Aus der klinik für Allgemein-, Viszeral- und Transplantationschirurgie
Klinikum der Ludwig-Maximilians-Universität München


# *The Feasibility to Exploit Fusion Protein Comprising Recoverin and HSP70 as Cancer Vaccine*

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität München

vorgelegt von
**Yue Zhao**

aus
Fujian, Volksrepublik China

Jahr
2022

Mit Genehmigung der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

| | |
|---|---|
| Erster Gutachter: | Prof. Dr. Alexandr V. Bazhin |
| Zweiter Gutachter: | Priv. Doz. Dr. Barbara Mayer |
| Dritter Gutachter: | Prof. Dr. Simon Rothenfußer |
| ggf. weitere Gutachter: | _____ |
| Mitbetreuung durch den promovierten Mitarbeiter: | |
| Dekan: | Prof. Dr. med. Thomas Gudermann |
| Tag der mündlichen Prüfung: | 26.07.2022 |

# Table of Contents

# Zusammenfassung:

Maligner Tumor ist weltweit die häufigste Todesursache. Trotz der Fortschritte bei konventionellen Krebsbehandlungen wie Chirurgie und Chemotherapie bleibt das Überleben der Krebspatienten unbefriedigend. Die Immuntherapie wäre eine neuartige therapeutische Alternative um eine große Wirksamkeit bei der Bekämpfung von Tumoren mit hoher Spezifität zu erzeugen. Unter den unterschiedlichen Immuntherapiearten zeichnen die Krebsvakzine sich durch ihre Fähigkeit aus, das Immunsystem zu trainieren und zu stärken, um gezielt maligne Zellen zu eliminieren. Die Auswahl eines hochimmunogenen und aviden Antigens ist jedoch ein kritischer Punkt bei der Entwicklung von Krebsvakzinen.

Aufgrund der Existenz der Blut-Retina-Schranke weist Recoverin, ein Ca2+-bindendes Protein, in der immunprivilegierten Zone – der Netzhaut, eine große Fähigkeit auf, Immunantwort auszulösen. Zahlreiche Studien belegten, dass Recoverin bei verschiedenen Krebszellen aberrant exprimiert werden kann. Daher besitzt Recoverin das Potenzial, als idealer Antigenkandidat für Vakzinen zu sein. Um die Immunogenität des Antigens zu verstärken, wurden Recoverin HLA Klasse I und II Epitope an HSP70 Protein fusioniert. HSP70 fungiert dabei als natürliches Adjuvans. In dieser Studie habe ich die Machbarkeit des Recoverin-Fusionsproteins als potenzielles Krebsvakzin untersucht.

In der Arbeit hat es mir gelungen zu zeigen, dass das Recoverin-Fusionsprotein die Reifung und Aktivierung von DCs durch Hochregulieren der Expression von CD83, CD80 und CD86 induziert. Darüber hinaus fand ich, dass das Fusionsprotein die Sekretion von proinflammatorischen Zytokinen von DCs, wie TNF-α und IL-6, stimuliert. Weitere Untersuchungen zeigten, dass mit dem Fusionsprotein gepulste DCs in der Lage sind, die Aktivierung autologer CD8+ T-Lymphozyten zu induzieren. Die immunsuppressiven Eigenschaften wurden jedoch auch durch Fusionsprotein nachgewiesen. Mit dem Fusionsprotein gepulste DCs weisen eine erhöhte Expression von PD-L1 auf und können die Expansion von regulatorischen T-Zellen anregen.

Zusammenfassend haben die durchgeführten Experimente die Fähigkeit des Fusionsproteins gezeigt, Immunantworten zu stimulieren, was die Möglichkeit nahelegt, das Fusionsprotein als Krebsvakzin zur Bekämpfung von Recoverin-exprimierenden Tumoren eingesetzt werden könnte.

# Abstract:

Cancer now is the leading cause of mortality worldwide. Despite the advances in conventional cancer treatments, like surgery and chemotherapy, the survival of cancer patients remains unsatisfying. Currently, immunotherapy, as a novel therapeutic alternative, exhibits a great efficacy to struggle against tumors with high specificity. Among immunotherapies, cancer vaccine stands out due to its capability to train and bolster the immune system to eliminate malignant cells specifically. However, selecting a high immunogenetic and avid antigen is a critical issue in the development of cancer vaccines.

Because of the existence of the blood-retina barrier, Recoverin, a $Ca^{2+}$-binding protein located in the immunoprivileged zone — retina, exhibit a great capability to prime immune responses against it when being exposed to the immune system. Accumulating studies revealed that Recoverin was found to be aberrantly expressed in various types of cancer. Therefore, Recoverin possesses the potential to serve as an ideal antigen candidate for the exploitation of cancer vaccines. To enhance the immunogenicity of antigen, Recoverin HLA class I and II epitopes were fused to HSP70 protein, which was reported to function as a natural adjuvant. In this study, I examined the feasibility of the Recoverin fusion protein as cancer vaccines *in vitro*.

Recoverin fusion protein was evidenced to induce the maturation and activation of DCs via upregulating the expression of CD83, CD80, and CD86. Moreover, I also found that the fusion protein stimulates the secretion of pro-inflammatory cytokines of DCs, like TNF-α and IL-6. Further investigation revealed that DCs pulsed with the fusion protein are capable to induce the activation of autologous $CD8^+$ T lymphocytes. However, the immunosuppressive properties were also demonstrated in the fusion protein. DCs pulsed with the fusion protein exhibit an increased level of PD-L1 and are able to prompt the expansion of Treg cells.

In conclusion, *in vitro* experiments demonstrated the ability of the fusion protein to stimulate immune responses, suggesting the possibility to apply the fusion protein as a cancer vaccine to struggle against Recoverin-expressing tumors.

# List of abbreviations

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microliter |
| ANOVA | Analysis of variance |
| APC | Antigen-presenting cell |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| C | Carbon |
| $Ca^{2+}$ | Calcium ion |
| CAR | Cancer-associated retinopathy |
| CAR T cells | Chimeric antigen receptor T cells |
| CD | Cluster of differentiation |
| CGA | Cancer-germline antigens |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| cyto | Cytokine |
| DC | Dendritic cell |
| DMSC | myeloid-derived suppressor cell |
| DNA | Deoxyribonucleic acid |
| EBV | Epstein–Barr virus |
| EDTA | Ethylenediaminetetraacetic acid |
| FACS | Fluorescence-activated cell sorting |
| FDA | Food and Drug Administration |
| FEEL-1 | Fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1 |
| FMO | Fluorescence minus one |
| FP | Fusion protein |
| g | Gram |

| | |
|---|---|
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GPCR | G protein-coupled receptors |
| GRK1 | Rhodopsin kinase |
| HLA | Human leukocyte antigen |
| HPV | Human papillomavirus |
| HSP | Heat shock protein |
| ICB | Immune checkpoint blockades |
| iDC | Immature DC |
| IHC | Immunohistochemistry |
| IFN-γb | Interferon-γb |
| IL | Interleukin |
| L | Liter |
| LAL | Limulus amebocyte lysate |
| LOX-1 | Lectin-like oxidized low-density lipoprotein receptor-1 |
| LPS | Lipopolysaccharide |
| MFI | Median fluorescence intensity |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| ml | Milliliter |
| N | Nitrogen |
| NK | Natural killer |
| NSCLC | Non-small cell lung cancer |
| OVA | Chicken ovalbumin |
| PBMC | Peripheral blood mononuclear cell |
| PD-1 | Programmed death 1 |
| PD-L1 | Programmed death ligand-1 |
| pept | peptide |
| PGE2 | Prostaglandin E2 |
| RNA | Ribonucleic acid |
| SCLC | Small cell lung cancer |

| | |
|---|---|
| SD | Standard deviation |
| SREC-1 | Scavenger receptor expressed by endothelial cell-1 |
| TAA | Tumor-associated antigen |
| TCR | T cell receptors |
| TGF-β | Transforming growth factor-beta |
| TIL | Tumor-infiltrating lymphocyte |
| TME | Tumor microenvironment |
| TNF-α | Tumor necrosis factor-α |
| Tregs | Regulatory T cells |
| TSA | Tumor-specific antigen |
| U | Unit |

# 1. Introduction

## 1.1 Current development of cancer immunotherapy

Cancer now is the leading cause of human deaths all over the world and poses a great threat to human health improvement, preventing life extension of humans and increasing patients', even the society's, economic burden. According to the report from Global Cancer Statistics 2020, approximately 19.3 million new cancer cases and almost 10.0 million cancer deaths took place worldwide in 2020 [1]. Cancer is the first or second cause of mortality in 112 out of 183 countries (Figure 1.1), even overwhelming the mortality rates of stroke and coronary heart disease. Furthermore, by 2040, the global burden of cancer incidence is expected to reach 28.4 million cases, an increase of 47% over 2020. Therefore, global efforts to prevent and control the occurrence and progression of cancers are urgently needed to be taken to increase human life expectancy, decrease society's financial burden and promote the sustainable development of the whole world.

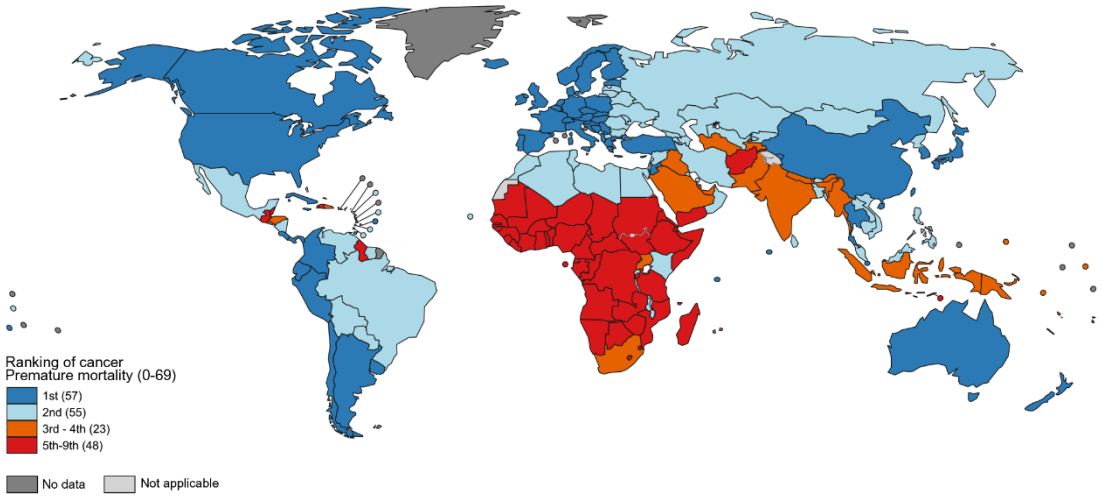


**Figure 1.1: Ranking of cancers as a cause of mortality all over the world in 2019 [1]. Data is originated from World Health Organization. The authority has been approved by John Wiley and Sons and Copyright Clearance Center.**

Traditional therapeutic approaches in oncology, including surgery, chemotherapy, and radiotherapy, are still the major measurements to battle against cancers clinically. However, the effectiveness of traditional therapies is still far from satisfactory. Moreover, inaccurate targeting of traditional therapy poses a cytotoxic influence on normal cells, even bringing damages to the immune system which plays a critical role in the struggle against cancers,

leading to serious side effects when being applied clinically. Thus, the development of accurate and personalized treatment approaches against cancers gain increasing attention in recent decades. Currently, a new field of medicine based on such trends called theranostics has appeared. Theranostics combines specific targeted therapy based on specific diagnostic tests, to fulfill the greatest anti-cancer function while with limited toxicity toward patients, opening opportunities for the utilization of modern methods of cancer therapy [2].

With the advance of oncology and immunology, the understanding of the relationship between tumors and the immune system has become clearer. Being driven by various kinds of mutations and epigenetic alterations, malignant cells express mutant or atypical proteins during oncogenesis and progression, making them immunogenic and detectable for the immune system. Nevertheless, malignant cells often evade recognition and elimination by the immune system mainly for the following reasons: (1) downregulation of detectable antigens processing and presentation procedures; (2) attraction of immune suppressor cells into the tumor microenvironment (TME), including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages; (3) secretion of immune-suppressing factors which inhibit the function of the immune response, such as interleukin-10 (IL-10), transforming growth factor-beta (TGF-β); (4) upregulation of co-inhibitory ligands which favor the immunosuppression of tumor-infiltrating lymphocytes (TILs), such as programmed death ligand-1 (PD-L1); (5) induction of co-inhibitory receptors on TILs, like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death 1 (PD-1) (Figure 1.2) [3,4]. Numerous inspiring progressions concerning cancer evasion in the immune system have been achieved in the past few decades, which in turn pave the avenue for the development of a new approach — cancer immunotherapy, to inhibit the immune evasion of cancers, leading to the elimination of malignant cells eventually.

Cancer immunotherapy harnesses high specificity and accuracy to combat malignancies, which implies antitumor immune response activation and/or immunosuppression inhibition. In 2013, “cancer immunotherapy” was selected as The Breakthrough of the Year by *Science* for its capacity to harness the immune system to battle cancers and its potential clinical benefits for cancer patients [5]. The current success of cancer immunotherapy by immune checkpoint blockades (ICB), by chimeric antigen receptor (CAR) T cells, and by cancer vaccines indicates the major research progression to re-edit the immune balance in favor of an activation of the immune system and prevention of the cancer evasion [6]. Recently, inspiring clinical results derived from cancer immunotherapy proved that the in-depth investigation of cancer

immunotherapy from global sites finally paid off. Nevertheless, in terms of utilizing cancer immunotherapy to eliminate cancers, making tumors completely free from patients, with limited cytotoxic side effects, we still have a long way to go.

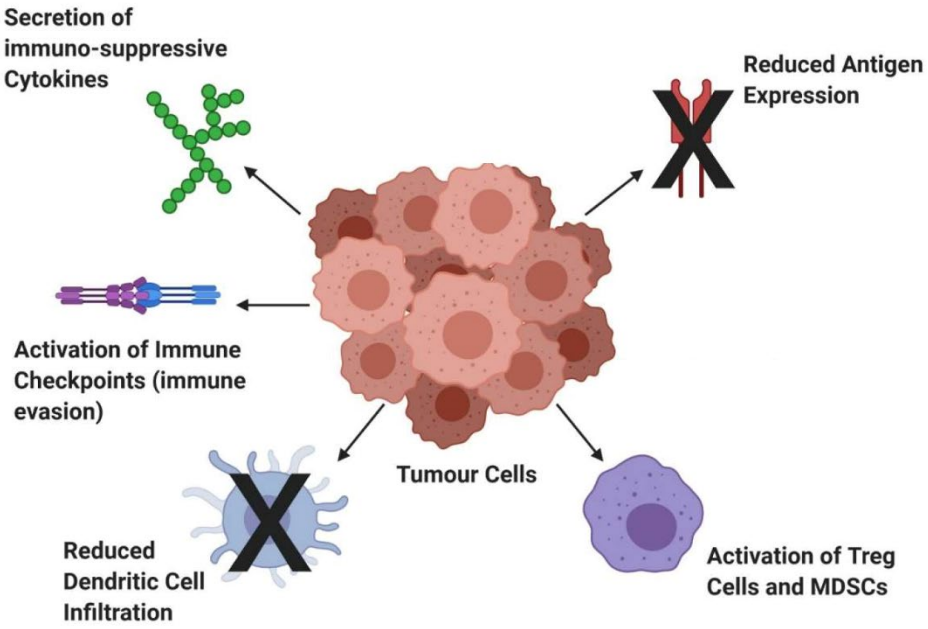


**Figure 1.2: The mechanism of cancer immunological evasion [4].**

Tumor cells are capable to reduce the antigen expression on the surface for the identification of immune cells. Meanwhile, they can also reduce the secretion of the pro-inflammatory cytokine or enhance the inhibitory cytokines secretion, to attenuate the antitumor activity of immune cells. Moreover, by upregulating the immune checkpoints, like PD-L1, they are able to trigger T cells‘ anergy and unresponsive status. Last but not least, tumor cells recruit immunosuppressive cells in the TME to inhibit the infiltration and activation of APCs and T lymphocytes. The authority has been approved by the corresponding author Zahidul Islam Pranjol.

### 1.1.1 Immune checkpoint blockade

The most well-developed and successful application of cancer immunotherapy recently is ICB. The method is to inhibit so call checkpoints of the immune system, including CTLA-4 and PD-1 expressed on T cells, or PD-L1 expressed on tumor cells. These proteins control the immune response and prevent T cells from cytotoxicity manifestation. ICB “releases the brake” of the immune system, making T cells lose the ability to receive a suppressive signal from mimicking tumor cells or immunotolerant antigen-presenting cells (APCs), which leads to their prolonged and unhindered cytotoxicity.

CTLA-4 was firstly discovered by Golstein and colleagues in 1987 [7]. Later on, several research teams investigate that CTLA-4 acts as an inhibitory receptor in the immune system, which further results in T cells inactivation and lymphoproliferative disorders [8-10]. Based on these milestones, in 1996, Allison and colleagues determined that *in vivo* injection of antibodies to CTLA-4 leads to tumor suppression, indicating the rationale of applying CTLA-4-targeting antibodies in clinical cancer treatment [11]. With accumulating clinical evidence that proves the efficacy and safety of CTLA-4 blockade, the United State Food and Drug Administration (FDA) approved the application of anti–CTLA-4 antibodies (ipilimumab) in melanoma treatment, opening a new era of cancer therapy.

Another important hallmark of the development of ICB is the investigation of antibodies targeting PD-1/PD-L1. PD-1 was originally discovered by Honjo and colleagues in 1992 [12] while PD-L1 was revealed by two independent research teams around 7 years later [13,14]. A further study reported that the expression of PD-L1 was elevated in tumors and after blocking the interaction of PD-1/PD-L1 by antibodies, activation of tumor-reactive T cells was demonstrated and a decrease in tumor burden was observed [15]. These findings provided a solid foundation for the application of PD-1 blocking in advanced solid tumor treatment, including melanoma and lung cancer, and the results turn out to be a great success, forever changing the balance in the choice of methods of anticancer therapy [16-18].

Although ICB has shown its dominant position in cancer immunotherapy, several clinical trials revealed that only a moderate proportion of patients benefits from ICB treatment [19]. Furthermore, some sets of cancer, like glioma and glioblastoma, particularly exhibit resistance to ICB treatment [20]. Therefore, it should be noted that ICB therapy is not a panacea for cancer treatment, innovative or improved approaches concerning this field still warrant an in-depth exploration for a better life-saving benefit.

### 1.1.2 Chimeric antigen receptor T cells

The notion of CAR T cells was firstly proposed by Schindler and colleagues in 1993 [21]. They designed and constructed chimeric genes encoding single-chain antibodies with an anti-trinitophenyl variable region and an intracellular constant region with signing adaptors, and transduced them into T cells, resulting in the expression of signal-receiving and transducing subunits on T cells. This technology endows T cells with antibody-type specificity to combat tumor cells expressing antibody-corresponding antigens, redirecting and enhancing the specific T lymphocytes killing against malignancies [22]. A further study revealed that

peripheral blood T cells genetically targeted to the CD19 are capable to eradicate B-cells malignancies *in vivo* and *in vitro*, highlighting the clinical feasibility to struggle against leukemia or lymphoma by CAR T cell therapeutic strategy [23]. Encouraging news was received in 2010 that a patient with advanced lymphoma was treated with autologous T cells genetically engineered to express CD19 and experienced a considerable tumor regression after administration. Eradication of B-lineage cells and low serum immunoglobulins levels were evidenced in this patient for at least 39 weeks after CAR T cell therapy. Since then, numerous clinical trials regarding CAR T cell therapy were initiated and impressive results were demonstrated [24-26]. In 2017, Novartis' CAR-T therapy was firstly approved by FDA for patients with B-cell precursor acute lymphoblastic leukemia [27].

The success of CAR-T therapy in hematopoietic malignancies arouse our expectations in the treatment of solid tumors. However, some clinical trials revealed that the efficacy of CAR-T therapy in solid tumor treatment is limited due to the following reasons: (1) tumor antigen loss after treatment, especially those antigen-null tumor cells, which is responsible for the therapy resistance; (2) CAR-T cells are hard to discriminate normal cells and tumor cells because few truly tumor-specific targets have been identified; (3) recognizing and trafficking to solid tumors is hard to reach; (4) The existence of TME prevent the interaction of CAR-T cells and tumor cells [28]. Thus, exploiting this approach for solid tumors treatment still requires efforts to overcome the hurdles posed by tumor heterogeneity and the immunosuppressive TME.

### 1.1.3 Cancer vaccines

In 1796, Edward Jenner extract the fluid from a pustule of a smallpox-infected patient and then administrated it into an 8 years old boy, preventing the occurrence of disease when the boy got an infection of the virus [29]. This miracle event unraveled the curtains of the development of vaccines, which save billions of people all over the world. In the past decades, the application of vaccines is no longer limited to infectious diseases prophylaxis. Investigation of cancer vaccines attracts the great interest of researchers globally, resulting in the rapid advancement of cancer vaccines recently. Cancer vaccines train and bolster the immune system of patients to initiate or strengthen an immune response against cancer cells by enhancing the identification of immune cells towards tumor antigens [30]. Therefore, the immune responses stimulated by cancer vaccines harness a highly accurate and specific potential, with limited cytotoxicity to normal cells.

The key point of cancer vaccines as immunotherapy is the delivery of tumor antigens to APCs, especially to dendritic cells (DCs), the most professional APCs. After being engulfed by DCs, tumor antigens will be processed and presented on the surface of DCs, which is also known as the maturation of DCs. Then matured and activated DCs migrate to the secondary lymphoid organs, where the tumor antigens are presented to T lymphocytes with subsequent activation occurrence, resulting in the elimination of cancer cells by activated cytotoxic T cells (Figure 1.3) [31]. Cancer vaccines show superiority in antigen recognition. Rather than receiving the tumor antigens in the TME, where the immunosuppressive cells or components attenuate the recognition of antigens. Cancer vaccines directly exposure tumor antigens to DCs outsides the TME, leading to a rapid and effective adaptive immunity against cancers.

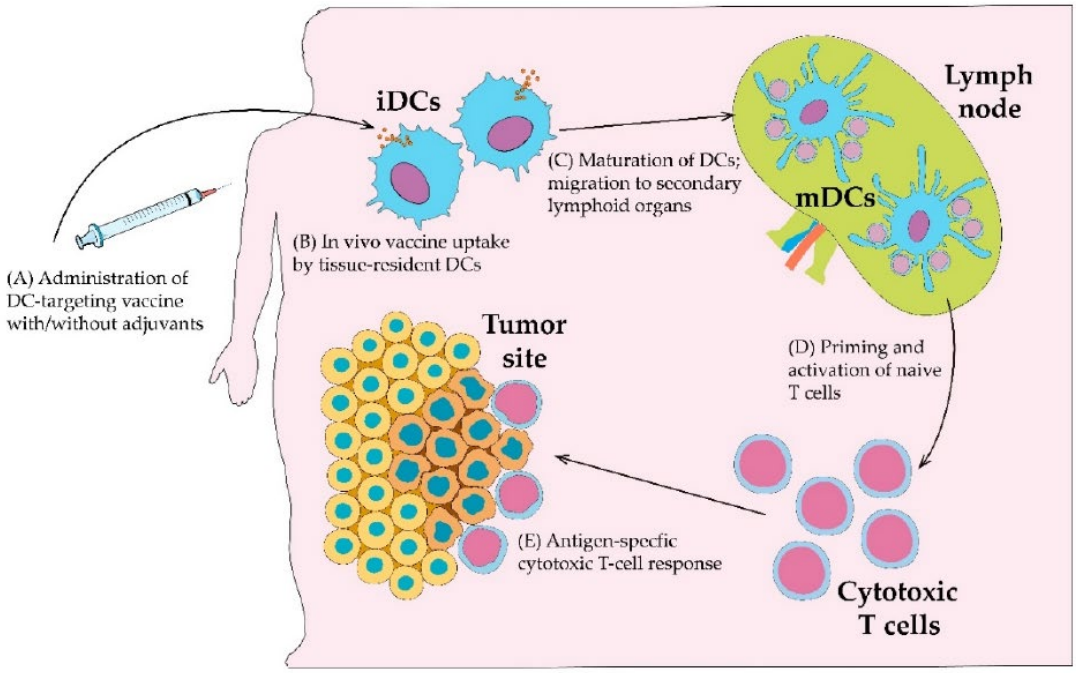


**Figure 1.3: Representation of *in vivo* cancer vaccine targeting strategies [31].**

(A) Administration of cancer vaccines into the body by various routes; (B) Cancer vaccines will be engulfed by APCs, especially DCs, for further process and presentation; (C) After maturation, DCs migrate to secondary lymphoid organs; (D) Once reach secondary lymphoid organs, DCs are capable to prime and activate T lymphocytes; (E) Fully functional effector T cells, especially cytotoxic T lymphocytes, induce antigen-specific cytotoxicity against tumor cells. The authority has been approved by the first author Alexey V Baldin.

Nowadays, various types of cancer vaccine platforms have been investigated, like peptides vaccines [32], DC vaccines [33], deoxyribonucleic acid (DNA) vaccines [34], and ribonucleic

acid (RNA) vaccines [35], to fulfill the greatest efficacy of antitumor responses. Clinical practice revealed that cancer patients benefit from the application of cancer vaccines with enhanced antitumor immunity and prolonged life [36-38], highlighting the outstanding role of cancer vaccines to struggle against malignancies as an immunotherapeutic strategy. However, the lack of specific and immunogenetic tumor antigens for selection, ineffective antigen delivery, and tumor heterogeneity are still the challenges that threaten the advances of cancer vaccines. How to address these issues has been a focused field that needs the efforts of researchers globally.

When in comparison to ICB, cancer vaccines proactively arouse the antitumor immune responses, while ICB only impedes immunosuppression via disrupting the interaction of inhibitory receptors. Therefore, cancer vaccines possess the initiative in adaptive antitumor immunity stimulation which ICB does not. As for CAR-T therapy, although it exhibits specificity and accuracy features in combating cancers, as an adoptive cell transfer therapy, the cost of manufacturing is unaffordable for most patients due to its labor-consuming procedures. Furthermore, the time length in the development of personalized CAR-T cells is also an obstacle for its application, which means patients might have a cancer progression during manufacturing, making him/her on longer suitable for this therapy, even die during the waiting period [39]. Therefore, cancer vaccines seem to have the potential to be exploited widely that CAR-T therapy does not. Cancer vaccines with specific tumor antigens can be manufactured in advance and when patients are diagnosed with cancers, cancer vaccines already stand by for employment. Altogether, cancer vaccines are greatly potential immunotherapy that can be applied widely with specificity, effectiveness, and low money and time consumption.

However, to achieve a comprehensive therapeutic efficacy, the immunotherapy strategy is not limited to monotherapy anymore. Combination approaches have been proposed to circumvent the limitations of immunotherapy and achieve the greatest effects against cancers. In pilot preclinical studies it has been shown that when immunotherapeutic agents were tested separately, each of them showed only average efficiency, while a combination of these components showed the greatest effect [40]. It has become clear that to fulfill the full potential of the immunotherapeutic approach in oncology, a combined strategy is required to impact the tumor and its TME in a comprehensive method. How to increase the contribution of cancer vaccines to the antitumor effect when combined with other immunotherapeutic agents, like ICBs, is an urgent task.

## 1.2 Antigen selection of cancer vaccines

The antigen selection is critical in the development of cancer vaccines. Various features of tumor antigens, like antigenicity, immunogenicity, and avidity, have a great influence on the capability of cancer vaccines to arouse an adaptive antitumor immunity. Tumor-associated antigens (TAAs), a set of proteins upregulated in cancer cells while exhibiting low levels in normal cells, have been well-studied and established in cancer vaccines [41]. Nevertheless, human leukocyte antigen (HLA) restriction, immune tolerance, and adverse events are still hurdles that hinder the advance of TAAs-based cancer vaccines due to the “self-proteins” feature of TAAs. With a further investigation of tumor antigens, a group of proteins, known as tumor-specific antigens (TSAs), come into view in the exploitation of cancer vaccines. As “foreign proteins”, the exposure of TSAs to the immune system triggers a robust antitumor immunity with high specificity while the immune tolerance is limited [42]. Therefore, the choice of tumor antigens in the design of cancer vaccines is a vital issue that affects the effectiveness and safety of cancer vaccines greatly.

### 1.2.1 Tumor-specific antigens

Thousands of genetic mutations occur in the tumor cells during carcinogenesis and progression, including single-nucleus substitution, insertion mutation, reading frame shift, etc. Among these mutations, most of them neither contribute to the initiation nor development of cancers, also known as “passenger mutations”. While a small proportion of mutations leads to tumorigenesis and advancement of malignancies, called “driver mutations” [43]. Theoretically, both of them, also regarded as “non-synonymous mutations”, generate abnormal antigens that are exclusively presented in cancer cells but not in normal healthy cells. This set of antigens are known as “neoantigens”. The first discovery of neoantigens traces back to the 1980s [44,45]. Bonn and colleagues found that mouse tumor cells express tum- variants after mutagen treatment and produce new antigens presented on the surface. These “tum- antigens” were recognized by T lymphocytes and rejected by alloimmune systems. Since then, accumulating studies have revealed that neoantigens resulting from somatic mutations can be strongly recognized by T cells and trigger a robust immune response [46,47].

Unlike TAAs, neoantigens generated during tumorigenesis and progression are sorely limited to tumor cells themselves, while undetectable in normal cells, which means immune responses triggered by neoantigens only direct tumor cells which express corresponding neoantigens, with no harms to those normal healthy cells. Furthermore, as “foreign proteins”, neoantigens

exhibit a high affinity to major histocompatibility complex (MHC) molecules and T cell receptors (TCR). The greater difference between altered amino acids sequence from the original one, the higher affinity to MHC and TCR neoantigens will be [48,49]. The high affinity between neoantigens and MHC or TCR results in a more vigorous antitumor elimination. Summarizing, these facts indicate the potential role of neoantigens in the development of immunotherapy, with high specificity, great ability to arouse antitumor immunity, and low cytotoxicity.

The manufacturing of neoantigens-based cancer vaccines require some major steps as shown in the following (Figure 1.4): (1) obtain a biopsy of tumor tissue and its corresponding healthy tissue from patients; (2) identify mutated genes in tumor biopsy via whole-genome or whole-exome sequencing in contrast to normal tissue; (3) select promising neoantigens candidates via bioinformatic algorithms; (4) validate the feasibility of predicted neoantigens by immunological assays. The application of neoantigens-based cancer vaccines in clinical trials has achieved encouraging results, especially in the form of mRNA vaccines [50-52].

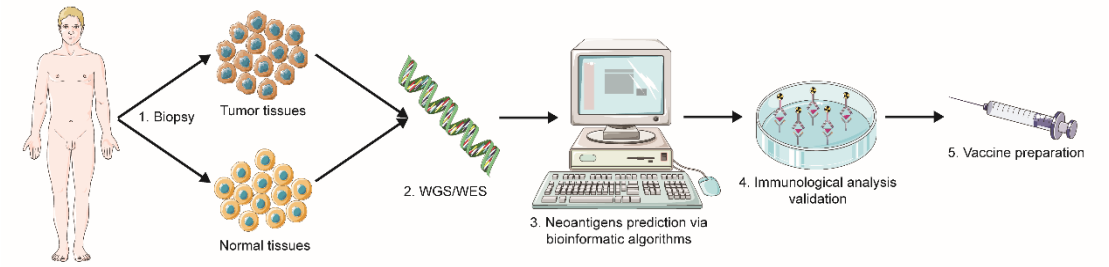


**Figure1.4: The procedures of neoantigens-based vaccines manufacturing [53].**

(1) Tumor tissues and corresponding normal tissues are obtained; (2) Biopsy is conducted to whole-genome or whole-exome sequencing for mutated genes identification; (3) Bioinformatic algorithms are carried out to filter the neoantigens candidates for vaccines; (4) the potential immunostimulatory properties of neoantigens are verified by immunological experiments.

The other TSAs, viral antigens, also exhibit great potential in the development of cancer vaccines. It has been reported that almost 15% of cancers are associated with the infection of the virus [54]. Epidemiological data demonstrated that some sets of viruses participate in the occurrence and progression of cancers critically via direct or indirect pathways. For example, some retroviruses integrate their viral genes into the genomes of host cells, especially in the vicinity of oncogenes, resulting in oncogene amplification, cellular transformation, and carcinogenesis [55]. Furthermore, viral oncoproteins translated from viral genes also have been proven to be a cancer-associated risk factor. Therefore, by targeting viral antigens, obstruction of the progression and even elimination of virus-associated malignancies are

realizable. The feature of “foreign antigens” also endows the viral antigens the capability to induce a powerful antitumor response, with limited cytotoxicity towards normal cells due to the absence of viral proteins in them [56]. Noteworthy, viral antigens can not only be investigated into therapeutic cancer vaccines but also prophylactic ones. By preventing the infection of cancer-associated viruses, the risk of virus-associated cancer occurrence will be considerably attenuated.

Some typical viruses were reported to be related to specific cancer types, like human papillomavirus (HPV) and cervical carcinoma, hepatitis viruses and hepatocellular carcinoma, Epstein–Barr virus (EBV) and nasopharyngeal carcinoma. Viral antigens, for instance, structure proteins E6 and E7 of HPV, can prime a tumor-specific immune response [57,58]. Based on this strategy concept, E6- or E7-based cancer vaccines were designed and applied in clinical practice [59,60]. Clinical results demonstrated that a considerable cellular immunity was stimulated after vaccination and patients achieve clinical benefits of prolonged survival. HBx oncoprotein from HBV and LMP oncoprotein from EBV are also attractive antigen candidates for the development of cancer vaccines against hepatocellular carcinoma and nasopharyngeal carcinoma, separately [61,62]. However, the high mutation rate is the major challenge that impedes the progression of viral antigen-based cancer vaccines. To select conserve but high immunogenetic viral antigens is an urgent issue for the advance of viral antigens-based cancer vaccines.

### 1.2.2 Tumor-associated antigens

TAAs usually exhibit an aberrant expression pattern in malignant cells, while a low level is evidenced in normal cells. Abnormal expression of TAAs is derived from genetic amplification or epigenetic alterations. According to the expression preference and location, TAAs can be divided into three types: overexpressed antigens, differentiation antigens, and cancer-germline antigens (CGAs) [53].

Overexpressed antigens refer to those proteins which are moderately expressed in most normal cells while abundantly in cancer cells. One presentative example, HER-2/neu was found to be decently expressed in most normal human epithelial cells but abnormally in most tumors, particularly in breast cancer [63]. Differentiation antigens indicate those proteins expressed by the cell lineage from which malignant cells evolved. For instance, the prostate-specific antigen is highly restricted in the prostate gland, as well as prostate carcinomas [64]. These two kinds of antigens have been well-studied in the exploitation of cancer vaccines.

However, as “self-proteins”, overexpressed antigens and differentiation antigens exhibit high immunological tolerance and when being applied in cancer vaccines, cytotoxicity towards normal cells is unignorable. Therefore, overexpressed antigens and differentiation antigens are not more suitable to be designed as targeting antigens in cancer vaccines, and interests concerning them showed a decreasing trend in the landscape of cancer vaccines.

The expression of CGAs is highly restricted in the human reproductive tissues. Because of the existence of the blood-testis barrier and the deficiency of HLA class I molecules on the surface of germ cells, the interaction between CGAs and immunity is prevented, which means the CGAs expressed in germ cells have not gone through the central immunologic tolerance, making them strongly recognizable for T lymphocytes (Figure 1.5) [65]. Once these antigens exposure to the immune system, a robust immune response against them will be triggered. Accumulating evidence demonstrated that CGAs show an upregulated pattern in various types of cancers via epigenetic alterations [66]. Furthermore, it has been proven that CGAs participate in the initiation and progression of tumors [67]. Collecting these facts together, CGAs reveal feasibility to be developed as cancer vaccines to trigger robust antitumor immune responses to eradicate malignant cells and obstruct the progression of tumors, with limited cytotoxicity to normal cells. Recently, more than 200 types of CGAs in the human genomes have been identified [68], and among these candidates, melanoma-associated antigen A3 (MAGE-A3) and New York esophageal squamous cell carcinoma-1 (NY-ESO-1) antigen have been extensively studied and maturely established in the development of cancer vaccines [69,70]. Numerous clinical trials regarding GCAs-based cancer vaccines have validated that CGAs can serve as ideal targeted markers to fulfill great anticancer functions [71-73].

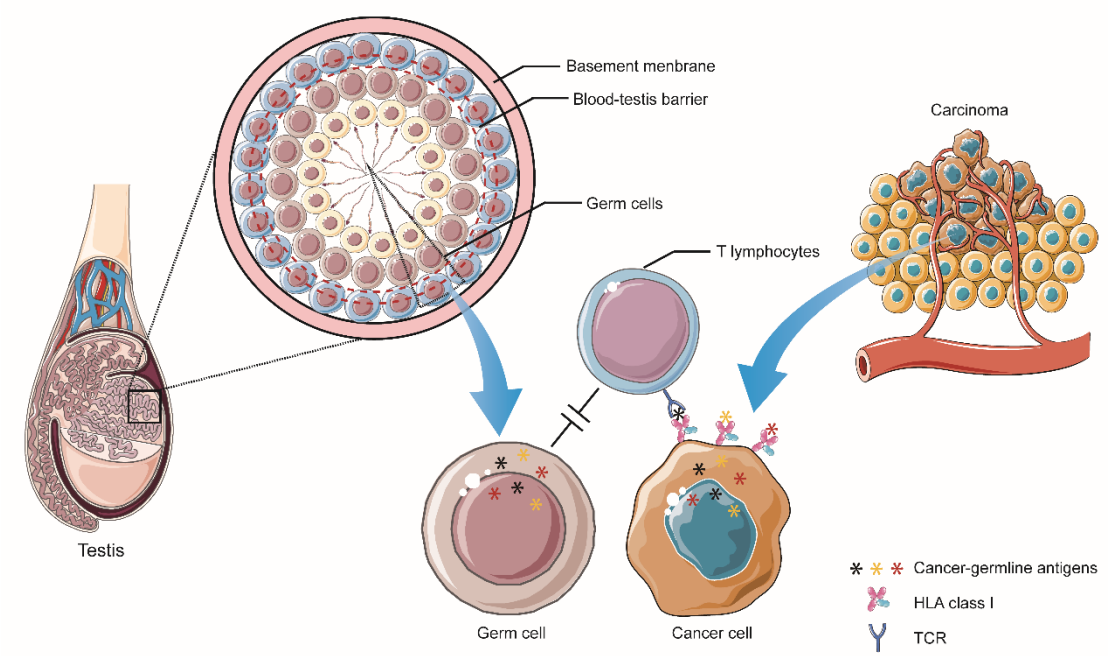


**Figure 1.5: the immunological characteristics of CGAs [53].**

The existence of CGAs normally located in germ cells. Because of the presence of the blood-testis barrier, testis becomes an immuno-privileged zone, which indicates CGAs expressed in germ cells are foreign to the immune system. However, CGAs are found to be aberrantly expressed in various kinds of tumors. Once being exposed to the immune system, CGAs will trigger a robust immunity against them, leading to the elimination of malignant cells eventually.

The blood-testis barrier endows CGAs with a highly immunogenic feature by which a vigorous antitumor immunity is initiated when CGAs are exposed to the immune system. However, except for the blood-testis barrier, there are two additional immune-privileged zones that exist in the human body — the blood-brain barrier and the blood-retina barrier. Proteins located behind these barriers exclusively can stimulate immune responses against them once exposed to the immune system. A group of proteins restricted to the retina called “cancer-retina antigens”, reported previously by our teams, exhibits great potential in the exploitation of immunotherapy. This group of tumor antigen candidates, especially one of the members — Recoverin, will be emphasized in our next introduction chapter.

## 1.3 The potential role of Recoverin in the development of cancer vaccines

Because of the existence of the blood-retina barrier, some proteins located exclusively in the human retina can not be engulfed by APCs and presented in the thymus, making them unrecognizable during the maturation of T lymphocytes. Therefore, once these proteins are exposed to the immune system, a robust immune response against them will be triggered, even though they are “self-proteins”. Recoverin, as one of those proteins, has been extensively studied by researchers before and its potential role in the application of immunotherapy has been highlighted.

### 1.3.1 The overview of Recoverin in cancers

The first discovery of Recoverin was a result of studies on paraneoplastic retina degradation or called “cancer-associated retinopathy” (CAR). The CAR refers to a syndrome that the immune system of cancer patients produces antibodies that target tumor cells, as well as photoceptor cells in the retina, leading to apoptosis of photoceptor cells, further loss of peripheral and color vision, even blindness [74]. Autoantibodies derived from cancer patients revealed that an unknown protein with a molecular weight of 23kDa was involved in the CAR progression [75,76]. Further studies identified this protein as Recoverin [77]. Recoverin is a $Ca^{2+}$ binding protein located in retina photoreceptor cells and fulfills its function via suppressing phosphorylation of the visual receptor rhodopsin in a $Ca^{2+}$-dependent pathway [78,79].

In addition to the retina, the level of Recoverin was found to be elevated abnormally in various kinds of cancer, including lung cancer, melanoma, breast adenocarcinoma, and head and neck cancer [80-82]. For instance, the frequency of recoverin expression was 68% in small cell lung cancer (SCLC) and 85% in non-small cell lung cancer (NSCLC) [83]. However, the function of Recoverin in cancers remains elusive. Whether the elevated expression of Recoverin participate in the initiation and progression of cancers? As a $Ca^{2+}$-dependent regulator of rhodopsin kinase (GRK1), we speculated that Recoverin might have a function in cancers. It has been reported that GRKs modulate the G protein-coupled receptors (GPCR) pathway in various cancers by interacting with the GPCR and phosphorylating its intracellular domain, leading to alteration of tumor biology, like vascular remolding, invasion, and migration [84]. Miyagawa and colleagues discovered that aberrantly expressed Recoverin gets involved in the GRK-dependent cellular regulation in cancer cells [85]. Indeep studies concerning the

mechanism of Recoverin in the regulation of cancers' initiation and progression are urgently needed.

Increasing studies showed that upregulated Recoverin is attributed to epigenetic alterations, especially DNA methylation. High methylation activity in the promoter regions of genes results in silencing while relatively low methylation leads to genes activation [86]. Previous research revealed that the methylation level of Recoverin in melanoma tissues and cell lines was 49-87% while 94% was evidenced in healthy skin [87]. Moreover, bisulfite sequencing data identified that the demethylation of definite nucleotides was located in the Recoverin promotor before the first exon and in the first exon itself [88]. After the treatment of 5-aza-2′-deoxycytidine, an inhibitor of DNA methyltransferase, the expression of Recoverin mRNA showed an upregulated trend corresponding to the decreased level of methylation in the bisulfite nucleotides in the promotor of the Recoverin gene.

### 1.3.2 Recoverin can serve as an antigen candidate in the immunotherapy

Aberrant expression of Recoverin was demonstrated to act as an autoantigen to trigger the CAR in cancer patients [89,90]. The molecular pathology that anti-Recoverin antibody causes CAR takes place in the following steps: (1) Recoverin abnormally expressed in tumor cells is recognized by the immune system; (2) antibody towards Recoverin are specifically generated by the immune system; (3) anti-Recoverin antibody reaches the retina and blocks Recoverin function (inhibition of rhodopsin phosphorylation in a calcium-dependent pathway) [91].

Autoantibodies against Recoverin were also evidenced in the sera of cancer patients with CAR, however, the frequency of such cases is pretty low with only 25 cases have been reported before and the majority of these cases occurred in SCLC [92]. Intriguingly, autoantibodies with low titers against Recoverin were demonstrated in 15 of 99 SCLC patients and 9 of 44 NSCLC patients from sera samples, but without the manifestation of CAR [83,93], indicating that autoimmunity against Recoverin can be observed in cancer patients even without the CAR syndrome. Administration of Recoverin in rat models resulted in the production of corresponding autoantibodies, leading to the degradation of the retina [94]. Furthermore, when injecting SCLC cell lines into a pig model, but not Recoverin proteins directly, spontaneous production of autoantibodies against Recoverin was still evidenced [95]. Collecting all these facts, Recoverin exhibits a strong capability to induce an anti-Recoverin immune response, highlighting its feasibility to be employed in immunotherapy as an antigen candidate.

A case with SCLC combined with CAR exhibited spontaneous regression without any anticancer treatments. Recoverin autoantigen was presented on the cancer cells and anti-Recoverin antibody was demonstrated in the patient's serum. Furthermore, activation of Recoverin-specific antitumor cytotoxic T lymphocytes was evidenced in this patient, highlighting the potential of Recoverin to stimulate antitumor immunity [96]. Moreover, by treating cancer patients with Recoverin peptide tetramer, specific anti-Recoverin cytotoxic responses were demonstrated to already exist in some cancer patients [97]. Interestingly, although recoverin exhibited an elevated level in cancers, elevated expression of Recoverin was correlated with a favorable prognosis for primary cancer in CAR patients, for instance, the aberrant expression of Recoverin was recognized in 6 out of 18 patients with different clinical stages of gastric cancer and among them, 3 out of 3 in stage I, 2 out of 3 in stage II, 0 out of 8 in stage III, and 1 out 4 in stage IV, which corresponds to the observations that recoverin-expressing cancer cells induced antitumor immunity favoring the prognosis of cancer patients [98,99].

Taken together, these facts revealed that Recoverin possesses a great capability to induce immune responses against Recoverin-expressing tumor cells. If we apply Recoverin in immunotherapy, then robust antitumor immunity can be expected to be triggered by Recoverin-based immunotherapy in cancer patients, leading to clinical regression eventually. Considering that Recoverin is widely and aberrantly expressed in various kinds of cancers, Recoverin-based immunotherapy is practicable and reliable, which could benefit cancer patients broadly.

## 1.4 Advances of heat shock protein (HSP)-based cancer vaccines

Administration of tumor antigens peptides alone as cancer vaccines to stimulate antitumor immune responses is still unsatisfying, cause the immunogenicity of peptides is not robust enough to invoke the greatest anti-cancer immunological effects. The application of adjuvants in cancer vaccines significantly enhances the immunogenicity of vaccines, as well as improves the quality of peptides engulfed by APCs, while do not confer immunity themselves [100]. A variety of adjuvants have been invented and employed in the design of cancer vaccines, such as aluminum adjuvants or oil adjuvants. In the past few decades, HSP families, especially HSP70, were revealed to function as adjuvants to enhance the immunogenicity of tumor antigen peptides attributing to their natural ability to interact with immune cells, especially APCs,

via scavenger receptors. Therefore, HSP70 can serve as an attractive platform for the development of cancer vaccines.

### 1.4.1 The HSP family and their functions

As a family of highly conservative proteins, HSPs can be detected in a wide range of living organisms from bacteria to mammals. The main function of HSPs is to maintain the homeostasis of proteins when cells are under stress intracellularly, preventing the harmful consequence caused by wrongly folded or denatured proteins [101]. According to the molecular weights, HSPs can be divided into small HSPs (from 15 to 43 kDa), HSP40, HSP60/HSP10, HSP70, HSP90, and HSP110. These proteins locate slightly different from each other but possess the similar capability to prevent proteotoxic effects via chaperone functions, enabling the maintenance of regular cellular processes [102].

The most well-studied member of HSPs families is HSP70. The critical role of HSP70 has been demonstrated in cellular activities, like folding and blocking degradation of proteins, transporting proteins to the endoplasmic reticulum, therefore preventing wrongly aggregation or translocation of proteins and regulating the cellular stress in a whole [103]. HSP70 contains two functional domains, including an N-terminal adenosine triphosphatase (ATPase) domain and a C-terminal substrate-binding domain. There is a hydrophobic cavity in the substrate-binding domain, which can capture peptides via various kinds of binding methods intracellularly as a physical carrier. Furthermore, the interaction of substrate-binding domain and peptides induces a conformational change of the N-terminal ATPase domain and further ATP hydrolysis, which in turn provides the energy to increase the affinity of the substrate-binding domain to peptides. These structural features endow HSP70 with the capability to act as antigen carriers.

During carcinogenesis, numerous mutations or misfolded cancer-related proteins occurred under extreme conditions, like oxidative stress or nutrient deficiency. These abnormal proteins disrupt the regular cellular proliferation and lead to apoptosis, but also stimulate the generation of HSPs to chaperone these mutated proteins. By stabilizing these aberrant proteins which might corrupt the proceeding of cancer cells, HSPs serve as cancer promotors to facilitate tumorigenesis and cancer progression [104]. However, after necrosis of cancer cells, multitudinous aberrant proteins, chaperoned by HSPs, are released to the microenvironment and recognized by the surrounding immune cells, especially APCs. Increasing studies have revealed that HSPs possess a natural ability to interact with immune cells via several receptors, therefore being attributed with an immunoregulatory characteristic (Figure 1.6).

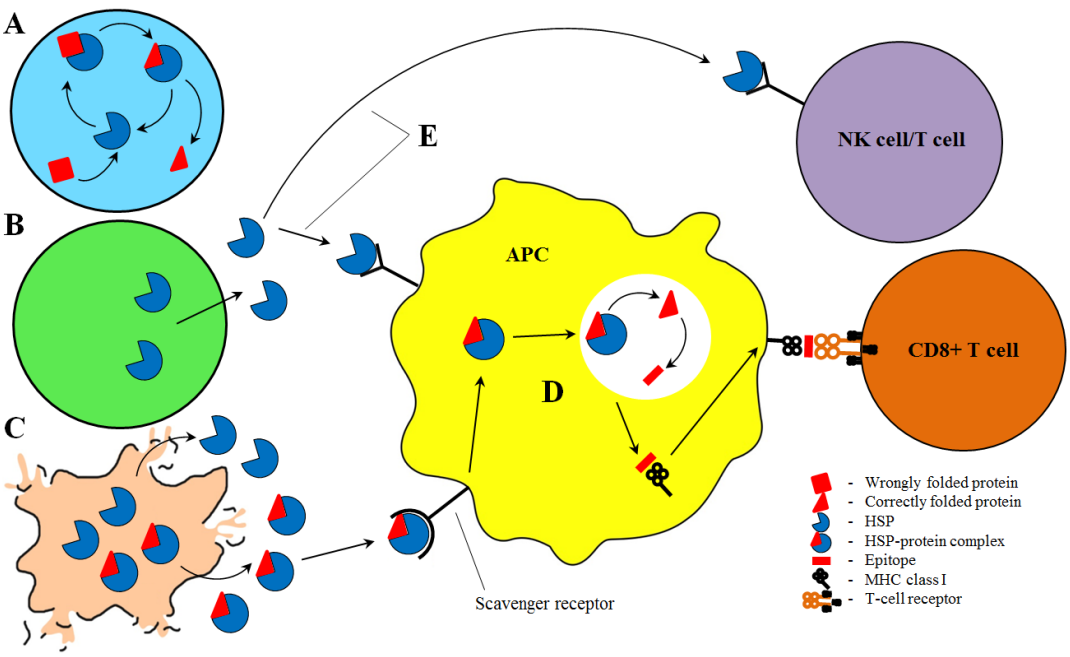


**Figure 1.6: The interaction between HSPs and immune cells [105].**

(A) The chaperon function of intracellular HSPs. (B) Free-form of HSPs are released from the intact cells. (C) Free-form or complexes of HSPs are released from necrotic cells. (D) Cross-presentation of peptides mediated by HSPs in APCs. (E) The immunoregulatory function of free-form HSPs extracellularly. The authority has been approved by Bentham Science Publishers Ltd.

### 1.4.2 Immunoregulatory properties of HSP70

The immunoregulatory potential of HSP70 was extensively investigated by Srivastava and colleagues [106]. As the pioneers in this field, they discovered that HSP70-peptide complexes induce antitumor immune responses strongly, highlighting the potential capacity of HSP70 to act as antigen carriers for specific vaccination against cancers. Under the highly stressed tumor condition, HSP70 will be released from tumor cells after necrosis disintegration. The HSP70-peptide complex will be uptook by APCs surrounding the necrotic cancer cells via endocytosis, followed by further process and cross-presentation (Figure 1.6) [107].

The receptors which mediate the interaction between HSP70 and APCs remain elusive so far. However, current evidence revealed that the scavenger receptors are responsible for the internalization of HSP70 into APCs. Scavenger receptors, including lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), scavenger receptor expressed by endothelial cell-1 (SREC-1), and fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1 (FEEL-1), were observed to bind mammalian HSP70. Furthermore,

regarding the recombinant HSP70, LOX-1, and FEEL-1 were proven to interact with, while SREC-1 poorly did. These receptors will help the engulfment of peptides into APCs, followed by the involvement of cross-presentation. After processing, the MHC class I and class II epitopes derived from the complexes will be presented on the surface of APCs, being recognized by T cells [108]. MHC class I epitopes induce the activation of cytotoxic T lymphocytes, while MHC class II epitopes trigger the activation of T helper cells, both contributing to the elimination of malignant cells synergetically. In addition to induction of T cells-specific immunity, HSP70 also exhibits its immunostimulatory functions via stimulating migratory and cytolytic activity of natural killer (NK) cells in the form of exosomes. Besides that, HSP70 within membranous structures can stimulate the activation of macrophages and lead to the production of tumor necrosis factor-α (TNF-α).

Despite being released in the complex formation as the major source extracellularly, HSP70 also can be secreted in free form by intact cells, displaying an immunosuppressive property different from other formulas. Free-form secreted HSP70 was evidenced to downregulate the expression of IL-6, IL-8, and MCP-1 induced by TNF-α in the rheumatoid arthritis model [109]. Furthermore, reduced T cell responses and stimulatory ability of monocyte-derived DCs were observed after a 24 hours treatment with recombinant HSP70 [110]. Additionally, recombinant HSP70 fails to induce the cytokine secretion of DCs and further activation, while peptides-HSP70 complexes directly activate DCs [111].

Therefore, the immunoregulatory properties of HSP70 seem to be a double-edged sword in cancers. On one hand, the immunosuppressive capacity of free-form HSP70, along with the intracellular stabilizing function, contribute to the initiation and progression of cancers. On the other hand, HSP70 binding with tumor antigens promotes the activation of T lymphocytes and NK cells via cross-presentation by APCs, triggering specific antitumor immune responses. By taking advantage of the latter one, the conception of HSP70-based immunotherapy is being tested in the past few decades, to activate specific antitumor immunity to struggle against malignant diseases.

### 1.4.3 Application of HSP70 in the design of cancer vaccines as an adjuvant

Isolation and further identification of peptides that HSP70 binds with have proven that those peptides were MHC class I epitopes derived from tumor antigens. However, not only cytotoxic immunity could be activated by peptide-HSP70 complexes, but also $CD4^+$ T cell-mediated

immune responses were evidenced [112]. This mechanism is also known as “cross-presentation”. Based on this conception, peptide-HSP70 complexes were designed as cancer vaccines to trigger a robust antitumor immunity, and clinical trials concerning this novel immunotherapy were continuously being tested to estimate its efficacy and safety.

There are two platforms for the development of peptides-HSP70 complexes as vaccines. The first one is to prepare autologous tumor-derived peptides-HSP70 complexes. This notion has been demonstrated to be effective in the mouce model via reduction of tumor progression and metastasis, and prolongation of the life span of mice [113]. Furthermore, the promising antitumor efficacy of tumor-derived peptide-HSP complexes has been evidenced in several clinical trials for the treatment of cancers, including melanoma [114], colorectal cancer [115], and renal cell carcinoma [116]. This tumor-derived platform provides a wide spectrum of tumor antigens to endow vaccines with a polyvalent potential, to eliminate malignant cells comprehensively. However, when preparing a tumor-derived peptides-HSP70 vaccine, a large number of tumor tissues are required. Most of the patients are excluded from clinical trials due to insufficient tumor volume for the manufacturing of vaccines [117]. Meanwhile, a large volume of tumor tissues is frequently associated with a late stage of disease, which means such therapy is already not suitable for patients. Therefore, it is better to apply autologous peptides-HSP70 complexes in the early stage of cancers, even for prophylactic purposes, from which cancer patients will benefit a lot.

The other strategy of HSP-based vaccines is to construct *in vitro* TAAs-HSP70 complexes as cancer vaccines. This notion has firstly been tested by Blachere and colleagues in 1997. They constructed a variety of synthetic tumor-associated peptides *in vitro* and bind with HSP70. These complexes showed immunogenic potential via eliciting antitumor immunity and specific $CD8^+$ cytotoxic T cell response [118]. Since then, various attempts have been taken to verify the efficacy of safety of *in vitro* constructed tumor antigen peptides-HSP70 complexes in cancer treatment. When comparing with the autologous complexes, this method exhibits several advantages: (1) avoid the requirement of tumor volume for tumor antigens (peptides) isolation; (2) feasibility to choose ideal antigens (peptides) that HSP70 binds with to form an immunogenetic complex, making it a more accurate and personalized cancer therapy [105]. However, unlike autologous HSP70-based complexes, in vitro complexes can not offer cross-presentation of a full palette of tumor antigens for T cells activation and further antitumor cytotoxicity. Therefore, it is of great importance to identify tumor-associated antigens with high

specificity and immunogenicity to make complexes for cancer treatment, to achieve the greatest effectiveness.

## 1.5 Aim of this study

The antigen candidate Recoverin was found to be aberrantly expressed in various types of cancers. The existence of Recoverin in the immune-privileged zone — retina endows it a great potential to elicit a robust immunity once being exposed to the immune system, highlighting the feasibility to apply Recoverin in immunotherapy development. Therefore, this study aimed to explore the capability of Recoverin in the exploitation of cancer vaccines. For this purpose, the fusion protein consisting of Recoverin epitopes and HSP70 was synthesized. Firstly, the capability of Recoverin fusion protein to induce the maturation, activation, and cytokine secretion of DCs should be determined. Then the activation status of autologous T lymphocytes co-cultured with DCs pulsed with Recoverin fusion protein should be assessed. Lastly, The immunosuppressive potential of Recoverin fusion protein will also be examined.

# 2. Materials and methods

## 2.1 Experimental 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 |
| Bio-Plex | Bio-Rad, 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 |
| Endosafe® nexgen-PTS™ | Charles River, USA |

### 2.1.2 Consumables

| | |
|---|---|
| 0.5-10µL L, Ep T.I.P.S.® Reloads | Eppendorf, Germany |
| 10-200µL L, Ep T.I.P.S.® Reloads | Eppendorf, Germany |
| 100-1000µL L, Ep T.I.P.S.® Reloads | Eppendorf, Germany |
| Stripette | Corning, USA |
| 7.5ml Lithium Heparin blood collection tube | S-Monovette®, Sarstedt, USA |

| | |
|---|---|
| 10ml Polystyrene Tube | Falcon, USA |
| 50ml Polystyrene Tube | Falcon, USA |
| Gloves | Eco Nitrile PF 250, ecoSHIELDTM, USA |
| Bio-Plex human inflammation cytokines kit | Bio-Rad, USA |
| Bio-Plex human chemokines kit | Bio-Rad, USA |
| Endosafe® cartridge | Charles River, USA |

### 2.1.3 Chemical

| | |
|---|---|
| Bovine serum albumin (BSA), Fraction V | Biomol, Germany |
| Natriumazid 10% | Morphisto, Germany |
| Millipore $H_2O$ | Advantage A10, Merck, Germany |
| Ethylenediaminetetraacetic acid (EDTA) | E5513, SIGMA-ALORICH, USA |
| Trypan blue | SIGMA, USA |
| PBS buffer (10X) | Power BC, PanReac AppliChem, Germany |

### 2.1.4 Buffers and Solutions

| Fluorescence-activated cell sorting (FACS) buffer | |
|---|---|
| pH | 7.4 |
| 1L | 1x Dulbecco's phosphate-buffered saline (DPBS) |
| 2ml | Natriumacid |
| 5g | BSA |

| T cells isolation buffer | |
|---|---|
| pH | 7.2 |
| 50mL | 1xDPBS |
| 250mg | BSA |
| 2mM | EDTA |

### 2.1.5 Antibodies

| Antibody | Isotype | Fluorochrome | Reactivity | Clone |
|---|---|---|---|---|
| Anti-CD45 | mouse IgG1, κ | BV650 | Human | HI30 |
| Anti-CD3 | mouse BALB/c IgG1, κ | PerCP Cy5.5 | Human | UCHT1 |
| Anti-CD14 | mouse BALB/c IgG2b, κ | BV510 | Human | MφP9 |
| Anti-CD33 | mouse BALB/c IgG1, κ | BV786 | Human | WM53 |
| Anti-CD11c | mouse BALB/c IgG1, κ | PE | Human | B-ly6 |
| Anti-CD11b | mouse IgG1, κ | PE-Cy7 | Human | ICRF44 |
| Anti-HLA-DR | mouse IgG2a, κ | APC-H7 | Human | G46-6 |
| Anti-CD80 | mouse C3H/Bi IgG1, κ | BB515 | Human | L307.4 |
| Anti-CD86 | mouse BALB/c IgG1, κ | BUV395 | Human | BU63 |
| Anti-CD83 | mouse IgG1, κ | BV421 | Human | HB15e |
| Anti-CD274 | mouse BALB/c IgG1, κ | APC | Human | MIH1 |
| Anti-CD4 | mouse BALB/c IgG1, κ | BUV395 | Human | SK3 |
| Anti-CD8 | mouse BALB/c IgG1, κ | APC-H7 | Human | SK1 |
| Anti-CD25 | mouse BALB/c IgG1, κ | BB515 | Human | 2A3 |
| Anti-CD38 | mouse IgG1, κ | BV605 | Human | HB7 |
| Anti-CD127 | mouse IgG1, κ | PE-CF594 | Human | HIL-7R-M21 |
| Anti-CD279 | mouse BALB/c IgG1, λ | APC | Human | SP34-2 |

### 2.1.6 Computer and Software

| | |
|---|---|
| Computer hardware | Z230 SFF workstation, HP, USA |
| FACSDIVA™ SOFTWARE | BD, USA |
| FlowJo™ version.10 | BD, USA |
| Bio-Plex Multiple Immunoassay system | Bio-Rad, USA |
| Prism | Version 8.0.2, GraphPad Software, USA |
| SPSS | Version 21.0, USA |

### 2.1.7 Blood donors

Five donors were included in this research project. Consent was received from all donors at first. Blood were collected and conducted to HLA diagnostics in Labor für Immungenetik und Molekulare Diagnostik, Klinikum Großhadern.

## 2.2 Experimental methods

### 2.2.1 The procedure of recombinant fusion protein manufacturing

This part of work is cooperated by the corporative partner Dr. Alexey V. Baldin, from Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University. An international scientific collective obtained a collection of bacterial E. Coli strains procedures of recombinant fusion proteins consisting of human HSP70 and epitopes of Recoverin. The collection includes 2 strain procedures, designated as follows:

1. E.coli JM109/pQE80 HSP70;

2. E.coli JM109/pQE80 H2epitope-HSP70-H1epitope rec;

All abbreviations are described in Table 2.1 below. Each strain producer is capable of synthesizing the corresponding unique recombinant fusion protein.

**Table 2.1: Abbreviations used in the name of bacterial strains, fusion proteins, plasmid vectors, etc., and their decoding.**

| | |
|---|---|
| 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) |
| rec | Recoverin |

The pQE80 system-based plasmids for each strain were designed to encode fusion proteins according to the general scheme described below. The central part of each protein is the amino

acid sequence corresponding to the human HSP70 protein encoded by the HSPA1B gene (Gene Bank NM005346): AKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYV AFTDTERLIGDAAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVINDGDKPKV QVSYKGETKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKDAGVIA GLNVLRIINEPTAAAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLG GEDFDNRLVNHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGI DFYTSITRARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPKVQKLLQDFF NGRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLETAGGVMTALIKR NSTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGRFELSGIPPAPRGVPQIEVTFD IDANGILNVTATDKSTGKASKITITNDKGRLSKEEIERMVQEAEKYKAEDEVQRERVSAKNAL ESYAFNMKSAVEDEGLKGKISEADKKKVLDKCQEVISWLDANTLAEKDEFEHKRKELEQVC NPIISGLYQGAGGPGPGGFGAQGPKGGSGSGPTIEE. For fusion proteins, amino acid sequences encoding the HLA II- and HLA I-specific recoverin epitopes were attached to the nitrogen (N)- and carbon (C)-terminus of HSP70, respectively, as shown in Figure 2.1. The HLA class I and class II Recoverin epitopes were selected for the following types of HLA: HLA-A*02:01, HLA-DRB1*11:01, HLA-DQA1*05:01/HLA-DQB1*03:01 (Table 2.2). Each protein has His-tag — amino acid sequence comprising 6 histidines, which can be applied for isolation and purification of protein using metal chelate affinity chromatography.

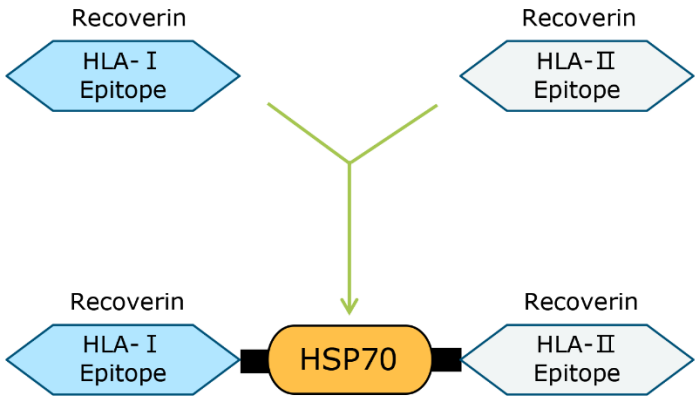


**Figure 2.1: The structure of Recoverin fusion protein.**

Recoverin HLA class I epitope was fused to the C-terminus of HSP70 while the HLA class II epitope was fused to the N-terminus.

**Table 2.2: Selected HLA class I and HLA class II epitopes of Recoverin.**

| Specificity of HLA | Designation | Amino acid sequence in FASTA format | Encoding nucleotide sequencing |
|---|---|---|---|
| HLA-A*02:01 | H1epitope | ALSKEILEEL | GCC CTG TCC AAG GAG ATC CTG GAG GAG CTG |
| HLA-DQA1*05:01/ HLA-DQB1*03:01 | H2epitope | YVIALHMTTAGKTNQ | TAC GTC ATC GCC CTG CAC ATG ACC ACC GCG GGC AAG ACC AAC CAG |

FASTA: A DNA and protein sequence alignment software package.

When all the plasmids encoding fusion proteins consisting of HSP70 and Recoverin epitopes are ready, they will be transfected into the bacterial E. coli JM109 strain and the resulting strains will be added to the collection for subsequent biosynthesis of the fusion protein with their help. Subsequently, these fusion proteins are isolated and purified using the technique established in the Laboratory of Molecular Biology and Biochemistry of Sechenov University. The technique consists in using the Triton X-114 reagent, the so-called “trap” for lipopolysaccharide (LPS), at the washing stage during the isolation of proteins using metal-chelate affinity chromatography with Ni2+-sepharose. Thus, fusion protein preparations are purified from the inevitably present LPS if using a bacterial recombinant protein expression system.

### 2.2.2 Endotoxins testing by limulus amebocyte lysate (LAL) reagents

1. Move the Endosafe® cartridge to room temperature in advance.
2. Turn on Portable Endotoxin Testing System Endosafe® nexgen-PTS™ and initiate the machine for 5 mins.
3. Once the temperature of the machine reaches 37°C, “Self Test OK” shows up on the screen.
4. Take the cartridge from the bag, only touch the handle or the edge of the cartridge.
5. Insert the cartridge into the endotoxin testing machine.
6. Enter relative assay data in the machine.
7. After finishing the data input, “Add Sample” shows up on the screen.
8. Carefully pipette 25 ml sample into 4 reservoirs of the cartridge. Avoid pipetting up and down to generate bubbles.
9. Press “Enter” to start the measurement.
10. After finishing the detection, download the results from the machine.

### 2.2.3 Isolation of peripheral blood mononuclear cells (PBMCs)

1. Prepare two 50ml Falcons. Fill each falcon with 15 ml Biocoll-Separating Solution in advance.
2. Dilute the whole blood sample collected from donors with 1x DPBS in 1:1 proportion and use peptide to mix the solution up and down gently.
3. Overlay blood/DPBS mixture to Biocoll-Separating Solution in 1:2 proportion carefully. Avoid mixing of blood/DPBS with Biocoll-Separating Solution (Biocoll is quite cytotoxic to PBMC). Noteworthy, the maximum maintenance time of the overlay is 45 minutes.
4. Centrifugate the blood/Biocoll-Separating Solution overlay 1200xg for 20 mins at room temperature without brake. Be careful that the brake mode after centrifugation will destroy the separation and result in difficulty of PBMCs collection.
5. After centrifugation, remove the cloudy interphase carefully (without Biocoll-Separating Solution) and transfer to a new falcon. It is no problem to collect serum but avoids collecting cytotoxic Biocoll-Separating Solution.
6. Wash out the interphase (PBMCs) by 1x DPBS in 1:4 proportion. Resuspension and then centrifugate PBMCs/DPBS mixture 300xg for 10 mins at room temperature. After centrifugation, reject the supernatant and leave the cell pellet in the falcon.
7. Resuspend the cell pellet with 1-2 ml of DPBS and then add DPBS up to 50ml to resuspend the cell pellet. Then centrifugate PBMCs/DPBS mixture 200xg for 10 mins at room temperature. Reject the supernatant and leave the cell pellet in the falcon.
8. Resuspend the cell pellet in RPMI1640/1% autologous plasma (same donor) medium. Prepare 10µl cell suspension for cell counting.

### 2.2.4 Differentiation induction from PBMCs to immature DCs (iDCs)

1. Collected and isolated PBMCs were seeded on a 6-well plate in the amount of $1x10^7$ cells per well with 4 ml RPMI1640/1% autologous plasma medium. Incubate cells in 37°C 5% $CO_2$ incubator.
2. During incubation, we prepare a medium with an addition of cytokines for iDCs induction: 1000U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1000 U/ml IL-4 RPMI1640/1% autologous plasma medium.
3. After 1.5 hours of incubation, cells were intensively washed with warmed-up RPMI1640 medium without plasma and cytokines.

4. The washing of cells that are not adherent was controlled by a microscope. Repeat washing three times until only adherent cells exit (Dark cells adherent to the bottom of the flask).
5. After final washing, add prepared RPMI1640/1% autologous plasma medium with cytokines to each well, 4ml for each well. Incubate cells in 37°C 5% $CO_2$ incubator for 5 days.
6. On day 2 of incubation, we prepared 500µl of RPMI1640/1% autologous plasma medium with 4000/ml IL-4 and 4000U/ml GM-CSF for each well (During 2 days of incubation, about 500µl medium will vapor). Then continue incubation in 37°C 5% $CO_2$ incubator.
7. At the end of day 5, cells were observed under the microscope. During the 5 days culture period, the monocytes differentiated into iDCs. iDCs should be easily detached from the plastic surface and relatively large (larger than lymphocytes), round with few or no cytoplasmic extension, loosely adherent.

### 2.2.5 Differentiation induction from iDCs to mature DCs (mDCs)

1. Harvest suspended cells and conditioned medium from each well to 50 ml falcon. Carefully and thoroughly wash wells with pre-warmed RPMI1640/1% autologous plasma medium to remove loosely adherent cells. The wash solution should be collected in the same falcon. The washing step may be repeated as wanted.
2. After washing and harvesting, there still will have some firmly adherent cells on the bottom of the flask. These firmly adherent cells are likely macrophages but no iDCs. So there is no need to collect them by scraping or other methods.
3. After the collection of suspended cells, centrifugate them 500xg for 5 mins at room temperature. Remove supernatant and leave cell pellet in falcon. Resuspend cells in RPMI1640/1% autologous plasma medium with 1000U/ml GM-CSF and 1000 U/ml IL-4. iDCs were seeded in a 24-well plate in the amount of $2.5x10^5$ cells in 1 ml per well.
4. Recoverin epitopes/HSP70 protein/fusion protein was added to corresponding wells in the concentration of 0.4 µM per well. The plate was gently swirled to mix and incubated in 37°C 5% $CO_2$ incubator for 3 hours.
5. During incubation, prepare RPMI1640/1% autologous plasma medium containing maturation cytokines cocktail (1000U/ml GM-CSF, 1000 U/ml IL-4, 2000U/ml interferon-γb (IFN-γb), 2000U/ml TNF-α, and 2ug/ml prostaglandin E2 (PGE2)). After 3 hours, add 1 ml prepared medium with cytokines to each corresponding well (The final concentration of medium in well is 1000U/ml GM-CSF, 1000 U/ml IL-4, 1000U/ml IFN-γb, 1000U/ml

TNF-α, and 1ug/ml PGE2). And then we add 2μl CD40L reagent to each well. Continue the incubation of iDCs in 37°C 5% $CO_2$ incubator for 6 days.

6. On day 6, under the stimulation of antigens and cytokines, iDCs differentiated into mDCs. Resuspend mDCs and collect them for further analysis of FACS or further experiments. The supernatant will be collected and frozen in -80°C fridge for further cytokines and chemokines analysis.

### 2.2.6 Isolation of T cells from PBMCs

**Reagents in Pan T Cell Isolation Kit required for Bio-Plex assay:**

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

**Steps:**

1. Isolate PBMCs from blood samples as mentioned above and determine cell numbers via the CASY machine.
2. Resuspend cell pellet in 40μl of T cell isolation buffer per $10^7$ total cells.
3. Add 10 μl of Pan T Cell Biotin-Antibody Cocktail to per $10^7$ total suspending cells.
4. Pipette cell solution gently and incubate in the fridge (2-8°C) for 5 mins.
5. Add 30μl of T cell isolation buffer per $10^7$ total cells.
6. Add 20μl of Pan T Cell MicroBead Cocktail per $10^7$ total cells.
7. Pipette cell solution gently and incubate in the fridge (2-8°C) for 5 mins.
8. Place the LS column in the MACS Separator, making sure that insert the LS column in the magnetic field.
9. Rinse the LS column with 3 ml of T cell isolation buffer.
10. Add the cell suspension onto the LS column and collect the flow-through unlabeled cells.
11. Wash the column with 3 ml of T cells isolation buffer. Collect the flow-through cells and then combine them with the effluent from step 10, representing the enriched T cells.

### 2.2.7 Activation of T cells co-cultured with mDCs

1. Prepare mDCs loaded with fusion protein or Recoverin epitopes as mentioned above.
2. Isolate T cells from PBMCs as described above.
3. Co-culture T cells and mDCs at a ratio of 10:1 ($5x10^5$ for T cells and $5x10^4$ for mDCs) in a flat bottom shaped 48-well plate.

4. T cells are stimulated in a 7-days cycle. On day 2 of the cycle, IL-2 was added to the cell culture media (25U/ml). Half of the cell culture medium was replaced with fresh IL-2 supplemented medium (50U/ml) on day 4 and day 6.
5. After day7, T cells were harvested and stained for FACS analysis.

### 2.2.8 Flow cytometry

**Extracellular staining:**

1. Prepare FACS tube with 100µl cell suspension (minimum with $5x10^4$ cells up to $1x10^6$ cells/tube).
2. Add Antibodies as panel showed, vortex, incubate sample for 15-30min in dark at room temperature.
3. Add 2 ml FACS buffer to each tube, vortex, centrifuge 500xg for 5 mins at room temperature, discard the supernatant.
4. Add 250µl FACS buffer to each tube, measure the sample by FACS machine.

### 2.2.9 Staining panel

The FACS analysis was designed in a modular system containing two different panels which examine DC and its subset (Supplement Table 1), and T cells and its subset (Supplement Table 1). Each panel consists of an unstained tube that serves as blank control, fluorescence minus one (FMO) control tubes, and sample tubes.

### 2.2.10 Bio-Plex human cytokines and chemokines assay

**Reagents in Bio-Plex kit required for Bio-Plex assay:**

1. Coupled magnetic beads (10x)
2. Detection antibodies (10x)
3. Standard samples
4. Quality control (Optional)
5. Sample diluent HB
6. Detection antibody diluent HB
7. Standard diluent HB
8. Assay buffer
9. Wash buffer (always prepare and store in 4°C fridge)
10. Streptavidin-PE (100x)

11. Assay plate (96-well flat bottom plate)
12. Sealing tape

**Step:**

1. Take standard samples outside and incubate them on ice. Start up/warm up the Bio-Plex Multiple Immunoassay System 30 mins in advance. Bring the kit to room temperature and begin to thaw frozen collected samples.
2. Prepare a dilution of beads, cover the diluted beads with foil (Always vortex the beads for 30 sec before use to make sure no deposit in the tube).
3. Reconstitute the vial of standards in 781µl standard diluent HB. Vortex at medium speed for 5 sec and incubate all vials on the ice for precisely 30 mins.
4. Prepare a threefold (Inflammation cytokines panel) or fourfold (Chemokines panel) dilution series and blank as shown in Figure 1. Vortex at medium speed for 5 sec between liquid transfers.

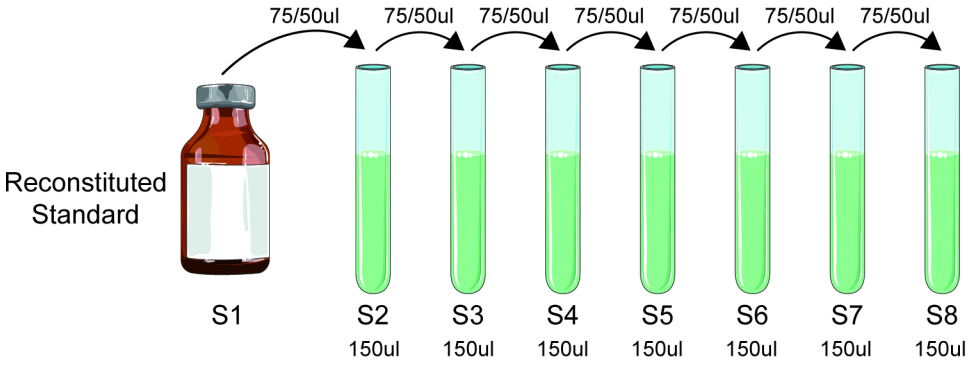


**Figure 2.4 Dilution of reconstituted standard samples.**

5. After use, standards need to be kept in a -80°C fridge. Standard diluent series need to be incubated on the ice.
6. Vortex the diluted (1x) beads. Add 50µl to each well of the assay plate.
7. Wash the plate two times with 100µl Bio-Plex wash buffer.
8. Centrifugate collected samples 1000xg for 15 mins at 4°C.
9. Vortex samples, standards, blanks, and control. Add 50µl to each well.
10. Cover the plate with sealing tape and protect it from light with aluminum foil. Incubate the plate on a shaker at 850 rpm for 1 hour at room temperature.
11. Start up Bio-Plex program. Choose a new protocol and have a description of the standard Lot#, Reaction Kit Lot#, treatment of the collected samples.

12. Select Panel and Cytokine in the program. Note that the region of cytokines or chemokines must match the Human Inflammation or Chemokine Panel.
13. Enter the concentration and Lot# of cytokines and chemokines.
14. Confirm that data acquisition is set to 50 beads per region. In advanced settings, confirm that the bead map is set to 100 regions, the sample size is set to 50µl, and the doublet discrimination gates are set to 5000 (low) and 25000 (high).
15. With 10 mins left in the incubation, vortex detection antibodies for 15 sec and quick-spin to collect liquid. Dilute to 1x according to requirement.
16. Wash the plate three times with 100µl wash buffer.
17. Vortex the diluted detection antibodies. Add 25µl to each well.
18. Cover with aluminum foil and incubate at 850 rpm in the dark for 30 mins at room temperature.
19. Select calibrate and confirm that the default values for CAL1 and CAL2 are the same as the values printed on the bottle of Bio-Plex calibration beads.
20. With 10 mins left in the incubation, vortex 100x streptavidin-PE for 5 sec and quick-spin to collect liquid. Dilute to 1x and protect from light.
21. Wash the plate three times with 100µl wash buffer.
22. Vortex the diluted 1x streptavidin-PE. Add 50µl to each well.
23. Cover with aluminum foil and incubate at 850 rpm in the dark for 10 mins at room temperature.
24. Wash the plate three times with 100µl wash buffer.
25. Resuspend the beads in 125µl assay buffer. Cover and shake at 850 rpm in the dark for 30 sec at room temperature.
26. Remove the sealing tape and read the plate.

### 2.2.11 HLA Diagnostics

HLA diagnostics was carried out by our cooperation partner - Labor für Immungenetik und Molekulare Diagnostik, Klinikum Großhadern. HLA low-resolution typing was performed using One Lambda rSSO typing kits (One Lambda, USA, CA). These LABType kits are reverse SSO DNA typing assays using an HLA locus-specific PCR amplification with labeled primers. The amplification product was hybridized to bead fixed oligonucleotides; Every bead of a distinct color is assigned to an oligonucleotide of a defined sequence. All reaction and washing steps were performed following the instructions of the manufacturer. A LABScan3D system, based on Luminex® xMAP® technology served as a detection platform and used Luminex®

xPONENT® software for data acquisition. xPONENT® raw data were imported in the One Lambda HLA Fusion software for calculating HLA typing suggestions. These typing results were manually confirmed and electronically imported by laboratory management software for report generation.

### 2.2.12 Statistical Analysis

Measurement data were presented as mean ± standard deviation (SD) of the mean. For comparison analysis, I divided the values of DCs treated with three different kinds of antigens by the value of iDC group and then get the ratio (For instance, the frequency of CD83 in iDCs group is 7.12% while the proportion is 36.4% in DCs treated with the fusion protein, then the ratio is 1:5.11). Statistical analyses were performed by using SPSS software (version 21.0). Differences between groups were analyzed by Student's t test or one-way analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

# 3. Results

## 3.1 Endotoxin level of the Recoverin fusion protein

After receiving the fusion protein synthesized by corporative partners, I measured the endotoxin level of the fusion protein and recombinant HSP70 by LAL test, as shown in Table 3.1. The LPS level of these two recombinant proteins was <10 EU/mg. The previous study has shown that HSP70 with an endotoxin level of <10 EU/mg is no longer to induce the secretion of cytokines by monocytes and monocytes-derived DCs [111]. Therefore, the immunogenicity of the residual endotoxins in the protein manufacturing can be excluded in the assessment of the immunogenicity of the fusion protein itself.

**Table 3.1: The presence of endotoxins in the preparations of isolated and purified fusion proteins consisting of HSP70 and Recoverin.**

| Designation of fusion protein | Concentration, mg/ml | Endotoxin level, EU/ml | Endotoxin level, EU/mg |
|---|---|---|---|
| HSP70 | 0.391 mg/ml | <0.5 EU/ml | <1.27 EU/mg |
| H2epitope-HSP70-H1epitope | 0.104 mg/ml | <0.5 EU/ml | <4.8 EU/mg |

## 3.2 The HLA diagnostics of the blood donors

In this project, I included 5 donors for blood collection. To make sure that the immunocytes derived from donors will react to the fusion protein, blood was collected from donors and conducted to HLA typing. All donors showed HLA-A*02, HLA-DQA1*05, and HLA-DQB1*03 positive, corresponding to the HLA specificity of the fusion protein that we designed (Table 3.2).

**Table 3.2: The HLA typing results of the blood donors**

| HLA-locus | Donor#1 | Donor#2 | Donor#3 | Donor#4 | Donor#5 |
|---|---|---|---|---|---|
| HLA-A | 02:02 | 02,25 | 02,02 | 02,33 | 02,02 |
| HLA-DQA1 | 05,05 | 02,03 | 01,05 | 02,05 | 02,05 |
| HLA-DQB1 | 03,03 | 03,03 | 03,06 | 02,03 | 03,01 |

## 3.3 Recoverin fusion protein induces morphological change of DCs

As described in the Materials and methods, we synthesized the bacterial E. Coli strains-derived recombinant fusion proteins consisting of human HSP70 and HLA class I and class II epitopes of Recoverin, which is expected to be applied as cancer vaccines to trigger antitumor immunity. When administered cancer vaccines into the body, they firstly will be recognized and engulfed by APCs, resulting in maturation and activation of DCs for further T lymphocytes activation. Therefore, firstly, I speculated that fusion proteins can induce the maturation and activation of DCs. To prove our hypothesis, I generated monocyte-derived DCs and processed them with four different kinds of treatment, as shown in Table 3.3. iDC: addition of IL-4 and GM-CSF only to maintain the differentiation of DCs; DC-antigens: monocyte-derived DCs pulsed with 3 different types of antigens, including Recoverin peptides, HSP70, and fusion proteins, respectively; DC cyto: addition of maturation cytokines cocktail, to serve as a positive control; DC-antigens cyto: monocyte-derived DCs treated with antigens and maturation cytokines simultaneously to examine the potential synergetic effects of antigens and maturation cytokines.

**Table 3.3 Four different types of DC treatment.**

| Group name | iDC | DC-antigens | DC cyto | DC-antigens cyto |
|---|---|---|---|---|
| Treatment | IL-4; GM-CSF | IL-4; GM-CSF<br>Antigens, including Recoverin peptides, HSP70, and Fusion protein, respectively. | IL-4; GM-CSF<br>Maturation cytokines cocktail: TNF-α; IFN-γ; CD40L; PGE2. | IL-4; GM-CSF<br>Antigens, including Recoverin peptides, HSP70, and fusion protein, respectively.<br>Maturation cocktail: TNF-α; IFN-γ; CD40L; PGE2. |

Most monocyte-derived DCs were relatively large and round in shape and loosely adherent, whereas a few proportions appeared to be spindle in shape under the microscope (Figure 3.1 A). However, when DCs pulsed with Recoverin fusion proteins, the appearance of most DCs turned into spindles (Figure 3.1 B). Addition of the maturation cytokines cocktail in the cultured medium induced characteristic cytoplasmic extensions of DCs. I observed antennas around the cytoplasm of DCs (Figure 3.1 C). When treating DC with fusion proteins and cytokines

simultaneously, spindle-shaped DCs with cytoplasmic extensions were noticed under the microscope (Figure 3.1 D). These results indicated that the fusion protein can induce the shape of DCs into spindles after co-cultivation, while maturation cytokines induced cytoplasmic extensions of DCs.

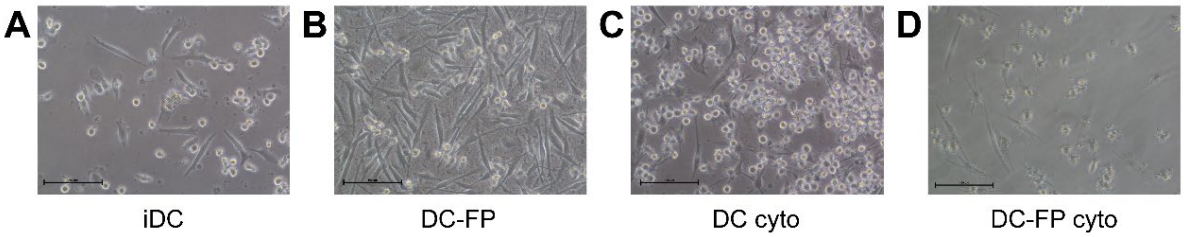


**Figure 3.1: The morphology of DCs after four different kinds of treatment.** (A-B) Image of immature DCs, DCs pulsed with fusion proteins, DCs treated with maturation cytokines, and DCs treated with fusion proteins and maturation cytokines under 200X microscope. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine.

## 3.4 Recoverin fusion protein induces the maturation of DCs

CD83, a surface immunoglobulin highly and stably expressed by mature DCs, represents the maturation status of DCs [119]. Monocytes-derived DCs were identified as a $CD45^+$, $CD14^-$ and $CD11c^+$ population, as shown in Figure 3.2. In Figure 3.3 A, It showed that when DCs pulsed with Recoverin peptides only, the frequency of $CD83^+$ DCs is 9.45%, remaining almost the same as the iDC group. However, when treating DCs with HSP70 and Recoverin fusion proteins, the percentage of $CD83^+$ DCs were 28.5% and 36.4%, respectively, which are much higher than the iDCs group. As the positive control group, the proportion of $CD83^+$ DCs ranged from 48.9% to 84.3%.

Meanwhile, I also detected the median fluorescence intensity (MFI) of CD83, as shown in Figure 3.3 B. When DCs pulsed with Recoverin peptides or HSP70, the fluorescence peak of CD83 remained the same as the iDC group, while the fluorescence peak showed a right shift in the DC-fusion protein group, which means higher amounts of CD83 were found in DCs pulsed with fusion proteins.

I performed three independent assays and integrated data for statistical analysis (Figure 3.3 C). From the bar graphs, It can be told that there is no difference between the iDC and DC-Recoverin peptides group in the $CD83^+$ frequency, while an upregulate $CD83^+$ frequency was evidenced in the DC-fusion protein group. Furthermore, I normalized the performance of three

types of antigens in the upregulation of CD83$^+$ frequency of DCs by comparing them with the CD83$^+$ percentage of iDCs. Although an increasing trend was observed in the DC-fusion protein group in comparison to the DC-Recoverin peptides group, there is no statistical difference. As for the MFI of CD83, no significant differences were found in four different treatment groups. These data together indicated that Recoverin fusion proteins can induce the maturation of DCs.

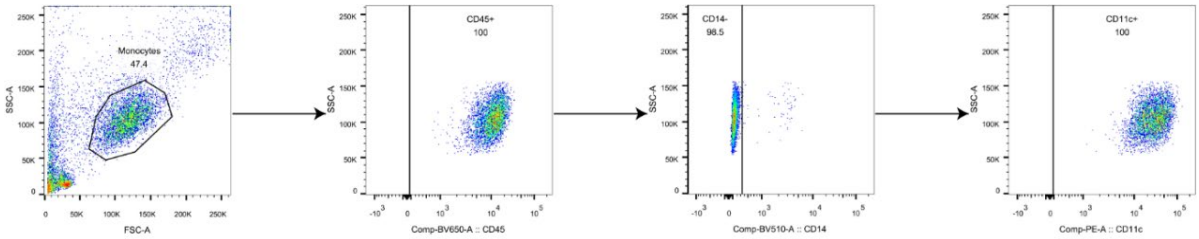


**Figure 3.2: Gating strategy of monocytes-derived DCs.** According to the dissimilar CD marker expressed on the surface of DCs, monocytes-derived DCs generated as mentioned above were CD45$^+$, CD14$^-$ and CD11c$^+$ population.

## 3.5 Recoverin fusion protein induces the activation of DCs

After maturation, the phenotype and function of DCs have changed, with elevated expression of MHC class I/II molecules and costimulatory receptors while a weak capacity to uptake and process antigens. Fully activated DCs possess a robust ability to present antigens and prime immune responses [120]. Costimulatory receptors, such as CD80 and CD86 (also known as B7.1 and B7.2), are critical signals for further T lymphocytes activation, representing the activation and functional status of DCs [121]. Therefore, I measured the percentage of CD80$^+$CD86$^+$ DCs after treatment of Recoverin fusion proteins by FACS. From Figure 3.4 A, after treatment of Recoverin peptides, the proportion of CD80$^+$CD86$^+$ DCs was 58.0%, a slight increase in comparison to the iDC group. However, when pulsed with HSP70 and Recoverin fusion proteins, the percentage of CD80$^+$CD86$^+$ DCs were 72.3% and 95.5%, respectively, a huge increase when compared with the iDC group. As the positive control, almost 100% of DCs treated with maturation cytokines were CD80$^+$CD86$^+$. Further statistical analysis revealed that DCs pulsed with fusion proteins exhibit a much higher frequency of CD80$^+$CD86$^+$ than the iDC group, while DCs pulsed with peptides do not (Figure 3.4 B).

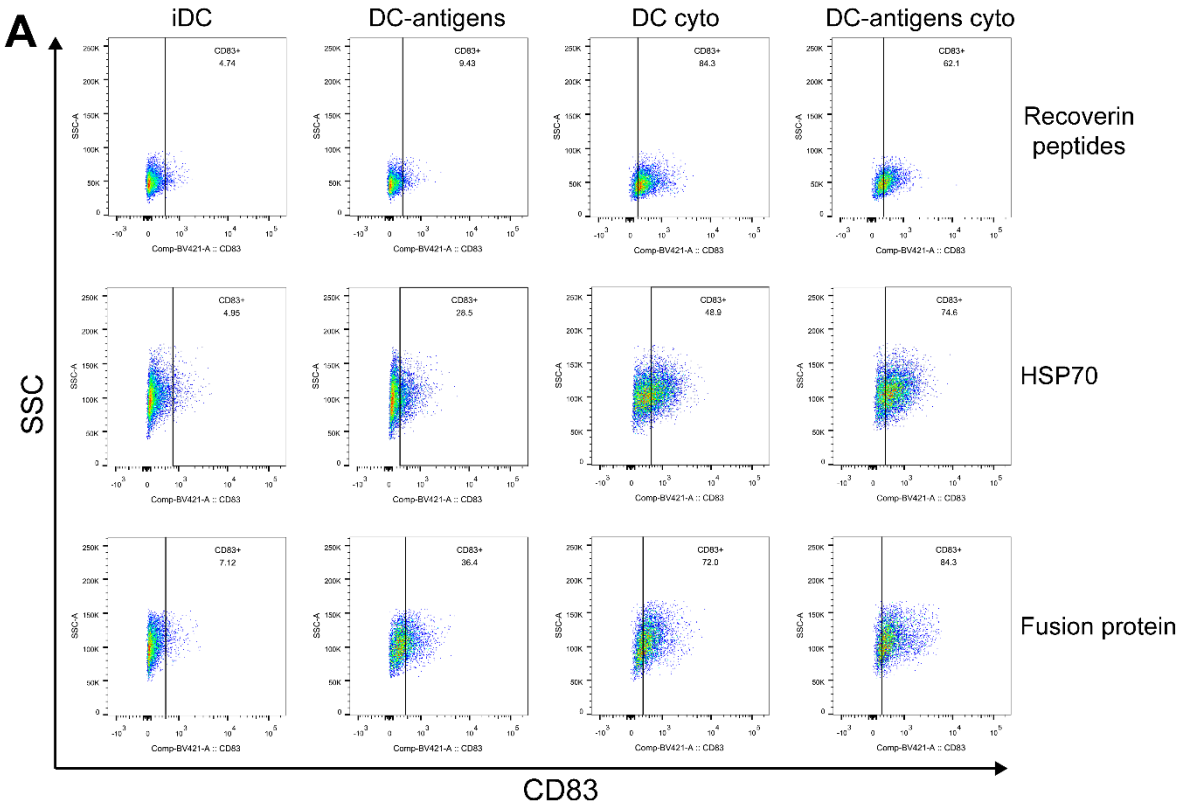
A
iDC
DC-antigens
DC cyto
DC-antigens cyto
Recoverin peptides
HSP70
Fusion protein
SSC
CD83
B
Recoverin peptides
HSP70
Fusion protein
Histogram
iDC
DC-antigens
DC cyto
DC-antigens cyto
CD83
C
Recoverin peptides
HSP70
Fusion protein
Comparison
CD83 expression
CD83 Frequency in CD11c+ cells (%)
CD83 MFI
CD83 Frequency Ratio
CD83 MFI Ratio
iDC
DC-pept
DC cyto
DC-pept cyto
DC-HSP70
DC-HSP70 cyto
DC-FP
DC-FP cyto

**Figure 3.3: Recoverin fusion proteins induce the maturation of DCs.** (A) The frequency of $CD83^+$ DCs after antigens and/or cytokines treatment. (B) The MFI of CD83 in DCs after antigens and/or cytokines treatment. (C) Statistical analysis of the proportion and MFI of CD83 in DCs after antigens and/or cytokines treatment from three independent samples. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by one-way ANOVA test followed by Tukey's multiple comparisons posttest, * $P$<0.05, ** $P$<0.01 and *** $P$<0.001).

Moreover, I also examined the MFI of CD80 and CD86 of DCs after the process. In Figure 3.5 A, It was observed that the fluorescence peak of CD80 shifts to the right obviously when DCs pulsed with fusion proteins, while the fluorescence peak of CD80 in the DC-peptides group remains almost the same as the iDC group. The same trend was also evidenced in the MFI of CD86 (Figure 3.5 B). From the statistical data (Figure 3.5 C), we can tell that Recoverin fusion proteins exhibit a superior ability to induce higher MFI of CD80 and CD86 than peptides after data normalization. Taken together, these results revealed that Recoverin fusion proteins can induce the activation of DCs.

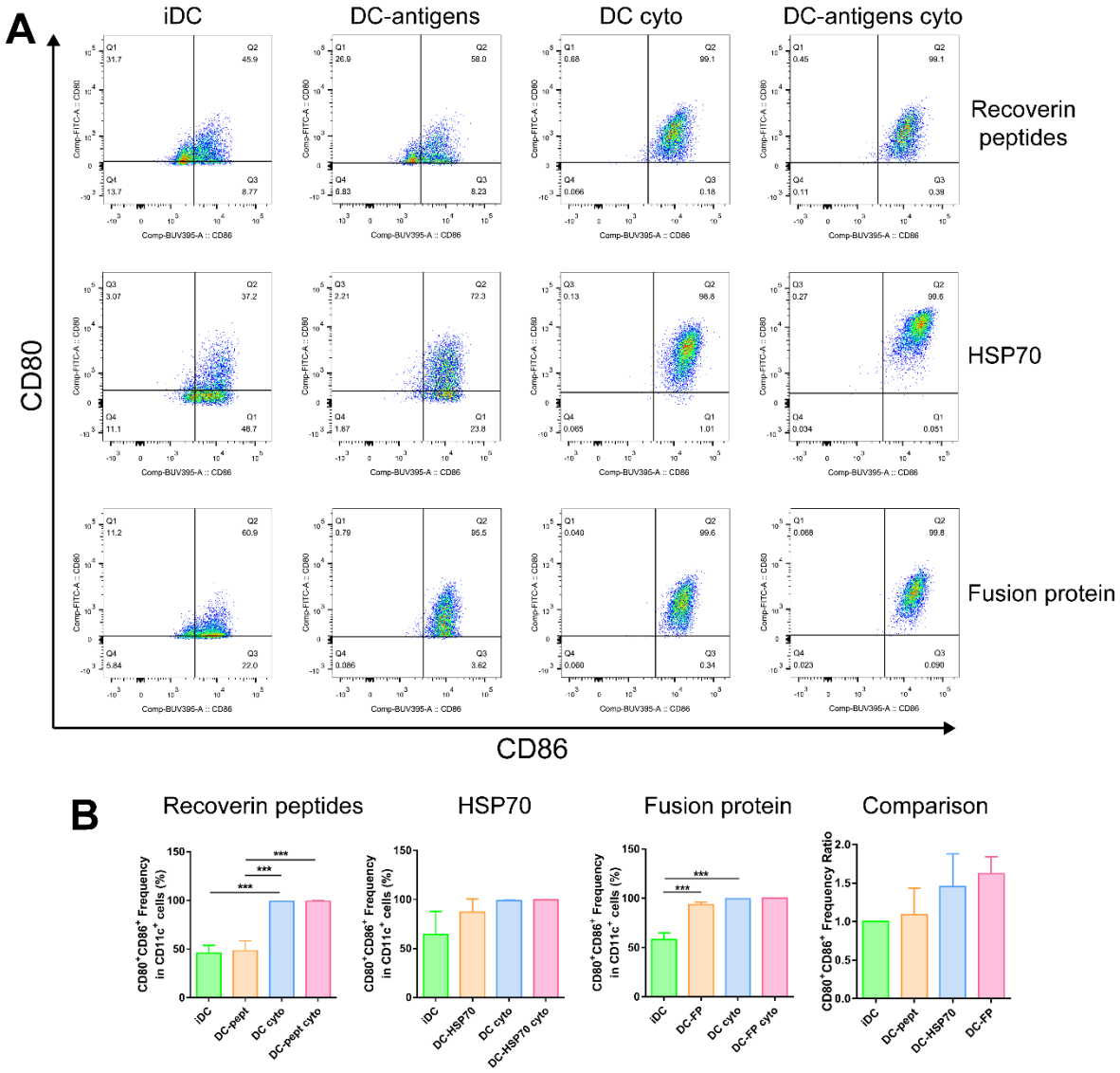


**Figure 3.4: Recoverin fusion proteins upregulated the frequency of activated DCs.**

(A) The frequency of $CD80^{+}CD86^{+}$ DCs after antigens and/or cytokines treatment. (B) Statistical analysis of the proportion of $CD80^{+}CD86^{+}$ DCs after antigens and/or cytokines treatment from three independent samples. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by one-way ANOVA test followed by Tukey's multiple comparisons posttest, *** $P$<0.001).

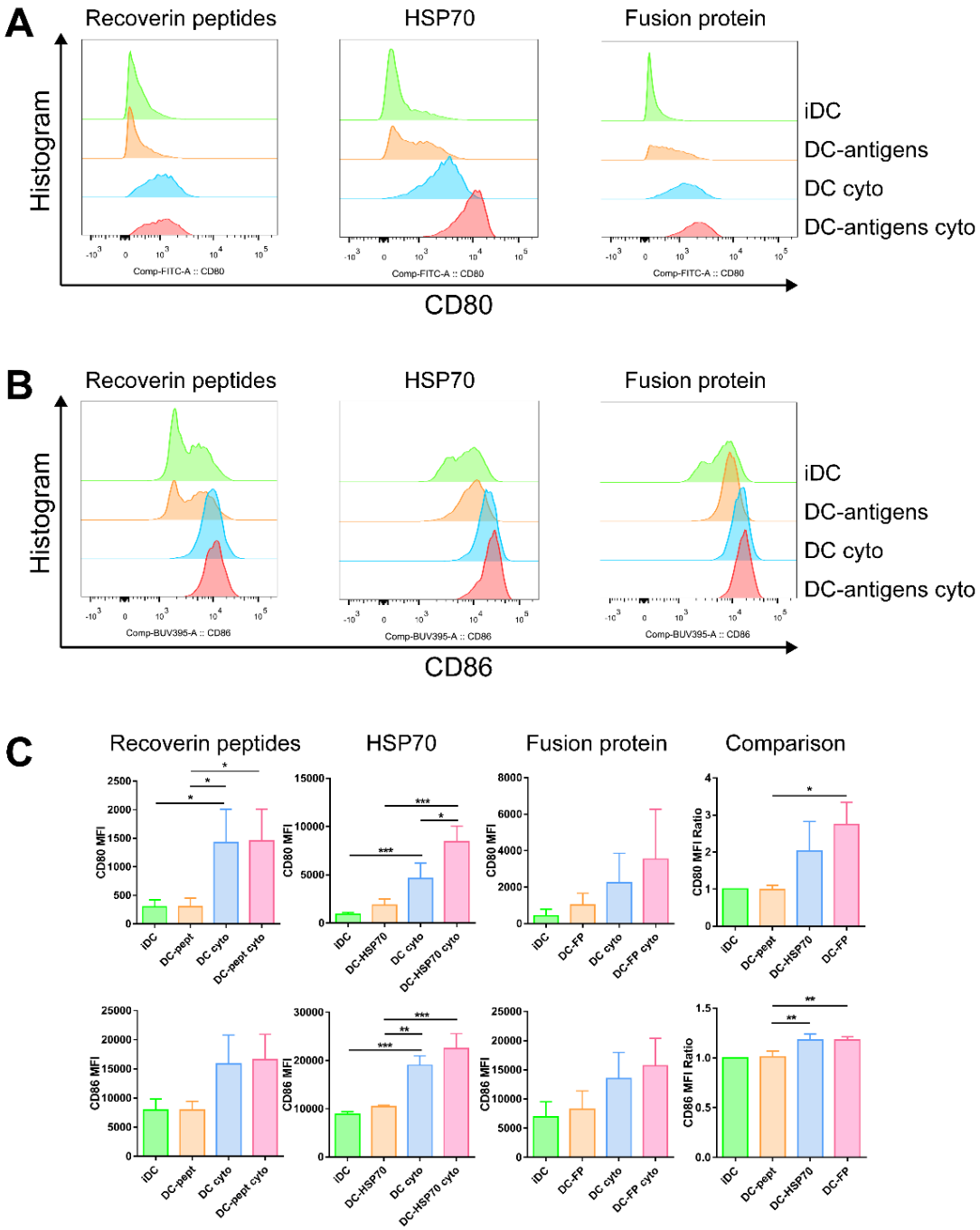


**Figure 3.5: Recoverin fusion proteins increased the number of CD80 and CD86 in DCs.** (A) The MFI of CD80 in DCs after antigens and/or cytokines treatment. (A) The MFI of CD86 in DCs after antigens and/or cytokines treatment. (C) Statistical analysis of the MFI of CD80 and CD86 in DCs after antigens and/or cytokines treatment from three independent samples. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by one-way ANOVA test followed by Tukey's multiple comparisons posttest, * $P$<0.05, ** $P$<0.01 and *** $P$<0.001).

## 3.6 Recoverin fusion protein induces the secretion of pro-inflammatory cytokines of DCs

The secretion of pro-inflammation cytokines from immune cells is critical for the regulation of innate immune response and cellular response. By acting through cell surface receptors, cytokines regulate the maturation, proliferation, and responsiveness of immune cells, contributing to host immune responses to infection, trauma, and cancer [122]. After maturation, DCs start to release various types of cytokine, favoring the activation of adaptive immunity. Furthermore, the cytokines secreted by DCs can bind with the surface receptors themselves reversely, leading to an inflammatory cascade of DCs [123]. Thus, I measured the concentration of different cytokines in the medium where DCs received the treatment of antigens and/or maturation cytokines.

Figure 3.6 A showed that when DCs pulsed with Recoverin peptides, the concentration of IL-6 was almost the same as the iDC group. DCs treated with fusion proteins exhibited an upregulated trend in the secretion of IL-6 in comparison to the iDC group or the DC- Recoverin peptides group. Intriguingly, when DCs treated with fusion proteins and maturation cytokines together, the secretion of IL-6 was much higher than fusion proteins or maturation cytokines alone treatment group, indicating the synergetic promoting effect of fusion proteins and maturation cytokines on the secretion of IL-6. The same trend was also evidenced in the HSP70 and maturation cytokines treatment group, revealing that the synergetic effect is attributed to the existence of HSP70 in the fusion protein.

I also measured the concentration of TNF-α in the medium, as shown in Figure 3.6 B. There is no difference between the iDC group and the DC-pept group. However, when DCs pulsed with HSP70 or fusion proteins, the concentration of TNF-α is considerably higher than iDCs. Because there is an overlap between the TNF-α that I added in the medium as one component of the maturation cytokines and the TNF-α secreted by DCs themselves, and it is hard to distinguish them clearly. Therefore, I did not present the TNF-α level of DCs treated with maturation cytokines. After data normalization, the DC-FP group exhibited a 20-fold upregulation compared with the DC-pept group in the concentration of TNF-α, but the statistical difference was not evidenced. The levels of IFN-γ, IL-12p40, IL-12p70, and IL-10 in the medium were also measured. However, the concentration of these cytokines in the iDC group was too low to be measured by Bio-Plex (data not shown).

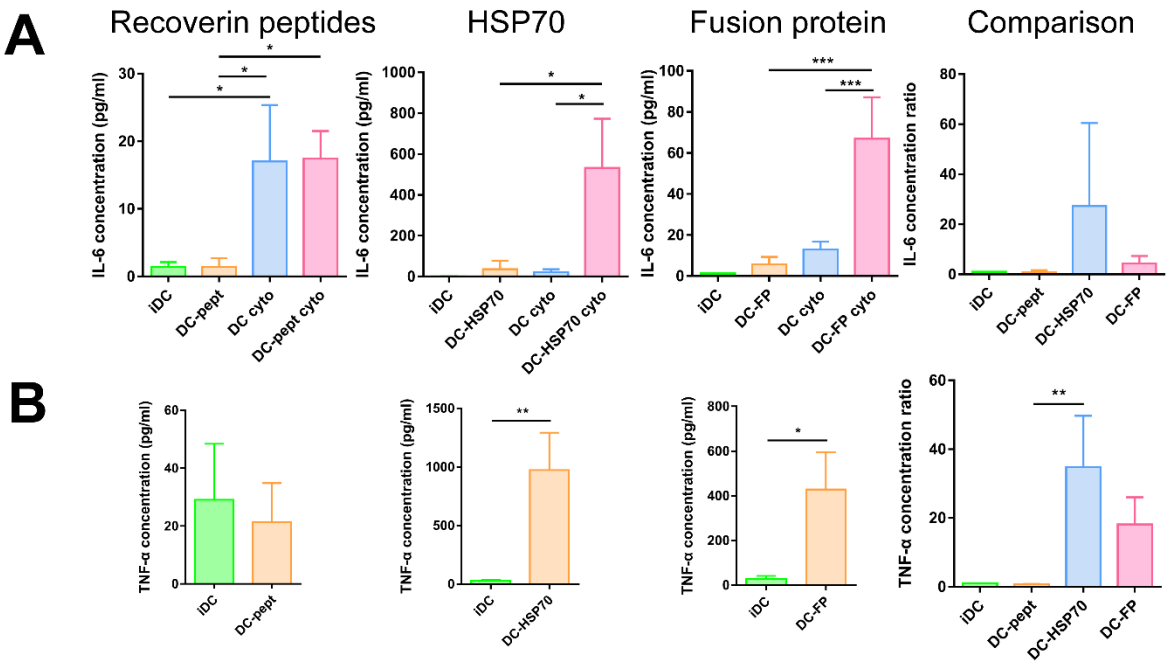


**Figure 3.6: Pro-inflammation cytokine secretion profile of DCs pulsed with Recoverin fusion proteins.** (A) The concentration of IL-6 in the medium where DCs received antigens and/or cytokines treatment. (B) The concentration of TNF-α in the medium where DCs received antigens and/or cytokines treatment. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by Student t test or one-way ANOVA test followed by Tukey's multiple comparisons posttest, * $P$<0.05, ** $P$<0.01 and *** $P$<0.001).

## 3.7 DCs pulsed with Recoverin fusion protein stimulates the activation of $CD8^+$ T cells, but not of $CD4^+$ T cells

The aforementioned results have proven the potential role of Recoverin fusion protein as a cancer vaccine to promote the maturation, activation, as well as pro-inflammatory cytokines secretion of DCs, highlighting its feasibility to further activate T lymphocytes to struggle against malignant cells. Therefore, I hypothesized that DCs pulsed with Recoverin fusion proteins can induce the activation of T cells. CD38, a transmembrane glycoprotein that is widely expressed in various cells, has been demonstrated to be an activation marker of T lymphocytes [124]. I generated DCs pulsed with Recoverin fusion protein and then co-cultured them with autologous T lymphocytes with an addition of IL-2. After two weeks, I harvested those cells and detected the CD38 marker in CD8 and CD4 T cells by FACS, respectively. $CD8^+$ T cells were

identified as a CD45⁺, CD3⁺ and CD8⁺ population, while CD4⁺ T cells were identified as a CD45⁺, CD3⁺ and CD4⁺ population, as shown in Figure 3.7.

Antitumor immune responses are mainly mediated by cytotoxic T lymphocytes, which can recognize and destruct malignant cells, leading to the collapse of tumor cells eventually. Thus, firstly, I measured the frequency of CD38⁺ in CD8⁺ T cells when co-cultured with DCs pulsed with different kinds of antigens and/or maturation cytokines. FACS results revealed that when T cells co-cultured with DCs pulsed with Recoverin peptides, the frequency of CD38⁺ in CD8⁺ T cells had a slight decrease in comparison to the iDC group. However, when T cells co-cultured with DCs pulsed with fusion proteins, the percentage of CD38⁺ in CD8⁺ T cells was almost twice as much as the T cells+iDC group (9.06% for T cells+iDC group versus 15.9% for T cells+DC-FP group, Figure 3.8 A). As for the MFI of CD38 in CD8⁺ T cells, no significant differences were observed in T cells co-cultured with DCs pulsed with the fusion protein in comparison to the T cells+iDC group (Figure 3.8 B). Statistical analysis showed that when T cells co-cultured with DCs pulsed with fusion proteins, the proportion of CD38⁺ in CD8⁺ T lymphocytes is higher than the T cells+iDC group, while the statistical difference was not evidenced. Nevertheless, when I normalized the data of three different types of antigens, I found that DCs pulsed with fusion proteins exhibit a more superior ability to activate CD8⁺ T cells than DCs pulsed with peptides do (Figure 3.8 C).

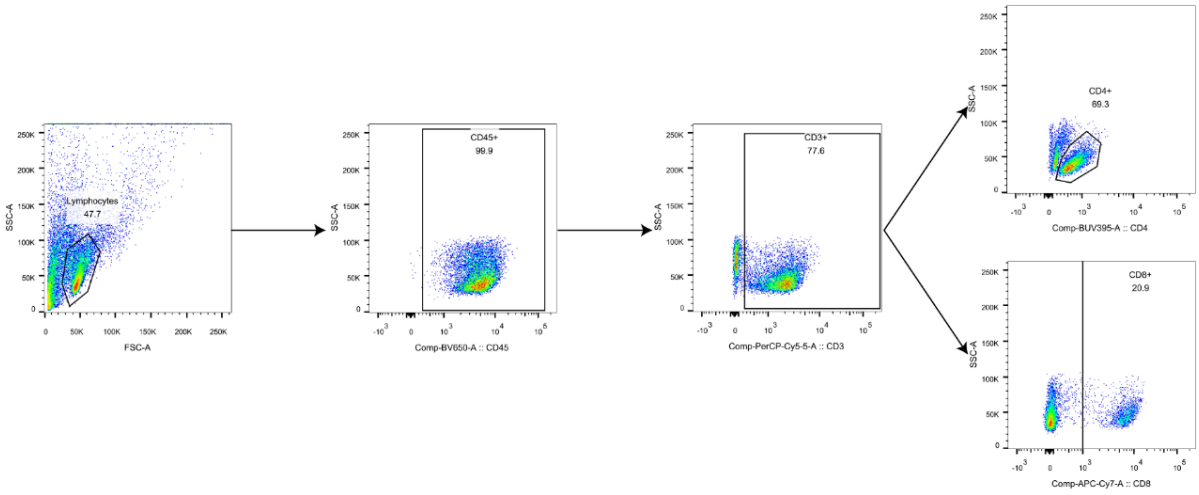


**Figure 3.7: Gating strategy of T cells.**

T cells isolated from PBMCs can be divided into CD4⁺ and CD8⁺ T cells. Population with CD45⁺, CD3⁺ and CD4⁺ were defined as CD4⁺ T cells, while population with CD45⁺, CD3⁺ and CD8⁺ were defined as CD8⁺ T cells.

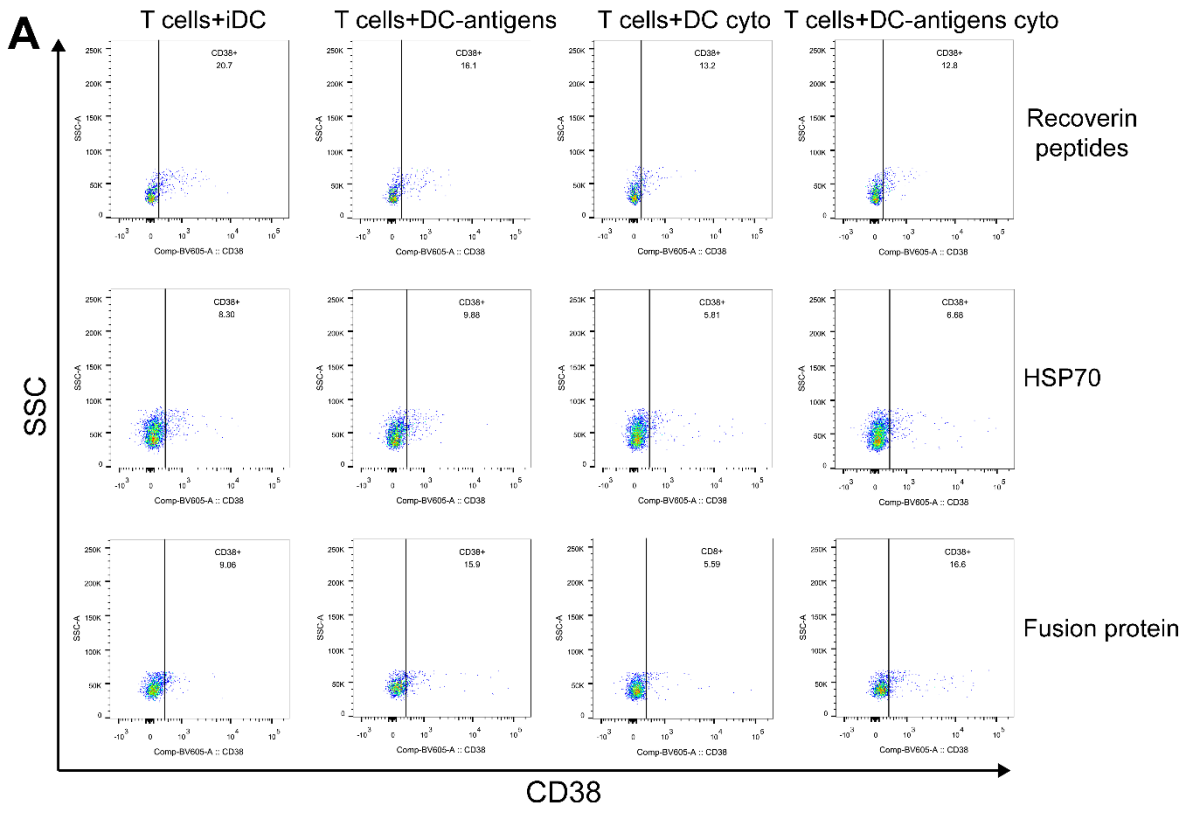
A
T cells+iDC
T cells+DC-antigens
T cells+DC cyto
T cells+DC-antigens cyto
Recoverin peptides
HSP70
Fusion protein
SSC
CD38
B
Recoverin peptides
HSP70
Fusion protein
Histogram
iDC
DC-antigens
DC cyto
DC-antigens cyto
CD38
C
Recoverin peptides
HSP70
Fusion protein
Comparison
CD38 expression in CD8+ cells
CD38 Frequency in CD8+ cells (%)
CD38 MFI
CD38 Frequency ratio
CD38 MFI ratio
T cells+iDC
T cells+DC-pept
T cells+DC cyto
T cells+DC-pept cyto
T cells+DC-HSP70
T cells+DC-HSP70 cyto
T cells+DC-FP
T cells+DC-FP cyto

**Figure 3.8: DCs pulsed with Recoverin fusion proteins primed the activation of CD8 T lymphocytes.** (A) The percentage of $CD38^+$ in $CD8^+$ T cells after co-cultured with DCs treated with antigens and/or cytokines. (B) The MFI of CD38 in $CD8^+$ T lymphocytes after co-cultured with DCs treated with antigens and/or cytokines. (C) Statistical analysis of the proportion and MFI of $CD38^+$ in $CD8^+$ T lymphocytes after co-cultured with DCs treated with antigens and/or cytokines. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by one-way ANOVA test followed by Tukey's multiple comparisons posttest, * $P<0.05$ and ** $P<0.01$).

Meanwhile, I also detected the frequency of $CD38^+$ in $CD4^+$ T lymphocytes when co-cultured with DCs pulsed with different kinds of antigens and/or maturation cytokines. FACS results demonstrated that there are no significant differences in the proportion and MFI of CD38 among four different treatment groups (Figure 3.9), indicating that DC pulsed with Recoverin fusion proteins had limited effects on the activation of $CD4^+$ T cells. Collectively, these results revealed that DCs treated with Recoverin fusion proteins can prime the activation of $CD8^+$ T lymphocytes but not $CD4^+$ T lymphocytes.

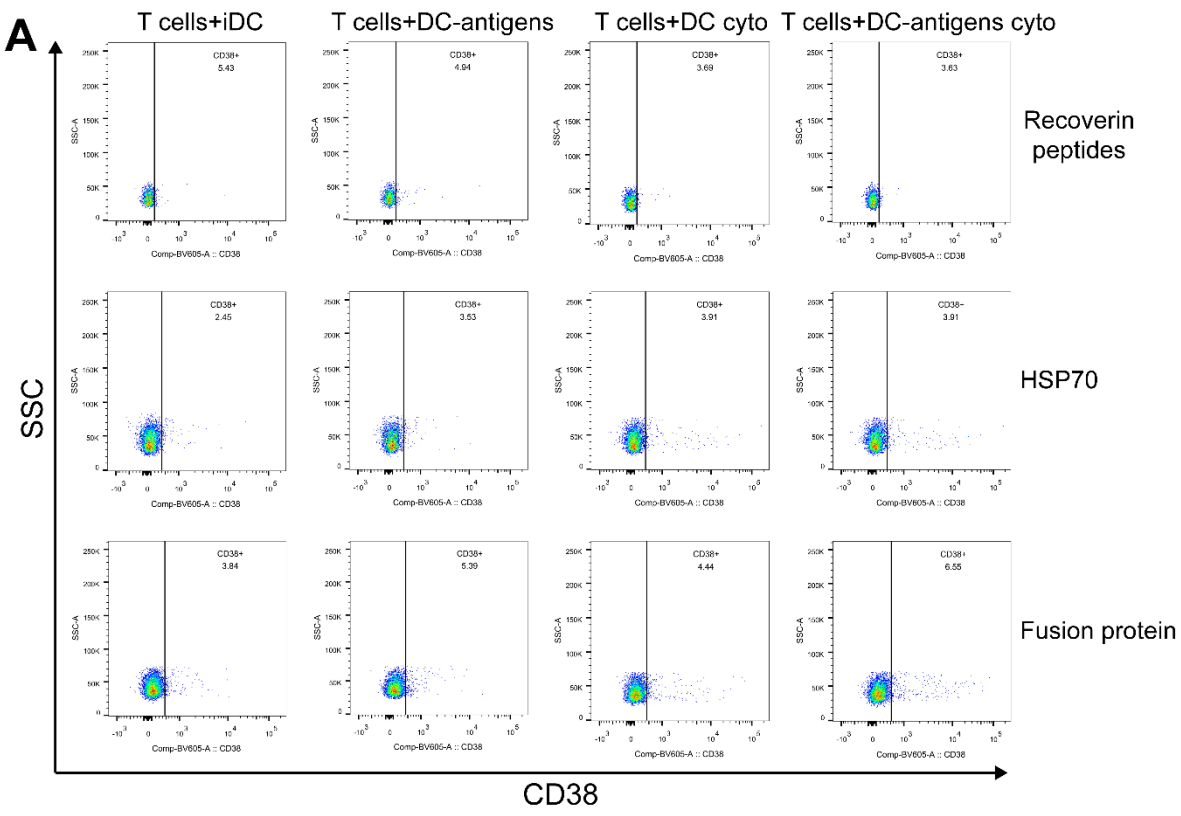
A
T cells+iDC
T cells+DC-antigens
T cells+DC cyto
T cells+DC-antigens cyto
Recoverin peptides
HSP70
Fusion protein
SSC
CD38
B
Recoverin peptides
HSP70
Fusion protein
Histogram
iDC
DC-antigens
DC cyto
DC-antigens cyto
CD38
C
Recoverin peptides
HSP70
Fusion protein
Comparison
CD38 expression in CD4+ cells
CD38 Frequency in CD4+ cells (%)
CD38 MFI
CD38 Frequency ratio
CD38 MFI ratio
T cells+iDC
T cells+DC-pept
T cells+DC cyto
T cells+DC-pept cyto
T cells+DC-HSP70
T cells+DC-HSP70 cyto
T cells+DC-FP
T cells+DC-FP cyto

**Figure 3.9: DCs pulsed with Recoverin fusion proteins failed to induce the activation of CD4 T lymphocytes.** (A) The percentage of CD38+ in $CD4^+$ T cells after co-cultured with DCs treated with antigens and/or cytokines. (B) The MFI of CD38 in $CD4^+$ T lymphocytes after co-cultured with DCs treated with antigens and/or cytokines. (C) Statistical analysis of the proportion and MFI of $CD38^+$ in $CD4^+$ T lymphocytes after co-cultured with DCs treated with antigens and/or cytokines. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by one-way ANOVA test followed by Tukey's multiple comparisons posttest).

## 3.8 Recoverin fusion protein induces immunosuppression

Above mentioned results have evidenced the potential role of Recoverin fusion proteins to act as cancer vaccines to trigger T cells activation for further tumor cells cytotoxicity. However, whether Recoverin fusion proteins possess immunosuppressive properties, which might attenuate the immunostimulatory function of fusion proteins, remains to be answered. Therefore, I also explored the immunosuppressive potentials of fusion proteins in our works. In Figure 3.10 A, I can tell that when DCs treated with fusion proteins, the percentage of $CD274^+$ DCs (also known as PD-L1) increased to 84.6% while the iDC group was only 45.4%. However, the proportion of $CD274^+$ DCs after peptides treatment was nearly the same as the iDC group. Intriguingly, although maturation cytokines induced the maturation and activation of DCs, they also upregulated the frequency of $CD274^+$ DCs to nearly 100%. Moreover, It was also observed that the fluorescence peak of CD274 shifted to the right greatly when compared with the iDC group, which indicates that fusion proteins can induce a higher amount of PD-L1 on DCs (Figure 3.10 B). Statistical analysis showed that fusion proteins can induce a higher frequency of $CD274^+$ DCs, whereas peptides can not, in comparison to the iDC group (Figure 3.10 C). Normalized data revealed that fusion proteins have a better performance to upregulate the frequency of $CD274^+$ DCs than peptides. An increase in the MFI of CD274 was also noticed in the DC-fusion protein group compared with the iDC group or DC-peptides group, even though the statistical difference was not demonstrated.

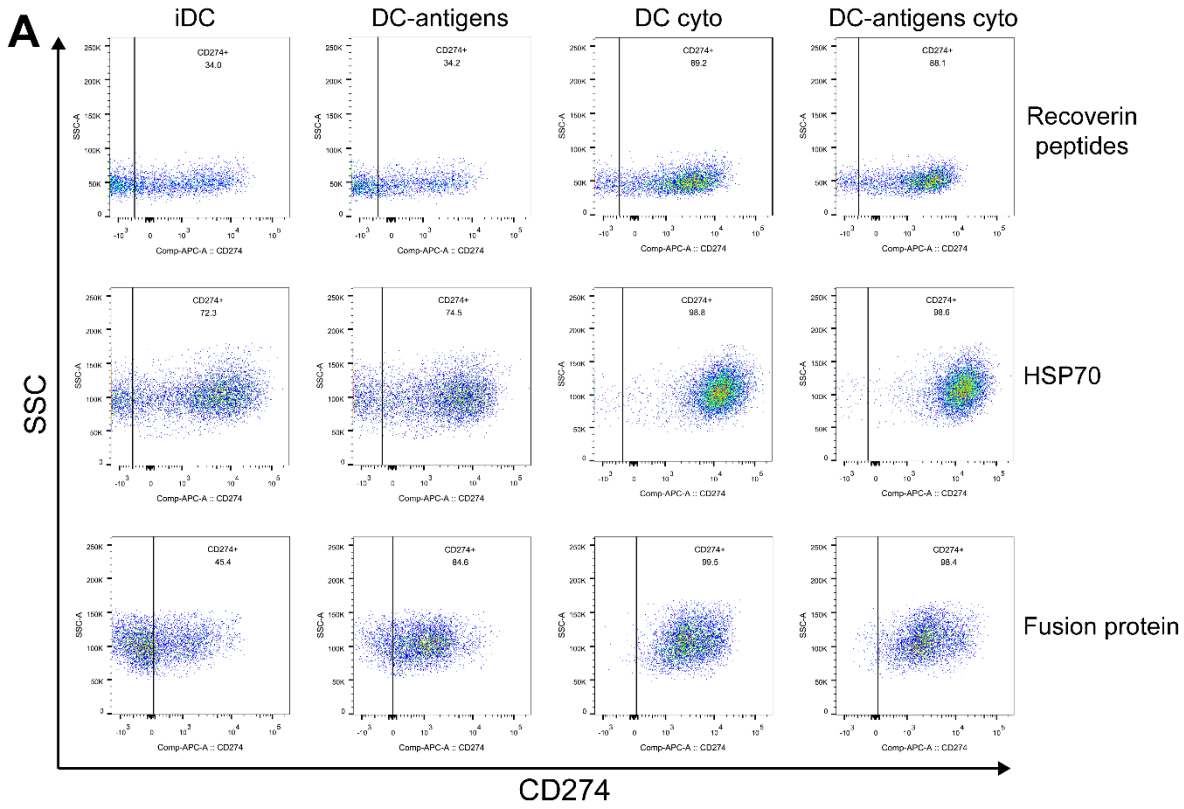
A
iDC
DC-antigens
DC cyto
DC-antigens cyto
Recoverin peptides
HSP70
Fusion protein
SSC
CD274
B
Recoverin peptides
HSP70
Fusion protein
Histogram
iDC
DC-antigens
DC cyto
DC-antigens cyto
CD274
C
Recoverin peptides
HSP70
Fusion protein
Comparison
CD274 expression
CD274 Frequency in CD11c+ cells (%)
CD274 MFI
CD274 Frequency Ratio
CD274 MFI Ratio
iDC
DC-pept
DC cyto
DC-pept cyto
DC-HSP70
DC-HSP70 cyto
DC-FP
DC-FP cyto

**Figure 3.10: Recoverin fusion proteins upregulated the expression of PD-L1 in DCs.** (A) The frequency of CD274$^{+}$ DCs after antigens and/or cytokines treatment. (B) The MFI of CD274 in DCs after antigens and/or cytokines treatment. (C) Statistical analysis of the percentage and MFI of CD274 in DCs after antigens and/or cytokines treatment from three independent samples. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by one-way ANOVA test followed by Tukey's multiple comparisons posttest, * $P$<0.05, ** $P$<0.01 and *** $P$<0.001).

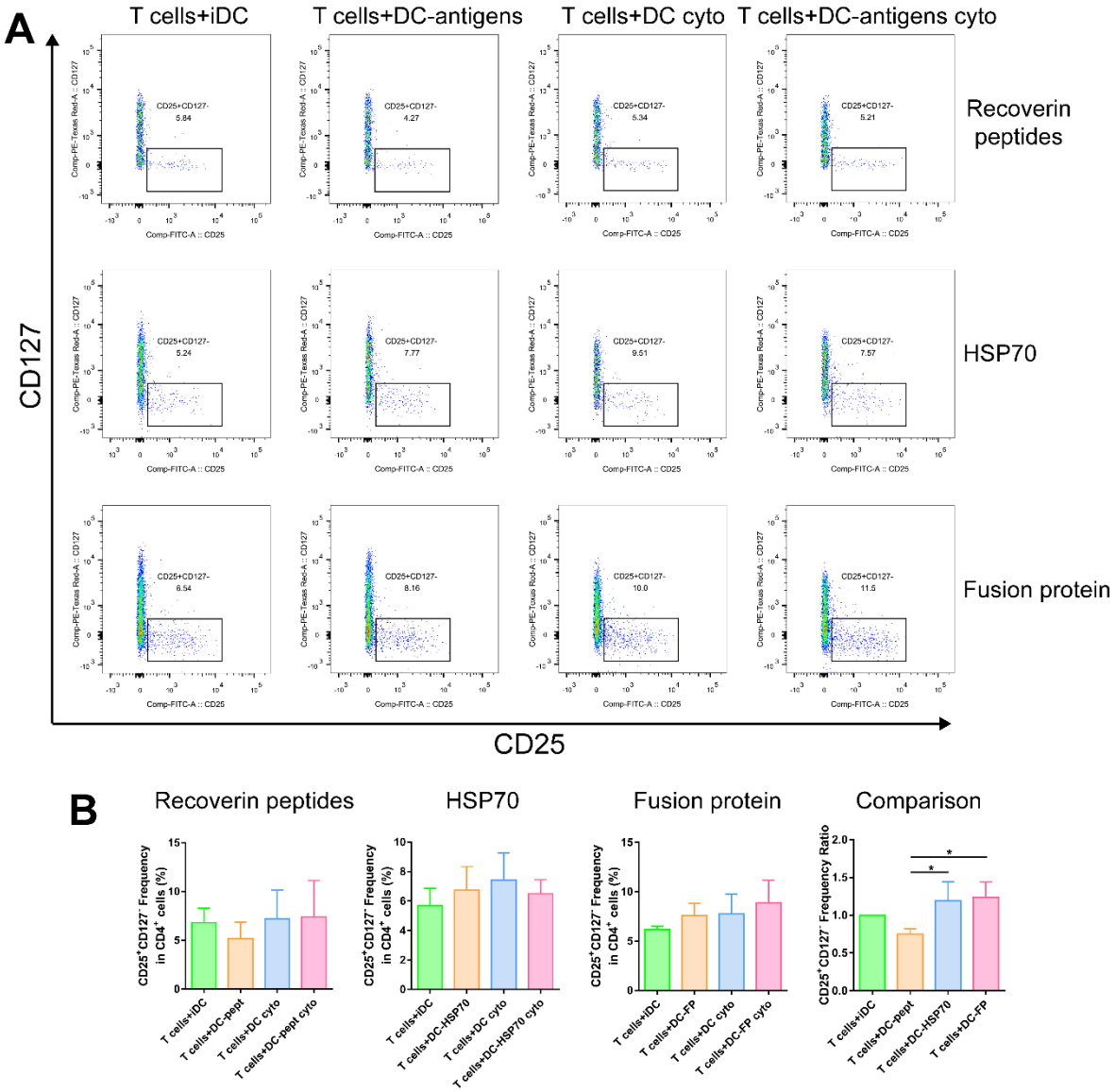


**Figure 3.11: DCs pulsed with Recoverin fusion proteins induced Treg cells.** (A) The percentage of CD25$^{+}$CD127$^{-}$ in CD4$^{+}$ T cells after co-cultured with DCs treated with antigens and/or cytokines. (B) Statistical analysis of the proportion of CD25$^{+}$CD127$^{-}$ in CD4$^{+}$ T

lymphocytes after co-cultured with DCs treated with antigens and/or cytokines. iDC: immature DC; pept: peptides; FP: fusion protein; cyto: cytokine; (Data are presented as mean±SD and analyzed by one-way ANOVA test followed by Tukey's multiple comparisons posttest, * $P<0.05$).

Subsequently, I examined the capacity of DCs pulsed with HSP70 fusion proteins to induce Treg cells. Treg cells are a subset of T lymphocytes possessing the ability to maintain immunological homeostasis and prevent excessive immune responses deleterious to the host. After activation, Treg cells fulfill their immunosuppressive effects via cell-cell contact and inhibitory cytokines secretion. In figure 3.11, FACS data demonstrated that fusion proteins can induce a slight increase in the frequency of Treg cells in comparison to the iDC group, while a slight decrease was observed in the DC-peptides group compared with the iDC group. Normalized data showed that DCs pulsed with fusion proteins can induce a higher frequency of Treg cells than DCs pulsed with peptides. Notably, DCs treated with HSP70 also exhibited a good potential to upregulate the percentage of Treg cells as DCs treated with fusion proteins do. These data suggested that Recoverin fusion proteins possess immunosuppressive properties when being applied as cancer vaccines.

# 4. Discussion

## 4.1 The immunoactive potency of Recoverin fusion protein

In this project, I applied fusion proteins comprising Recoverin epitopes and HSP70 as a cancer vaccine, to examine its potential capability to trigger immune responses against Recoverin-expressing tumors *in vitro*. Recoverin proteins can stimulate a strong immunity when being exposed to the immune system. However, by reducing the DCs‘ infiltration in the TME, cancer cells prevent the recognition of tumor antigens, including Recoverin, by the immune system and promote immunological evasion. Therefore, the aim of the fusion protein as a cancer vaccine is to train and bolster the immune system against Recoverin-expressing tumors. Moreover, HSP70 was fused with Recoverin epitopes to enhance the recognition of Recoverin by APCs and prime the cross-presentation of Recoverin on the surface of APCs for T lymphocytes activation.

As the most professional APC, DCs participate in antitumor immunity critically. When administrating the cancer vaccine into hosts, tissue-resident DCs engulf the vaccine, becoming matured and activated. Then these DCs circulate to secondary lymphoid organs, where DCs confer activation signals to T cells to trigger cytotoxicity against tumors. To mimic the situation that DCs are stimulated by the cancer vaccine, monocytes-derived DCs were generated and co-cultured with the fusion protein. It has been shown that the Recoverin fusion protein is capable to induce the maturation of DCs by upregulating the proportion of CD83 in DCs, but not the amounts of CD83 in this study. Moreover, the fusion protein can prime the activation of DCs. A high frequency of CD80 and CD86 was evidenced in DCs treated with the fusion protein while elevated numbers of CD80 and CD86 on the surface of DCs were also demonstrated. Notably, when DCs pulsed with the fusion protein, the percentage of CD80 and CD86 was as high as DCs treated with maturation cytokines, which serves as a positive control in the results, highlighting the robust ability of Recoverin fusion protein to induce the activation of DCs. As the co-stimulatory receptors, CD80 and CD86 can bind with CD28 on the surface of naïve T cells. The communication of CD80 and CD86 with CD28 confers a co-stimulatory signal, also known as a secondary signal, to promote the activation of T lymphocytes. A high expression of CD80 and CD86 in DCs treated with the fusion protein indicates a potential strong capacity of DCs to trigger antitumor immunity by activating Recoverin-targeting T cells. Interestingly, there is no difference between immatured DCs and DCs pulsed with Recoverin peptides in the

expression of CD83, CD80, and CD86. The underlying mechanism, supposedly, is the absence of HSP70. As an ideal antigen carrier, HSP70 possesses a natural ability to present antigens to APCs via scavenger receptors, promoting the endocytosis and process of antigens in APCs efficiently. However, the recognition of Recoverin peptides by DCs attenuates greatly without the chaperone of HSP70. The chaperone function of HSP70 in other cancer vaccines to induce the activation of DCs has been demonstrated previously. Gao and colleagues found that DCs pulsed with HSP70 and tumor peptides complex exhibits a great up-regulation of CD80 and CD86 on DCs [125]. Coincidentally, Cao and colleagues synthesized fusion protein comprising HSP70 and FPR1, a protein overexpressed in cervical cancer, and tested its ability to activate DCs. Their experiments revealed that the fusion protein remarkably upregulates the expression of CD80 and CD83 on the surface of DCs in comparison to the FPR1 alone group [126]. These studies indicate the remarkable impacts of HSP70 on the maturation and activation of DCs when being fused or complexed to tumor antigens. In other words, a small amount of Recoverin peptides are already enough to activate the DCs with the assistance of HSP70 whereas peptides alone fail to do so, suggesting that small doses of Recoverin peptides are sufficient to develop an anti-Recoverin cancer vaccine with the help of HSP70, avoiding the potential harms derived from large doses of Recoverin to the host.

In addition to inducing the maturation and activation of DCs, the fusion protein also stimulate the secretion of pro-inflammatory cytokines in DCs, including TNF-α and IL-6. when DCs pulsed with the fusion protein, I observed an upregulated trend in the secretion of TNF-α in comparison to iDCs, while the statistical difference was not evidenced. A greatly increased secretion of TNF-α in DCs was identified when treated with the fusion protein and maturation cytokines simultaneously, in comparison to the fusion protein or maturation cytokines alone treatment group. This suggests the synergetic effects of the fusion protein and maturation cytokines on the secretion of TNF-α in DCs. Taken the fact that the fusion protein and maturation cytokines also induce maturation and activation of DCs considerably, the feasibility to apply both of them together to achieve robust antitumor immune responses is highlighted. The efficacy of HSP70-based cancer vaccines to induce the secretion of cytokines of DCs also has been revealed by other research teams before. Cao et al. found that the HSP70 and FPR1 fusion protein promotes the secretion of IL-12p70 and IFN-γ of DCs [126], while Gao et al. demonstrated that HSP70 and tumor peptides complex induces the secretion of IL-12p70 and TNF-$\alpha$ of DCs [125]. These results indicate that HSP70-based complex or fusion protein both have a favorable capability to induce the secretion of pro-inflammatory cytokines of DCs. Interestingly, I also found that DCs pulsed with HSP70 secrete higher levels of IL-6 and TNF-

α when compared with DCs pulsed with the fusion protein. The underlying mechanism might be the fusion of Recoverin to HSP70 changes the conformation of HSP70 and affect the functional domains which might be correlated with the potency to induce the secretion of cytokines of DCs. However, this remains to be verified.

Further studies proved that DCs pulsed with the fusion protein are capable to induce the activation of $CD8^+$ T lymphocytes, but not $CD4^+$ T lymphocytes. Antitumor cytotoxicity is dominantly mediated by $CD8^+$ T cells, which eliminate tumor cells via various pathways, like perforin-mediated cellular lysis and ligation of FasL-Fas mediated apoptosis [127]. Activated $CD8^+$ T cells triggered by DCs pulsed with the fusion protein are expected to generate anti-Recoverin killing activity, leading to the destruction of Recoverin-expressing tumor cells. Regretfully, an upregulation of activated $CD4^+$ T cells was not evidenced when co-cultured with DCs pulsed with the fusion protein. $CD4^+$ T cells, also known as helper T cells (Th cells), play a vital role in antitumor immune responses, especially $CD4^+$ Th 1 cells, which mainly participate in cell-mediated immunity. Without the support from activated $CD4^+$ T cells, whether the antitumor cytotoxicity of CD8 T cells *in vivo* will be attenuated remains to be answered. Maeda et al. have ever immunized mice with Recoverin peptides and then Recoverin-specific cytotoxic T lymphocytes (CTL) were demonstrated *in vivo* after vaccination, which results in tumor regression [128]. Furthermore, Nikoopour and colleagues also found that Recoverin-specific T cells responses were evidenced in mice after vaccination with Reocverin peptides [129], indicating the robust ability of Recoverin to trigger T cells immune responses and its potential role to serve as a cancer vaccine candidate. However, in this study, DCs pulsed with a low dose of Recoverin peptides fail to induce the activation of $CD8^+$ T cells, whereas a higher proportion of activated $CD8^+$ T cells was demonstrated when co-cultured with DCs pulsed with the same dose of Recoverin fusion protein, highlighting the immunostimulatory potential of HSP70 to assist the occurrence of Recoverin-specific T cells immune responses. Interestingly, when DCs treated with maturation cytokines only, they had limited effects on the activation of $CD8^+$ T lymphocytes, even though those DCs were fully matured and activated. The possible underlying mechanism is the lack of peptide-MHC complexes expressed on the surface of DCs, which means those cells can not provide the first activation signals for T lymphocytes, even though they expressed abundant costimulatory receptors, like CD80 and CD86. In this study, even though I found that DCs pulsed with Recoverin fusion protein are capable to induce the activation of $CD8^+$ T cells, the cytotoxicity of those T cells against Recoverin-expressing tumor cells remains elusive. Whether a Recoverin- and HLA-A2-specific cytotoxicity will be triggered by Recoverin fusion protein warrants further exploration.

A robust antitumor immunity is essential to struggle against cancer, preventing the corruptive damages to hosts originating from cancers. However, to achieve a persistent antitumor immune response to safeguard the host from a cancer recurrence, one-time antitumor immunity is insufficient. Practically, at present, it is hard to establish an immunotherapeutic approach that could eliminate cancer cells thoroughly in patients. Therefore, a long-lasting antitumor immunity is indispensable to suppress the growth and metastasis of tumors, keeping the balance between cancer and the host, leading to the prolonged life expectancy of cancer patients. It would be of great significance for cancer patients to receive consecutive cancer vaccine administration, rather than one-time vaccination, to attain a long-lasting antitumor immune response. During the establishment of antitumor immunological persistence after consecutive cancer vaccines administration, a subgroup of T lymphocytes termed “memory T cells”, plays a critical role. In contrast to naïve T cells, memory T cells exhibit enhanced proliferative and survival potential, offering strong and enduring protection against cancers. Accumulating studies have revealed that memory T cells are superior to terminally differentiated effectors in mediating cancer clearance [130,131]. A long-term antitumor immunity is mainly attributed to the function of memory T lymphocytes after vaccination and the reduction of memory T cells results in a lower efficacy of cancer vaccine [132]. Furthermore, a clinical trial demonstrated that expansion of HER2-specific memory $CD8^+$ T lymphocytes in vaccinated patients was significantly associated with prolonged progression-free survival, highlighting the unreplaceable role of memory T cells in long-term antitumor immunity [133]. Therefore, it is worthy to detect that whether the fusion protein can upregulate the frequency of memory T cells and induce the re-expansion of memory T cells in the following vaccination. Constructing a cancer vaccine that is able to upregulate the population of memory T cells against tumors can serve as a novel strategy in cancer vaccine development. However, how to design the antigen, what is the core part in the development of cancer vaccines to generate a higher frequency of memory T cells against cancers, is still unknown. Another approach to generating long-lived T cells with a memory phenotype is the addition of memory cytokines, such as IL-7, IL-15 [134]. For instance, when coculturing splenocytes with chicken ovalbumin (OVA) peptides and addition of IL-15 *in vitro*, a memory-like status of spleen cells was evidenced. Adoptive transfer of these cells *in vivo* can form a persistent memory-like population and reject OVA-expressing EG7 tumors in mice [135]. A combination of the fusion protein and memory cytokines would be efficacious in triggering a long-lasting immunity against Reocverin-expressing tumors. However, this notion needs to be verified by further *in vivo* and *in vitro* assays.

## 4.2 The immunosuppressive properties of the Recoverin fusion protein

In addition to the immunostimulatory properties of the fusion protein, I also detected the immunosuppressive potentials of the fusion protein. When DCs were treated with Recoverin fusion protein, upregulation of PD-L1 was demonstrated in comparison to the iDC group. PD-L1 expressed on the surface of DCs will confer an immunosuppressive signal to T cells via binding with PD-1, resulting in downregulated T cells function and even anergy. However, DCs pulsed with the fusion protein still exhibit a superior in inducing the activation of $CD8^+$ T cells, suggesting that the function of PD-L1 expressed on the surface of DCs does not overwhelm the immunostimulatory activity of DCs pulsed with the fusion protein. DCs pulsed with the fusion protein hold the potential to induce Treg cells, even though the increase is less than two-fold in comparison to DCs pulsed with Recoverin peptides. However, it was been found that the percentage of Treg cells upregulated by DCs pulsed with Recoverin fusion protein was the same as DCs pulsed with HSP70, indicating that the Treg cells-stimulating function of the fusion protein is attributed to the existence of HSP70, but not Recoverin. Previous studies have reported that HSP70 is capable to enhance the immunosuppressive function of Treg cells, preventing exaggerated immune responses [136]. Moreover, Liu et al. demonstrated that mice vaccinated with HSP70 were correlated with an induction of tolerogenic immune responses and an expansion of functional Treg cells [137], which corresponds to the Treg cells-inducing capability of HSP70 that I found in this study.

However, if robust antitumor cytotoxicity against Recoverin-expressing tumors can be evidenced with the fusion protein, then the immunosuppressive properties of the fusion protein are acceptable. Furthermore, the limitations derived from the immunosuppressive properties of the fusion protein are surmountable if other immunotherapies are applied simultaneously, such as ICB, to overcome immunosuppression. By combining Recoverin fusion protein and ICB together for cancer treatment, it is expected to elicit a stronger antitumor immune response in patients. Nonetheless, the rationale of such a combinate strategy is still worth further verification and discussion.

## 4.3 The bio-safety of Recoverin fusion protein

In addition to the efficacy of the fusion protein as a cancer vaccine to combat tumors, the bio-safety of the fusion protein *in vivo* also attracts attention. The most concerning issue in cancer vaccines is the inaccurate cytotoxicity, which might damage normal healthy cells and result in severe adverse events. However, except for the existence in tumors, Recoverin was only found to be expressed in the retina. The presence of the blood-retina barrier prevents the interaction of T lymphocytes and the retina, avoiding the anti-Recoverin cytotoxic effects of T cells on the retina. Therefore, anti-Recoverin cellular immune responses are secure theoretically. Previous studies reported that CAR occurred in some cancer cases because autoantibodies can pass through the blood-retina barrier in high titers, leading to loss of peripheral and color vision and even blindness [92,138]. Nonetheless, the epitopes we designed in the fusion protein were T cells epitopes, which means the fusion protein is unable to stimulate humoral immunity to produce anti-Reocverin autoantibodies, preventing the occurrence of CAR.

However, some bio-safety issues of the Recoverin fusion protein also need to be clarified. *In vitro* assays, I only co-cultured the fusion protein with DCs in the medium without the presence of other normal human cells, therefore, whether the fusion protein will pose toxicity against normal cells when being administered to the host warrants exploration. Furthermore, off-target effects of activated T lymphocytes primed by DCs pulsed with the fusion protein can not be ignored. If activated T lymphocytes exhibit inaccurate killing activities, then normal healthy cells are exposed to cytotoxic risks mediated by activated T cells when the host receives fusion protein administration. Thus, evaluation of the bio-safety of the fusion protein in mouse models, even in clinical trials, is essential to establish Recoverin fusion protein as a cancer vaccine.

## 4.4 The rationale of combination strategy of Recoverin-based immunotherapy

The single utilization of immunotherapeutic approaches, including pro-inflammatory cytokines, antibodies against tumor antigens, ICB, and cancer vaccines, showed a relatively low efficiency for cancer treatment clinically, while a combination of them led to a complete persistent clinical response in large immunosuppressive tumors [40]. To overcome the restriction of single immunotherapy in cancer treatment, the rationale of a combination approach has been proposed [19]. To achieve the greatest therapeutic effects, it is required to use a combined

method to combat the tumor and its microenvironment comprehensively. Currently, clinical trials concerning a combined immunotherapeutic approach against oncological diseases are under test, especially the combination of ICB and cancer vaccines [139]. The effectiveness and safety of ICBs now have been extensively examined, how to enhance the contribution of cancer vaccines to the efficacy of the combined strategy, is urgent work for researchers. In this project, it has been revealed the potential of the Reocverin fusion protein as a cancer vaccine to struggle against malignancies, however, some limitations need to be addressed. For instance, an elevated level of PD-L1 on the surface of DCs pulsed with the fusion protein has been demonstrated, which might lead to the anergy of T cells. The utilization of ICBs, which block the interaction of PD-1 and PD-L1 and prevent the dysfunction of T lymphocytes, seems to be an ideal complementary component in combination with anti-Recoverin cancer vaccines, to impact the tumor in a comprehensive manner. This combined approach is expected to remodel the TME where multiple branches of the antitumor immune responses are integrated.

## 4.5 Conclusion and prospect

To summarize, in this project, I examined the possibility to apply the fusion protein comprising Recoverin and HSP70 as a cancer vaccine to trigger antitumor immunity. I found that the fusion protein could induce the maturation and activation of DCs by upregulating the expression of CD80, CD83, and CD86 on the surface of DCs. Moreover, the fusion protein was also demonstrated to promote the secretion of pro-inflammatory cytokines, including TNF-α and IL-6. Further investigation revealed that DCs pulsed with the fusion protein possess the capability to stimulate the activation of $CD8^+$ T lymphocytes. However, the immunosuppressive properties were also evidenced in the fusion protein. These results together highlighted the feasibility to develop the fusion protein as a cancer vaccine, to trigger immune responses against Recoverin-expressing tumor cells.

The major work in this manuscript focus on the development of anti-Recoverin cancer vaccines to combat tumors, however, it does not mean the application of Recoverin in immunotherapy is limited to the exploitation of cancer vaccines. For instance, the advance of CAR T cell therapy currently highlights the feasibility to develop an adoptive transfer of anti-Reocverin CAR T cells for cancer treatment. The CAR T cell therapy has been proved to be effective for cancer treatment and five of them have been approved by FDA for clinical therapy [140]. Therefore, to construct T cells expressing Recoverin-targeting receptors is practicable to struggle against

Recoverin-expressing tumors. How can we utilize Recoverin in immunotherapy, I believe, is only limited to our imagination.

# References


1. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **2021**, *71*, 209-249, doi:10.3322/caac.21660.

2. Ramaswami, R.; Bayer, R.; Galea, S. Precision Medicine from a Public Health Perspective. *Annu Rev Public Health* **2018**, *39*, 153-168, doi:10.1146/annurev-publhealth-040617-014158.

3. Vinay, D.S.; Ryan, E.P.; Pawelec, G.; Talib, W.H.; Stagg, J.; Elkord, E.; Lichtor, T.; Decker, W.K.; Whelan, R.L.; Kumara, H.; et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Semin Cancer Biol* **2015**, *35 Suppl*, S185-s198, doi:10.1016/j.semcancer.2015.03.004.

4. Alard, E.; Butnariu, A.B.; Grillo, M.; Kirkham, C.; Zinovkin, D.A.; Newnham, L.; Macciochi, J.; Pranjol, M.Z.I. Advances in Anti-Cancer Immunotherapy: Car-T Cell, Checkpoint Inhibitors, Dendritic Cell Vaccines, and Oncolytic Viruses, and Emerging Cellular and Molecular Targets. *Cancers (Basel)* **2020**, *12*, doi:10.3390/cancers12071826.

5. Couzin-Frankel, J. Cancer immunotherapy. **2013**.

6. Yang, Y. Cancer immunotherapy: harnessing the immune system to battle cancer. *J Clin Invest* **2015**, *125*, 3335-3337, doi:10.1172/JCI83871.

7. Brunet, J.F.; Denizot, F.; Luciani, M.F.; Roux-Dosseto, M.; Suzan, M.; Mattei, M.G.; Golstein, P. A new member of the immunoglobulin superfamily--CTLA-4. *Nature* **1987**, *328*, 267-270, doi:10.1038/328267a0.

8. Walunas, T.L.; Lenschow, D.J.; Bakker, C.Y.; Linsley, P.S.; Freeman, G.J.; Green, J.M.; Thompson, C.B.; Bluestone, J.A. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1994**, *1*, 405-413, doi:10.1016/1074-7613(94)90071-x.

9. Krummel, M.F.; Allison, J.P. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **1995**, *182*, 459-465, doi:10.1084/jem.182.2.459.

10. Waterhouse, P.; Penninger, J.M.; Timms, E.; Wakeham, A.; Shahinian, A.; Lee, K.P.; Thompson, C.B.; Griesser, H.; Mak, T.W. Lymphoproliferative disorders with early

lethality in mice deficient in Ctla-4. *Science* **1995**, *270*, 985-988, doi:10.1126/science.270.5238.985.

11. Leach, D.R.; Krummel, M.F.; Allison, J.P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* **1996**, *271*, 1734-1736, doi:10.1126/science.271.5256.1734.

12. Ishida, Y.; Agata, Y.; Shibahara, K.; Honjo, T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *Embo j* **1992**, *11*, 3887-3895.

13. Dong, H.; Zhu, G.; Tamada, K.; Chen, L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* **1999**, *5*, 1365-1369, doi:10.1038/70932.

14. Freeman, G.J.; Long, A.J.; Iwai, Y.; Bourque, K.; Chernova, T.; Nishimura, H.; Fitz, L.J.; Malenkovich, N.; Okazaki, T.; Byrne, M.C.; et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* **2000**, *192*, 1027-1034, doi:10.1084/jem.192.7.1027.

15. Dong, H.; Strome, S.E.; Salomao, D.R.; Tamura, H.; Hirano, F.; Flies, D.B.; Roche, P.C.; Lu, J.; Zhu, G.; Tamada, K.; et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* **2002**, *8*, 793-800, doi:10.1038/nm730.

16. Topalian, S.L.; Hodi, F.S.; Brahmer, J.R.; Gettinger, S.N.; Smith, D.C.; McDermott, D.F.; Powderly, J.D.; Carvajal, R.D.; Sosman, J.A.; Atkins, M.B.; et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **2012**, *366*, 2443-2454, doi:10.1056/NEJMoa1200690.

17. Brahmer, J.R.; Tykodi, S.S.; Chow, L.Q.; Hwu, W.J.; Topalian, S.L.; Hwu, P.; Drake, C.G.; Camacho, L.H.; Kauh, J.; Odunsi, K.; et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* **2012**, *366*, 2455-2465, doi:10.1056/NEJMoa1200694.

18. Wolchok, J.D.; Kluger, H.; Callahan, M.K.; Postow, M.A.; Rizvi, N.A.; Lesokhin, A.M.; Segal, N.H.; Ariyan, C.E.; Gordon, R.A.; Reed, K.; et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* **2013**, *369*, 122-133, doi:10.1056/NEJMoa1302369.

19. Palucka, K.; Banchereau, J. Diversity and collaboration for effective immunotherapy. *Nat Med* **2016**, *22*, 1390-1391, doi:10.1038/nm.4249.

20. Garg, A.D.; Vandenberk, L.; Van Woensel, M.; Belmans, J.; Schaaf, M.; Boon, L.; De Vleeschouwer, S.; Agostinis, P. Preclinical efficacy of immune-checkpoint monotherapy does not recapitulate corresponding biomarkers-based clinical predictions in glioblastoma. *Oncoimmunology* **2017**, *6*, e1295903, doi:10.1080/2162402x.2017.1295903.

21. Eshhar, Z.; Waks, T.; Gross, G.; Schindler, D.G. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A* **1993**, *90*, 720-724, doi:10.1073/pnas.90.2.720.

22. Feins, S.; Kong, W.; Williams, E.F.; Milone, M.C.; Fraietta, J.A. An introduction to chimeric antigen receptor (CAR) T-cell immunotherapy for human cancer. *Am J Hematol* **2019**, *94*, S3-s9, doi:10.1002/ajh.25418.

23. Brentjens, R.J.; Latouche, J.B.; Santos, E.; Marti, F.; Gong, M.C.; Lyddane, C.; King, P.D.; Larson, S.; Weiss, M.; Rivière, I.; et al. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med* **2003**, *9*, 279-286, doi:10.1038/nm827.

24. Grupp, S.A.; Kalos, M.; Barrett, D.; Aplenc, R.; Porter, D.L.; Rheingold, S.R.; Teachey, D.T.; Chew, A.; Hauck, B.; Wright, J.F.; et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* **2013**, *368*, 1509-1518, doi:10.1056/NEJMoa1215134.

25. Brentjens, R.J.; Rivière, I.; Park, J.H.; Davila, M.L.; Wang, X.; Stefanski, J.; Taylor, C.; Yeh, R.; Bartido, S.; Borquez-Ojeda, O.; et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* **2011**, *118*, 4817-4828, doi:10.1182/blood-2011-04-348540.

26. Lee, D.W.; Kochenderfer, J.N.; Stetler-Stevenson, M.; Cui, Y.K.; Delbrook, C.; Feldman, S.A.; Fry, T.J.; Orentas, R.; Sabatino, M.; Shah, N.N.; et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* **2015**, *385*, 517-528, doi:10.1016/s0140-6736(14)61403-3.

27. Sheridan, C. First approval in sight for Novartis' CAR-T therapy after panel vote. *Nat Biotechnol* **2017**, *35*, 691-693, doi:10.1038/nbt0817-691.

28. Srivastava, S.; Riddell, S.R. Chimeric Antigen Receptor T Cell Therapy: Challenges to Bench-to-Bedside Efficacy. *J Immunol* **2018**, *200*, 459-468, doi:10.4049/jimmunol.1701155.

29. Barquet, N.; Domingo, P. Smallpox: the triumph over the most terrible of the ministers of death. *Ann Intern Med* **1997**, *127*, 635-642, doi:10.7326/0003-4819-127-8_part_1-199710150-00010.

30. de Gruijl, T.D.; Curiel, D.T. Cancer vaccine strategies get bigger and better. *Nat Med* **1999**, *5*, 1124-1125, doi:10.1038/13443.

31. Baldin, A.V.; Savvateeva, L.V.; Bazhin, A.V.; Zamyatnin, A.A., Jr. Dendritic Cells in Anticancer Vaccination: Rationale for Ex Vivo Loading or In Vivo Targeting. *Cancers (Basel)* **2020**, *12*, doi:10.3390/cancers12030590.

32. Calvo Tardón, M.; Allard, M.; Dutoit, V.; Dietrich, P.Y.; Walker, P.R. Peptides as cancer vaccines. *Curr Opin Pharmacol* **2019**, *47*, 20-26, doi:10.1016/j.coph.2019.01.007.

33. Bol, K.F.; Schreibelt, G.; Gerritsen, W.R.; de Vries, I.J.; Figdor, C.G. Dendritic Cell-Based Immunotherapy: State of the Art and Beyond. *Clin Cancer Res* **2016**, *22*, 1897-1906, doi:10.1158/1078-0432.Ccr-15-1399.

34. Lopes, A.; Vandermeulen, G.; Préat, V. Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. *J Exp Clin Cancer Res* **2019**, *38*, 146, doi:10.1186/s13046-019-1154-7.

35. Pardi, N.; Hogan, M.J.; Porter, F.W.; Weissman, D. mRNA vaccines - a new era in vaccinology. *Nat Rev Drug Discov* **2018**, *17*, 261-279, doi:10.1038/nrd.2017.243.

36. Maslak, P.G.; Dao, T.; Bernal, Y.; Chanel, S.M.; Zhang, R.; Frattini, M.; Rosenblat, T.; Jurcic, J.G.; Brentjens, R.J.; Arcila, M.E.; et al. Phase 2 trial of a multivalent WT1 peptide vaccine (galinpepimut-S) in acute myeloid leukemia. *Blood Adv* **2018**, *2*, 224-234, doi:10.1182/bloodadvances.2017014175.

37. Wen, P.Y.; Reardon, D.A.; Armstrong, T.S.; Phuphanich, S.; Aiken, R.D.; Landolfi, J.C.; Curry, W.T.; Zhu, J.J.; Glantz, M.; Peereboom, D.M.; et al. A Randomized Double-Blind Placebo-Controlled Phase II Trial of Dendritic Cell Vaccine ICT-107 in Newly

Diagnosed Patients with Glioblastoma. *Clin Cancer Res* **2019**, *25*, 5799-5807, doi:10.1158/1078-0432.Ccr-19-0261.

38. Sahin, U.; Oehm, P.; Derhovanessian, E.; Jabulowsky, R.A.; Vormehr, M.; Gold, M.; Maurus, D.; Schwarck-Kokarakis, D.; Kuhn, A.N.; Omokoko, T.; et al. An RNA vaccine drives immunity in checkpoint-inhibitor-treated melanoma. *Nature* **2020**, *585*, 107-112, doi:10.1038/s41586-020-2537-9.

39. Hartmann, J.; Schüßler-Lenz, M.; Bondanza, A.; Buchholz, C.J. Clinical development of CAR T cells-challenges and opportunities in translating innovative treatment concepts. *EMBO Mol Med* **2017**, *9*, 1183-1197, doi:10.15252/emmm.201607485.

40. Moynihan, K.D.; Opel, C.F.; Szeto, G.L.; Tzeng, A.; Zhu, E.F.; Engreitz, J.M.; Williams, R.T.; Rakhra, K.; Zhang, M.H.; Rothschilds, A.M.; et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat Med* **2016**, *22*, 1402-1410, doi:10.1038/nm.4200.

41. Haen, S.P.; Rammensee, H.G. The repertoire of human tumor-associated epitopes--identification and selection of antigens and their application in clinical trials. *Curr Opin Immunol* **2013**, *25*, 277-283, doi:10.1016/j.coi.2013.03.007.

42. Lu, Y.C.; Robbins, P.F. Cancer immunotherapy targeting neoantigens. *Semin Immunol* **2016**, *28*, 22-27, doi:10.1016/j.smim.2015.11.002.

43. Garraway, L.A.; Lander, E.S. Lessons from the cancer genome. *Cell* **2013**, *153*, 17-37, doi:10.1016/j.cell.2013.03.002.

44. De Plaen, E.; Lurquin, C.; Van Pel, A.; Mariamé, B.; Szikora, J.P.; Wölfel, T.; Sibille, C.; Chomez, P.; Boon, T. Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum- antigen P91A and identification of the tum- mutation. *Proc Natl Acad Sci U S A* **1988**, *85*, 2274-2278, doi:10.1073/pnas.85.7.2274.

45. Sibille, C.; Chomez, P.; Wildmann, C.; Van Pel, A.; De Plaen, E.; Maryanski, J.L.; de Bergeyck, V.; Boon, T. Structure of the gene of tum- transplantation antigen P198: a point mutation generates a new antigenic peptide. *J Exp Med* **1990**, *172*, 35-45, doi:10.1084/jem.172.1.35.

46. Monach, P.A.; Meredith, S.C.; Siegel, C.T.; Schreiber, H. A unique tumor antigen produced by a single amino acid substitution. *Immunity* **1995**, *2*, 45-59, doi:10.1016/1074-7613(95)90078-0.

47. Coulie, P.G.; Lehmann, F.; Lethé, B.; Herman, J.; Lurquin, C.; Andrawiss, M.; Boon, T. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc Natl Acad Sci U S A* **1995**, *92*, 7976-7980, doi:10.1073/pnas.92.17.7976.

48. Besser, H.; Yunger, S.; Merhavi-Shoham, E.; Cohen, C.J.; Louzoun, Y. Level of neo-epitope predecessor and mutation type determine T cell activation of MHC binding peptides. *J Immunother Cancer* **2019**, *7*, 135, doi:10.1186/s40425-019-0595-z.

49. Stone, J.D.; Harris, D.T.; Kranz, D.M. TCR affinity for p/MHC formed by tumor antigens that are self-proteins: impact on efficacy and toxicity. *Curr Opin Immunol* **2015**, *33*, 16-22, doi:10.1016/j.coi.2015.01.003.

50. Ott, P.A.; Hu, Z.; Keskin, D.B.; Shukla, S.A.; Sun, J.; Bozym, D.J.; Zhang, W.; Luoma, A.; Giobbie-Hurder, A.; Peter, L.; et al. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **2017**, *547*, 217-221, doi:10.1038/nature22991.

51. Keskin, D.B.; Anandappa, A.J.; Sun, J.; Tirosh, I.; Mathewson, N.D.; Li, S.; Oliveira, G.; Giobbie-Hurder, A.; Felt, K.; Gjini, E.; et al. Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature* **2019**, *565*, 234-239, doi:10.1038/s41586-018-0792-9.

52. Sahin, U.; Derhovanessian, E.; Miller, M.; Kloke, B.P.; Simon, P.; Löwer, M.; Bukur, V.; Tadmor, A.D.; Luxemburger, U.; Schrörs, B.; et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **2017**, *547*, 222-226, doi:10.1038/nature23003.

53. Zhao, Y.; Baldin, A.V.; Isayev, O.; Werner, J.; Zamyatnin, A.A., Jr.; Bazhin, A.V. Cancer Vaccines: Antigen Selection Strategy. *Vaccines (Basel)* **2021**, *9*, doi:10.3390/vaccines9020085.

54. Boccardo, E.; Villa, L.L. Viral origins of human cancer. *Curr Med Chem* **2007**, *14*, 2526-2539, doi:10.2174/092986707782023316.

55. Kassiotis, G. Endogenous retroviruses and the development of cancer. *J Immunol* **2014**, *192*, 1343-1349, doi:10.4049/jimmunol.1302972.

56. Attermann, A.S.; Bjerregaard, A.M.; Saini, S.K.; Grønbæk, K.; Hadrup, S.R. Human endogenous retroviruses and their implication for immunotherapeutics of cancer. *Ann Oncol* **2018**, *29*, 2183-2191, doi:10.1093/annonc/mdy413.

57. Morrow, M.P.; Yan, J.; Sardesai, N.Y. Human papillomavirus therapeutic vaccines: targeting viral antigens as immunotherapy for precancerous disease and cancer. *Expert Rev Vaccines* **2013**, *12*, 271-283, doi:10.1586/erv.13.23.

58. van der Burg, S.H.; Melief, C.J. Therapeutic vaccination against human papilloma virus induced malignancies. *Curr Opin Immunol* **2011**, *23*, 252-257, doi:10.1016/j.coi.2010.12.010.

59. Rahma, O.E.; Herrin, V.E.; Ibrahim, R.A.; Toubaji, A.; Bernstein, S.; Dakheel, O.; Steinberg, S.M.; Abu Eid, R.; Mkrtichyan, M.; Berzofsky, J.A.; et al. Pre-immature dendritic cells (PIDC) pulsed with HPV16 E6 or E7 peptide are capable of eliciting specific immune response in patients with advanced cervical cancer. *J Transl Med* **2014**, *12*, 353, doi:10.1186/s12967-014-0353-4.

60. Kenter, G.G.; Welters, M.J.; Valentijn, A.R.; Lowik, M.J.; Berends-van der Meer, D.M.; Vloon, A.P.; Essahsah, F.; Fathers, L.M.; Offringa, R.; Drijfhout, J.W.; et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* **2009**, *361*, 1838-1847, doi:10.1056/NEJMoa0810097.

61. Ding, F.X.; Wang, F.; Lu, Y.M.; Li, K.; Wang, K.H.; He, X.W.; Sun, S.H. Multiepitope peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular carcinoma. *Hepatology* **2009**, *49*, 1492-1502, doi:10.1002/hep.22816.

62. Pan, J.; Zhang, Q.; Zhou, J.; Ma, D.; Xiao, X.; Wang, D.W. Recombinant adeno-associated virus encoding Epstein-Barr virus latent membrane proteins fused with heat shock protein as a potential vaccine for nasopharyngeal carcinoma. *Mol Cancer Ther* **2009**, *8*, 2754-2761, doi:10.1158/1535-7163.Mct-08-1176.

63. Iqbal, N.; Iqbal, N. Human Epidermal Growth Factor Receptor 2 (HER2) in Cancers: Overexpression and Therapeutic Implications. *Mol Biol Int* **2014**, *2014*, 852748, doi:10.1155/2014/852748.

64. Pinzani, P.; Lind, K.; Malentacchi, F.; Nesi, G.; Salvianti, F.; Villari, D.; Kubista, M.; Pazzagli, M.; Orlando, C. Prostate-specific antigen mRNA and protein levels in laser microdissected cells of human prostate measured by real-time reverse transcriptase-quantitative polymerase chain reaction and immuno-quantitative polymerase chain reaction. *Hum Pathol* **2008**, *39*, 1474-1482, doi:10.1016/j.humpath.2008.02.012.

65. Fijak, M.; Meinhardt, A. The testis in immune privilege. *Immunol Rev* **2006**, *213*, 66-81, doi:10.1111/j.1600-065X.2006.00438.x.

66. Fratta, E.; Coral, S.; Covre, A.; Parisi, G.; Colizzi, F.; Danielli, R.; Nicolay, H.J.; Sigalotti, L.; Maio, M. The biology of cancer testis antigens: putative function, regulation and therapeutic potential. *Mol Oncol* **2011**, *5*, 164-182, doi:10.1016/j.molonc.2011.02.001.

67. Salmaninejad, A.; Zamani, M.R.; Pourvahedi, M.; Golchehre, Z.; Hosseini Bereshneh, A.; Rezaei, N. Cancer/Testis Antigens: Expression, Regulation, Tumor Invasion, and Use in Immunotherapy of Cancers. *Immunol Invest* **2016**, *45*, 619-640, doi:10.1080/08820139.2016.1197241.

68. Almeida, L.G.; Sakabe, N.J.; deOliveira, A.R.; Silva, M.C.; Mundstein, A.S.; Cohen, T.; Chen, Y.T.; Chua, R.; Gurung, S.; Gnjatic, S.; et al. CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. *Nucleic Acids Res* **2009**, *37*, D816-819, doi:10.1093/nar/gkn673.

69. Esfandiary, A.; Ghafouri-Fard, S. MAGE-A3: an immunogenic target used in clinical practice. *Immunotherapy* **2015**, *7*, 683-704, doi:10.2217/imt.15.29.

70. Thomas, R.; Al-Khadairi, G.; Roelands, J.; Hendrickx, W.; Dermime, S.; Bedognetti, D.; Decock, J. NY-ESO-1 Based Immunotherapy of Cancer: Current Perspectives. *Front Immunol* **2018**, *9*, 947, doi:10.3389/fimmu.2018.00947.

71. Vansteenkiste, J.F.; Cho, B.C.; Vanakesa, T.; De Pas, T.; Zielinski, M.; Kim, M.S.; Jassem, J.; Yoshimura, M.; Dahabreh, J.; Nakayama, H.; et al. Efficacy of the MAGE-A3 cancer immunotherapeutic as adjuvant therapy in patients with resected MAGE-A3-positive non-small-cell lung cancer (MAGRIT): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* **2016**, *17*, 822-835, doi:10.1016/s1470-2045(16)00099-1.

72. Dreno, B.; Thompson, J.F.; Smithers, B.M.; Santinami, M.; Jouary, T.; Gutzmer, R.; Levchenko, E.; Rutkowski, P.; Grob, J.J.; Korovin, S.; et al. MAGE-A3 immunotherapeutic as adjuvant therapy for patients with resected, MAGE-A3-positive, stage III melanoma (DERMA): a double-blind, randomised, placebo-controlled, phase 3 trial. *Lancet Oncol* **2018**, *19*, 916-929, doi:10.1016/s1470-2045(18)30254-7.

73. Gasser, O.; Sharples, K.J.; Barrow, C.; Williams, G.M.; Bauer, E.; Wood, C.E.; Mester, B.; Dzhelali, M.; Caygill, G.; Jones, J.; et al. A phase I vaccination study with dendritic cells loaded with NY-ESO-1 and α-galactosylceramide: induction of polyfunctional T cells in high-risk melanoma patients. *Cancer Immunol Immunother* **2018**, *67*, 285-298, doi:10.1007/s00262-017-2085-9.

74. Adamus, G. Autoantibody targets and their cancer relationship in the pathogenicity of paraneoplastic retinopathy. *Autoimmun Rev* **2009**, *8*, 410-414, doi:10.1016/j.autrev.2009.01.002.

75. Grunwald, G.B.; Kornguth, S.E.; Towfighi, J.; Sassani, J.; Simmonds, M.A.; Housman, C.M.; Papadopoulos, N. Autoimmune basis for visual paraneoplastic syndrome in patients with small cell lung carcinoma. Retinal immune deposits and ablation of retinal ganglion cells. *Cancer* **1987**, *60*, 780-786, doi:10.1002/1097-0142(19870815)60:4<780::aid-cncr2820600413>3.0.co;2-9.

76. Kornguth, S.E.; Klein, R.; Appen, R.; Choate, J. Occurrence of anti-retinal ganglion cell antibodies in patients with small cell carcinoma of the lung. *Cancer* **1982**, *50*, 1289-1293, doi:10.1002/1097-0142(19821001)50:7<1289::aid-cncr2820500711>3.0.co;2-9.

77. Polans, A.S.; Witkowska, D.; Haley, T.L.; Amundson, D.; Baizer, L.; Adamus, G. Recoverin, a photoreceptor-specific calcium-binding protein, is expressed by the tumor of a patient with cancer-associated retinopathy. *Proc Natl Acad Sci U S A* **1995**, *92*, 9176-9180, doi:10.1073/pnas.92.20.9176.

78. Senin, II; Dean, K.R.; Zargarov, A.A.; Akhtar, M.; Philippov, P.P. Recoverin inhibits the phosphorylation of dark-adapted rhodopsin more than it does that of bleached rhodopsin: a possible mechanism through which rhodopsin kinase is prevented from participation in a side reaction. *Biochem J* **1997**, *321 ( Pt 2)*, 551-555, doi:10.1042/bj3210551.

79. Milam, A.H.; Dacey, D.M.; Dizhoor, A.M. Recoverin immunoreactivity in mammalian cone bipolar cells. *Vis Neurosci* **1993**, *10*, 1-12, doi:10.1017/s0952523800003175.

80. Maeda, A.; Ohguro, H.; Maeda, T.; Wada, I.; Sato, N.; Kuroki, Y.; Nakagawa, T. Aberrant expression of photoreceptor-specific calcium-binding protein (recoverin) in cancer cell lines. *Cancer Res* **2000**, *60*, 1914-1920.

81. Bazhin, A.V.; Dalke, C.; Willner, N.; Abschütz, O.; Wildberger, H.G.; Philippov, P.P.; Dummer, R.; Graw, J.; de Angelis, M.H.; Schadendorf, D.; et al. Cancer-retina antigens as potential paraneoplastic antigens in melanoma-associated retinopathy. *Int J Cancer* **2009**, *124*, 140-149, doi:10.1002/ijc.23909.

82. Bazhin, A.V.; Schadendorf, D.; Willner, N.; De Smet, C.; Heinzelmann, A.; Tikhomirova, N.K.; Umansky, V.; Philippov, P.P.; Eichmüller, S.B. Photoreceptor proteins as cancer-retina antigens. *Int J Cancer* **2007**, *120*, 1268-1276, doi:10.1002/ijc.22458.

83. Bazhin, A.V.; Savchenko, M.S.; Shifrina, O.N.; Demoura, S.A.; Chikina, S.Y.; Jaques, G.; Kogan, E.A.; Chuchalin, A.G.; Philippov, P.P. Recoverin as a paraneoplastic antigen in lung cancer: the occurrence of anti-recoverin autoantibodies in sera and recoverin in tumors. *Lung Cancer* **2004**, *44*, 193-198, doi:10.1016/j.lungcan.2003.10.006.

84. Yu, S.; Sun, L.; Jiao, Y.; Lee, L.T.O. The Role of G Protein-coupled Receptor Kinases in Cancer. *Int J Biol Sci* **2018**, *14*, 189-203, doi:10.7150/ijbs.22896.

85. 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, doi:10.1016/s0006-291x(02)02888-7.

86. Esteller, M. Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum Mol Genet* **2007**, *16 Spec No 1*, R50-59, doi:10.1093/hmg/ddm018.

87. Golovastova, M.O.; Bazhin, A.V.; Philippov, P.P. Cancer-retina antigens -- a new group of tumor antigens. *Biochemistry (Mosc)* **2014**, *79*, 733-739, doi:10.1134/S000629791408001X.

88. Bazhin, A.V.; De Smet, C.; Golovastova, M.O.; Schmidt, J.; Philippov, P.P. Aberrant demethylation of the recoverin gene is involved in the aberrant expression of recoverin in cancer cells. *Exp Dermatol* **2010**, *19*, 1023-1025, doi:10.1111/j.1600-0625.2010.01126.x.

89. Matsubara, S.; Yamaji, Y.; Sato, M.; Fujita, J.; Takahara, J. Expression of a photoreceptor protein, recoverin, as a cancer-associated retinopathy autoantigen in human lung cancer cell lines. *Br J Cancer* **1996**, *74*, 1419-1422, doi:10.1038/bjc.1996.558.

90. Ohguro, H.; Ogawa, K.; Nakagawa, T. Recoverin and Hsc 70 are found as autoantigens in patients with cancer-associated retinopathy. *Invest Ophthalmol Vis Sci* **1999**, *40*, 82-89.

91. Ohguro, H.; Nakazawa, M. Pathological roles of recoverin in cancer-associated retinopathy. *Adv Exp Med Biol* **2002**, *514*, 109-124, doi:10.1007/978-1-4615-0121-3_7.

92. Bazhin, A.V.; Schadendorf, D.; Philippov, P.P.; Eichmüller, S.B. Recoverin as a cancer-retina antigen. *Cancer Immunol Immunother* **2007**, *56*, 110-116, doi:10.1007/s00262-006-0132-z.

93. Bazhin, A.V.; Shifrina, O.N.; Savchenko, M.S.; Tikhomirova, N.K.; Goncharskaia, M.A.; Gorbunova, V.A.; Senin, II; Chuchalin, A.G.; Philippov, P.P. Low titre autoantibodies against recoverin in sera of patients with small cell lung cancer but without a loss of vision. *Lung Cancer* **2001**, *34*, 99-104, doi:10.1016/s0169-5002(01)00212-4.

94. Adamus, G.; Ortega, H.; Witkowska, D.; Polans, A. Recoverin: a potent uveitogen for the induction of photoreceptor degeneration in Lewis rats. *Exp Eye Res* **1994**, *59*, 447-455, doi:10.1006/exer.1994.1130.

95. Thirkill, C.E. Experimental, cancer-induced retinopathy. *Ocul Immunol Inflamm* **1997**, *5*, 55-65, doi:10.3109/09273949709085051.

96. Kitai, H.; Sakakibara-Konishi, J.; Oizumi, S.; Hirohashi, Y.; Saito, W.; Kanda, A.; Sato, N.; Nishimura, M. Spontaneous regression of small cell lung cancer combined with cancer associated retinopathy. *Lung Cancer* **2015**, *87*, 73-76, doi:10.1016/j.lungcan.2014.10.015.

97. Rong, S.; Ikeda, H.; Sato, Y.; Hirohashi, Y.; Takamura, Y.; Sahara, H.; Sato, T.; Maeda, A.; Ohguro, H.; Sato, N. Frequent detection of anti-recoverin cytotoxic T-lymphocyte precursors in peripheral blood of cancer patients by using an HLA-A24-recoverin tetramer. *Cancer Immunol Immunother* **2002**, *51*, 282-290, doi:10.1007/s00262-002-0282-6.

98. 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, doi:10.1620/tjem.202.213.

99. Matsuo, S.; Ohguro, H.; Ohguro, I.; Nakazawa, M. Clinicopathological roles of aberrantly expressed recoverin in malignant tumor cells. *Ophthalmic Res* **2010**, *43*, 139-144, doi:10.1159/000253486.

100. Edelman, R. An overview of adjuvant use. *Vaccine Adjuvants* **2000**, 1-27.

101. Ellis, R.J. Protein misassembly: macromolecular crowding and molecular chaperones. *Adv Exp Med Biol* **2007**, *594*, 1-13, doi:10.1007/978-0-387-39975-1_1.

102. Jee, H. Size dependent classification of heat shock proteins: a mini-review. *J Exerc Rehabil* **2016**, *12*, 255-259, doi:10.12965/jer.1632642.321.

103. Rosenzweig, R.; Nillegoda, N.B.; Mayer, M.P.; Bukau, B. The Hsp70 chaperone network. *Nat Rev Mol Cell Biol* **2019**, *20*, 665-680, doi:10.1038/s41580-019-0133-3.

104. Calderwood, S.K.; Gong, J. Heat Shock Proteins Promote Cancer: It's a Protection Racket. *Trends Biochem Sci* **2016**, *41*, 311-323, doi:10.1016/j.tibs.2016.01.003.

105. Baldin, A.V.; Zamyatnin, A.A., Jr.; Bazhin, A.V.; Xu, W.H.; Savvateeva, L.V. Advances in the Development of Anticancer HSP-based Vaccines. *Curr Med Chem* **2019**, *26*, 427-445, doi:10.2174/0929867325666180129100015.

106. Udono, H.; Srivastava, P.K. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* **1993**, *178*, 1391-1396, doi:10.1084/jem.178.4.1391.

107. Thériault, J.R.; Adachi, H.; Calderwood, S.K. Role of scavenger receptors in the binding and internalization of heat shock protein 70. *J Immunol* **2006**, *177*, 8604-8611, doi:10.4049/jimmunol.177.12.8604.

108. Stocki, P.; Morris, N.J.; Preisinger, C.; Wang, X.N.; Kolch, W.; Multhoff, G.; Dickinson, A.M. Identification of potential HLA class I and class II epitope precursors associated with heat shock protein 70 (HSPA). *Cell Stress Chaperones* **2010**, *15*, 729-741, doi:10.1007/s12192-010-0184-z.

109. Luo, X.; Zuo, X.; Zhou, Y.; Zhang, B.; Shi, Y.; Liu, M.; Wang, K.; McMillian, D.R.; Xiao, X. Extracellular heat shock protein 70 inhibits tumour necrosis factor-alpha induced proinflammatory mediator production in fibroblast-like synoviocytes. *Arthritis Res Ther* **2008**, *10*, R41, doi:10.1186/ar2399.

110. Stocki, P.; Wang, X.N.; Dickinson, A.M. Inducible heat shock protein 70 reduces T cell responses and stimulatory capacity of monocyte-derived dendritic cells. *J Biol Chem* **2012**, *287*, 12387-12394, doi:10.1074/jbc.M111.307579.

111. Bausinger, H.; Lipsker, D.; Ziylan, U.; Manié, S.; Briand, J.P.; Cazenave, J.P.; Muller, S.; Haeuw, J.F.; Ravanat, C.; de la Salle, H.; et al. Endotoxin-free heat-shock protein 70 fails to induce APC activation. *Eur J Immunol* **2002**, *32*, 3708-3713, doi:10.1002/1521-4141(200212)32:12<3708::Aid-immu3708>3.0.Co;2-c.

112. Ishii, T.; Udono, H.; Yamano, T.; Ohta, H.; Uenaka, A.; Ono, T.; Hizuta, A.; Tanaka, N.; Srivastava, P.K.; Nakayama, E. Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. *J Immunol* **1999**, *162*, 1303-1309.

113. Tamura, Y.; Peng, P.; Liu, K.; Daou, M.; Srivastava, P.K. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* **1997**, *278*, 117-120, doi:10.1126/science.278.5335.117.

114. Belli, F.; Testori, A.; Rivoltini, L.; Maio, M.; Andreola, G.; Sertoli, M.R.; Gallino, G.; Piris, A.; Cattelan, A.; Lazzari, I.; et al. Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes: clinical and immunologic findings. *J Clin Oncol* **2002**, *20*, 4169-4180, doi:10.1200/jco.2002.09.134.

115. Mazzaferro, V.; Coppa, J.; Carrabba, M.G.; Rivoltini, L.; Schiavo, M.; Regalia, E.; Mariani, L.; Camerini, T.; Marchianò, A.; Andreola, S.; et al. Vaccination with autologous tumor-derived heat-shock protein gp96 after liver resection for metastatic colorectal cancer. *Clin Cancer Res* **2003**, *9*, 3235-3245.

116. Jonasch, E.; Wood, C.; Tamboli, P.; Pagliaro, L.C.; Tu, S.M.; Kim, J.; Srivastava, P.; Perez, C.; Isakov, L.; Tannir, N. Vaccination of metastatic renal cell carcinoma patients with autologous tumour-derived vitespen vaccine: clinical findings. *Br J Cancer* **2008**, *98*, 1336-1341, doi:10.1038/sj.bjc.6604266.

117. Reitsma, D.J.; Combest, A.J. Challenges in the development of an autologous heat shock protein based anti-tumor vaccine. *Hum Vaccin Immunother* **2012**, *8*, 1152-1155, doi:10.4161/hv.21382.

118. Blachere, N.E.; Li, Z.; Chandawarkar, R.Y.; Suto, R.; Jaikaria, N.S.; Basu, S.; Udono, H.; Srivastava, P.K. Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J Exp Med* **1997**, *186*, 1315-1322, doi:10.1084/jem.186.8.1315.

119. Li, Z.; Ju, X.; Silveira, P.A.; Abadir, E.; Hsu, W.H.; Hart, D.N.J.; Clark, G.J. CD83: Activation Marker for Antigen Presenting Cells and Its Therapeutic Potential. *Front Immunol* **2019**, *10*, 1312, doi:10.3389/fimmu.2019.01312.

120. Reis e Sousa, C. Activation of dendritic cells: translating innate into adaptive immunity. *Curr Opin Immunol* **2004**, *16*, 21-25, doi:10.1016/j.coi.2003.11.007.

121. Sharpe, A.H. Mechanisms of costimulation. *Immunol Rev* **2009**, *229*, 5-11, doi:10.1111/j.1600-065X.2009.00784.x.

122. Borish, L.C.; Steinke, J.W. 2. Cytokines and chemokines. *J Allergy Clin Immunol* **2003**, *111*, S460-475, doi:10.1067/mai.2003.108.

123. Leonard, W.J.; Lin, J.X. Cytokine receptor signaling pathways. *J Allergy Clin Immunol* **2000**, *105*, 877-888, doi:10.1067/mai.2000.106899.

124. Li, Y.; Yang, R.; Chen, L.; Wu, S. CD38 as an immunomodulator in cancer. *Future Oncol* **2020**, *16*, 2853-2861, doi:10.2217/fon-2020-0401.

125. Gao, J.; Luo, S.M.; Peng, M.L.; Deng, T. Enhanced immunity against hepatoma induced by dendritic cells pulsed with Hsp70-H22 peptide complexes and CD40L. *J Cancer Res Clin Oncol* **2012**, *138*, 917-926, doi:10.1007/s00432-012-1166-6.

126. Cao, G.; Cui, R.; Liu, C.; Zhang, G.; Zhang, Z. MTBHsp70-exFPR1-pulsed Dendritic Cells Enhance the Immune Response against Cervical Cancer. *J Cancer* **2019**, *10*, 6364-6373, doi:10.7150/jca.29779.

127. Golstein, P.; Griffiths, G.M. An early history of T cell-mediated cytotoxicity. *Nat Rev Immunol* **2018**, *18*, 527-535, doi:10.1038/s41577-018-0009-3.

128. 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, doi:10.1002/1521-4141(200208)32:8<2300::Aid-immu2300>3.0.Co;2-7.

129. Nikoopour, E.; Lin, C.M.; Sheskey, S.; Heckenlively, J.R.; Lundy, S.K. Immune Cell Infiltration into the Eye Is Controlled by IL-10 in Recoverin-Induced Autoimmune Retinopathy. *J Immunol* **2019**, *202*, 1057-1068, doi:10.4049/jimmunol.1800574.

130. Perret, R.; Ronchese, F. Memory T cells in cancer immunotherapy: which CD8 T-cell population provides the best protection against tumours? *Tissue Antigens* **2008**, *72*, 187-194, doi:10.1111/j.1399-0039.2008.01088.x.

131. Park, S.L.; Gebhardt, T.; Mackay, L.K. Tissue-Resident Memory T Cells in Cancer Immunosurveillance. *Trends Immunol* **2019**, *40*, 735-747, doi:10.1016/j.it.2019.06.002.

132. Nizard, M.; Roussel, H.; Diniz, M.O.; Karaki, S.; Tran, T.; Voron, T.; Dransart, E.; Sandoval, F.; Riquet, M.; Rance, B.; et al. Induction of resident memory T cells enhances the efficacy of cancer vaccine. *Nat Commun* **2017**, *8*, 15221, doi:10.1038/ncomms15221.

133. Crosby, E.J.; Gwin, W.; Blackwell, K.; Marcom, P.K.; Chang, S.; Maecker, H.T.; Broadwater, G.; Hyslop, T.; Kim, S.; Rogatko, A.; et al. Vaccine-Induced Memory CD8(+) T Cells Provide Clinical Benefit in HER2 Expressing Breast Cancer: A Mouse

to Human Translational Study. *Clin Cancer Res* **2019**, *25*, 2725-2736, doi:10.1158/1078-0432.Ccr-18-3102.

134. Schluns, K.S.; Lefrançois, L. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* **2003**, *3*, 269-279, doi:10.1038/nri1052.

135. Rolle, C.E.; Carrio, R.; Malek, T.R. Modeling the CD8+ T effector to memory transition in adoptive T-cell antitumor immunotherapy. *Cancer Res* **2008**, *68*, 2984-2992, doi:10.1158/0008-5472.Can-07-3040.

136. Wachstein, J.; Tischer, S.; Figueiredo, C.; Limbourg, A.; Falk, C.; Immenschuh, S.; Blasczyk, R.; Eiz-Vesper, B. HSP70 enhances immunosuppressive function of CD4(+)CD25(+)FoxP3(+) T regulatory cells and cytotoxicity in CD4(+)CD25(-) T cells. *PLoS One* **2012**, *7*, e51747, doi:10.1371/journal.pone.0051747.

137. Liu, A.; Ferretti, C.; Shi, F.D.; Cohen, I.R.; Quintana, F.J.; La Cava, A. DNA Vaccination With Hsp70 Protects Against Systemic Lupus Erythematosus in (NZB x NZW)F1 Mice. *Arthritis Rheumatol* **2020**, *72*, 997-1002, doi:10.1002/art.41202.

138. Keltner, J.L.; Thirkill, C.E. Cancer-associated retinopathy vs recoverin-associated retinopathy. *Am J Ophthalmol* **1998**, *126*, 296-302, doi:10.1016/s0002-9394(98)00212-8.

139. Sprooten, J.; Ceusters, J.; Coosemans, A.; Agostinis, P.; De Vleeschouwer, S.; Zitvogel, L.; Kroemer, G.; Galluzzi, L.; Garg, A.D. Trial watch: dendritic cell vaccination for cancer immunotherapy. *Oncoimmunology* **2019**, *8*, e1638212, doi:10.1080/2162402x.2019.1638212.

140. Boyiadzis, M.M.; Dhodapkar, M.V.; Brentjens, R.J.; Kochenderfer, J.N.; Neelapu, S.S.; Maus, M.V.; Porter, D.L.; Maloney, D.G.; Grupp, S.A.; Mackall, C.L.; et al. Chimeric antigen receptor (CAR) T therapies for the treatment of hematologic malignancies: clinical perspective and significance. *J Immunother Cancer* **2018**, *6*, 137, doi:10.1186/s40425-018-0460-5.

**Supplement Table 1: Overview of the 6 staining tubes that indicate DC and its subsets.**

Unstained tube: cells without FACS antibodies staining; FMO1 tube: cells stained with all antibodies except for anti-CD14 and CD274 antibodies; FMO2 tube: cells stained with all antibodies except for anti-CD33 antibody; FMO3 tube: cells stained with all antibodies except for anti-CD11c antibody; FMO4 tube: cells stained with anti-CD45, CD3, CD14, CD33, CD11c, CD274 antibodies; Sample tube: cells stained with all antibodies as mentioned below. (--, no antibody was added).

| DC characterization/ function | BV650 | PerCP Cy5.5 | BV510 | BV786 | PE | PE-Cy7 | APC-H7 | BB515 | BUV395 | BV421 | APC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Unstained | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| FMO 1 | CD45 | CD3 | -- | CD33 | CD11c | CD11b | HLA-DR | CD80 | CD86 | CD83 | -- |
| FMO 2 | CD45 | CD3 | CD14 | -- | CD11c | CD11b | HLA-DR | CD80 | CD86 | CD83 | CD274 |
| FMO 3 | CD45 | CD3 | CD14 | CD33 | -- | CD11b | HLA-DR | CD80 | CD86 | CD83 | CD274 |
| FMO 4 | CD45 | CD3 | CD14 | CD33 | CD11c | -- | -- | -- | -- | -- | CD274 |
| Sample | CD45 | CD3 | CD14 | CD33 | CD11c | CD11b | HLA-DR | CD80 | CD86 | CD83 | CD274 |

**Supplement Table 2: Overview of the 6 staining tubes that indicate T cell and its subsets.**

Unstained tube: cells without FACS antibodies staining; FMO1 tube: cells stained with all antibodies except for anti-CD3 antibodiy; FMO2 tube: cells stained with all antibodies except for anti-CD4 antibody; FMO3 tube: cells stained with all antibodies except for anti-CD8 antibody; FMO4 tube: cells stained with anti-CD45, CD3, CD4, CD8, antibodies; Sample tube: cells stained with all antibodies as mentioned below. (--, no antibody was added).

| T cell activation | BV650 | PerCP Cy5.5 | BUV395 | APC-H7 | BB515 | BV605 | PE-CF594 | APC |
|---|---|---|---|---|---|---|---|---|
| unstained | -- | -- | -- | -- | -- | -- | -- | -- |
| FMO 1 | CD45 | -- | CD4 | CD8 | CD25 | CD38 | CD127 | CD279 |
| FMO 2 | CD45 | CD3 | -- | CD8 | CD25 | CD38 | CD127 | CD279 |
| FMO 3 | CD45 | CD3 | CD4 | -- | CD25 | CD38 | CD127 | CD279 |
| FMO4 | CD45 | CD3 | CD4 | CD8 | -- | -- | -- | -- |
| Sample | CD45 | CD3 | CD4 | CD8 | CD25 | CD38 | CD127 | CD279 |

# Acknowledgment

Firstly, I would like to thank my supervisor, Prof. Dr. Alexandr Bazhin, who is a very gentle and warm person. I really cherish the opportunity to work with him and appreciate everything he provides to support this research work. Whenever I encountered research obstacles or scientific questions, he can always offer me helpful suggestions for my project and encourage me a lot to move forward. It is an unforgettable period to accomplish this study under the guidance of Prof. Dr. Alexandr Bazhin.

I also would like to thank my co-supervisor, Dr. Alexey V Baldin, who established this project and synthesized the Recoverin fusion protein for further research. He provides me with plenty of instructions to improve this study. Without his previous fundamental investigation, this work could not be completed nicely.

Many thanks to the technicians in our lab, including Michaela Svihla, Johanna Knallinger, and Nicole Strobl. They generally provided me with scientific technic and research methods, especially in the field of immunological assays. With their patient and detailed training, I mastered research skills and am capable to perform experiments independently to complete this project.

Grateful to the China Scholarship Councill, which grants me personal financial support to cover my daily spendings in Munich (reference number: CSC201906310088).

Lastly, deep gratitude was given to my parents, Jianhe Zhao and Xuefang Yang, as well as my girlfriend, Yan Zhang. Their warm spirit encouragements are one of the most important motivations for me to finish this study.

These years millions of valuable lives in the world were taken because of the widely spread of pandemic Covid-19. Numerous researchers contributed to combating the pandemic via investigating prophylactic or therapeutic approaches, especially the exploitation of vaccines. Even though I did not work in the field of prophylactic vaccines against infectious diseases, I believe that our final goal is the same — to improve human health and prolong the lifespan as a whole. Gladly, I am a part of the scientific researchers and I am willing to devote my career to this great cause.

# Affidavit

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dean's Office
Faculty of Medicine

MMRS

## Affidavit

Zhao Yue

Surname, first name

Street

Zip code, town

Country

I hereby declare, that the submitted thesis entitled

The feasibility to exploit fusion protein comprising Recoverin and HSP70 as cancer vaccine

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Planegg, 28.07.2022

Place, date

Yue Zhao

Signature doctoral candidate

# List of publications


1. **Zhao, Y.**; Baldin, A.V.; Isayev, O.; Werner, J.; Zamyatnin, A.A., Jr.; Bazhin, A.V. Cancer Vaccines: Antigen Selection Strategy. Vaccines (Basel) 2021, 9, doi:10.3390/vaccines9020085.
2. Soltani, M*; **Zhao, Y.***; Xia, Z; Ganjalikhani-Hakemi, M; Bazhin, A.V. The importance of cellular metabolic pathways in pathogenesis and selective treatments of hematological malignancies. Front Oncol 2021, 11, doi:10.3389/fonc.2021.767026.
3. Bidram, M.; **Zhao, Y.**; Shebardina, N.G.; Baldin, A.V.; Bazhin, A.V.; Ganjalikhany, M.R.; Zamyatnin, A.A., Jr.; Ganjalikhani-Hakemi, M. mRNA-Based Cancer Vaccines: A Therapeutic Strategy for the Treatment of Melanoma Patients. Vaccines (Basel) 2021, 9, doi:10.3390/vaccines9101060.