell proliferation (**Figure 3-5**).

Following the stimulation of iDCs from various blood donors with the four treatment strategies outlined in **Table 3.2.1**, we co-cultured these iDCs with CFSE-labeled T cells for seven days and subsequently analyzed the CFSE signal intensity (**Figure 3 and Figure 4**). Our results exhibited variations in the response of T cells from different donors to DCs pulsed with Recoverin peptide, with some donors showing comparable T cell proliferation to iDC-induced T cells (**Figure 3A, B**), while others displayed slightly higher (**Figure 3C, D**). or lower T cell proliferation (**Figure 3E, F**).
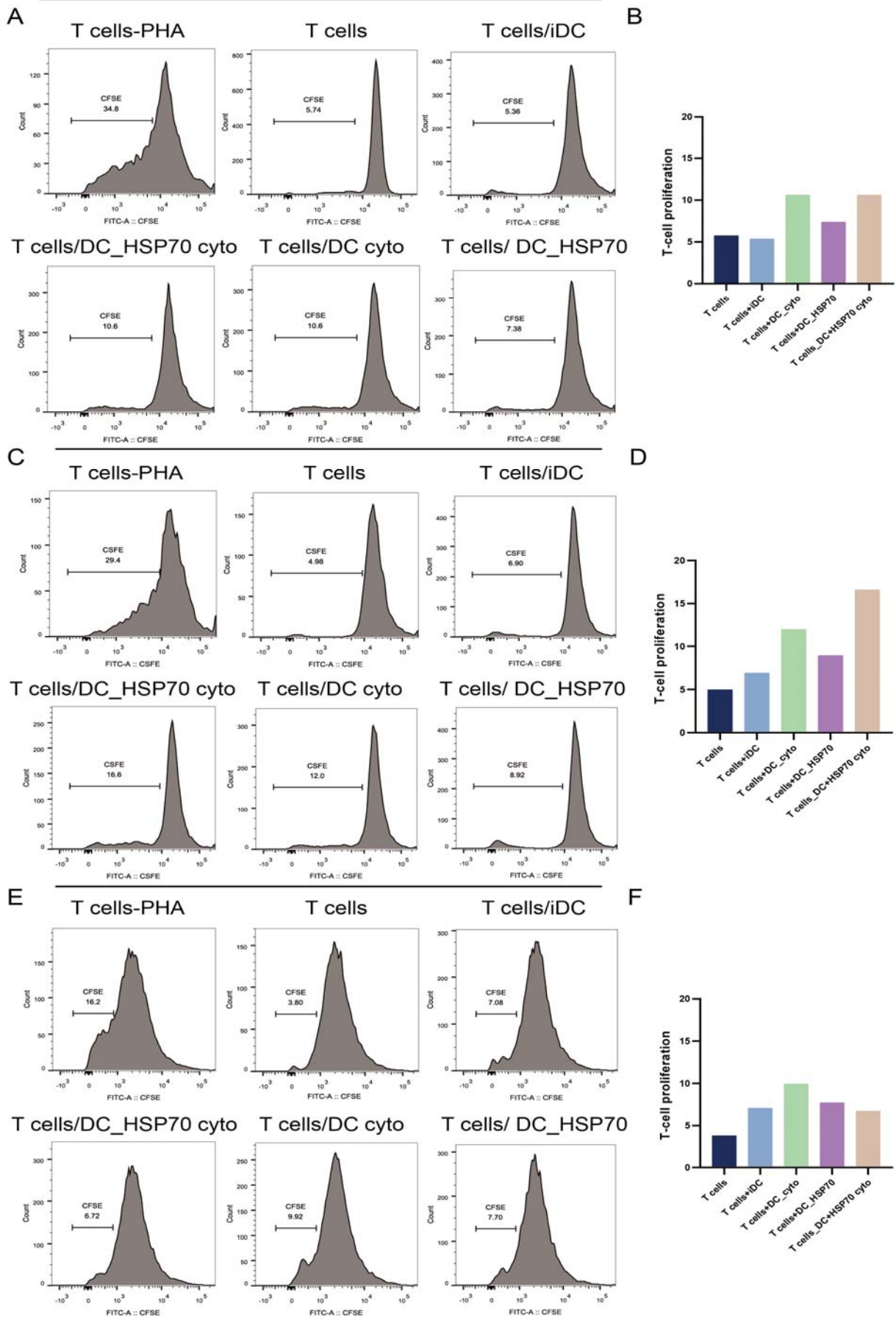
Proliferation of T cells
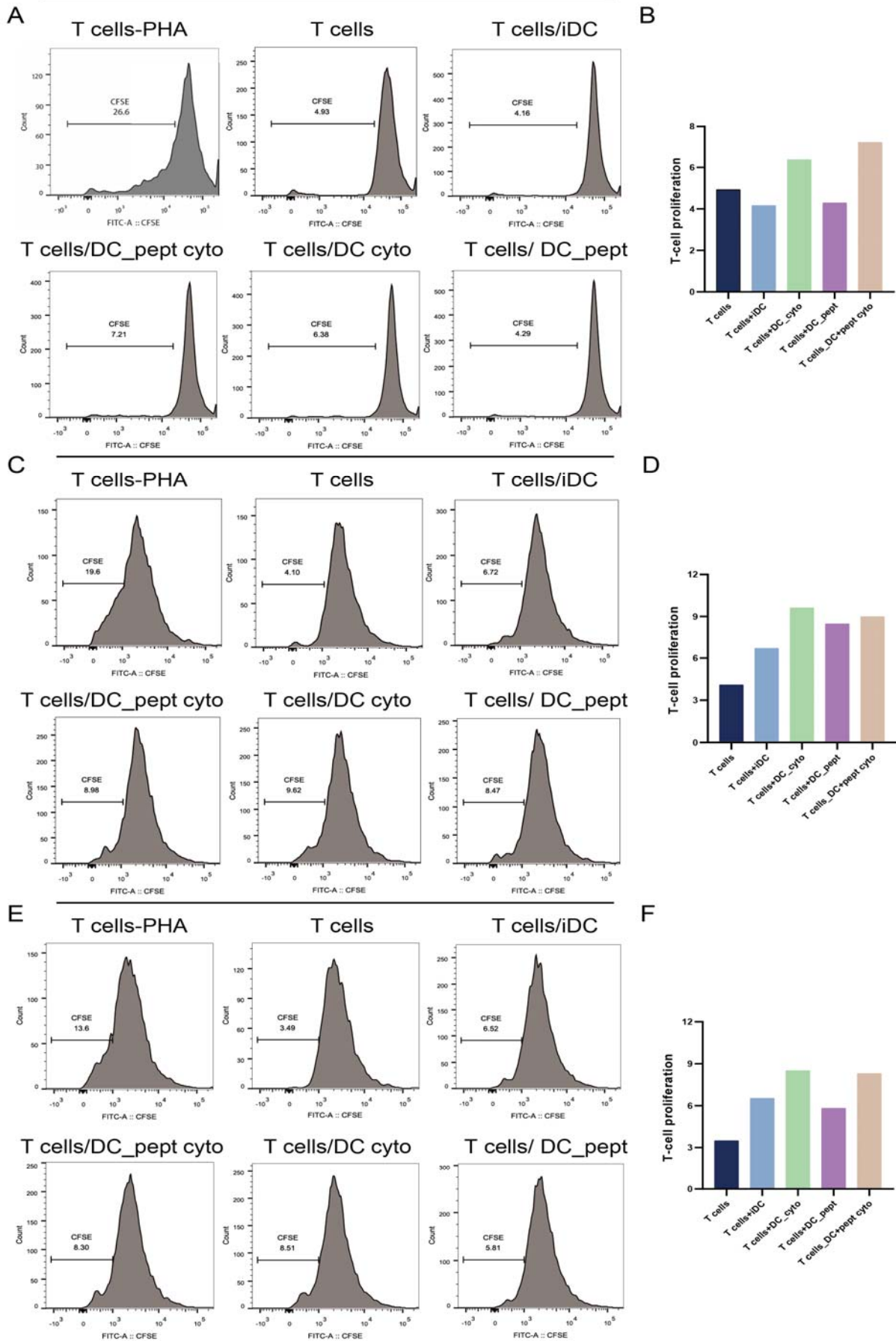
Figure 3: Impact of Recoverin peptide-induced DCs on T cell proliferation. (**A, C, E**) Detection of T cell proliferation signals upon stimulation with Recoverin peptide-loaded DCs using flow cytometry from 3 different blood donors. (**B, D, F**) Column graph of T cell proliferation signals in different treatment groups. Three independent repeated experiments were performed.

Similarly, stimulation of DCs with HSP70 led to slightly higher T cell proliferation in all donors compared to iDC-induced T cell proliferation (**Figure 4**). Interestingly, stimulation of DCs with individual cytokines demonstrated a more pronounced effect on promoting T cell proliferation than when combined with Recoverin peptide or HSP70 (**Figure 3, 4**).
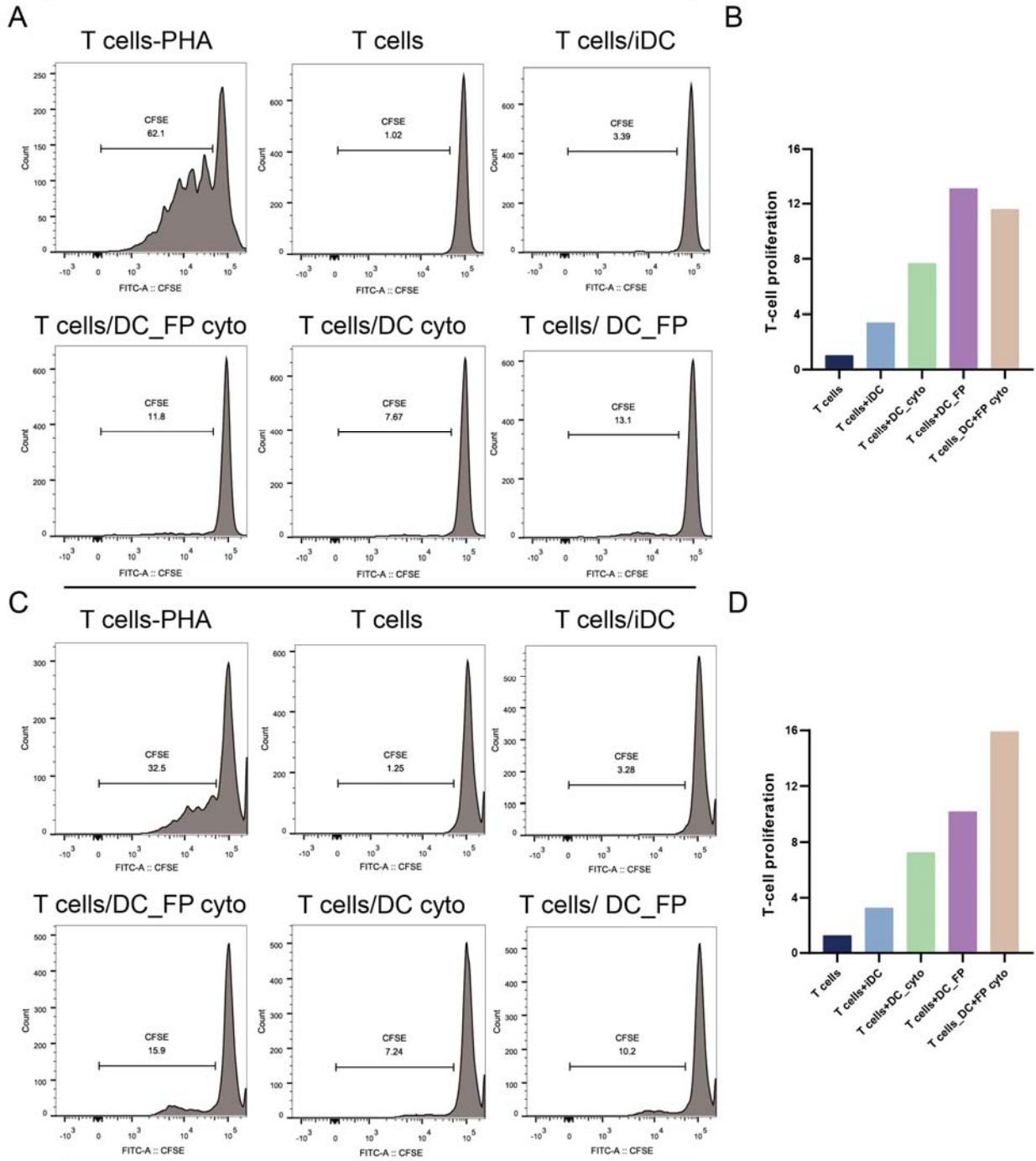
Proliferation of T cells

Figure 4: Impact of HSP70-induced DCs on T cell proliferation. (**A, C, E**) Detection of T cell proliferation signals upon stimulation with HSP70-induced DCs using flow cytometry from 3 different blood donors. (**B, D, F**) Column graph of T cell proliferation signals in different treatment groups. Three independent repeated experiments were performed.

Given HSP70's well-established role as an immune adjuvant, we constructed the Recoverin fusion protein to endow Recoverin with stronger immunogenicity. Utilizing a process similar to that described in **Figure 3** and **Figure 4**, we employed the Recoverin fusion protein to stimulate DCs for subsequent co-culturing with T cells. However, due to practical limitations, we were only able to perform the DCs pulsed with Recoverin fusion protein experiment twice (**Figure 5A, C**). Despite this limitation, the results showed significantly higher T cell proliferation when stimulated by DCs pulsed with Recoverin fusion protein compared to iDC-induced T cell proliferation (**Figure 5B, D**).

Figure 5: Impact of Recoverin fusion protein-induced DCs on T cell proliferation. (**A, C**) Detection of T cell proliferation signals upon stimulation with Recoverin fusion protein-induced DCs using flow cytometry from 2 different blood donors. (**B, D**) Column graph of T cell proliferation signals in different treatment groups. Two independent repeated experiments were performed.

To comprehensively evaluate the impact of the three different antigens on T cell proliferation induced by DCs, we calculated the ratio of T cells_DCs antigen-stimulated T cell proliferation values to T cells_iDC-stimulated T cell proliferation values (e.g., T cells/iDCs proliferation frequency: 3.39%, and T cells/DCs_FP proliferation frequency: 13.1%, resulting in a ratio of 1:3.86). As demonstrated in **Figure 6**, Recoverin fusion protein significantly promoted T cell proliferation compared to iDC, while DCs stimulated by Recoverin peptide or HSP70 alone did not significantly enhance T cell proliferation. This supports the notion that HSP70, as an immune adjuvant, effectively enhances the immunogenicity of Recoverin peptide, thereby inducing T cell proliferation.
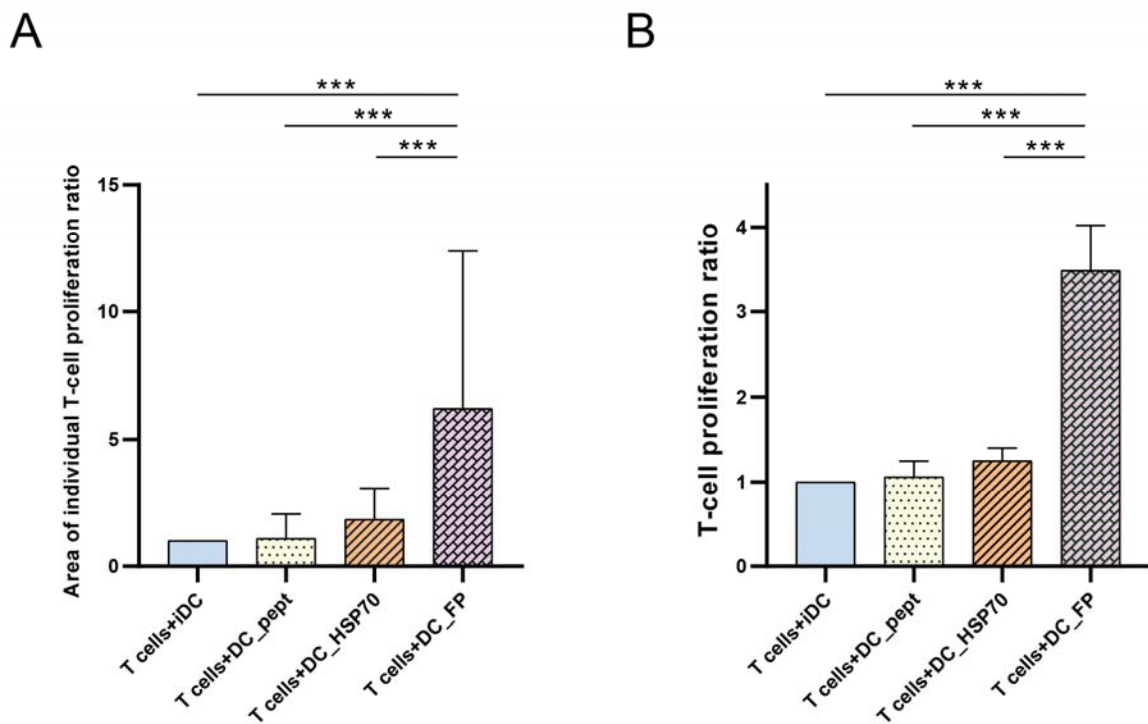
Figure 6: Impact of 3 antigens on T cell proliferation induced by DCs. (**A**) T cell colony formation size induced by DCs pulsed with 3 antigens. (**B**) T cell proliferation levels induced by DCs pulsed with 3 antigens. (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

3.3 Recoverin expression patterns in retinoblastoma via single-cell analysis

Recoverin is known to be primarily expressed in the ocular region, particularly in various

types of photosensitive cells within the retina. As part of our study aiming to explore the cytotoxic ability of T cells activated by dendritic cells (DCs) pulsed with Recoverin fusion protein against tumor cells, I have chosen retinoblastoma cells as the target for our subsequent anti-tumor research. Prior to this investigation, we employed single-cell analysis techniques to explore the expression patterns of Recoverin in different cell types within retinoblastoma (**Figure 7**). Initially, all cells were annotated based on specific markers expressed by the cells. Ultimately, retinoblastoma cells were categorized into 33 subgroups (**Figure 7A**), and subsequently classified into 8 distinct cell types, including Amacrine cells, Cones, Endothelial cells, Goblet cells, Horizontal cells, Microglia, Muller Glia, and Retinal cells (**Figure 7B**). I visualized the distribution of RCVRN (Recoverin) across these various cell subgroups. Interestingly, I observed that RCVRN is predominantly expressed at high levels in Retinal cells and Goblet cells (**Figure 7C**). Following this, RCVRN expression levels in each cell subgroup were depicted using violin plots. Notably, Retinal cells were further subdivided into distinct clusters, including C2, 4, 9, 13, 17, 18, 24, 25, and 31, all of which exhibited high levels of RCVRN expression collectively (**Figure 7D**). Goblet cells, on the other hand, were divided into two subclusters, C12 and C28, both showing elevated levels of RCVRN expression (**Figure 7G**). These findings suggest a potential association between RCVRN expression and the Retinal cells and Goblet cells, prompting further investigation into the intercellular communication between these two cell types. Interestingly, we found that two Goblet cell subgroups (C12, 28) and certain Retinal cell subgroups (C2, 4, 9, 17, 20, 24, 25, 27, 31) exhibited remarkably similar communication patterns (**Figure 7E, F**). Considering the significant enrichment of RCVRN in Retinal cells of retinoblastoma, we selected the Y79 cell line (a retinoblastoma cell line) as a working model for studying the anti-tumor cytotoxicity of T cells activated by Recoverin antigen.
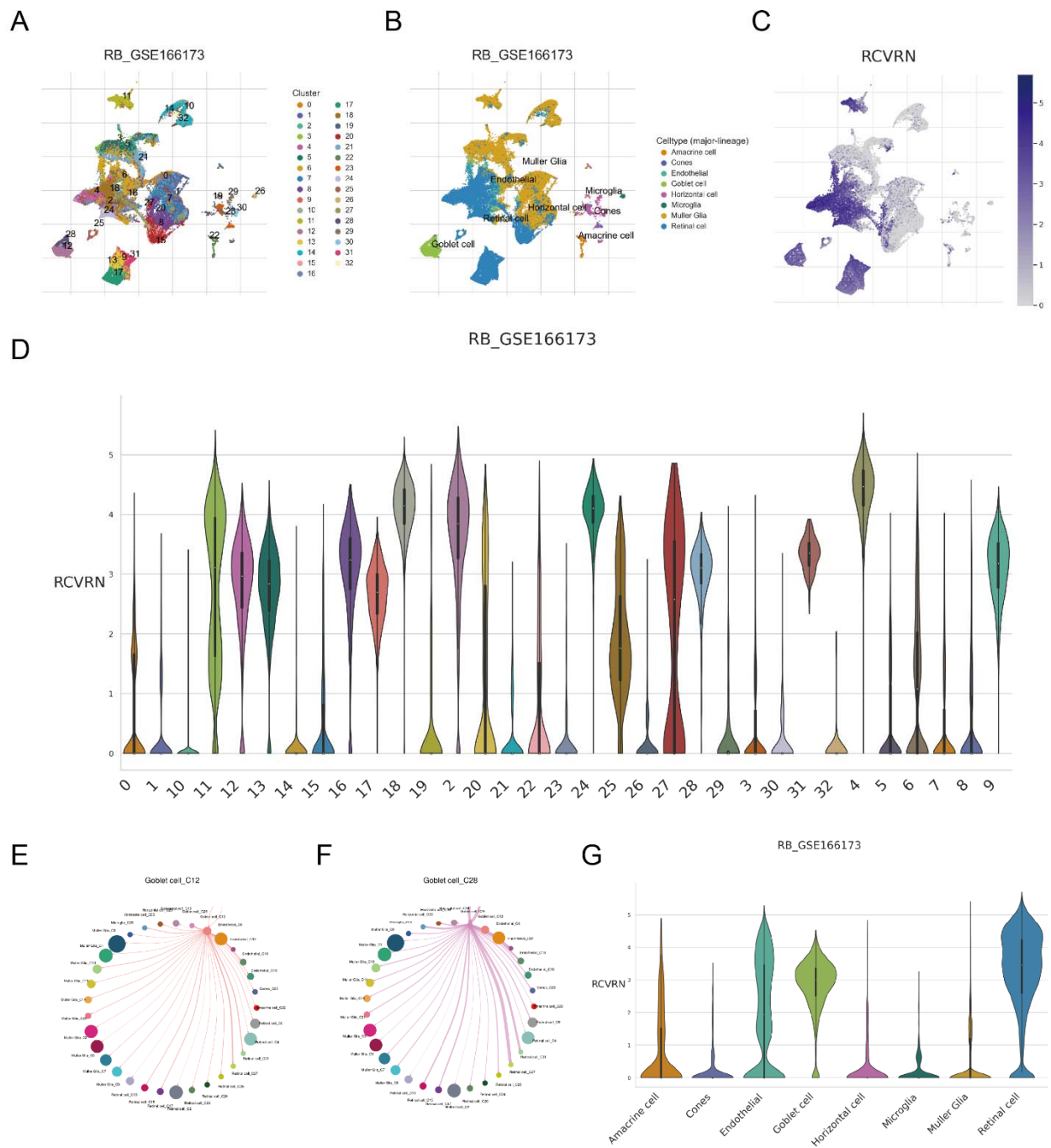
Figure 7: Recoverin expression patterns in retinoblastoma via single-cell analysis. (**A**) The UMAP plot revealed that all cells were classified into 33 distinct clusters**.** (**B**) The UMAP map was used to identify 8 different cell types in the TME, as represented by different colors. (**C**) RCVRN expression pattern in retinoblastoma. (**D**) RCVRN expression level in 33 retinoblastoma clusters**.** (**E, F**) Cell-cell interaction (CCI) between Goblet cell and other clusters. (**G**) Distribution of RCVRN expression in 8 cell types from retinoblastoma.

3.4 Recoverin protein expression level in Y79 cell line

Single-cell analysis has revealed an elevated transcription level of Recoverin mRNA within retinoblastoma retinal cells, indicating their active engagement within the pathological milieu. To extend this comprehension, I conducted an investigation into the expression profile of the Recoverin protein within Y79 cell line, a retinoblastoma cell line. Instances of CAR have been observed in certain cases of HCC patients, implying the existence of Recoverin expression within HCC cells. Consequently, I employed HCC cell line, Huh7, as control group to scrutinize the levels of Recoverin protein in Y79 cells.

The outcomes derived from western blot analyses have been illuminating. Particularly, the zenith of Recoverin protein expression has been conspicuously demonstrated within Y79 cells, showcasing a marked contrast against HCC cell line (P<0.001) (**Figure 8A, B**). In summary, the results of the western blot ascertain one of the pivotal prerequisites for Y79 cell line as a suitable working model for cytotoxicity experiments. Another vital precondition is the expression of HLA-A2 in Y79, which I will assess in the forthcoming experiments.
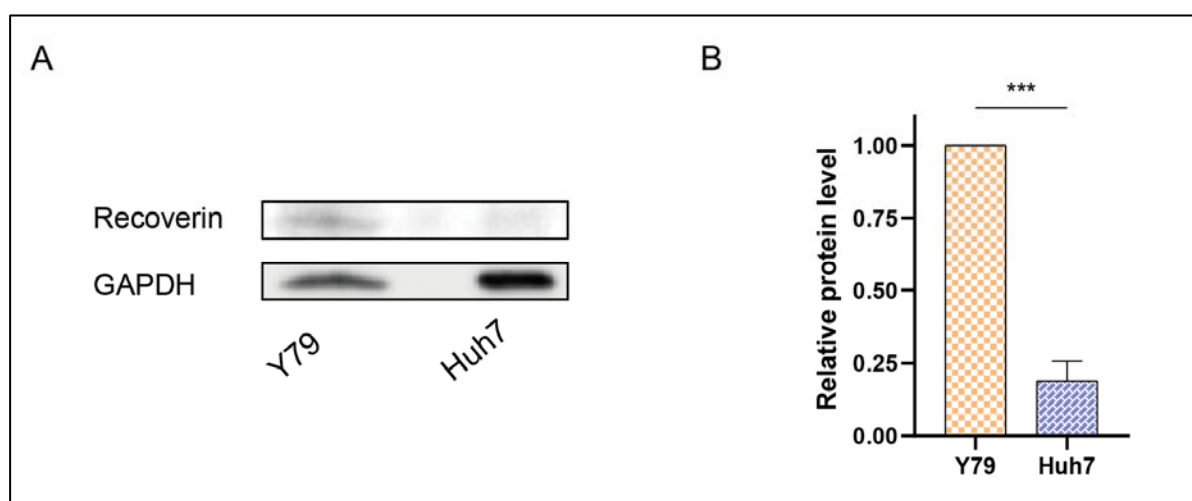
Figure 8: Recoverin protein expression levels in Y79 cell line. (**A**) Detection of Recoverin protein expression via western blot in different cell lines. (**B**) Statistical analysis of three independent repeated experiments showed that Y79 significantly expressed Recoverin protein, compared to Huh7 cell line. (*** P<0.001).

3.5 HLA-A2 expression level in Y79 cell line

HLA-A2, a subtype of human leukocyte antigen (HLA), plays a crucial role in presenting antigen fragments to CD8+ T cells on the cell surface. HLA-A2 molecules bind to

intracellularly produced antigen fragments, such as intracellular aberrant proteins, and present these fragments on the cell surface for detection and recognition by T cells of the immune system. Upon detection of antigen fragments presented by HLA-A2 molecules, T cells become activated through binding with these antigen fragments via the T cell receptor (TCR), subsequently leading to the release of cytotoxins that target cells expressing these antigens. Thus, before confirming Y79 as the model cell line, it is essential to assess the HLA-A2 expression level in Y79 cells. As shown in **Figure 9**, approximately half of the Y79 cells are HLA-A2 positive, indicating that Y79 cells can serve as a suitable model cell line for detecting the cytotoxic effects of T cells.
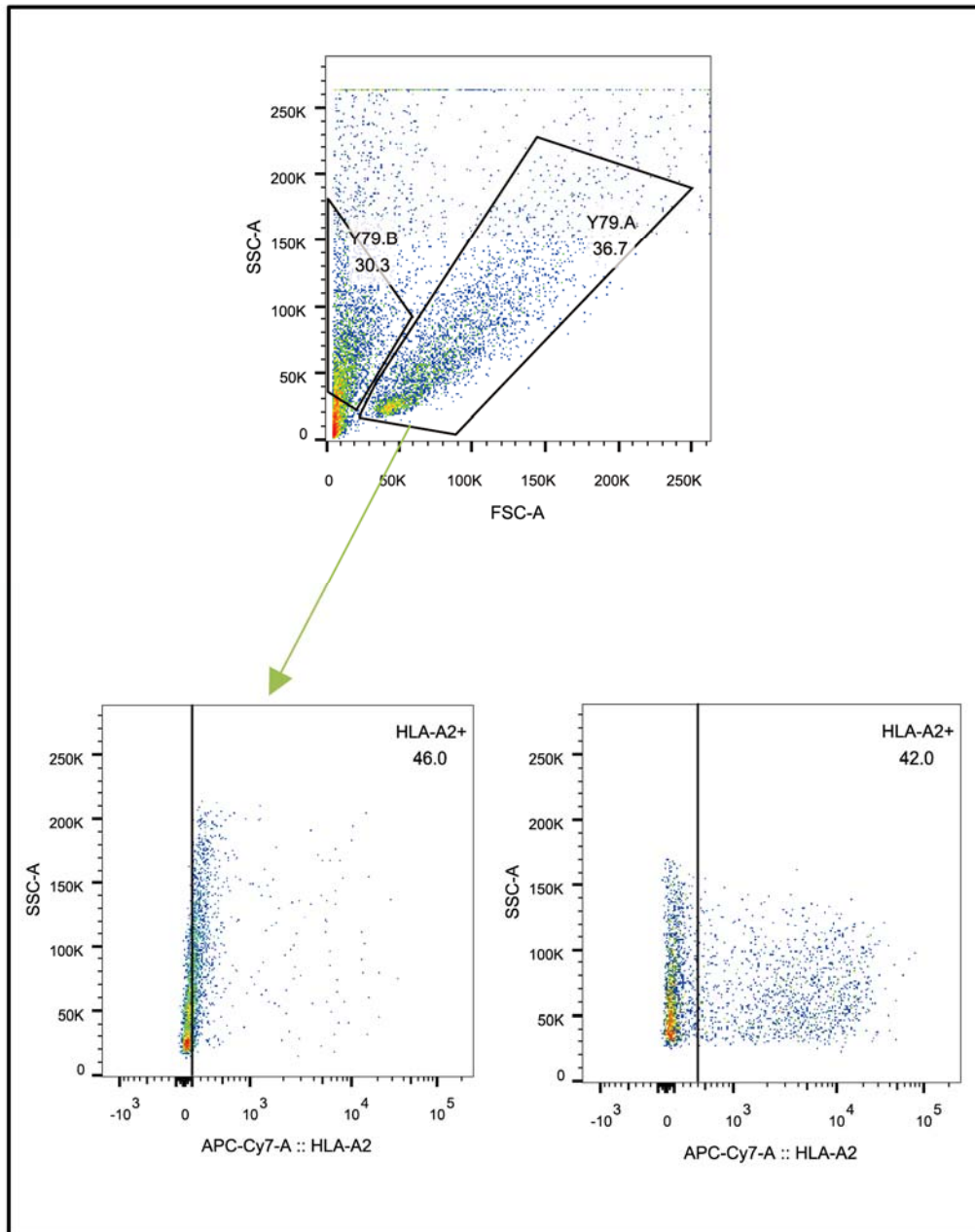
Figure 9: HLA-A2 expression level in Y79 cell line.

3.6 Dynamic survival states of T cells co-cultured with DCs at various time points within two weeks

A sufficient number of healthy T cells is a prerequisite for effective anti-tumor action. Prior to co-culturing T cells with Y79 cells, T cells need to be co-cultured with antigen-activated DCs for 2 weeks. Hence, we chose different time points (days 1, 7, 13, 14, and 15) to assess T cell viability. Most of the T cells were found to be viable at the time points before T cell activation by DCs (days 1, 7, 13) (**Figure 10 A-C**). However, with increasing T cell culture time, more T cells exhibited a dispersed distribution. Therefore, we re-evaluated T cell viability during the first two days of co-culturing T cells with Y79 cells (**Figure 10 D, E**). Regarding cluster "T cells 1," the proportion of T cells decreased in a time-dependent manner (from 80.9% to 10.7%). Nonetheless, it is worth noting that this subset of T cells remained mostly viable and potentially capable of exerting anti-tumor effects. After 14 days of T cell culture, some T cells in cluster T cells 1 started to move towards cluster T cells 2, and these T cells showed a higher percentage of 7-AAD positivity. However, considering the overall cluster T cells 3, approximately 70% of the T cells retained their vitality, suggesting potential tumor-killing activity.
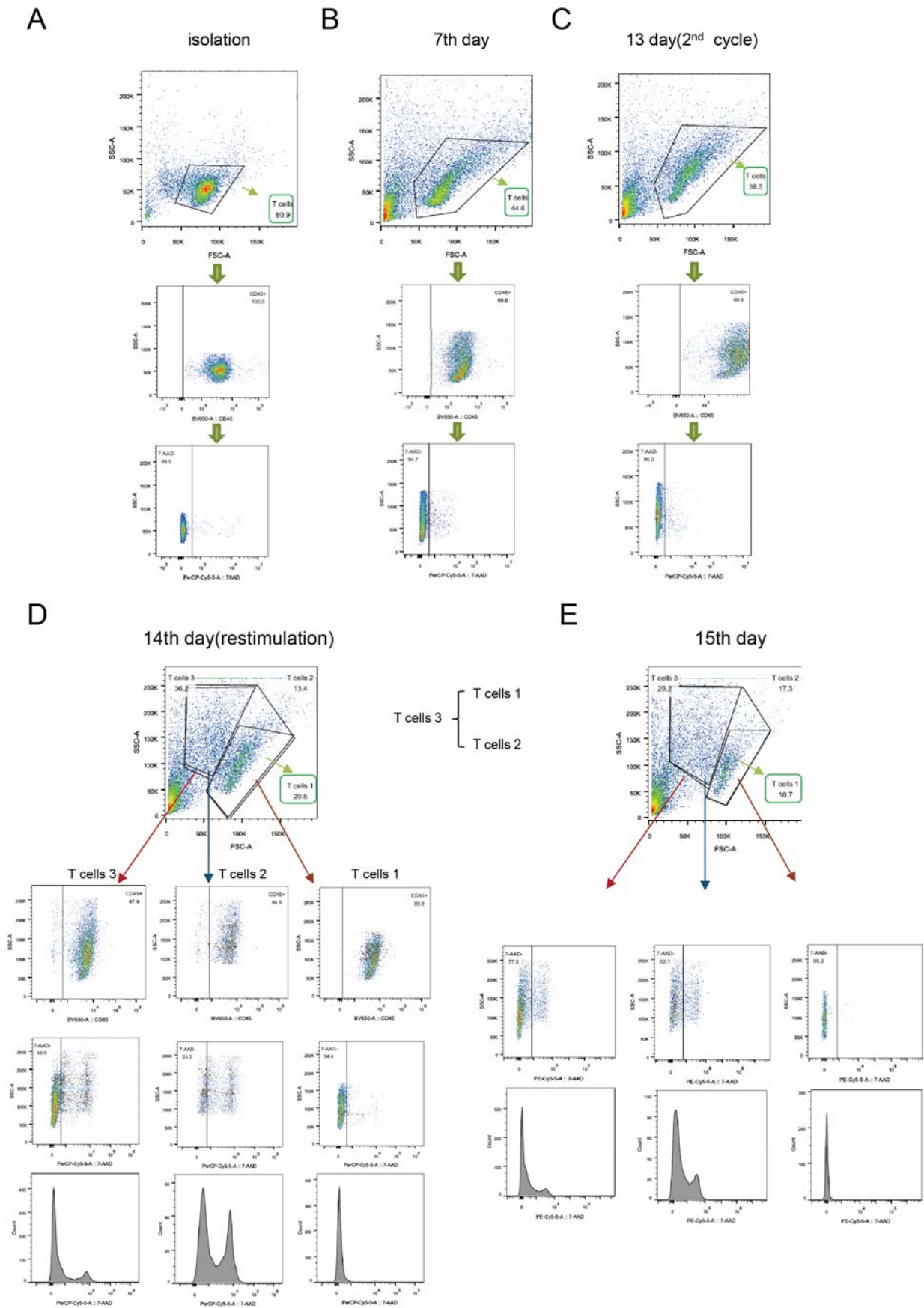
Figure 10: Assessment of T cell viability during co-culture with antigen-activated DCs.

(**A, B, C**) Quantify the surviving T cells on days 1, 7, and 13 after co-culture with antigen-activated DCs. During this timeframe, approximately 95% proportion of T cells remained viable. (**D**) Upon receipt of DCs re-stimulation at day 14, T cell viability was assessed. (**E**) T cell viability was assessed the day before co-culture with Y79 (day 15).

3.7 Cytotoxicity assessment of T cells against Y79 induced by DCs loaded with Recoverin fusion protein

Following the determination of the appropriate model cell (Y79) and the assessment of T cell viability before co-culturing with Y79 cells, we initiated the assessment of T cell cytotoxicity against Y79 cells induced by DCs, as described in Methods 2.2.11, to clarify the potential of the three antigens as tumor vaccines.

3.7.1 DCs pulsed with Recoverin fusion protein stimulate T cell colony formation

After co-culturing T cells with DCs from different treatment groups for 2 weeks, we observed and recorded the formation of T cell colonies under a microscope. The "ImageJ" and "GraphPad Prism 8" software were used for measuring the size of T cell colonies (**Figure 11A, C, E**) and for statistical analysis (**Figure 11B, D, F**). Compared to the iDC group, T cell colonies from the Recoverin fusion protein and HSP70 groups exhibited larger sizes ($P<0.01$) (**Figure 11A-D**). However, no statistically significant difference in T cell colony size was observed between the Recoverin peptide group and the iDC group ($P>0.05$) (**Figure 11E, F**).
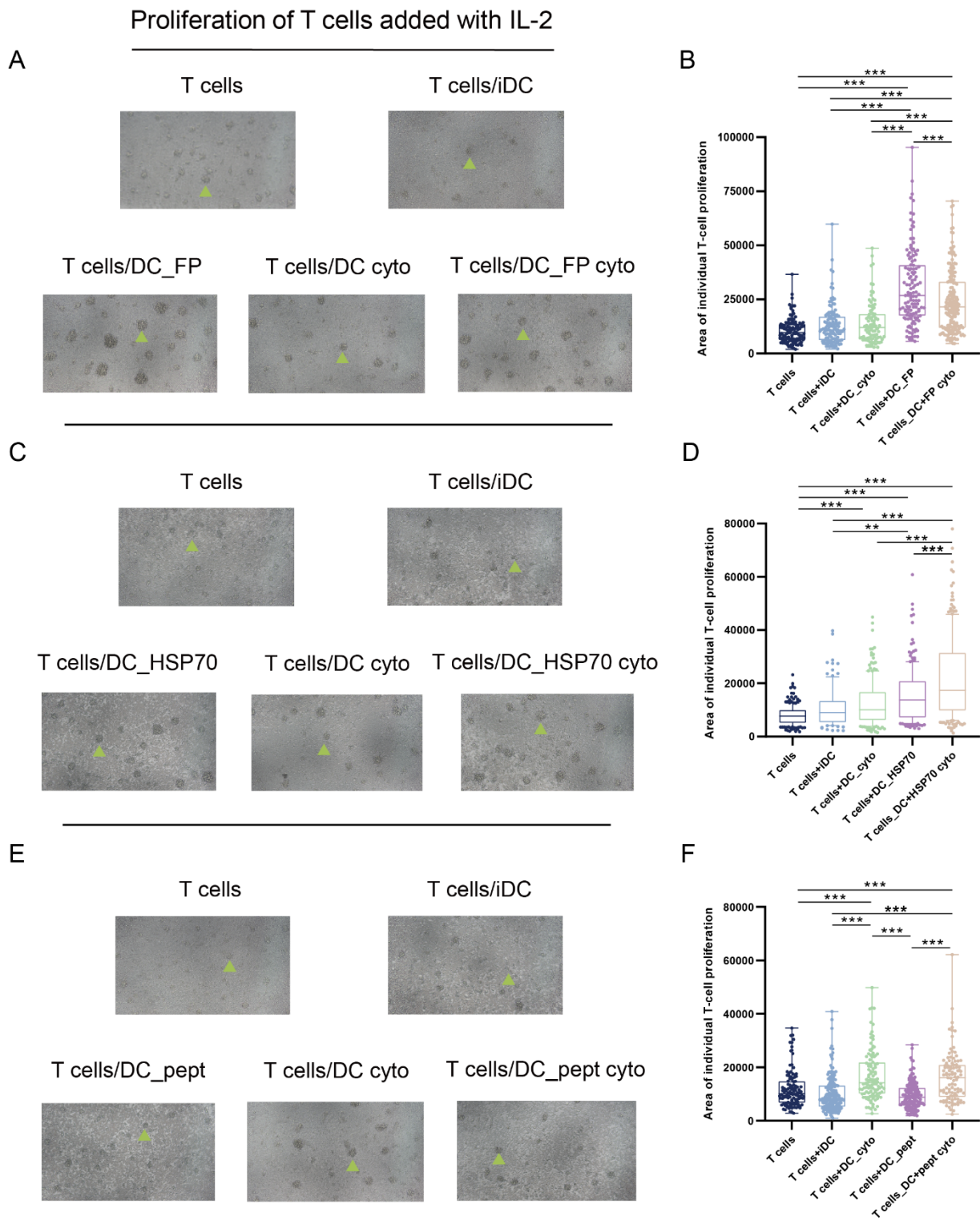
Figure 11: Measurement of T cell colony formation induced by DCs after 14 days. (**A, B**) T cell colony formation after co-culturing with activated DCs pulsed with Recoverin fusion protein. (**C, D**) T cell colony formation after co-culturing with activated DCs pulsed with HSP70. (**E, F**) T cell colony formation after co-culturing with activated DCs pulsed with Recoverin peptide. Three independent repeated experiments were performed (* P<0.05, **

P<0.01, and *** *P*<0.001).

3.7.2 DCs pulsed with Recoverin fusion protein strengthen T cell cytotoxicity against Y79 cell line

Subsequently, we collected these T cells and co-cultured them with Y79 cells at a ratio of 10:1 for 24 hours, followed by flow cytometry to detect the percentage of dead Y79 cells. **Figure 12** demonstrates the cytotoxic effects of T cells stimulated by DCs loaded with Recoverin fusion protein against Y79 cells from two different blood donors. The results indicated that T cells from the Recoverin fusion protein group exhibited significantly higher cytotoxicity against Y79 cells, with increases of 24.27% and 26.9% compared to the T cell group (**Figure 12A, D**). Furthermore, compared to the iDC control group, T cells stimulated by Recoverin fusion protein induced higher Y79 cell death, suggesting stronger anti-Y79 cytotoxicity (**Figure 12C, F**).
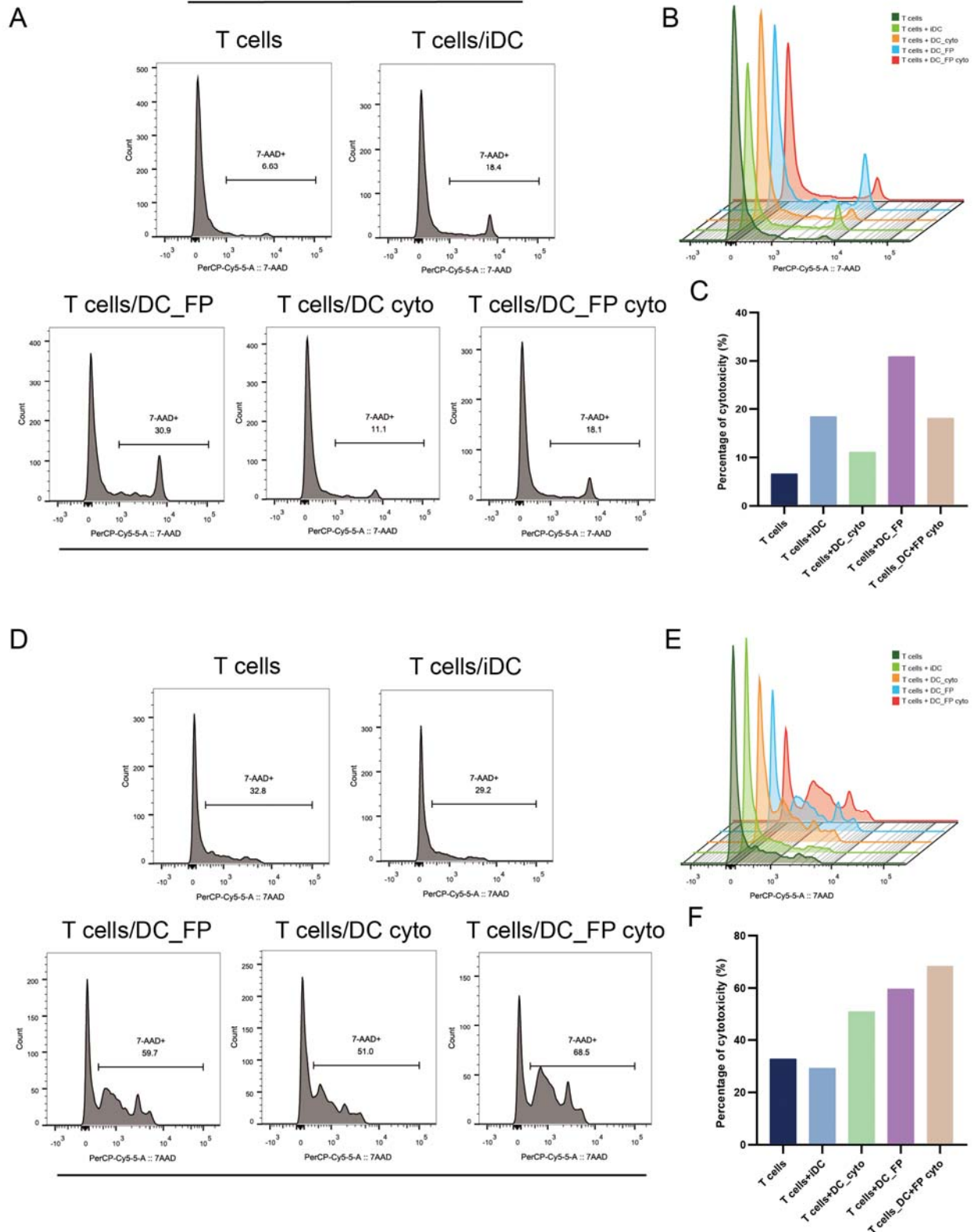
Figure 12: T cell cytotoxicity induced by DCs loaded with Recoverin fusion protein against Y79 cells. (**A, B, D, E**) Detection of Y79 dead signals after 24h co-culture with T cells induced by Recoverin peptide-loaded DCs using flow cytometry. (**C, F**) Bar charts

depicting the proportions of Y79 cell death from distinct treatment groups, each derived from two separate blood donors. Two independent repeated experiments were performed.

As the Recoverin fusion protein consists of HSP70 and Recoverin peptide, we continued to evaluate the ability of DCs stimulated with HSP70 and Recoverin peptide separately to induce T cell killing of Y79 cells. As shown in **Figure 13**, T cells stimulated by DCs loaded with HSP70 exhibited a cytotoxic effect on Y79 cells similar to that of the iDC group (10.7% and 10.2%) (**Figure 13A, D**). Moreover, the cytokine group seemed to display better anti-Y79 ability; however, this effect varied greatly between different blood donors (25.9% and 11.8%) (**Figure 13C, F**).

Figure 13: T cell cytotoxicity induced by DCs pulsed with HSP70 against Y79 cells. (**A, B, D, E**) Detection of Y79 dead signals after 24h co-culture with T cells induced by HSP70-pulsed DCs using flow cytometry. (**C, F**) Bar charts depicting the proportions of Y79 cell

death from distinct treatment groups, each derived from two separate blood donors. Two independent repeated experiments were performed.

The above research has already indicated that the Recoverin peptide group failed to induce significant T cell proliferation compared to the iDC group. However, the cytotoxic effect of these T cells against Y79 cells remains unknown. Three different blood donors' T cells participated in this experiment (**Figure 14; Figure 15**). T cells stimulated by DCs loaded with Recoverin peptide from these three donors demonstrated killing percentages of 12.3%, 19.4%, and 12.2% against Y79 cells (**Figure 14A, D; Figure 15A**). Compared to the iDC group, Recoverin peptide-stimulated T cells exhibited higher cytotoxicity against Y79 cells (**Figure 14C, F; Figure 15C**).
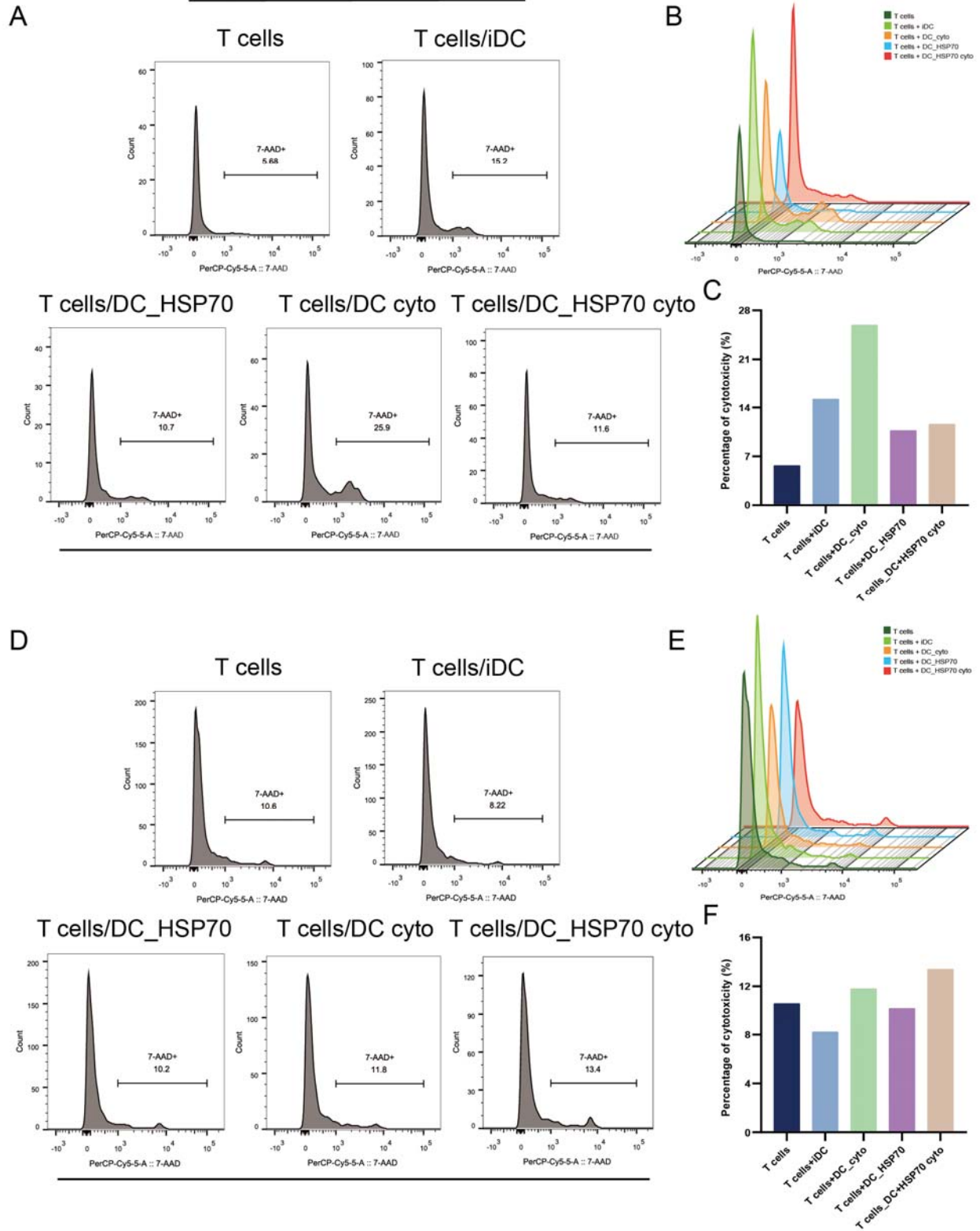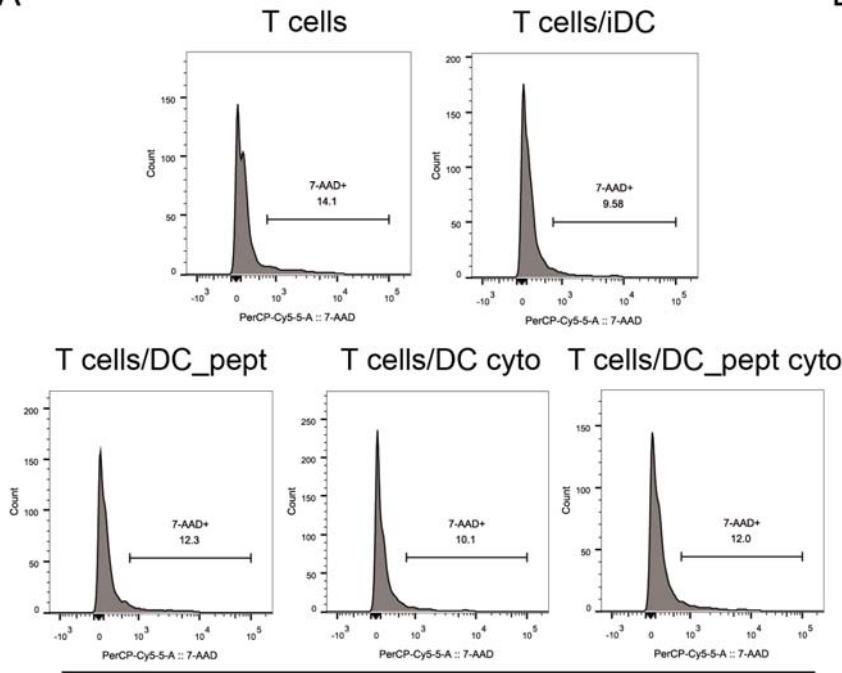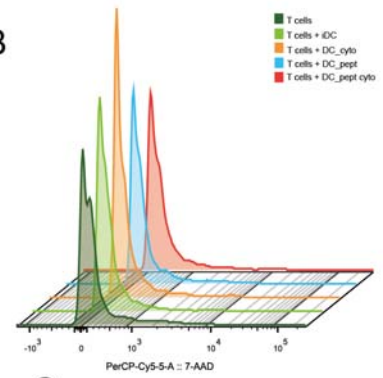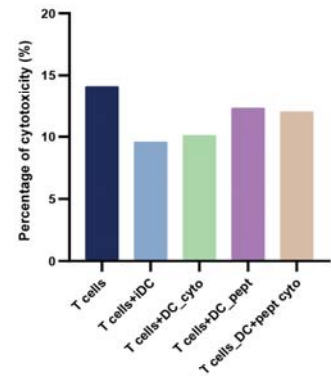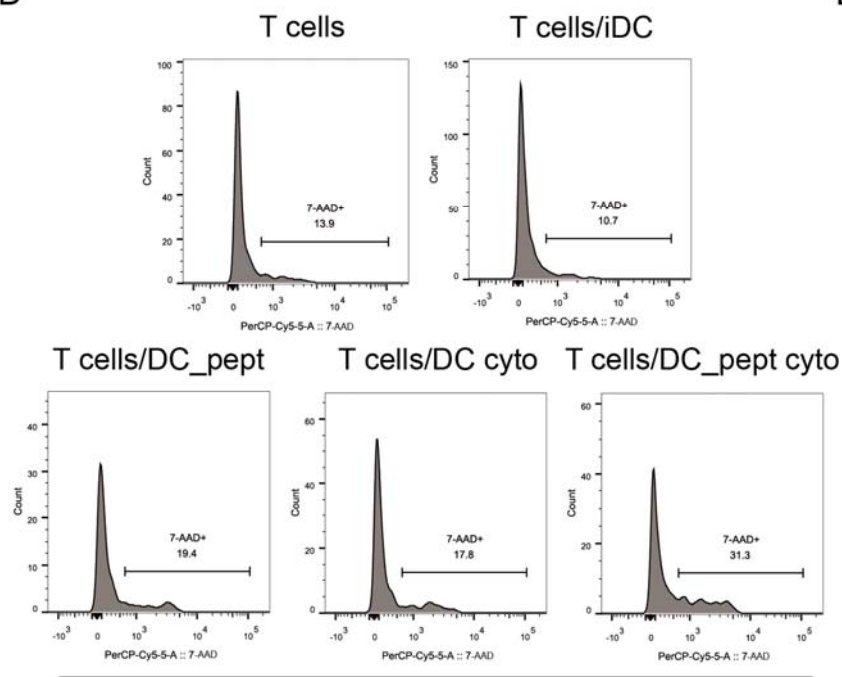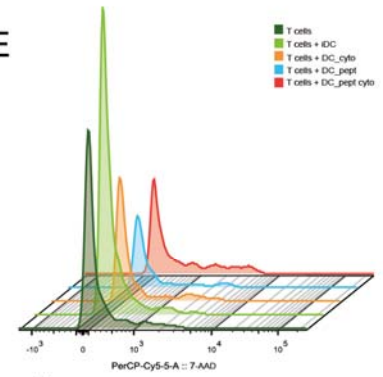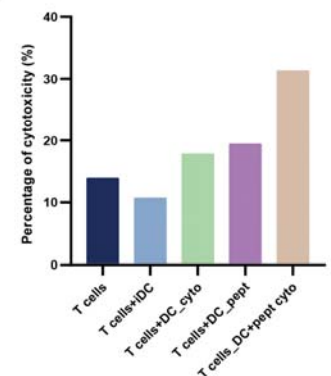
Figure 14: T cell cytotoxicity induced by DCs loaded with Recoverin peptide against Y79 cells. (**A, B, D, E**) Detection of Y79 dead signals after 24h co-culture with T cells induced by Recoverin peptide-loaded DCs using flow cytometry. (**C, F**) Bar charts depicting

the proportions of Y79 cell death from distinct treatment groups, each derived from two separate blood donors. Three independent repeated experiments were performed.
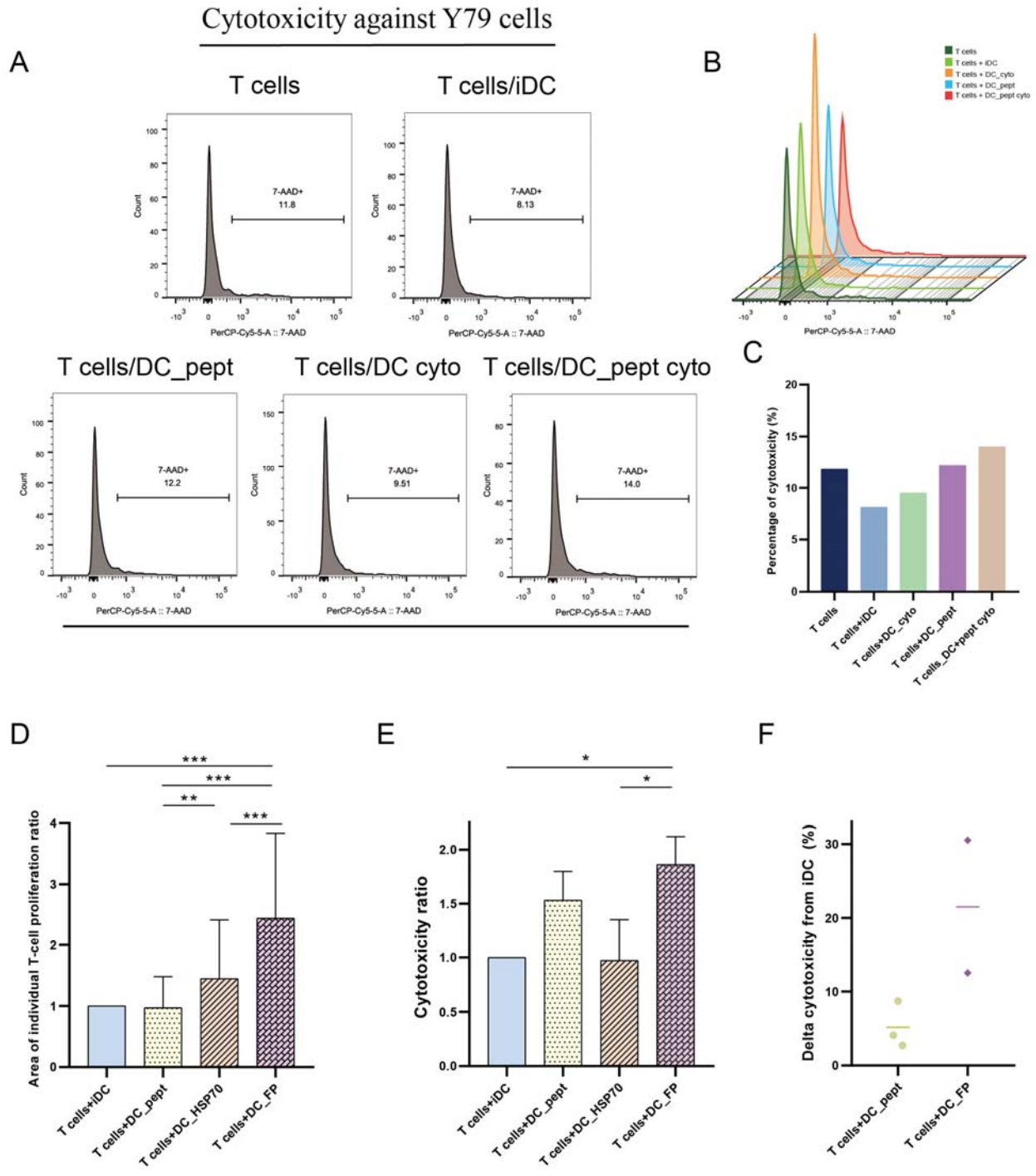
Figure 15: Comparative Analysis of T cell cytotoxicity ratios from DCs pulsed with 3 antigens. (**A-C**) Detection of Y79 dead signals after 24h co-culture with T cells induced by Recoverin peptide-loaded DCs using flow cytometry. (**D**) T cell colony formation size induced

by DCs pulsed with 3 antigens for 14 days. (**E**) T cell cytotoxicity ability against Y79 cells after stimulating by DCs pulsed with 3 antigens. (**F**) The delta value of T-cell cytotoxicity against Y79 cells between the Recoverin peptide and Recoverin fusion protein groups, as compared to the T-cell cytotoxicity in the iDC group. (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

Furthermore, we compared the colony size and cytotoxicity of T cells induced by DCs loaded with the three antigens. The results showed that only T cells from the Recoverin fusion protein group exhibited larger colony sizes and more significant cytotoxic effects against Y79 cells compared to the iDC group (**Figure 15D, E**). However, no statistically significant difference was observed between the Recoverin fusion protein group and the Recoverin peptide group, which may be attributed to the limited number of experiments. Nevertheless, the difference in cytotoxicity between the Recoverin fusion protein group and the iDC group was markedly higher than that between the Recoverin peptide group and the iDC group (**Figure 15F**). Further experiments with increased replication are required to clarify the difference in T cell cytotoxicity between the Recoverin fusion protein group and the Recoverin peptide group.

In summary, our findings demonstrate that DCs loaded with Recoverin fusion protein can effectively stimulate T cell proliferation and enhance T cell cytotoxicity against Y79 cells.

4. Discussion

In recent years, the efficacy of immunotherapy in tumor resistance has been consistently revealed, including immune checkpoint blockade (ICB) and chimeric antigen receptor T cell therapy (CAR-T). Despite these advancements, the effectiveness of current immunotherapies still faces limitations, emphasizing the urgent need to identify new tumor antigens with strong immunogenicity. Recoverin, a widely distributed protein in the retina, is mainly located in the photoreceptor outer segments of vertebrate retinas and is scarcely expressed in other normal tissues due to the presence of the blood-retinal barrier. However, in various tumor tissues, especially in retinoblastoma, breast cancer, lung cancer, and prostate cancer, there is a significant upregulation of Recoverin expression [139-144]. This confers strong immunogenicity upon the Recoverin protein, making it a crucial component for immune vaccination. In this context, my research team has directed its attention to the potential of Recoverin as a cancer vaccine.

Cancer-associated retinopathy (CAR) has been reported as a rare paraneoplastic autoimmune disease, characterized by painless vision loss [141]. Autoantibodies generated against cancer-associated retinal antigens cross-react with proteins located at the blood-retinal barrier, ultimately leading to retinal damage and severe vision loss [148, 149]. Patients with small cell lung cancer exhibiting CAR demonstrate improved prognosis over their counterparts lacking CAR [259]. Maeda et al. postulate this advantage could stem from the activity of Recoverin-specific cytotoxic T lymphocytes, which target tumor cells [260]. This insight suggests a unique immunological interaction where Recoverin-specific CTLs play a pivotal role in mediating tumor cell eradication. Further, they employed a subcutaneous tumor model in mice to assess the in vivo anti-tumoral efficacy of Recoverin peptide. The study revealed that, although the Recoverin peptide exhibited significant antitumor activity, it concurrently precipitated retinal degeneration in the mice [261]. This has deterred the serious consideration of using Recoverin protein as a tumor vaccine. Drawing on this experience, my research team has fused Recoverin peptide with HSP70 protein, a well-established and efficacious immune adjuvant as reported in earlier investigations [262]. The primary focus of this design is the precise targeting of T cell epitopes, leading to a potentiation of immunostimulatory responses. This strategic integration aims to circumvent the potential initiation of autoantibody generation against the Recoverin peptide through humoral immune

reactions. This effectively prevents the occurrence of CAR associated with the Recoverin vaccine and reduces damage to normal tissues. It is noteworthy that the Recoverin fusion protein exhibits antitumor activity exclusively in recoverin-positive patients, who may have already developed cancer-associated retinopathy (CAR) prior to vaccination with a recoverin vaccine. For example, Luis et al. reported a case of CAR where the patient exhibited only rapid bilateral visual acuity decline without other symptoms, ultimately being diagnosed with non-small cell lung cancer [263]. Fortunately, recent findings by Mudri and Kim et al. demonstrate that intervention with intravitreal dexamethasone implants in patients with CAR can effectively improve visual acuity [190, 204]. Consequently, for tumor patients already afflicted with CAR, a combined therapeutic approach involving Recoverin fusion protein and dexamethasone presents a potential treatment strategy.

Understanding the immunogenicity of Recoverin and evaluating its potential as a tumor vaccine is the primary focus of our current project. Earlier investigations by team members, particularly Yue Zhao et al., have convincingly demonstrated that Recoverin fusion protein efficiently induces the maturation and activation of dendritic cells (DCs), subsequently leading to effective T cell activation [262]. Building upon this foundation, our project aims to further explore the impact of DCs loaded with Recoverin fusion protein on T cell proliferation, comparing its effects to those of individual Recoverin peptide and HSP70. Additionally, we seek to evaluate the anti-tumor cytotoxicity of T cells stimulated by DCs pulsed with three distinct antigens.

In the immune response, T cells play a critical role, with CD8+ T cells being a subset referred to as cytotoxic T lymphocytes (CTLs) [264, 265]. These CD8+ T cells carry CD8 receptors and specifically recognize and eliminate infected or abnormal cells by binding to antigenic fragments presented by HLA-I molecules. Activation of CD8+ T cells occurs when the HLA-I molecule binds to an appropriate antigenic fragment, forming an HLA-antigen complex recognized by the CD8+ T cells. If the antigenic fragment originates from abnormal cells, the CD8+ T cells will be activated to attack these aberrant cells [266]. Among the most common HLA-I molecules, HLA-A2 plays a significant role in presenting endogenous antigens, such as proteins produced by virus-infected or cancer cells, to T cells in the immune system, directing immune cells to attack these abnormal cells [267]. In the study by Knuth et al., a recombinant protein vaccine incorporating HLA-A2 and peptides was developed and trialed in patients with various metastatic tumors expressing HLA-A2 [268]. This investigation revealed that 75% (9 out of 12) of the antigen-positive cancer patients exhibited a specific

immune response, demonstrating the potential of HLA-A2-based peptide-targeted tumor vaccines to induce significant antitumor responses. However, the study highlighted a critical issue: the recombinant protein not only provoked peptide-specific CD8+ T cell activation but also initiated humoral immune responses, potentially leading to adverse effects on normal tissue due to autoimmune reactions. In contrast, our research introduces an innovative approach by integrating the HLA-A2-based Recoverin peptide with HSP70 protein into a fusion protein. This strategy effectively mitigates the risk of autoantibody production associated with immunogenic peptides, therefore minimizing the damage to normal tissues, addressing a significant limitation observed in the use of conventional recombinant protein-based vaccines.

However, most impressively, post-vaccination, 60% (3 out of 5) of the antibody-positive patients exhibited disease stabilization and regression of individual metastases. In 5 out of 7 antibody-negative patients, both disease stabilization and strong immunoreactivity were observed. Nonetheless, three of the patients experiencing disease stabilization eventually showed disease progression, which is attributed to antigen loss [268], highlighting the pivotal role of continuous antigen delivery in eliciting a durable immune response. Notably, HSP70, known for its natural ability to present antigens to dendritic cells, effectively promotes antigen internalization and presentation by DCs, and subsequently promotes the proliferation of T cells stimulated by co-administered peptides [269, 270]. The investigation conducted by Hui Wang et al. revealed that matured dendritic cells (mDCs) loaded with the Hsp70/HBxAg complex elicited a considerable degree of autologous T-cell proliferation when contrasted against scenarios involving standalone Hsp70 or HBxAg [271]. Similarly, I analyzed the impact of three distinct antigens on the formation of T cell colonies, thereby reflecting their potential to induce T cell proliferation. Compared to stimulation by HSP70 or Recoverin peptide alone, the Recoverin fusion protein significantly enhanced the formation of T cell colonies. While studies have demonstrated that antigen peptide-loaded dendritic cells (DCs) can stimulate T cell colony formation and induce cytotoxic T lymphocytes (CTLs) [272], in my research, Recoverin-loaded DCs alone did not significantly stimulate T cell colony formation. This discrepancy could be attributed to variations in antigen peptide concentration or the state of immune cells derived from the volunteers' blood, including dendritic and T cells. Another reason could be that the immunogenicity of the Recoverin peptide is inferior to other cancer antigens, such as cancer-testis antigens [261]. Despite the recognition of various tumor antigens for their capacity to stimulate T-cell proliferation [273, 274], investigations concerning their potential to incite the T-cell colony formation, such as the Recoverin antigen,

remain notably underrepresented. My experimental findings suggested that the quantification of T-cell colony formation size could potentially offer a rapid and uncomplicated avenue for assessing the extent of antigen-driven T-cell proliferation. Nevertheless, it is important to acknowledge that this methodology might exhibit limitations in accurately discerning subtle enhancements in T-cell expansion.

The quantity of activated T cells is crucial for an effective immune response [275]. Intracellular levels of CSFE diminish gradually with cell division, making it a common tool for assessing cellular proliferation levels. Subsequently, I used CFSE-labeled T cells to track their proliferation. In my results, T cells in the Recoverin fusion protein group exhibited stronger proliferation compared to those in the groups with DCs pulsed with HSP70 and Recoverin peptide, providing further support for the potent immunogenic potential of Recoverin fusion protein and confirming HSP70 as an immune adjuvant for Recoverin peptide. Combining these findings with early experimental data, we have shown that DCs loaded with Recoverin fusion protein can effectively induce T cell activation and proliferation under in vitro conditions. However, despite the HSP70 group promoting larger T cell colony sizes compared to the iDC group, there was no significant impact on overall T cell proliferation. This could be attributed to the relatively lower number of T cell colonies induced by the HSP70 group. Hence, the role of HSP70 becomes evident in enhancing peptide-driven T cell proliferation through augmentation of HSP70/peptide complexes, with HSP70 itself not inciting T cell proliferation [276]. Notably, clinical studies have already demonstrated the potential value of HSP70 as a cancer vaccine adjuvant with promising clinical outcomes [248]. Therefore, the immunoadjuvant function of HSP70 may not operate through its direct promotion of T cell proliferation, but rather through alternative pathways.

Cytokines can activate T cells, enhance their recognition of antigens, and attack tumor cells expressing specific antigens [277]. Additionally, cytokines can contribute to the formation of immune memory, which is crucial in the design of preventive vaccines [278]. Seyed et al. observed a profound stimulation of T cell proliferation in the spleens of mice upon the combined administration of vaccine and cytokines, whereas the individual use of either vaccine or IL-24 failed to promote T cell proliferation [279]. However, I did not observe any additional cytokine-induced promotion of T cell proliferation when combined with the three antigens mentioned above. Researchers indicated that in the presence of IL-4 during pre-cultivation, DCs express relatively lower levels of MHC and co-stimulatory molecules, suggesting an immature phenotype. Furthermore, these cells exhibit a diminished response to IFNα [280]. Therefore, supplementing with other cytokines, such as IL-24, may help to

facilitate T cell proliferation. Notably, the proliferation levels of T cells from different donors in the antigen-combined cytokine group exhibited considerable variability, suggesting that the antigen-combined cytokine strategy may not necessarily lead to superior T cell proliferation compared to individual antigen groups. It is essential to acknowledge that our experimental data are derived solely from in vitro experiments and do not encompass the complex tumor microenvironment within the human body. Therefore, further in vivo studies are imperative to comprehensively validate these findings.

In addition to eliciting the proliferation of T cells, researchers have observed that these expanded T cells, prompted by HSP70/peptide complexes, demonstrate targeted cytotoxicity against tumor cells expressing the associated antigens [270, 271]. Li and Ye et al. discovered that DCs pulsed with HSP70-antigen complexes significantly induce anti-tumor cytotoxicity in T cells [281], while single HSP70 demonstrates no efficacy against any cancer, a phenomenon attributed to the tight binding of HSP70 with antigens [282]. Although these HSP70-based vaccines have demonstrated the potential to elicit clinically meaningful immune responses in cancer, HSP70-peptide complexes derived from the patient's own tumor face challenges of production cost. Differently, fusion proteins produced by engineered bacteria offer significantly lower production costs [283]. However, the anti-tumor efficacy of fusion proteins requires further examination.

My experiment showed that T cells induced by DCs pulsed with Recoverin fusion protein exhibited stronger cytotoxicity against Y79 cells compared to T cells induced by DCs pulsed with HSP70. Consistent with our findings, Chen et al. also constructed a fusion protein of NY-ESO-1 epitope with HSP70. Their study similarly did not observe a stimulatory effect of HSP70 on T cells in vitro [284]. Therefore, HSP70 acting as an immunoadjuvant enhances the immunogenicity of peptides, thereby further promoting T cell-mediated anti-tumor activity. This implies that Recoverin fusion protein has greater potential for tumor antigen activation and can enhance T cell anti-tumor capabilities. When compared with iDC groups, the Recoverin fusion protein group exhibited significantly increased cytotoxicity against Y79 cells compared to the Recoverin peptide group. However, no significant difference was observed in T cell anti-Y79 cell ability between the Recoverin fusion protein group and the Recoverin peptide group. In contrast to our findings, Chen et al. observed a significantly higher specific T cell immune response induced by their fusion protein compared to peptide-induced responses [284]. This discrepancy may be due to the limited number of experimental repetitions in my study, potentially introducing bias. Further optimization of experimental repetitions is necessary to obtain more reliable and consistent results. Moreover, Chen et al.

employed different co-culture ratios (T cells: tumor cells) to assess T cells' cytotoxicity under varying ratios. Interestingly, when the co-culture ratio was below 5:1, the fusion protein did not demonstrate better cytotoxicity compared to using peptide alone. The advantage of the fusion protein became evident only when the proportion of T cells was increased [284]. This suggests that although T cells stimulated by DCs pulsed with the fusion protein exhibit stronger cytotoxicity than those stimulated by DCs pulsed with peptide alone, a higher proportion of T cells is needed to manifest this difference. In my experiment, each group of T cells was co-cultured with Y79 cells at a ratio of 10:1 in the incubator. Considering the results of Chen et al., further increasing the relative proportion of T cells and Y79 cells may be an area for improvement. Additionally, increasing amount of effector cells, especially tumor-specific T cells, has been reported in studies to be positively correlated with the prognosis of cancer patients [285]. Considering this, this certain cell ratio ignores the impact of proliferating T cells induced by DCs stimulated with different antigens on the anti-tumor effect against Y79 cells. As mentioned above, the Recoverin fusion protein group induced significant T cell proliferation. it is possible that the T cells in the Recoverin fusion protein may exhibit a more significant anti-Y79 capacity relative to the Recoverin peptide alone.

It is noteworthy that the literature has documented similar significant promotion of T-cell proliferation for several other antigens or vaccines, along with robust immune responses demonstrated in in vivo experiments [286-290]. For example, akin to the blood-retinal barrier, the presence of the blood-testis barrier allows certain proteins to exist exclusively in testicular tissue, such as Mage, a frequently expressed cancer-testis antigen within numerous human tumors [291]. Murine experimentation alludes to the capacity of the HSP70/Mage3 fusion protein to elicit a robust antigen-specific immune retort in vivo, effectually curbing the proliferation of Mage3-expressing tumor cells [292-294]. This foreshadows the potential for Recoverin to exert a potent immune reaction and impede tumorigenesis within in vivo experiments, although my further in vivo evaluations are pending at present. Many existing results from preclinical tests and clinical trials indicate that HSP-based cancer vaccines, including HSP-peptide fusion protein cancer vaccines, hold promising prospects in cancer therapy [295]. This collectively instills confidence in the ongoing exploration of Recoverin fusion protein as a promising candidate for a potential tumor vaccine. Surprisingly, Zhang et al. demonstrated a clear anti-tumor immune effect in mice by using nano-materials to encapsulate HSP70-complexed multi-peptide vaccines [296]. This provides a new approach, suggesting the construction of composite vaccines containing multiple retinal antigens or different Recoverin epitopes simultaneously, thereby generating a

potent anti-tumor immune response. Additionally, Gao et al. utilized gas-filled ultrasound microbubbles to deliver HSP70-peptide fusion proteins. Compared to standalone fusion proteins, precise control of fusion protein release sites in mice through ultrasound imaging significantly inhibited melanoma growth and extended the survival time of mice [297]. This novel vaccine design suggests a future focus on the controlled engineering of tumor vaccines to further enhance the anti-tumor effects of Recoverin fusion proteins.

Ultimately, it is imperative to acknowledge the inherent constraints encompassing my research investigation. While Y79 serves as a representative retinoblastoma cell line, its fidelity in faithfully emulating the complete spectrum of retinoblastoma attributes remains a subject of uncertainty. Moreover, the finite availability of blood donors introduces an additional layer of complexity. Consequently, variations observed in T cell proliferation prompted by divergent antigens or variations in countering Y79 cytotoxicity could conceivably be attributed to the intrinsic inter-individual diversity within the donor cohort. It is noteworthy that my methodology for evaluating the cytotoxic potential of distinct antigen-induced T cells involved a co-culturing approach entailing equal proportions of T cells and Y79 cells. However, this approach inadvertently overlooks potential disparities in the antigen-stimulated T cell proliferation capacity, thereby potentially yielding statistically insignificant outcomes. It is paramount to emphasize that the scope of my experimental framework exclusively encompasses ex-vivo observations involving DCs pulsed with Recoverin fusion protein, orchestrating T cell proliferation and onco-suppressive potential. In reality, the intricate milieu of the tumor microenvironment often undermines the efficacy of numerous immunotherapeutic agents, impeding their optimal onco-suppressive efficacy. Consequently, a comprehensive series of experiments is requisite to holistically assess the potential viability of Recoverin fusion protein as an efficacious tumor vaccine, considering the intricate interplay between its immunogenic properties and the complex tumor milieu. Although my results demonstrate the great potential of Recoverin fusion protein as a tumor vaccine, as it exerts strong immunogenicity, stimulates T cell proliferation, and exhibits significant anti-tumor effects against Y79 cells expressing Recoverin. However, given the variability in immune responses among different donors, it is essential to validate our experimental findings in a larger sample of blood donors in future studies. Furthermore, it remains to be assessed whether the Recoverin fusion protein can effectively induce an immune response in vivo and trigger a sustained immune response against Recoverin-expressing tumor cells. It must be emphasized that, before considering the Recoverin fusion protein as a potential tumor vaccine, its safety in vivo still requires careful evaluation.

5.   Conclusion

In summary, this study aimed to evaluate the immunogenicity of the Recoverin fusion protein and explore the level of immune response it elicits, providing a solid experimental basis for future preclinical investigations. Through our investigations, we observed that dendritic cells (DCs) loaded with the Recoverin fusion protein effectively promoted T-cell proliferation. Moreover, the T-cells stimulated by the Recoverin-loaded DCs exhibited potent cytotoxicity against Y79 cells expressing Recoverin in vitro, confirming the immunogenicity of the Recoverin fusion protein and its potential as a viable tumor vaccine. The significance of our findings lies in the potential application of the Recoverin fusion protein as a novel antigen, which can be further synergistically combined with other therapeutic modalities, such as radiotherapy, chemotherapy, and various immune-based treatments, to enhance anti-tumor efficacy. This multi-faceted approach holds promise for improving the outcomes of clinical anti-tumor therapies, particularly benefiting patients with tumors characterized by abnormal Recoverin protein expression.

In conclusion, our study provides a promising foundation for advancing novel immunotherapeutic approaches, and we are optimistic that our findings will contribute significantly to the development of innovative and personalized anti-tumor treatments for patients with Recoverin-expressing tumors.

References

1. Tiong IS, Hiwase DK, Abro E, Bajel A, Palfreyman E, Beligaswatte A, Reynolds J, Anstee N, Nguyen T, Loo S, et al: **Targeting Molecular Measurable Residual Disease and Low-Blast Relapse in AML With Venetoclax and Low-Dose Cytarabine: A Prospective Phase II Study (VALDAC).** *J Clin Oncol* 2024:JCO2301599.
2. Marano L, Polom K, Patriti A, Roviello G, Falco G, Stracqualursi A, De Luca R, Petrioli R, Martinotti M, Generali D, et al: **Surgical management of advanced gastric cancer: An evolving issue.** *Eur J Surg Oncol* 2016, **42:**18-27.
3. Willett CG, Czito BG, Tyler DS: **Intraoperative radiation therapy.** *J Clin Oncol* 2007, **25:**971-977.
4. Walburn T, Chen MH, Loffredo M, McMahon E, Orio PF, Nguyen PL, D'Amico AV, Sayan M: **Secondary analysis of late major gastrointestinal and genitourinary toxicities in unfavorable-risk prostate cancer patients receiving docetaxel: Insights from a randomized trial.** *Cancer* 2024.
5. Yao Y, Li B, Song R, Yang L, Zou B, Wang L: **Efficacy and safety of thoracic radiotherapy in extensive-stage small-cell lung cancer patients receiving first-line immunotherapy plus chemotherapy: a propensity score matched multicentre retrospective analysis.** *Radiat Oncol* 2024, **19:**25.
6. Cheung TT, Yu SC, Chan SL, Poon RTP, Kwok P, Lee AS, Tai A, Tam D, Cheung CC, Lai TW, et al: **The Hong Kong consensus statements on unresectable hepatocellular carcinoma: narrative review and update for 2021.** *Hepatobiliary Surg Nutr* 2023, **12:**366-385.
7. Shen PC, Chang WC, Lo CH, Yang JF, Lee MS, Dai YH, Lin CS, Fan CY, Huang WY: **Comparison of Stereotactic Body Radiation Therapy and Transarterial Chemoembolization for Unresectable Medium-Sized Hepatocellular Carcinoma.** *Int J Radiat Oncol Biol Phys* 2019, **105:**307-318.
8. He Z, Xie H, Xu H, Wu J, Zeng W, He Q, Jobin C, Jin S, Lan P: **Chemotherapy-induced microbiota exacerbates the toxicity of chemotherapy through the suppression of interleukin-10 from macrophages.** *Gut Microbes* 2024, **16:**2319511.
9. Kawasaki K, Kai K, Minesaki A, Maeda S, Yamauchi M, Kuratomi Y: **Chemoradiotherapy and Lymph Node Metastasis Affect Dendritic Cell Infiltration and Maturation in Regional Lymph Nodes of Laryngeal Cancer.** *Int J Mol Sci* 2024, **25**.
10. Brosseau S, Abreu P, Bouchez C, Charon L, Kieffer Y, Gentric G, Picant V, Veith I, Camonis J, Descroix S, et al: **YAP/TEAD involvement in resistance to paclitaxel chemotherapy in lung cancer.** *Mol Cell Biochem* 2024.
11. Lin X, Kong D, Chen ZS: **Editorial: Chemo-Radiation-Resistance in Cancer Therapy.** *Front Pharmacol* 2022, **13:**904063.
12. Wang Z, Strasser A, Kelly GL: **Should mutant TP53 be targeted for cancer therapy?** *Cell Death Differ* 2022, **29:**911-920.
13. Swain SM, Shastry M, Hamilton E: **Targeting HER2-positive breast cancer: advances and future directions.** *Nat Rev Drug Discov* 2023, **22:**101-126.
14. Kho DH, Nangia-Makker P, Balan V, Hogan V, Tait L, Wang Y, Raz A: **Autocrine motility factor promotes HER2 cleavage and signaling in breast cancer cells.** *Cancer Res* 2013, **73:**1411-1419.
15. Zhai X, Xia Z, Du G, Zhang X, Xia T, Ma D, Li X, Jin B, Zhang H: **LRP1B suppresses HCC progression through the NCSTN/PI3K/AKT signaling axis and affects doxorubicin resistance.** *Genes Dis* 2023, **10:**2082-2096.
16. Chi H, Gao X, Xia Z, Yu W, Yin X, Pan Y, Peng G, Mao X, Teichmann AT, Zhang J, et al: **FAM family gene prediction model reveals heterogeneity, stemness and immune microenvironment of UCEC.** *Front Mol Biosci* 2023,

10:1200335.

17. Li Z, Zhou H, Xia Z, Xia T, Du G, Franziska SD, Li X, Zhai X, Jin B: **HMGA1 augments palbociclib efficacy via PI3K/mTOR signaling in intrahepatic cholangiocarcinoma.** *Biomark Res* 2023, **11:**33.

18. Gutzmer R, Stroyakovskiy D, Gogas H, Robert C, Lewis K, Protsenko S, Pereira RP, Eigentler T, Rutkowski P, Demidov L, et al: **Atezolizumab, vemurafenib, and cobimetinib as first-line treatment for unresectable advanced BRAF(V600) mutation-positive melanoma (IMspire150): primary analysis of the randomised, double-blind, placebo-controlled, phase 3 trial.** *Lancet* 2020, **395:**1835-1844.

19. Proietti I, Skroza N, Michelini S, Mambrin A, Balduzzi V, Bernardini N, Marchesiello A, Tolino E, Volpe S, Maddalena P, et al: **BRAF Inhibitors: Molecular Targeting and Immunomodulatory Actions.** *Cancers (Basel)* 2020, **12**.

20. Mainini F, Eccles MR: **Lipid and Polymer-Based Nanoparticle siRNA Delivery Systems for Cancer Therapy.** *Molecules* 2020, **25**.

21. Sendi H, Yazdimamaghani M, Hu M, Sultanpuram N, Wang J, Moody AS, McCabe E, Zhang J, Graboski A, Li L, et al: **Nanoparticle Delivery of miR-122 Inhibits Colorectal Cancer Liver Metastasis.** *Cancer Res* 2022, **82:**105-113.

22. Lu ZR, Laney VEA, Hall R, Ayat N: **Environment-Responsive Lipid/siRNA Nanoparticles for Cancer Therapy.** *Adv Healthc Mater* 2021, **10:**e2001294.

23. Kara G, Calin GA, Ozpolat B: **RNAi-based therapeutics and tumor targeted delivery in cancer.** *Adv Drug Deliv Rev* 2022, **182:**114113.

24. Karimian A, Azizian K, Parsian H, Rafieian S, Shafiei-Irannejad V, Kheyrollah M, Yousefi M, Majidinia M, Yousefi B: **CRISPR/Cas9 technology as a potent molecular tool for gene therapy.** *J Cell Physiol* 2019, **234:**12267-12277.

25. Katti A, Diaz BJ, Caragine CM, Sanjana NE, Dow LE: **CRISPR in cancer biology and therapy.** *Nat Rev Cancer* 2022, **22:**259-279.

26. Luo Z, Gao M, Huang N, Wang X, Yang Z, Yang H, Huang Z, Feng W: **Efficient disruption of bcr-abl gene by CRISPR RNA-guided FokI nucleases depresses the oncogenesis of chronic myeloid leukemia cells.** *J Exp Clin Cancer Res* 2019, **38:**224.

27. Xu P, Liang F: **Nanomaterial-Based Tumor Photothermal Immunotherapy.** *Int J Nanomedicine* 2020, **15:**9159-9180.

28. Niu Q, Sun Q, Bai R, Zhang Y, Zhuang Z, Zhang X, Xin T, Chen S, Han B: **Progress of Nanomaterials-Based Photothermal Therapy for Oral Squamous Cell Carcinoma.** *Int J Mol Sci* 2022, **23**.

29. Mao D, Dong Z, Liu X, Li W, Li H, Gu C, Chen G, Zhu X, Yang Y: **An Intelligent DNA Nanoreactor for Easy-to-Read In Vivo Tumor Imaging and Precise Therapy.** *Angew Chem Int Ed Engl* 2024, **63:**e202311309.

30. Gyamfi J, Kim J, Choi J: **Cancer as a Metabolic Disorder.** *Int J Mol Sci* 2022, **23**.

31. Pal S, Sharma A, Mathew SP, Jaganathan BG: **Targeting cancer-specific metabolic pathways for developing novel cancer therapeutics.** *Front Immunol* 2022, **13:**955476.

32. Ishay-Ronen D, Diepenbruck M, Kalathur RKR, Sugiyama N, Tiede S, Ivanek R, Bantug G, Morini MF, Wang J, Hess C, Christofori G: **Gain Fat-Lose Metastasis: Converting Invasive Breast Cancer Cells into Adipocytes Inhibits Cancer Metastasis.** *Cancer Cell* 2019, **35:**17-32 e16.

33. Weng J, Li S, Zhu Z, Liu Q, Zhang R, Yang Y, Li X: **Exploring immunotherapy in colorectal cancer.** *J Hematol Oncol* 2022, **15:**95.

34. Zhu S, Zhang T, Zheng L, Liu H, Song W, Liu D, Li Z, Pan CX: **Combination strategies to maximize the benefits of cancer immunotherapy.** *J Hematol Oncol* 2021, **14:**156.

35. Lv M, Chen M, Zhang R, Zhang W, Wang C, Zhang Y, Wei X, Guan Y, Liu J, Feng K, et al: **Manganese is critical for antitumor immune responses via cGAS-STING and improves the efficacy of clinical immunotherapy.** *Cell Res* 2020, **30:**966-979.

36. Patil VM, Noronha V, Menon N, Rai R, Bhattacharjee A, Singh A, Nawale K, Jogdhankar S, Tambe R, Dhumal S, et al: **Low-Dose Immunotherapy in Head and Neck Cancer: A Randomized Study.** *J Clin Oncol* 2023, **41:**222-232.

37. Peng M, Mo Y, Wang Y, Wu P, Zhang Y, Xiong F, Guo C, Wu X, Li Y, Li X, et al: **Neoantigen vaccine: an emerging tumor immunotherapy.** *Mol Cancer* 2019, **18:**128.

38. Xie N, Shen G, Gao W, Huang Z, Huang C, Fu L: **Neoantigens: promising targets for cancer therapy.** *Signal Transduct Target Ther* 2023, **8:**9.

39. Liu CC, Yang H, Zhang R, Zhao JJ, Hao DJ: **Tumour-associated antigens and their anti-cancer applications.** *Eur J Cancer Care (Engl)* 2017, **26**.

40. Curigliano G, Bagnardi V, Ghioni M, Louahed J, Brichard V, Lehmann FF, Marra A, Trapani D, Criscitiello C, Viale G: **Expression of tumor-associated antigens in breast cancer subtypes.** *Breast* 2020, **49:**202-209.

41. Rashidijahanabad Z, Huang X: **Recent advances in tumor associated carbohydrate antigen based chimeric antigen receptor T cells and bispecific antibodies for anti-cancer immunotherapy.** *Semin Immunol* 2020, **47:**101390.

42. Yang T, Zhang S, Yuan H, Wang Y, Cai L, Chen H, Wang X, Song D, Wang X, Guo Z, Wang X: **Platinum-Based TREM2 Inhibitor Suppresses Tumors by Remodeling the Immunosuppressive Microenvironment.** *Angew Chem Int Ed Engl* 2023, **62:**e202213337.

43. Jhunjhunwala S, Hammer C, Delamarre L: **Antigen presentation in cancer: insights into tumour immunogenicity and immune evasion.** *Nat Rev Cancer* 2021, **21:**298-312.

44. Yang H, Xue M, Su P, Zhou Y, Li X, Li Z, Xia Y, Zhang C, Fu M, Zheng X, et al: **RNF31 represses cell progression and immune evasion via YAP/PD-L1 suppression in triple negative breast Cancer.** *J Exp Clin Cancer Res* 2022, **41:**364.

45. Mathew D, Torres RM: **Lysophosphatidic Acid Is an Inflammatory Lipid Exploited by Cancers for Immune Evasion via Mechanisms Similar and Distinct From CTLA-4 and PD-1.** *Front Immunol* 2020, **11:**531910.

46. Zhang X, Zhu L, Zhang H, Chen S, Xiao Y: **CAR-T Cell Therapy in Hematological Malignancies: Current Opportunities and Challenges.** *Front Immunol* 2022, **13:**927153.

47. Alizadeh D, Wong RA, Yang X, Wang D, Pecoraro JR, Kuo CF, Aguilar B, Qi Y, Ann DK, Starr R, et al: **IL15 Enhances CAR-T Cell Antitumor Activity by Reducing mTORC1 Activity and Preserving Their Stem Cell Memory Phenotype.** *Cancer Immunol Res* 2019, **7:**759-772.

48. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, Bader P, Verneris MR, Stefanski HE, Myers GD, et al: **Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia.** *N Engl J Med* 2018, **378:**439-448.

49. Wahid B, Ali A, Rafique S, Waqar M, Wasim M, Wahid K, Idrees M: **An overview of cancer immunotherapeutic strategies.** *Immunotherapy* 2018, **10:**999-1010.

50. Propper DJ, Balkwill FR: **Harnessing cytokines and chemokines for cancer therapy.** *Nat Rev Clin Oncol* 2022, **19:**237-253.

51. Carlino MS, Larkin J, Long GV: **Immune checkpoint inhibitors in melanoma.** *Lancet* 2021, **398:**1002-1014.

52. Restifo NP, Dudley ME, Rosenberg SA: **Adoptive immunotherapy for cancer:**

harnessing the T cell response. *Nat Rev Immunol* 2012, **12:**269-281.

53. Saxena M, van der Burg SH, Melief CJM, Bhardwaj N: **Therapeutic cancer vaccines.** *Nat Rev Cancer* 2021, **21:**360-378.

54. Hunter R, Imbach KJ, Zhou C, Dougan J, Hamilton JAG, Chen KZ, Do P, Townsel A, Gibson G, Dreaden EC, et al: **B-cell acute lymphoblastic leukemia promotes an immune suppressive microenvironment that can be overcome by IL-12.** *Sci Rep* 2022, **12:**11870.

55. Kurz E, Hirsch CA, Dalton T, Shadaloey SA, Khodadadi-Jamayran A, Miller G, Pareek S, Rajaei H, Mohindroo C, Baydogan S, et al: **Exercise-induced engagement of the IL-15/IL-15Ralpha axis promotes anti-tumor immunity in pancreatic cancer.** *Cancer Cell* 2022, **40:**720-737 e725.

56. Dickerson LK, Carter JA, Kohli K, Pillarisetty VG: **Emerging interleukin targets in the tumour microenvironment: implications for the treatment of gastrointestinal tumours.** *Gut* 2023, **72:**1592-1606.

57. Waldmann TA: **Cytokines in Cancer Immunotherapy.** *Cold Spring Harb Perspect Biol* 2018, **10**.

58. Hernandez R, Poder J, LaPorte KM, Malek TR: **Engineering IL-2 for immunotherapy of autoimmunity and cancer.** *Nat Rev Immunol* 2022, **22:**614-628.

59. Zhang Q, Hresko ME, Picton LK, Su L, Hollander MJ, Nunez-Cruz S, Zhang Z, Assenmacher CA, Sockolosky JT, Garcia KC, Milone MC: **A human orthogonal IL-2 and IL-2Rbeta system enhances CAR T cell expansion and antitumor activity in a murine model of leukemia.** *Sci Transl Med* 2021, **13:**eabg6986.

60. Shemesh A, Pickering H, Roybal KT, Lanier LL: **Differential IL-12 signaling induces human natural killer cell activating receptor-mediated ligand-specific expansion.** *J Exp Med* 2022, **219**.

61. Ma S, Caligiuri MA, Yu J: **Harnessing IL-15 signaling to potentiate NK cell-mediated cancer immunotherapy.** *Trends Immunol* 2022, **43:**833-847.

62. Yu R, Zhu B, Chen D: **Type I interferon-mediated tumor immunity and its role in immunotherapy.** *Cell Mol Life Sci* 2022, **79:**191.

63. Han J, Wu M, Liu Z: **Dysregulation in IFN-gamma signaling and response: the barricade to tumor immunotherapy.** *Front Immunol* 2023, **14:**1190333.

64. Bae J, Liu L, Moore C, Hsu E, Zhang A, Ren Z, Sun Z, Wang X, Zhu J, Shen J, et al: **IL-2 delivery by engineered mesenchymal stem cells re-invigorates CD8(+) T cells to overcome immunotherapy resistance in cancer.** *Nat Cell Biol* 2022, **24:**1754-1765.

65. Xiao Z, Tan Y, Cai Y, Huang J, Wang X, Li B, Lin L, Wang Y, Shuai X, Zhu K: **Nanodrug removes physical barrier to promote T-cell infiltration for enhanced cancer immunotherapy.** *J Control Release* 2023, **356:**360-372.

66. Cha E, Graham L, Manjili MH, Bear HD: **IL-7 + IL-15 are superior to IL-2 for the ex vivo expansion of 4T1 mammary carcinoma-specific T cells with greater efficacy against tumors in vivo.** *Breast Cancer Res Treat* 2010, **122:**359-369.

67. Gao J, Zhao L, Wan YY, Zhu B: **Mechanism of Action of IL-7 and Its Potential Applications and Limitations in Cancer Immunotherapy.** *Int J Mol Sci* 2015, **16:**10267-10280.

68. Kulkarni U, Herrmenau C, Win SJ, Bauer M, Kamradt T: **IL-7 treatment augments and prolongs sepsis-induced expansion of IL-10-producing B lymphocytes and myeloid-derived suppressor cells.** *PLoS One* 2018, **13:**e0192304.

69. Vogt TK, Link A, Perrin J, Finke D, Luther SA: **Novel function for interleukin-7 in dendritic cell development.** *Blood* 2009, **113:**3961-3968.

70. Meazza R, Azzarone B, Orengo AM, Ferrini S: **Role of common-gamma chain**

cytokines in NK cell development and function: perspectives for immunotherapy. *J Biomed Biotechnol* 2011, **2011:**861920.

71. Zhou T, Damsky W, Weizman OE, McGeary MK, Hartmann KP, Rosen CE, Fischer S, Jackson R, Flavell RA, Wang J, et al: **IL-18BP is a secreted immune checkpoint and barrier to IL-18 immunotherapy.** *Nature* 2020, **583:**609-614.

72. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Vetto JT, et al.: **Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer.** *N Engl J Med* 1985, **313:**1485-1492.

73. Garris CS, Arlauckas SP, Kohler RH, Trefny MP, Garren S, Piot C, Engblom C, Pfirschke C, Siwicki M, Gungabeesoon J, et al: **Successful Anti-PD-1 Cancer Immunotherapy Requires T Cell-Dendritic Cell Crosstalk Involving the Cytokines IFN-gamma and IL-12.** *Immunity* 2022, **55:**1749.

74. Hargadon KM, Gyorffy B, McGee TJ: **Genomic and transcriptional changes in IFNgamma pathway genes are putative biomarkers of response to ipilimumab immunotherapy in melanoma patients.** *Expert Rev Clin Immunol* 2020, **16:**1099-1103.

75. Quetglas JI, Labiano S, Aznar MA, Bolanos E, Azpilikueta A, Rodriguez I, Casales E, Sanchez-Paulete AR, Segura V, Smerdou C, Melero I: **Virotherapy with a Semliki Forest Virus-Based Vector Encoding IL12 Synergizes with PD-1/PD-L1 Blockade.** *Cancer Immunol Res* 2015, **3:**449-454.

76. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, Greenberg PD: **Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells.** *Proc Natl Acad Sci U S A* 2002, **99:**16168-16173.

77. Petitprez F, Meylan M, de Reynies A, Sautes-Fridman C, Fridman WH: **The Tumor Microenvironment in the Response to Immune Checkpoint Blockade Therapies.** *Front Immunol* 2020, **11:**784.

78. Cha JH, Chan LC, Li CW, Hsu JL, Hung MC: **Mechanisms Controlling PD-L1 Expression in Cancer.** *Mol Cell* 2019, **76:**359-370.

79. Genova C, Dellepiane C, Carrega P, Sommariva S, Ferlazzo G, Pronzato P, Gangemi R, Filaci G, Coco S, Croce M: **Therapeutic Implications of Tumor Microenvironment in Lung Cancer: Focus on Immune Checkpoint Blockade.** *Front Immunol* 2021, **12:**799455.

80. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Lao CD, Cowey CL, Schadendorf D, Wagstaff J, Dummer R, et al: **Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma.** *N Engl J Med* 2019, **381:**1535-1546.

81. Ledford H: **Melanoma drug wins US approval.** *Nature* 2011, **471:**561.

82. Wei SC, Duffy CR, Allison JP: **Fundamental Mechanisms of Immune Checkpoint Blockade Therapy.** *Cancer Discov* 2018, **8:**1069-1086.

83. Paik J: **Nivolumab Plus Relatlimab: First Approval.** *Drugs* 2022, **82:**925-931.

84. Doki Y, Ajani JA, Kato K, Xu J, Wyrwicz L, Motoyama S, Ogata T, Kawakami H, Hsu CH, Adenis A, et al: **Nivolumab Combination Therapy in Advanced Esophageal Squamous-Cell Carcinoma.** *N Engl J Med* 2022, **386:**449-462.

85. Kirchhammer N, Trefny MP, Auf der Maur P, Laubli H, Zippelius A: **Combination cancer immunotherapies: Emerging treatment strategies adapted to the tumor microenvironment.** *Sci Transl Med* 2022, **14:**eabo3605.

86. Morad G, Helmink BA, Sharma P, Wargo JA: **Hallmarks of response, resistance, and toxicity to immune checkpoint blockade.** *Cell* 2021, **184:**5309-5337.

87. Irani YD, Kok CH, Clarson J, Shanmuganathan N, Branford S, Yeung DT, Ross DM, Hughes TP, Yong ASM: **Association of TIM-3 checkpoint receptor expression on T cells with treatment-free remission in chronic myeloid leukemia.** *Blood Adv* 2023, **7:**2364-2374.

88. Jeon SH, Kang M, Jeon M, Chung Y, Kim AR, Lee YJ, Kim ES, Nam H, Park J, Lee JY, et al: **CEACAM1 Marks Highly Suppressive Intratumoral Regulatory T Cells for Targeted Depletion Therapy.** *Clin Cancer Res* 2023, **29:**1794-1806.

89. Andrews LP, Marciscano AE, Drake CG, Vignali DA: **LAG3 (CD223) as a cancer immunotherapy target.** *Immunol Rev* 2017, **276:**80-96.

90. Robert C: **A decade of immune-checkpoint inhibitors in cancer therapy.** *Nat Commun* 2020, **11:**3801.

91. Darvin P, Toor SM, Sasidharan Nair V, Elkord E: **Immune checkpoint inhibitors: recent progress and potential biomarkers.** *Exp Mol Med* 2018, **50:**1-11.

92. Marin-Acevedo JA, Kimbrough EO, Lou Y: **Next generation of immune checkpoint inhibitors and beyond.** *J Hematol Oncol* 2021, **14:**45.

93. Tawbi HA, Schadendorf D, Lipson EJ, Ascierto PA, Matamala L, Castillo Gutierrez E, Rutkowski P, Gogas HJ, Lao CD, De Menezes JJ, et al: **Relatlimab and Nivolumab versus Nivolumab in Untreated Advanced Melanoma.** *N Engl J Med* 2022, **386:**24-34.

94. Horndalsveen H, Alver TN, Dalsgaard AM, Rogg LV, Helbekkmo N, Gronberg BH, Halvorsen TO, Ramberg C, Haakensen VD, Ojlert AK, et al: **Atezolizumab and stereotactic body radiotherapy in patients with advanced non-small cell lung cancer: safety, clinical activity and ctDNA responses-the ComIT-1 trial.** *Mol Oncol* 2023, **17:**487-498.

95. Ascierto PA, Stroyakovskiy D, Gogas H, Robert C, Lewis K, Protsenko S, Pereira RP, Eigentler T, Rutkowski P, Demidov L, et al: **Overall survival with first-line atezolizumab in combination with vemurafenib and cobimetinib in BRAF(V600) mutation-positive advanced melanoma (IMspire150): second interim analysis of a multicentre, randomised, phase 3 study.** *Lancet Oncol* 2023, **24:**33-44.

96. Jin W, Yang Q, Chi H, Wei K, Zhang P, Zhao G, Chen S, Xia Z, Li X: **Ensemble deep learning enhanced with self-attention for predicting immunotherapeutic responses to cancers.** *Front Immunol* 2022, **13:**1025330.

97. Bareche Y, Kelly D, Abbas-Aghababazadeh F, Nakano M, Esfahani PN, Tkachuk D, Mohammad H, Samstein R, Lee CH, Morris LGT, et al: **Leveraging big data of immune checkpoint blockade response identifies novel potential targets.** *Ann Oncol* 2022, **33:**1304-1317.

98. Liu B, Zhang Y, Wang D, Hu X, Zhang Z: **Single-cell meta-analyses reveal responses of tumor-reactive CXCL13(+) T cells to immune-checkpoint blockade.** *Nat Cancer* 2022, **3:**1123-1136.

99. Miao L, Zhang Y, Huang L: **mRNA vaccine for cancer immunotherapy.** *Mol Cancer* 2021, **20:**41.

100. Wang DY, Salem JE, Cohen JV, Chandra S, Menzer C, Ye F, Zhao S, Das S, Beckermann KE, Ha L, et al: **Fatal Toxic Effects Associated With Immune Checkpoint Inhibitors: A Systematic Review and Meta-analysis.** *JAMA Oncol* 2018, **4:**1721-1728.

101. Vesely MD, Zhang T, Chen L: **Resistance Mechanisms to Anti-PD Cancer Immunotherapy.** *Annu Rev Immunol* 2022, **40:**45-74.

102. Breitenecker K, Homolya M, Luca AC, Lang V, Trenk C, Petroczi G, Mohrherr J, Horvath J, Moritsch S, Haas L, et al: **Down-regulation of A20 promotes immune escape of lung adenocarcinomas.** *Sci Transl Med* 2021, **13**.

103. Saez-Ibanez AR, Upadhaya S, Partridge T, Shah M, Correa D, Campbell J: **Landscape of cancer cell therapies: trends and real-world data.** *Nat Rev*

Drug Discov 2022, **21:**631-632.

104. Hiltensperger M, Krackhardt AM: **Current and future concepts for the generation and application of genetically engineered CAR-T and TCR-T cells.** *Front Immunol* 2023, **14:**1121030.

105. Zhang Y, Zhang Z: **The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications.** *Cell Mol Immunol* 2020, **17:**807-821.

106. Olson DJ, Odunsi K: **Adoptive Cell Therapy for Nonhematologic Solid Tumors.** *J Clin Oncol* 2023, **41:**3397-3407.

107. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, et al: **CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia.** *Sci Transl Med* 2013, **5:**177ra138.

108. Dagar G, Gupta A, Masoodi T, Nisar S, Merhi M, Hashem S, Chauhan R, Dagar M, Mirza S, Bagga P, et al: **Harnessing the potential of CAR-T cell therapy: progress, challenges, and future directions in hematological and solid tumor treatments.** *J Transl Med* 2023, **21:**449.

109. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, Robertson C, Gray TL, Diouf O, Wakefield A, et al: **HER2-Specific Chimeric Antigen Receptor-Modified Virus-Specific T Cells for Progressive Glioblastoma: A Phase 1 Dose-Escalation Trial.** *JAMA Oncol* 2017, **3:**1094-1101.

110. Neelapu SS, Dickinson M, Munoz J, Ulrickson ML, Thieblemont C, Oluwole OO, Herrera AF, Ujjani CS, Lin Y, Riedell PA, et al: **Axicabtagene ciloleucel as first-line therapy in high-risk large B-cell lymphoma: the phase 2 ZUMA-12 trial.** *Nat Med* 2022, **28:**735-742.

111. Harris DT, Hager MV, Smith SN, Cai Q, Stone JD, Kruger P, Lever M, Dushek O, Schmitt TM, Greenberg PD, Kranz DM: **Comparison of T Cell Activities Mediated by Human TCRs and CARs That Use the Same Recognition Domains.** *J Immunol* 2018, **200:**1088-1100.

112. Salter AI, Rajan A, Kennedy JJ, Ivey RG, Shelby SA, Leung I, Templeton ML, Muhunthan V, Voillet V, Sommermeyer D, et al: **Comparative analysis of TCR and CAR signaling informs CAR designs with superior antigen sensitivity and in vivo function.** *Sci Signal* 2021, **14**.

113. Stadtmauer EA, Fraietta JA, Davis MM, Cohen AD, Weber KL, Lancaster E, Mangan PA, Kulikovskaya I, Gupta M, Chen F, et al: **CRISPR-engineered T cells in patients with refractory cancer.** *Science* 2020, **367**.

114. Foy SP, Jacoby K, Bota DA, Hunter T, Pan Z, Stawiski E, Ma Y, Lu W, Peng S, Wang CL, et al: **Non-viral precision T cell receptor replacement for personalized cell therapy.** *Nature* 2023, **615:**687-696.

115. Shafer P, Kelly LM, Hoyos V: **Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects.** *Front Immunol* 2022, **13:**835762.

116. Okumura S, Ishihara M, Kiyota N, Yakushijin K, Takada K, Kobayashi S, Ikeda H, Endo M, Kato K, Kitano S, et al: **Chimeric antigen receptor T-cell therapy targeting a MAGE A4 peptide and HLA-A*02:01 complex for unresectable advanced or recurrent solid cancer: protocol for a multi-institutional phase 1 clinical trial.** *BMJ Open* 2022, **12:**e065109.

117. Nyberg WA, Ark J, To A, Clouden S, Reeder G, Muldoon JJ, Chung JY, Xie WH, Allain V, Steinhart Z, et al: **An evolved AAV variant enables efficient genetic engineering of murine T cells.** *Cell* 2023, **186:**446-460 e419.

118. Fenton GA, Mitchell DA: **Cellular Cancer Immunotherapy Development and Manufacturing in the Clinic.** *Clin Cancer Res* 2023, **29:**843-857.

119. Teppert K, Wang X, Anders K, Evaristo C, Lock D, Kunkele A: **Joining Forces for Cancer Treatment: From "TCR versus CAR" to "TCR and CAR".** *Int J*

Mol Sci 2022, **23**.

120. Labanieh L, Mackall CL: **CAR immune cells: design principles, resistance and the next generation.** *Nature* 2023, **614:**635-648.

121. Abramson JS, Palomba ML, Gordon LI, Lunning MA, Wang M, Arnason J, Mehta A, Purev E, Maloney DG, Andreadis C, et al: **Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study.** *Lancet* 2020, **396:**839-852.

122. Frey N, Porter D: **Cytokine Release Syndrome with Chimeric Antigen Receptor T Cell Therapy.** *Biol Blood Marrow Transplant* 2019, **25:**e123-e127.

123. Allen ES, Stroncek DF, Ren J, Eder AF, West KA, Fry TJ, Lee DW, Mackall CL, Conry-Cantilena C: **Autologous lymphapheresis for the production of chimeric antigen receptor T cells.** *Transfusion* 2017, **57:**1133-1141.

124. Sellars MC, Wu CJ, Fritsch EF: **Cancer vaccines: Building a bridge over troubled waters.** *Cell* 2022, **185:**2770-2788.

125. Igarashi Y, Sasada T: **Cancer Vaccines: Toward the Next Breakthrough in Cancer Immunotherapy.** *J Immunol Res* 2020, **2020:**5825401.

126. Garbuglia AR, Lapa D, Sias C, Capobianchi MR, Del Porto P: **The Use of Both Therapeutic and Prophylactic Vaccines in the Therapy of Papillomavirus Disease.** *Front Immunol* 2020, **11:**188.

127. Nguyen MH, Wong G, Gane E, Kao JH, Dusheiko G: **Hepatitis B Virus: Advances in Prevention, Diagnosis, and Therapy.** *Clin Microbiol Rev* 2020, **33**.

128. Sobhani N, Scaggiante B, Morris R, Chai D, Catalano M, Tardiel-Cyril DR, Neeli P, Roviello G, Mondani G, Li Y: **Therapeutic cancer vaccines: From biological mechanisms and engineering to ongoing clinical trials.** *Cancer Treat Rev* 2022, **109:**102429.

129. Wang Y, Xiang Y, Xin VW, Wang XW, Peng XC, Liu XQ, Wang D, Li N, Cheng JT, Lyv YN, et al: **Dendritic cell biology and its role in tumor immunotherapy.** *J Hematol Oncol* 2020, **13:**107.

130. Saadeldin MK, Abdel-Aziz AK, Abdellatif A: **Dendritic cell vaccine immunotherapy; the beginning of the end of cancer and COVID-19. A hypothesis.** *Med Hypotheses* 2021, **146:**110365.

131. Gatti-Mays ME, Redman JM, Collins JM, Bilusic M: **Cancer vaccines: Enhanced immunogenic modulation through therapeutic combinations.** *Hum Vaccin Immunother* 2017, **13:**2561-2574.

132. Harari A, Graciotti M, Bassani-Sternberg M, Kandalaft LE: **Antitumour dendritic cell vaccination in a priming and boosting approach.** *Nat Rev Drug Discov* 2020, **19:**635-652.

133. Cannon MJ, Block MS, Morehead LC, Knutson KL: **The evolving clinical landscape for dendritic cell vaccines and cancer immunotherapy.** *Immunotherapy* 2019, **11:**75-79.

134. Mohamad Razif MI, Nizar N, Zainal Abidin NH, Muhammad Ali SN, Wan Zarimi WNN, Khotib J, Susanti D, Mohd Jailani MT, Taher M: **Emergence of mRNA vaccines in the management of cancer.** *Expert Rev Vaccines* 2023, **22:**629-642.

135. Huang X, Zhang G, Tang TY, Gao X, Liang TB: **Personalized pancreatic cancer therapy: from the perspective of mRNA vaccine.** *Mil Med Res* 2022, **9:**53.

136. Lopes A, Vandermeulen G, Preat V: **Cancer DNA vaccines: current preclinical and clinical developments and future perspectives.** *J Exp Clin Cancer Res* 2019, **38:**146.

137. Jahanafrooz Z, Baradaran B, Mosafer J, Hashemzaei M, Rezaei T, Mokhtarzadeh A, Hamblin MR: **Comparison of DNA and mRNA vaccines**

against cancer. *Drug Discov Today* 2020, **25:**552-560.

138.   Morse MA, Gwin WR, 3rd, Mitchell DA: **Vaccine Therapies for Cancer: Then and Now.** *Target Oncol* 2021, **16:**121-152.

139.   Ma M, Liu J, Jin S, Wang L: **Development of tumour peptide vaccines: From universalization to personalization.** *Scand J Immunol* 2020, **91:**e12875.

140.   Dougan M, Luoma AM, Dougan SK, Wucherpfennig KW: **Understanding and treating the inflammatory adverse events of cancer immunotherapy.** *Cell* 2021, **184:**1575-1588.

141.   Zaidi N: **Can Personalized Neoantigens Raise the T Cell Bar?** *Cell* 2020, **183:**301-302.

142.   Hilf N, Kuttruff-Coqui S, Frenzel K, Bukur V, Stevanovic S, Gouttefangeas C, Platten M, Tabatabai G, Dutoit V, van der Burg SH, et al: **Publisher Correction: Actively personalized vaccination trial for newly diagnosed glioblastoma.** *Nature* 2019, **566:**E13.

143.   Ott PA, Hu-Lieskovan S, Chmielowski B, Govindan R, Naing A, Bhardwaj N, Margolin K, Awad MM, Hellmann MD, Lin JJ, et al: **A Phase Ib Trial of Personalized Neoantigen Therapy Plus Anti-PD-1 in Patients with Advanced Melanoma, Non-small Cell Lung Cancer, or Bladder Cancer.** *Cell* 2020, **183:**347-362 e324.

144.   Awad MM, Govindan R, Balogh KN, Spigel DR, Garon EB, Bushway ME, Poran A, Sheen JH, Kohler V, Esaulova E, et al: **Personalized neoantigen vaccine NEO-PV-01 with chemotherapy and anti-PD-1 as first-line treatment for non-squamous non-small cell lung cancer.** *Cancer Cell* 2022, **40:**1010-1026 e1011.

145.   Lin MJ, Svensson-Arvelund J, Lubitz GS, Marabelle A, Melero I, Brown BD, Brody JD: **Cancer vaccines: the next immunotherapy frontier.** *Nat Cancer* 2022, **3:**911-926.

146.   Hollingsworth RE, Jansen K: **Turning the corner on therapeutic cancer vaccines.** *NPJ Vaccines* 2019, **4:**7.

147.   Flemming A: **Tumour heterogeneity determines immune response.** *Nat Rev Immunol* 2019, **19:**662-663.

148.   Anagnostou V, Smith KN, Forde PM, Niknafs N, Bhattacharya R, White J, Zhang T, Adleff V, Phallen J, Wali N, et al: **Evolution of Neoantigen Landscape during Immune Checkpoint Blockade in Non-Small Cell Lung Cancer.** *Cancer Discov* 2017, **7:**264-276.

149.   Wolf Y, Sameuls Y: **Neoantigens in cancer immunotherapy: quantity vs. quality.** *Mol Oncol* 2023.

150.   Nicolas-Morales ML, Luisa-Sanjuan A, Gutierrez-Torres M, Vences-Velazquez A, Ortuno-Pineda C, Espinoza-Rojo M, Navarro-Tito N, Cortes-Sarabia K: **Peptide-Based Vaccines in Clinical Phases and New Potential Therapeutic Targets as a New Approach for Breast Cancer: A Review.** *Vaccines (Basel)* 2022, **10**.

151.   Guan H, Wu Y, Li LU, Yang Y, Qiu S, Zhao Z, Chu X, He J, Chen Z, Zhang Y, et al: **Tumor neoantigens: Novel strategies for application of cancer immunotherapy.** *Oncol Res* 2023, **31:**437-448.

152.   Conarty JP, Wieland A: **The Tumor-Specific Immune Landscape in HPV+ Head and Neck Cancer.** *Viruses* 2023, **15**.

153.   Schumacher TN, Scheper W, Kvistborg P: **Cancer Neoantigens.** *Annu Rev Immunol* 2019, **37:**173-200.

154.   Ho SY, Chang CM, Liao HN, Chou WH, Guo CL, Yen Y, Nakamura Y, Chang WC: **Current Trends in Neoantigen-Based Cancer Vaccines.** *Pharmaceuticals (Basel)* 2023, **16**.

155.   Chiang CL, Coukos G, Kandalaft LE: **Whole Tumor Antigen Vaccines: Where Are We?** *Vaccines (Basel)* 2015, **3:**344-372.

156.   Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA,

Davis JL, Morgan RA, Merino MJ, Sherry RM, et al: **T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis.** *Mol Ther* 2011, **19:**620-626.

157. Chan TA, Yarchoan M, Jaffee E, Swanton C, Quezada SA, Stenzinger A, Peters S: **Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic.** *Ann Oncol* 2019, **30:**44-56.

158. Strickler JH, Hanks BA, Khasraw M: **Tumor Mutational Burden as a Predictor of Immunotherapy Response: Is More Always Better?** *Clin Cancer Res* 2021, **27:**1236-1241.

159. Chick RC, Faries MB, Hale DF, Kemp Bohan PM, Hickerson AT, Vreeland TJ, Myers JW, 3rd, Cindass JL, Brown TA, 2nd, Hyngstrom J, et al: **Multi-institutional, prospective, randomized, double-blind, placebo-controlled phase IIb trial of the tumor lysate, particle-loaded, dendritic cell (TLPLDC) vaccine to prevent recurrence in high-risk melanoma patients: A subgroup analysis.** *Cancer Med* 2021, **10:**4302-4311.

160. Tran E, Robbins PF, Rosenberg SA: **'Final common pathway' of human cancer immunotherapy: targeting random somatic mutations.** *Nat Immunol* 2017, **18:**255-262.

161. Sahin U, Tureci O: **Personalized vaccines for cancer immunotherapy.** *Science* 2018, **359:**1355-1360.

162. Jung M, Lee JB, Kim HS, Kwon WS, Kim HO, Kim S, Park M, Kim W, Choi KY, Oh T, et al: **First-in-Human Phase 1 Study of a B Cell- and Monocyte-Based Immunotherapeutic Vaccine Against HER2-Positive Advanced Gastric Cancer.** *Cancer Res Treat* 2023.

163. Schonharting W, Roehnisch T, Manoochehri M, Christoph J, Sieger M, Nogueira M, Martos-Contreras MC, Kunz M: **Improved Survival of a HER2-Positive Metastatic Breast Cancer Patient Following a Personalized Peptide Immunization.** *Vaccines (Basel)* 2023, **11**.

164. Jiang T, Shi T, Zhang H, Hu J, Song Y, Wei J, Ren S, Zhou C: **Tumor neoantigens: from basic research to clinical applications.** *J Hematol Oncol* 2019, **12:**93.

165. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T: **Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy.** *Nat Rev Cancer* 2014, **14:**135-146.

166. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N: **Molecular and genetic properties of tumors associated with local immune cytolytic activity.** *Cell* 2015, **160:**48-61.

167. Alsalloum A, Shevchenko JA, Sennikov S: **The Melanoma-Associated Antigen Family A (MAGE-A): A Promising Target for Cancer Immunotherapy?** *Cancers (Basel)* 2023, **15**.

168. Weon JL, Potts PR: **The MAGE protein family and cancer.** *Curr Opin Cell Biol* 2015, **37:**1-8.

169. Schooten E, Di Maggio A, van Bergen En Henegouwen PMP, Kijanka MM: **MAGE-A antigens as targets for cancer immunotherapy.** *Cancer Treat Rev* 2018, **67:**54-62.

170. Wei X, Chen F, Xin K, Wang Q, Yu L, Liu B, Liu Q: **Cancer-Testis Antigen Peptide Vaccine for Cancer Immunotherapy: Progress and Prospects.** *Transl Oncol* 2019, **12:**733-738.

171. Fan C, Qu H, Wang X, Sobhani N, Wang L, Liu S, Xiong W, Zeng Z, Li Y: **Cancer/testis antigens: from serology to mRNA cancer vaccine.** *Semin Cancer Biol* 2021, **76:**218-231.

172. Hong DS, Van Tine BA, Biswas S, McAlpine C, Johnson ML, Olszanski AJ, Clarke JM, Araujo D, Blumenschein GR, Jr., Kebriaei P, et al: **Autologous T cell therapy for MAGE-A4(+) solid cancers in HLA-A*02(+) patients: a**

phase 1 trial. *Nat Med* 2023, **29:**104-114.

173. Lu YC, Parker LL, Lu T, Zheng Z, Toomey MA, White DE, Yao X, Li YF, Robbins PF, Feldman SA, et al: **Treatment of Patients With Metastatic Cancer Using a Major Histocompatibility Complex Class II-Restricted T-Cell Receptor Targeting the Cancer Germline Antigen MAGE-A3.** *J Clin Oncol* 2017, **35:**3322-3329.

174. Blumenschein GR, Devarakonda S, Johnson M, Moreno V, Gainor J, Edelman MJ, Heymach JV, Govindan R, Bachier C, Doger de Speville B, et al: **Phase I clinical trial evaluating the safety and efficacy of ADP-A2M10 SPEAR T cells in patients with MAGE-A10(+) advanced non-small cell lung cancer.** *J Immunother Cancer* 2022, **10**.

175. Ai H, Yang H, Li L, Ma J, Liu K, Li Z: **Cancer/testis antigens: promising immunotherapy targets for digestive tract cancers.** *Front Immunol* 2023, **14:**1190883.

176. Golovastova MO, Bazhin AV, Philippov PP: **Cancer-retina antigens -- a new group of tumor antigens.** *Biochemistry (Mosc)* 2014, **79:**733-739.

177. Savvateeva L, Zernii E, Zamyatnin A: **Current Perspectives in Ultra-Sensitive Detection of Cancer-Specific Biomarkers and Therapeutic Targets.** *Curr Cancer Drug Targets* 2023.

178. Laban S, Gangkofner DS, Holzinger D, Schroeder L, Eichmuller SB, Zornig I, Jager D, Wichmann G, Dietz A, Broglie MA, et al: **Antibody Responses to Cancer Antigens Identify Patients with a Poor Prognosis among HPV-Positive and HPV-Negative Head and Neck Squamous Cell Carcinoma Patients.** *Clin Cancer Res* 2019, **25:**7405-7412.

179. Bazhin AV, Schadendorf D, Philippov PP, Eichmuller SB: **Recoverin as a cancer-retina antigen.** *Cancer Immunol Immunother* 2007, **56:**110-116.

180. Eynard AR, Repossi G: **Role of omega3 polyunsaturated fatty acids in diabetic retinopathy: a morphological and metabolically cross talk among blood retina barriers damage, autoimmunity and chronic inflammation.** *Lipids Health Dis* 2019, **18:**114.

181. Bondareva IB, Narkevich B: **[Mathematical model of radioactive colloid transport in the evaluation of the functional state of the reticuloendothelial system].** *Med Radiol (Mosk)* 1990, **35:**52-55.

182. Caspi RR: **Ocular autoimmunity: the price of privilege?** *Immunol Rev* 2006, **213:**23-35.

183. Dutta Majumder P, Marchese A, Pichi F, Garg I, Agarwal A: **An update on autoimmune retinopathy.** *Indian J Ophthalmol* 2020, **68:**1829-1837.

184. Tanaka T, Ames JB, Harvey TS, Stryer L, Ikura M: **Sequestration of the membrane-targeting myristoyl group of recoverin in the calcium-free state.** *Nature* 1995, **376:**444-447.

185. Komolov KE, Senin, II, Kovaleva NA, Christoph MP, Churumova VA, Grigoriev, II, Akhtar M, Philippov PP, Koch KW: **Mechanism of rhodopsin kinase regulation by recoverin.** *J Neurochem* 2009, **110:**72-79.

186. Strissel KJ, Lishko PV, Trieu LH, Kennedy MJ, Hurley JB, Arshavsky VY: **Recoverin undergoes light-dependent intracellular translocation in rod photoreceptors.** *J Biol Chem* 2005, **280:**29250-29255.

187. Chen CK, Woodruff ML, Fain GL: **Rhodopsin kinase and recoverin modulate phosphodiesterase during mouse photoreceptor light adaptation.** *J Gen Physiol* 2015, **145:**213-224.

188. Zang J, Neuhauss SCF: **The Binding Properties and Physiological Functions of Recoverin.** *Front Mol Neurosci* 2018, **11:**473.

189. Sakurai K, Chen J, Khani SC, Kefalov VJ: **Regulation of mammalian cone phototransduction by recoverin and rhodopsin kinase.** *J Biol Chem* 2015, **290:**9239-9250.

190. Mudri J, Phagura RS, Lam WS, Wu XN: **Intravitreal dexamethasone implant use as first-line therapy for cancer-associated retinopathy.** *BMJ Case Rep* 2021, **14**.

191. Sayman C, Tireli H, Gurleyik GM: **Cancer-Associated Retinopathy in Patients with Newly Diagnosed Breast Tumor.** *Noro Psikiyatr Ars* 2021, **58**:278-282.

192. Klieme B, Viestenz A, Eisenmann S, Wienrich R: **[Acute progressive loss of vision as first manifestation of cancer-associated retinopathy (CAR) in neuroendocrine small cell lung cancer].** *Ophthalmologie* 2022, **119**:737-740.

193. Mueller CM, Hojjatie SL, Lawson DH, Jain N, Robinson J, Khan MK, Yushak ML, G ADOK: **Clinical Correlation between Acute Exudative Polymorphous Paraneoplastic Vitelliform Maculopathy and Metastatic Melanoma Disease Activity: A 48-month Longitudinal Case Report.** *Ocul Immunol Inflamm* 2022, **30**:330-337.

194. Tang Z, Ma H, Mao Y, Ai S, Zhang P, Nie C, Gao Y, Lu R: **Identification of stemness in primary retinoblastoma cells by analysis of stem-cell phenotypes and tumorigenicity with culture and xenograft models.** *Exp Cell Res* 2019, **379**:110-118.

195. Ohguro H, Odagiri H, Miyagawa Y, Ohguro I, Sasaki M, Nakazawa M: **Clinicopathological features of gastric cancer cases and aberrantly expressed recoverin.** *Tohoku J Exp Med* 2004, **202**:213-219.

196. Aslebagh R, Whitham D, Channaveerappa D, Mutsengi P, Pentecost BT, Arcaro KF, Darie CC: **Mass Spectrometry-Based Proteomics of Human Milk to Identify Differentially Expressed Proteins in Women with Breast Cancer versus Controls.** *Proteomes* 2022, **10**.

197. Shlyapnikov YM, Malakhova EA, Potoldykova NV, Svetocheva YA, Vinarov AZ, Zinchenko DV, Zernii EY, Zamyatnin AA, Jr., Shlyapnikova EA: **Non-Invasive Diagnostics of Renal Cell Carcinoma Using Ultrasensitive Immunodetection of Cancer-Retina Antigens.** *Biochemistry (Mosc)* 2022, **87**:658-666.

198. Shlyapnikov YM, Malakhova EA, Vinarov AZ, Potoldykova NV, Vladimirov VI, Zernii EY, Zamyatnin AA, Jr., Shlyapnikova EA: **Cancer-Retina Antigens in the Urine of Bladder and Prostate Cancer Patients.** *Biochemistry (Mosc)* 2022, **87**:1268-1276.

199. Chan JW: **Paraneoplastic retinopathies and optic neuropathies.** *Surv Ophthalmol* 2003, **48**:12-38.

200. Adamus G, Karren L: **Autoimmunity against carbonic anhydrase II affects retinal cell functions in autoimmune retinopathy.** *J Autoimmun* 2009, **32**:133-139.

201. Chauhan MZ, Mansour HA, Zafar MK, Uwaydat SH: **Anti-Programmed Death Ligand-1 Induced Acute Vision Loss in a Patient With Cancer-Associated Retinopathy.** *Cureus* 2022, **14**:e21709.

202. Chen Q, Feng C, Sun C, Wang W, Wang M, Chen L, Sun X, Tian G: **Cancer-associated retinopathy after anti-programmed death 1 (PD-1) antibody for treating hepatocellular carcinoma--a case report of a Chinese patient.** *Am J Ophthalmol Case Rep* 2022, **25**:101370.

203. Adamus G, Champaigne R, Yang S: **Occurrence of major anti-retinal autoantibodies associated with paraneoplastic autoimmune retinopathy.** *Clin Immunol* 2020, **210**:108317.

204. Kim MS, Hong HK, Park KH, Woo SJ: **Intravitreal Dexamethasone Implant with Plasma Autoantibody Monitoring for Cancer-associated Retinopathy.** *Korean J Ophthalmol* 2019, **33**:298-300.

205. Frese KK, Simpson KL, Dive C: **Small cell lung cancer enters the era of precision medicine.** *Cancer Cell* 2021, **39**:297-299.

206. Kobayashi M, Ikezoe T, Uemura Y, Ueno H, Taguchi H: **Long-term survival of**

a patient with small cell lung cancer associated with cancer-associated retinopathy. *Lung Cancer* 2007, **57:**399-403.

207. Oohira A: **Fifteen-year follow-up of patient with cancer-associated retinopathy.** *Jpn J Ophthalmol* 2007, **51:**74-75.

208. Stanescu-Segall D, Wilson C, Bonnel S, Heligon JP: **Seventeen-year survival of small-cell lung carcinoma presenting as carcinoma-associated retinopathy.** *J Clin Oncol* 2011, **29:**e566-568.

209. Miyagawa Y, Ohguro H, Odagiri H, Maruyama I, Maeda T, Maeda A, Sasaki M, Nakazawa M: **Aberrantly expressed recoverin is functionally associated with G-protein-coupled receptor kinases in cancer cell lines.** *Biochem Biophys Res Commun* 2003, **300:**669-673.

210. Yu S, Sun L, Jiao Y, Lee LTO: **The Role of G Protein-coupled Receptor Kinases in Cancer.** *Int J Biol Sci* 2018, **14:**189-203.

211. Nogues L, Palacios-Garcia J, Reglero C, Rivas V, Neves M, Ribas C, Penela P, Mayor F, Jr.: **G protein-coupled receptor kinases (GRKs) in tumorigenesis and cancer progression: GPCR regulators and signaling hubs.** *Semin Cancer Biol* 2018, **48:**78-90.

212. Sun WY, Wu JJ, Peng WT, Sun JC, Wei W: **The role of G protein-coupled receptor kinases in the pathology of malignant tumors.** *Acta Pharmacol Sin* 2018, **39:**1699-1705.

213. Ichioka H, Hirohashi Y, Sato T, Furuhashi M, Watanabe M, Ida Y, Hikage F, Torigoe T, Ohguro H: **G-Protein-Coupled Receptors Mediate Modulations of Cell Viability and Drug Sensitivity by Aberrantly Expressed Recoverin 3 within A549 Cells.** *Int J Mol Sci* 2023, **24**.

214. Gupta M, Wahi A, Sharma P, Nagpal R, Raina N, Kaurav M, Bhattacharya J, Rodrigues Oliveira SM, Dolma KG, Paul AK, et al: **Recent Advances in Cancer Vaccines: Challenges, Achievements, and Futuristic Prospects.** *Vaccines (Basel)* 2022, **10**.

215. Paston SJ, Brentville VA, Symonds P, Durrant LG: **Cancer Vaccines, Adjuvants, and Delivery Systems.** *Front Immunol* 2021, **12:**627932.

216. Kim H, Kwon B, Sin JI: **Combined stimulation of IL-2 and 4-1BB receptors augments the antitumor activity of E7 DNA vaccines by increasing Ag-specific CTL responses.** *PLoS One* 2013, **8:**e83765.

217. Li X, Wang X, Ito A: **Tailoring inorganic nanoadjuvants towards next-generation vaccines.** *Chem Soc Rev* 2018, **47:**4954-4980.

218. Maisonneuve C, Bertholet S, Philpott DJ, De Gregorio E: **Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants.** *Proc Natl Acad Sci U S A* 2014, **111:**12294-12299.

219. Ho NI, Huis In 't Veld LGM, Raaijmakers TK, Adema GJ: **Adjuvants Enhancing Cross-Presentation by Dendritic Cells: The Key to More Effective Vaccines?** *Front Immunol* 2018, **9:**2874.

220. Lee W, Suresh M: **Vaccine adjuvants to engage the cross-presentation pathway.** *Front Immunol* 2022, **13:**940047.

221. Schmidt ST, Olsen CL, Franzyk H, Worzner K, Korsholm KS, Rades T, Andersen P, Foged C, Christensen D: **Comparison of two different PEGylation strategies for the liposomal adjuvant CAF09: Towards induction of CTL responses upon subcutaneous vaccine administration.** *Eur J Pharm Biopharm* 2019, **140:**29-39.

222. Seya T, Tatematsu M, Matsumoto M: **Toward Establishing an Ideal Adjuvant for Non-Inflammatory Immune Enhancement.** *Cells* 2022, **11**.

223. Gu Y, Yang J, He C, Zhao T, Lu R, Liu J, Mo X, Wen F, Shi H: **Incorporation of a Toll-like receptor 2/6 agonist potentiates mRNA vaccines against cancer and infectious diseases.** *Signal Transduct Target Ther* 2023, **8:**273.

224. Eton O, Ross MI, East MJ, Mansfield PF, Papadopoulos N, Ellerhorst JA,

Bedikian AY, Lee JE: **Autologous tumor-derived heat-shock protein peptide complex-96 (HSPPC-96) in patients with metastatic melanoma.** *J Transl Med* 2010, **8:**9.

225. Maki RG, Livingston PO, Lewis JJ, Janetzki S, Klimstra D, Desantis D, Srivastava PK, Brennan MF: **A phase I pilot study of autologous heat shock protein vaccine HSPPC-96 in patients with resected pancreatic adenocarcinoma.** *Dig Dis Sci* 2007, **52:**1964-1972.

226. Lang BJ, Guerrero-Gimenez ME, Prince TL, Ackerman A, Bonorino C, Calderwood SK: **Heat Shock Proteins Are Essential Components in Transformation and Tumor Progression: Cancer Cell Intrinsic Pathways and Beyond.** *Int J Mol Sci* 2019, **20**.

227. Karapanagiotou EM, Syrigos K, Saif MW: **Heat shock protein inhibitors and vaccines as new agents in cancer treatment.** *Expert Opin Investig Drugs* 2009, **18:**161-174.

228. Chatterjee S, Burns TF: **Targeting Heat Shock Proteins in Cancer: A Promising Therapeutic Approach.** *Int J Mol Sci* 2017, **18**.

229. Ma B, Ju A, Zhang S, An Q, Xu S, Liu J, Yu L, Fu Y, Luo Y: **Albumosomes formed by cytoplasmic pre-folding albumin maintain mitochondrial homeostasis and inhibit nonalcoholic fatty liver disease.** *Signal Transduct Target Ther* 2023, **8:**229.

230. Hebishy M, Shintouo CM, Dufait I, Debacq-Chainiaux F, Bautmans I, Njemini R: **Heat shock proteins and cellular senescence in humans: A systematic review.** *Arch Gerontol Geriatr* 2023, **113:**105057.

231. Rosenzweig R, Nillegoda NB, Mayer MP, Bukau B: **The Hsp70 chaperone network.** *Nat Rev Mol Cell Biol* 2019, **20:**665-680.

232. Ampie L, Choy W, Lamano JB, Fakurnejad S, Bloch O, Parsa AT: **Heat shock protein vaccines against glioblastoma: from bench to bedside.** *J Neurooncol* 2015, **123:**441-448.

233. De Maio A, Vazquez D: **Extracellular heat shock proteins: a new location, a new function.** *Shock* 2013, **40:**239-246.

234. Linder M, Pogge von Strandmann E: **The Role of Extracellular HSP70 in the Function of Tumor-Associated Immune Cells.** *Cancers (Basel)* 2021, **13**.

235. Yamaguchi-Tanaka M, Takagi K, Miki Y, Sato A, Iwabuchi E, Miyashita M, Suzuki T: **The Pro-Tumorigenic Role of Chemotherapy-Induced Extracellular HSP70 from Breast Cancer Cells via Intratumoral Macrophages.** *Cancers (Basel)* 2023, **15**.

236. Wong SHD, Yin B, Li Z, Yuan W, Zhang Q, Xie X, Tan Y, Wong N, Zhang K, Bian L: **Mechanical manipulation of cancer cell tumorigenicity via heat shock protein signaling.** *Sci Adv* 2023, **9:**eadg9593.

237. Zhao K, Zhou G, Liu Y, Zhang J, Chen Y, Liu L, Zhang G: **HSP70 Family in Cancer: Signaling Mechanisms and Therapeutic Advances.** *Biomolecules* 2023, **13**.

238. Albakova Z, Armeev GA, Kanevskiy LM, Kovalenko EI, Sapozhnikov AM: **HSP70 Multi-Functionality in Cancer.** *Cells* 2020, **9**.

239. Yin Y, Chen C, Chen J, Zhan R, Zhang Q, Xu X, Li D, Li M: **Cell surface GRP78 facilitates hepatoma cells proliferation and migration by activating IGF-IR.** *Cell Signal* 2017, **35:**154-162.

240. Theivanthiran B, Yarla N, Haykal T, Nguyen YV, Cao L, Ferreira M, Holtzhausen A, Al-Rohil R, Salama AKS, Beasley GM, et al: **Tumor-intrinsic NLRP3-HSP70-TLR4 axis drives premetastatic niche development and hyperprogression during anti-PD-1 immunotherapy.** *Sci Transl Med* 2022, **14:**eabq7019.

241. Rapp UK, Kaufmann SH: **DNA vaccination with gp96-peptide fusion proteins induces protection against an intracellular bacterial pathogen.** *Int*

Immunol 2004, **16:**597-605.

242. Sanchez-Lopez EF, Corigliano MG, Oliferuk S, Ramos-Duarte VA, Rivera M, Mendoza-Morales LF, Angel SO, Sander VA, Clemente M: **Oral Immunization With a Plant HSP90-SAG1 Fusion Protein Produced in Tobacco Elicits Strong Immune Responses and Reduces Cyst Number and Clinical Signs of Toxoplasmosis in Mice.** *Front Plant Sci* 2021, **12:**726910.

243. Oki Y, Younes A: **Heat shock protein-based cancer vaccines.** *Expert Rev Vaccines* 2004, **3:**403-411.

244. Corigliano MG, Sander VA, Sanchez Lopez EF, Ramos Duarte VA, Mendoza Morales LF, Angel SO, Clemente M: **Heat Shock Proteins 90 kDa: Immunomodulators and Adjuvants in Vaccine Design Against Infectious Diseases.** *Front Bioeng Biotechnol* 2020, **8:**622186.

245. Arnold-Schild D, Hanau D, Spehner D, Schmid C, Rammensee HG, de la Salle H, Schild H: **Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells.** *J Immunol* 1999, **162:**3757-3760.

246. Hagymasi AT, Dempsey JP, Srivastava PK: **Heat-Shock Proteins.** *Curr Protoc* 2022, **2:**e592.

247. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H: **HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway.** *J Biol Chem* 2002, **277:**15107-15112.

248. Einstein MH, Roden RBS, Ferrall L, Akin M, Blomer A, Wu TC, Chang YN: **Safety Run-in of Intramuscular pNGVL4a-Sig/E7(detox)/HSP70 DNA and TA-CIN Protein Vaccination as Treatment for HPV16+ ASC-US, ASC-H, or LSIL/CIN1.** *Cancer Prev Res (Phila)* 2023, **16:**219-227.

249. Li J, Xing Y, Zhou Z, Yao W, Cao R, Li T, Xu M, Wu J: **Microbial HSP70 peptide epitope 407-426 as adjuvant in tumor-derived autophagosome vaccine therapy of mouse lung cancer.** *Tumour Biol* 2016, **37:**15097-15105.

250. Ge C, Xing Y, Wang Q, Xiao W, Lu Y, Hu X, Gao Z, Xu M, Ma Y, Cao R, Liu J: **Improved efficacy of therapeutic vaccination with dendritic cells pulsed with tumor cell lysate against hepatocellular carcinoma by introduction of 2 tandem repeats of microbial HSP70 peptide epitope 407-426 and OK-432.** *Int Immunopharmacol* 2011, **11:**2200-2207.

251. Yuan J, Kashiwagi S, Reeves P, Nezivar J, Yang Y, Arrifin NH, Nguyen M, Jean-Mary G, Tong X, Uppal P, et al: **A novel mycobacterial Hsp70-containing fusion protein targeting mesothelin augments antitumor immunity and prolongs survival in murine models of ovarian cancer and mesothelioma.** *J Hematol Oncol* 2014, **7:**15.

252. Calderwood SK, Gong J, Stevenson MA, Murshid A: **Cellular and molecular chaperone fusion vaccines: targeting resistant cancer cell populations.** *Int J Hyperthermia* 2013, **29:**376-379.

253. Zong J, Wang C, Liu B, Liu M, Cao Y, Sun X, Yao Y, Sun G: **Human hsp70 and HPV16 oE7 fusion protein vaccine induces an effective antitumor efficacy.** *Oncol Rep* 2013, **30:**407-412.

254. Specht HM, Ahrens N, Blankenstein C, Duell T, Fietkau R, Gaipl US, Gunther C, Gunther S, Habl G, Hautmann H, et al: **Heat Shock Protein 70 (Hsp70) Peptide Activated Natural Killer (NK) Cells for the Treatment of Patients with Non-Small Cell Lung Cancer (NSCLC) after Radiochemotherapy (RCTx) - From Preclinical Studies to a Clinical Phase II Trial.** *Front Immunol* 2015, **6:**162.

255. Krause SW, Gastpar R, Andreesen R, Gross C, Ullrich H, Thonigs G, Pfister K, Multhoff G: **Treatment of colon and lung cancer patients with ex vivo heat shock protein 70-peptide-activated, autologous natural killer cells: a clinical phase i trial.** *Clin Cancer Res* 2004, **10:**3699-3707.

256. Bausinger H, Lipsker D, Ziylan U, Manie S, Briand JP, Cazenave JP, Muller S, Haeuw JF, Ravanat C, de la Salle H, Hanau D: **Endotoxin-free heat-shock protein 70 fails to induce APC activation.** *Eur J Immunol* 2002, **32:**3708-3713.

257. Sun D, Wang J, Han Y, Dong X, Ge J, Zheng R, Shi X, Wang B, Li Z, Ren P, et al: **TISCH: a comprehensive web resource enabling interactive single-cell transcriptome visualization of tumor microenvironment.** *Nucleic Acids Res* 2021, **49:**D1420-D1430.

258. Collin J, Queen R, Zerti D, Steel DH, Bowen C, Parulekar M, Lako M: **Dissecting the Transcriptional and Chromatin Accessibility Heterogeneity of Proliferating Cone Precursors in Human Retinoblastoma Tumors by Single Cell Sequencing-Opening Pathways to New Therapeutic Strategies?** *Invest Ophthalmol Vis Sci* 2021, **62:**18.

259. Darnell RB, DeAngelis LM: **Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies.** *Lancet* 1993, **341:**21-22.

260. Maeda A, Ohguro H, Nabeta Y, Hirohashi Y, Sahara H, Maeda T, Wada Y, Sato T, Yun C, Nishimura Y, et al: **Identification of human antitumor cytotoxic T lymphocytes epitopes of recoverin, a cancer-associated retinopathy antigen, possibly related with a better prognosis in a paraneoplastic syndrome.** *Eur J Immunol* 2001, **31:**563-572.

261. Maeda A, Maeda T, Ohguro H, Palczewski K, Sato N: **Vaccination with recoverin, a cancer-associated retinopathy antigen, induces autoimmune retinal dysfunction and tumor cell regression in mice.** *Eur J Immunol* 2002, **32:**2300-2307.

262. Zhao Y: **The feasibility to exploit fusion protein comprising Recoverin and HSP70 as cancer vaccine.** Imu, 2022.

263. Ramos-Ruperto L, Busca-Arenzana C, Boto-de Los Bueis A, Schlincker A, Arnalich-Fernandez F, Robles-Marhuenda A: **Cancer-Associated Retinopathy and Treatment with Intravenous Immunoglobulin Therapy. A Seldom Used Approach?** *Ocul Immunol Inflamm* 2021, **29:**399-402.

264. Takeuchi A, Saito T: **CD4 CTL, a Cytotoxic Subset of CD4(+) T Cells, Their Differentiation and Function.** *Front Immunol* 2017, **8:**194.

265. Raskov H, Orhan A, Christensen JP, Gogenur I: **Cytotoxic CD8(+) T cells in cancer and cancer immunotherapy.** *Br J Cancer* 2021, **124:**359-367.

266. Hayashi Y, Hoon DS, Park MS, Terasaki PI, Morton DL: **Cytotoxic T cell lines recognize autologous and allogeneic melanomas with shared or cross-reactive HLA-A.** *Cancer Immunol Immunother* 1992, **34:**419-423.

267. Chen KY, Liu J, Ren EC: **Structural and functional distinctiveness of HLA-A2 allelic variants.** *Immunol Res* 2012, **53:**182-190.

268. Jager E, Gnjatic S, Nagata Y, Stockert E, Jager D, Karbach J, Neumann A, Rieckenberg J, Chen YT, Ritter G, et al: **Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers.** *Proc Natl Acad Sci U S A* 2000, **97:**12198-12203.

269. Millar DG, Garza KM, Odermatt B, Elford AR, Ono N, Li Z, Ohashi PS: **Hsp70 promotes antigen-presenting cell function and converts T-cell tolerance to autoimmunity in vivo.** *Nat Med* 2003, **9:**1469-1476.

270. Wang XH, Qin Y, Hu MH, Xie Y: **Dendritic cells pulsed with hsp70-peptide complexes derived from human hepatocellular carcinoma induce specific anti-tumor immune responses.** *World J Gastroenterol* 2005, **11:**5614-5620.

271. Wang H, Feng F, Wang XP, Wang RS, Wu Y, Zhu MG, Zhang H, Zhuang ZX: **Dendritic cells pulsed with Hsp70 and HBxAg induce specific antitumor immune responses in hepatitis B virus-associated hepatocellular carcinoma.** *Mol Med Rep* 2016, **13:**1077-1082.

272. Zeng X, Nong WX, Zou XQ, Li F, Ge YY, Zhang QM, Luo B, Huang W, Zou JX,

Fan R, Xie XX: **Prediction and identification of HLA-A*0201-restricted epitopes from cancer testis antigen CT23.** *Hum Vaccin Immunother* 2023, **19:**2293299.

273. Chai D, Qiu D, Shi X, Ding J, Jiang N, Zhang Z, Wang J, Yang J, Xiao P, Wang G, Zheng J: **Dual-targeting vaccine of FGL1/CAIX exhibits potent anti-tumor activity by activating DC-mediated multi-functional CD8 T cell immunity.** *Mol Ther Oncolytics* 2022, **24:**1-13.

274. Zheng Y, Lu Z, Ding J, Jiang N, Wang J, Yang J, Song J, Chen H, Fang L, Li H, et al: **Therapeutic Adenovirus Vaccine Combined Immunization with IL-12 Induces Potent CD8(+) T Cell Anti-Tumor Immunity in Hepatocellular Carcinoma.** *Cancers (Basel)* 2022, **14**.

275. Wang Q, Qin Y, Li B: **CD8(+) T cell exhaustion and cancer immunotherapy.** *Cancer Lett* 2023, **559:**216043.

276. McLaughlin K, Seago J, Robinson L, Kelly C, Charleston B: **Hsp70 enhances presentation of FMDV antigen to bovine CD4+ T cells in vitro.** *Vet Res* 2010, **41:**36.

277. Zhao Y, Wei K, Chi H, Xia Z, Li X: **IL-7: A promising adjuvant ensuring effective T cell responses and memory in combination with cancer vaccines?** *Front Immunol* 2022, **13:**1022808.

278. Gu YZ, Fan CW, Lu R, Shao B, Sang YX, Huang QR, Li X, Meng WT, Mo XM, Wei YQ: **Forced co-expression of IL-21 and IL-7 in whole-cell cancer vaccines promotes antitumor immunity.** *Sci Rep* 2016, **6:**32351.

279. Miri SM, Pourhossein B, Hosseini SY, Keshavarz M, Shahmahmoodi S, Zolfaghari MR, Mohebbi SR, Gorji A, Ghaemi A: **Enhanced synergistic antitumor effect of a DNA vaccine with anticancer cytokine, MDA-7/IL-24, and immune checkpoint blockade.** *Virol J* 2022, **19:**106.

280. Sriram U, Biswas C, Behrens EM, Dinnall JA, Shivers DK, Monestier M, Argon Y, Gallucci S: **IL-4 suppresses dendritic cell response to type I interferons.** *J Immunol* 2007, **179:**6446-6455.

281. Li Y, Wang D, Fang X: **In Vitro Generation of Anti-Osteosarcoma Cytotoxic Activity Using Dendritic Cells Loaded with Heat Shock Protein 70-Peptide Complexes.** *Fetal Pediatr Pathol* 2019, **38:**387-398.

282. Ye JJ, Bao P, Deng K, Dong X, He J, Xia Y, Wang Z, Liu X, Tang Y, Feng J, Zhang XZ: **Engineering cancer cell membranes with endogenously upregulated HSP70 as a reinforced antigenic repertoire for the construction of material-free prophylactic cancer vaccines.** *Acta Biomater* 2024, **174:**386-399.

283. Brauns T, Leblanc P, Gelfand JA, Poznanski M: **Could mycobacterial Hsp70-containing fusion protein lead the way to an affordable therapeutic cancer vaccine?** *Expert Rev Vaccines* 2015, **14:**435-446.

284. Chen Y, Zheng L, Hua W, Wang J, Chen L, Huang A, Zhang W: **Fusion of NY-ESO-1 epitope with heat shock protein 70 enhances its induced immune responses and antitumor activity against glioma in vitro.** *Transl Cancer Res* 2024, **13:**191-201.

285. Kim JH, Lee KJ, Lee SW: **Cancer immunotherapy with T-cell targeting cytokines: IL-2 and IL-7.** *BMB Rep* 2021, **54:**21-30.

286. Xu C, Marelli B, Qi J, Qin G, Yu H, Wang H, Jenkins MH, Lo KM, Lan Y: **NHS-IL12 and bintrafusp alfa combination therapy enhances antitumor activity in preclinical cancer models.** *Transl Oncol* 2022, **16:**101322.

287. Gatti-Mays ME, Tschernia NP, Strauss J, Madan RA, Karzai FH, Bilusic M, Redman J, Sater HA, Floudas CS, Toney NJ, et al: **A Phase I Single-Arm Study of Biweekly NHS-IL12 in Patients With Metastatic Solid Tumors.** *Oncologist* 2023, **28:**364-e217.

288. Legat A, Maby-El Hajjami H, Baumgaertner P, Cagnon L, Abed Maillard S,

Geldhof C, Iancu EM, Lebon L, Guillaume P, Dojcinovic D, et al: **Vaccination with LAG-3Ig (IMP321) and Peptides Induces Specific CD4 and CD8 T-Cell Responses in Metastatic Melanoma Patients--Report of a Phase I/IIa Clinical Trial.** *Clin Cancer Res* 2016, **22:**1330-1340.

289. Romano E, Michielin O, Voelter V, Laurent J, Bichat H, Stravodimou A, Romero P, Speiser DE, Triebel F, Leyvraz S, Harari A: **MART-1 peptide vaccination plus IMP321 (LAG-3Ig fusion protein) in patients receiving autologous PBMCs after lymphodepletion: results of a Phase I trial.** *J Transl Med* 2014, **12:**97.

290. Zhang X, Yu C, Zhao J, Fu L, Yi S, Liu S, Yu T, Chen W: **Vaccination with a DNA vaccine based on human PSCA and HSP70 adjuvant enhances the antigen-specific CD8+ T-cell response and inhibits the PSCA+ tumors growth in mice.** *J Gene Med* 2007, **9:**715-726.

291. Ren S, Zhang Z, Li M, Wang D, Guo R, Fang X, Chen F: **Cancer testis antigen subfamilies: Attractive targets for therapeutic vaccine (Review).** *Int J Oncol* 2023, **62**.

292. Duperret EK, Liu S, Paik M, Trautz A, Stoltz R, Liu X, Ze K, Perales-Puchalt A, Reed C, Yan J, et al: **A Designer Cross-reactive DNA Immunotherapeutic Vaccine that Targets Multiple MAGE-A Family Members Simultaneously for Cancer Therapy.** *Clin Cancer Res* 2018, **24:**6015-6027.

293. Wang L, Rollins L, Gu Q, Chen SY, Huang XF: **A Mage3/Heat Shock Protein70 DNA vaccine induces both innate and adaptive immune responses for the antitumor activity.** *Vaccine* 2009, **28:**561-570.

294. Ma JH, Sui YF, Ye J, Huang YY, Li ZS, Chen GS, Qu P, Song HP, Zhang XM: **Heat shock protein 70/MAGE-3 fusion protein vaccine can enhance cellular and humoral immune responses to MAGE-3 in vivo.** *Cancer Immunol Immunother* 2005, **54:**907-914.

295. Wang HH, Mao CY, Teng LS, Cao J: **Recent advances in heat shock protein-based cancer vaccines.** *Hepatobiliary Pancreat Dis Int* 2006, **5:**22-27.

296. Zhang X, Huang Y, Li X, Wang Y, Yuan Y, Li M: **Preparation of a new combination nanoemulsion-encapsulated MAGE1-MAGE3-MAGEn/HSP70 vaccine and study of its immunotherapeutic effect.** *Pathol Res Pract* 2020, **216:**152954.

297. Gao X, Nan Y, Yuan Y, Gong X, Sun Y, Zhou H, Zong Y, Zhang L, Yu M: **Gas‑filled ultrasound microbubbles enhance the immunoactivity of the HSP70‑MAGEA1 fusion protein against MAGEA1‑expressing tumours.** *Mol Med Rep* 2018, **18:**315-321.

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Cancer, an enduring purveyor of human mortality, has exacted a toll by snatching cherished lives. My aspiration is to evolve into an adept scientific luminary, dedicating a lifetime's devotion to the canvas of oncological exploration. The burgeoning realm of immunotherapy stands as a formidable armamentarium in humanity's crusade against malignancy. My steadfast dedication to the precincts of immunotherapeutic exploration will persevere, with sanguine anticipation for forthcoming breakthroughs.

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List of publications

1. Soltani M, Zhao Y, **Xia Z**, Ganjalikhani Hakemi M, Bazhin AV. The Importance of Cellular Metabolic Pathways in Pathogenesis and Selective Treatments of Hematological Malignancies. Front Oncol. 2021;11:767026. doi: 10.3389/fonc.2021.767026.