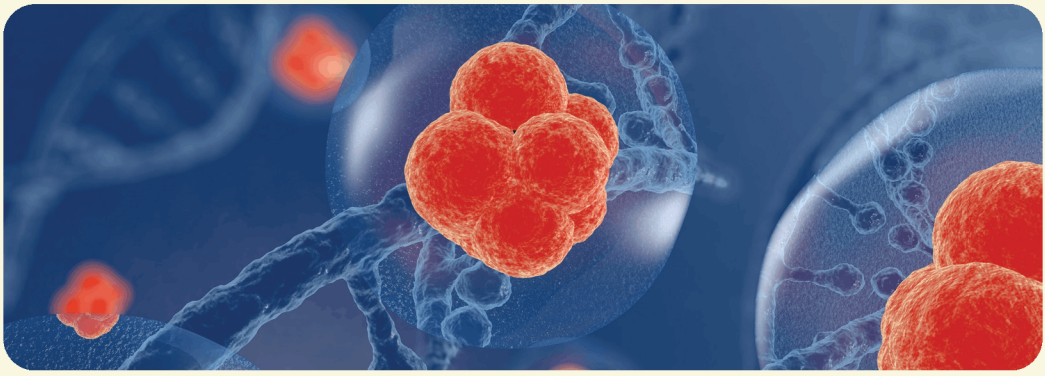
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Advances in Cancer Immunotherapy and Stem Cell Research



EDITORS
Zeynep KARAKAŞ
Günnur DENİZ
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ADVANCES IN CANCER IMMUNOTHERAPY AND STEM CELL RESEARCH

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FOREWORD

I am honored to present to you this book, collaboratively written by doctoral students and advisors on current research issues at the institute. This work commemorates the 100th Anniversary of the Republic of Turkey and to the Istanbul University's Institute of Graduate Studies in Health Sciences, which earned the ORPHEUS (Organisation of PhD in Biomedicine and Health Sciences European System) label in its 41st year. I would like to thank the rectorate of Istanbul University for their contribution to this project's inception and to the Istanbul University Press for bringing it to fruition. As an institution that prioritizes the goal of raising scientists and competent people in their fields, I would like to express my gratitude to the authors who have enriched our book with important topics and the ever-expanding body of knowledge in the field.

Prof. Dr. Zeynep Karakaş

PREFACE

Stem cells are undifferentiated cells and newly developed molecular techniques have made significant advances in our ability to study the development of stem cells. An important stimulus for the development of these techniques has been the identification of specific stem cells for gene therapy. Stem cell research plays an important role in tissue regeneration, treatment of neurological disorders, treatment of cardiovascular diseases and treatment of brain diseases. Some studies have shown that the effects of stem cell therapy can last for several years or even indefinitely, while other studies have shown that the results may be more short-lived. Bringing such complex procedures for revolutionary and reproducible cell replacement therapies into general, common regenerative medicine will require interdisciplinary and international co-operation.

This book, Stem Cell Immunology, consists of the studies prepared within the scope of the Health Sciences PhD theses and the experience and knowledge of the researchers in this field within the scope of the '100th Anniversary of Republic of Türkiye Project' designed by Istanbul University. All chapters were prepared with students continuing their PhD education and/or graduates and their advisors.

This book provides up-to-date reviews on a wide range of stem cell immunology. Chapters range from a description of current knowledge of the biology of stem cells to current molecular biological approaches and clinical applications. We hope it will be useful to all readers.

Prof. Dr. Günnur Deniz and Prof. Dr. Fatma Savran Oğuz

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We would like to express our sincere thanks to Assoc. Prof. Dr. Nurcan Orhan as assistant directors of Institute of Graduate Studies in Health Science (June 2022-August 2023), Prof. Dr. Merva Soluk Tekkeşin, Assoc. Prof. Dr. Meryem Sedef Erdal and Prof. Dr. Ayşe Evrim Bayrak as members of Institute Director Board for their contribution to the formation and preparation of the books. We would also like to extend special thanks to Barış Ertuğrul, PhD, for the images of the book covers.

CHAPTER 1

THE BASICS OF CANCER IMMUNOTHERAPY

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ABSTRACT

Cancer is an umbrella term for more than 100 different malignant diseases. Global burden of cancer remains high necessating more effective treatment modalities. Although the concept of cancer immunotherapy is a relatively old view, recent developments in this field in the past years have revolutionized cancer management. Compared to conventional cancer therapies such chemotherapy and radiotherapy, immunotherapy is a more dynamic treatment modality and comprises a broad and rapidly growing group of therapies and therapeutic agents. The strength of immunotherapy lies in its ability to activate the immune system to specifically target cancer cells with less side effects. Cellular therapies with modified/engineered T cells and the immune checkpoint blockade currently represent most emerging and promising treatment options. In this Chapter. we describe the basic concepts and principles of cancer immunotherapy with a spotlight on the immune checkpoint inhibition therapy.

Keywords: Cancer, immunotherapy, cellular therapy, monoclonal antibodies, immune checkpoint inhibition

1. Introduction

Cancer is a complex disease that includes more than 100 distinct diseases of different tissues and organs, which considerably differ in tumor biology, response to treatment and prognosis. Global burden of cancer is high and tends to increase as it is estimated that by 2030 about 25 million new cases and 17 million deaths will be recorded each year (1). Cancer is the first or second main cause of death in individuals under the age of 70 in most countries. This trend in cancer mortality worldwide also reflects notable declines in mortality rates by circulatory system and coronary heart disease in many countries (1). Incidence and mortality rates of individual cancer types vary considerably. Prostate, lung and colorectal cancers are the most frequently diagnosed maligant tumors in men worldwide and account for approximately. 45% of all cancers. In women, breast cancer is the most frequent malignancy accounting for 30% of female cancers followed by lung and colorectal cancers (2). Lung cancer remains the leading cause of cancer deaths accounting for 18% of all cancer-related deaths, followed by colorectal (9.4%), liver (8.3%), gastric (7.7%) and female breast cancers (6.9%).

Surgery, chemotherapy, radiation therapy are traditional cancer treatments while their combination is often used in the management of cancer patients. Hormone therapy, stem cell and bone marrow transplantation represent other types of therapies utilized to treat patients with various malignancies. In last decades, a deeper understanding of mechanisms underlying cancer development and progression led to development of more recent and effective treatment options such as targeted therapies which are being applied more frequently. Similarly, the elucidation of anti-tumor immune responses and mechanisms underlying immune escape of cancer cells enabled scientists to establish immunotherapeutic treatments that represent the most recent treatment strategy in oncology. Compared to radiotherapy and chemotherapy, immunotherapy is a more dynamic treatment modality and may modulate and strengthen the immune system in multiple routes to counteract cancer cells (3). The research on the mechanisms of immune escape and immunotherapy-related drugs is expanding and the application of validated drugs in different cancer types is gradually increasing. Combination of immunotherapy with traditional cancer therapies (e.g. chemotherapy and radiotherapy) represents a further option to achieve an enhanced effect. In this Chapter, after a brief description of the role of immune cells in anti-tumor immunity we describe the basic concepts of cancer immunotherapy with a focus on the immune checkpoint inhibition.

2. The Immune System Components and Their Role in Anti-tumor Immunity

The immune system with all its components fights cancer cells, and both innate and adaptive immune responses are involved in the anti-tumor immunity. Several cell types of innate immune system such as macrophages, neutrophils, natural killer (NK) cells, dendritic cells, and the cells of adaptive immune system such as T and B lymphocytes are engaged in the anti-tumor immune responses. Macrophages are differentiated forms of circulating

monocytes in tissues and involved in tissue homeostasis (4). Activated macrophages could be as either proinflammatory (M1 type) or anti-inflammatory (M2-type). Of these, M1-type macrophages have anti-tumor properties. As the tumor progresses, M1-type macrophages undergo a polarization (e.g. M2-like) that confers them pro-tumorigenic characteristics in tumor microenvironment. Neutrophils as key players of inflammation also possess anti-tumor activities. However, similar to macrophage polarization, neutrophils exist in two polarization states (N1and N2), which harbor anti-tumor and pro-tumor activities, respectively (5). High levels of tumor-associated neutrophils have been shown to be indicative of poor prognosis in different malignances.

NK cells also have a proven anti-tumor effect. They are an essential component of innate system in the immune surveillance and possess inhibitory and stimulatory receptors that act differentially (6). The inhibitory receptors target cancer cells, which hide their major histocompatibility, class I (MHC-I) to evade immune surveillance and kill them with their potent cytolytic activity. In contrast, in healthy cells expressing MHC-I molecules, the interaction of MHC-I molecules with their receptors on NK cells plays an inhibitory effect on the NK cell function. NK cell infiltration in tumors has been shown to correlate with a favorable outcome in a variety of cancers including colorectal and gastric tumors (6). Dendritic cells, which exist in every tissue across the body, are the specialized antigen-presenting cells intervening the interface between innate and adaptive immunity. These cells are committed to present cancer antigens to naïve and memory T cells via their MHC molecules which results in triggering an effector T cell response. Tumor-infiltrating dendritic cells have been documented in many cancers (7).

T lymphocytes are principial components of the adaptive immune system, acting as orchestrators and effector cells of cellular anti-tumor immunity. Of two conventional T cell types, $CD4^+$ T acquire a functional role in the immunity whereas $CD8^+$ T cells are the most notable anti-tumor cells which upon priming, differentiate into cytotoxic T lymphocytes. Presentation of cancer antigens by antigen-presenting cells to $CD4^+$ and $CD8^+$ T cells and the killing of the target tumor cells in an antigen specific way by the activated effector $CD8^+$ T cells is the main route of anti-tumor immune response (8). Cancer antigens can be tumor-specific or tumor-associated. Tumor-specific antigens are unique to tumor cells and derive from mutated proteins, cancer-germline proteins or oncogenic viruses. In contrast, tumor-associated antigens are not unique to tumor cells and include various types of antigens including differentiation antigens such as prostate-specific antigen, tyrosinase, carcinoembryonic antigen, and proteins that are overexpressed in tumors like survivin, erbB-2 (9).

3. Strategies of Cancer Immunotherapy

Cancer immunotherapy is based on two broad strategies: i) targeting tumor directly and ii) activating or modulating immune cells that in turn target the tumor. Targeting tumors by monoclonal antibodies developed against specific targets on tumor cells falls within the first category. Cellular therapies and immune checkpoint blockade by specific inhibitors are immunotherapy approaches in the second category (10).

3.1. Tumor-targeting Monoclonal Antibodies

Antibodies generated by unique B cell clones and bind to specific portions of an antigen (e.g. epitope) are called monoclonal antibodies (mAbs). The large scale production of mAbs by hybridoma technology in 1970s has been a breakthrough in the cancer immunotherapy (11). Since the initial description of mAb production by the hybridomas (e.g. the mouse-human hybrid cells) in 1975, significant advances have been made in the generation and clinical application of mAbs. First hybridoma-based antibodies developed in 1980s had extensive limitations as due to their murine origin they were immunogenic in humans, and induced poor immunity in patients. In the late 1980s, researches succeeded in developing techniques to humanize murine-derived antibodies and eliminate such limitations (12). Further advances have enabled to design fully humanized antibodies using transgenic mice or *in vitro* yeast or phage display assays. These technical innovations made mAbs a valuable component of cancer immunotherapy. mAbs can be classified according to their respective targets or functions, and direct tumor-targeting mAbs and checkpoint inhibitors are two main classes with a wide use in oncology. Additionally, mAbs can be modified by adding specific conjugates to enhance tumor-killing activity. Agents frequently conjugated to mAbs include immunotoxins, radioisotopes or cytotoxic drugs (13).

Tumor-targeting antibodies accomplish their effector mechanisms via multiple ways which include ligand blocking, blocking cell signaling, the induction of tumor-killing through antibody-dependent cellular cytotoxicity (ADCC) by NK cells or macrophages, complement activation and also through the activation of cytotoxic T cells (14). Over the past thirty years, many mAbs against different targets have been developed and approved as cancer therapeutics. CD20 molecule which is expressed on B lymphocytes of patients with non-Hodgkin's lymphoma (NHL), but not on healthy immature B cells, was the first target for a mAb therapy. Rituximab is a chimeric mouse/human mAb which specifically binds to CD20 and the first tumor-targeting antibody to be approved in oncology (15). Even if it has been initally approvaed in indolent lymphomas in 1997, rituximab is being used in both indolent and aggressive lymphomas, and several clinical trails showed the superiority of its combination with chemotherapy in first-line treatmet of $CD20^+$ NHL (16).

Some of mAbs developed over last 2-3 decades were directed to antigens overexpressed on cancer cells of solid tumors. Such targets, among others, include the receptor tyrosine

kinase HER2 (also known as erbB2 or neu) and epidermal growth receptor (EGFR), and mAbs directed them are approved for the treatment of patients with breast and colorectal cancer, respectively (17, 18). HER2 proto-oncogene is overexpressed in approximately. 20%-30% of breast tumors and in lower percentages of ovarian and gastric tumors, and HER2 overexpression is generally associated with poor clinical outcomes. Trastuzumab, the anti-HER2 mAb, is a humanized IgG1 murine monoclonal antibody targeting the extracellular domain of HER2. This antibody is being used in neoadjuvant, adjuvant and metastatic settings and has been the first approved treatment for patients with early stage HER2-positive breast tumors in combination with chemotherapy (19). In addition, trastuzumab is applied as a monotherapy after adjuvant treatment and improves survival for patients with advanced HER2-positive breast cancer (19). EGFR pathway is deregulated in many cancer types. Cetuximab, a recombinant chimeric monoclonal IgG1 antibody, inhibits EGFR and is approved for the first-line treatment of patients with metastatic colorectal cancer whose tumor cells express EGFR without any RAS gene mutations. It has been documented that cetuximab is more effective in combination with chemotherapy in advanced patients without RAS mutations (20).

Cancer cells secrete many pro-angiogenic factors. Among these, vascular endothelial growth factors (VEGFs, especially VEGF-A) are key molecules for inducing tumor angiogenesis. VEGFs activate angiogenic signaling in endothelial cells by binding to VEGF receptors which harbor tyrosine kinase activity. Bevacizumab, the first available anti-angiogenic therapy, is a humanized monoclonal antibody binding to soluble VEGF forms and blocks the interaction with their receptors and thereby inhibits the activation of angiogenic signaling and neo-vascularization. This monoclonal antibody was first approved for the treatment of patients with advanced colorectal cancer in 2004, followed by approval in advanced lung and kidney cancer and glioblastoma in the following years (21).

3.2. Adoptive Cell Therapy

Adoptive cell therapy (ACT), also referred to as cellular immunotherapy, is a treatment modality wherein effector immune cells are injected systemically to a patient to eradicate whose tumor. All ACT protocols employ cells harvested from the patient's blood or tumor which are activated and expanded in *ex vivo* conditions (22). ACT may be nonspecific or specific; in non-specific type, immune cells such dendritic cells, NK cells, tumor-infiltrating lymphocytes (TILs) or lymphocyte activated killer cells are employed. Howewer, due to its non-specific nature, this type of cellular therapy is associated with a poor targeting and killing of tumor cells. In specific ACT, two main approaches are utilized to redirect cytotoxic T cells for an efficient anti-tumor response. In the first approach TILs induced by a specific tumor antigen *ex vivo* are given back to patient without any genetic manipulation. Even if the immunotherapy by TILs is an old view, thanks to recent advances in understanding of tumor

microenvironment, immune surveillance and immune checkpoint biology, it has regained attention as a safe personalized immunotherapy, and currently, the efficacy of TIL therapy is being evaluated in several clinical trials (23). The second scheme of specific cellular therapies involves genetic engineering of patient-derived T cells to potentiate cellular immunity against a specific target, with CAR-T cells being best example.

Chimeric antigen receptor T cells (CAR-T cells) are typically genetically manipulated T cells (24). CARs are bioengineered synthetic receptors and possess the specificity of a mAb and a signaling domain required for inducing a signaling cascade in the CAR-engineered T cells. Genetic engineering of CAR-T cells is achieved by inserting protein-coding sequences of target antigen using lentiviral or retroviral vectors. Structurally, CARs have 3 domains: the extracellular domain which represents an antigen-specific immunoglobin and recognizes antigen; the transmembrane domain is a hydrophobic alpha helix and an essential part for receptor stability. The intracellular domain is the functional unit of CARs; upon stimulation it undergoes conformational changes which enable the recruitment and phosphorylation of downstream signaling proteins. Since the initial construction, CARs have undergone several modifications made in intracellular domain over last 30 years and so multiple generations of CAR-T cells have been achieved (24).

CAR-T cells are similar to activated T cells in many of their immunological characteristics as they are able to generate a specific immune response with a long-term protection in patient. However, CARs offer some advanced and superior properties. Firstly, CAR-T cells are more flexible in recognition and killing of tumor cells as they are capable of recognizing antigens in the absence of MHC presentation (24). Secondly, the combination of the antigen-binding ability of a monoclonal antiboy with the cytotoxic effect of T cells make these cells very attractive tools in cancer immunotherapy. Also, unlike TCR which can recognize short peptides upon presentation, CAR-T cells are capable of recognizing tumor antigens as whole proteins, carbohydrates and glycolipids.

Currently, CAR-T based cellular treatments are primarily used in hematological malignancies. The first target of engineered T cells was CD19 which is a transmembrane glycoprotein highly expressed on B cells and involved in B cell activation (25). CD19-specific CAR-T cells have been showed to have efficacy in acute lymphoblastic leukemia (ALL), a malignancy characterized by a rapid proliferation and accumulation of naïve B cells in the bone marrow and also in patients with relapsed or refractory B-ALL. In addition to ALL, this therapy is approved for diffuse large cell lymphoma (DLBCL). Tisagenlecleucel, a second generation CAR-T cell product targeting CD19 antigen, was approved for ALL in 2017 as clinical studies indicated a high remission rate (81%) in children and young adults with relapsed or refractory disease (26). Even if the relapse is a frequent event in patients with hematological malignancies recieving CAR-T cell therapy, the relative success of this

immunotherapy in hematological cancers encourages oncologists to use CAR-T cells for the treatment of solid tumors. However, the efficacy of CAR-T cell-based immunotherapy in solid cancers is currently not satisfying (27).

3.3. Immune Modulation in Cancer Patients by Checkpoint Inhibitors

During cancer progression tumor cells acquire many abilities which enable them to evade immunosurveillance. Immune evasion of tumor cells is achieved through different mechanisms including signaling suppression. Signaling suppression may occur at two levels: supressing the stimulatory signaling and upregulating the inhibitory immunoreceptors. Downregulating surface MHC-I levels by tumor cells is a good example for inhibiting TCR-mediated signaling leading to the escape from immunosurveillance (28). A number of inhibitory immunoreceptors have been identified and studied in cancer over the past few decades, with cytotoxic T-lymphocyte antigen-4 (CTLA-4) and PD-1 being two most clinically relevant targets. Such inhibitory immunoreceptors have been termed as "immune checkpoints" as they act 'gatekeepers' of immune responses. Under normal circumstances inhibitory checkpoints are involved in preventing overactive immune signals or autoimmune disease; however in solid tumors their deregulation prevents the tumor infiltration of T cells leading to the weakening of immune response against tumor cells (29). The concept that blocking the co-inhibitory signaling pathways can restore anti-tumor activity of immune cells has been validated experimentally and clinically. Thus, the development of immune checkpoint inhibitors with a proven efficay in a variety of cancers is regarded a revolutionary breakthrough in the field of immuno-oncology. Additionally, the memory feature of the immune system facilitates a more durable response to immune checkpoint blockade compared with chemotherapy or targeted therapies. However, the major obstacle of this targeted immune therapy is the low response rate (approximately 10% to 30%) in most cancers (30).

3.3.1. CTLA-4 Pathway

The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a co-inhibitory receptor on activated T cells is one of the most studied checkpoints. For an efficent T cell response to tumors, two stimulatory signals are needed: the recognition of MHC-presented antigen by T cell receptor ensures the specificity, and the binding of CD28 receptor on T cells with B7 (CD86) on antigen presenting cell is needed for activation (Figure 1). On the basis of a high structural homology to the CD28, CTLA-4 binds to B7 more effectively than CD28. However, unlike CD28, the binding of CTLA-4 to B7 produces a inhibitory signal and abolishes T cell activation. Ipilimumab, the monoclonal antibody that hinders the signal induced by CTLA-4-B7 interaction was the first approved immune checkpoint inhibitor in advanced melanoma in 2011 (31). Blockade of CTLA-4-B7 interaction by ipilimumab results in the activation and proliferation of effector T cells. In patients with renal cell carcinoma, treatment with ipilimumab has been shown to reduce tumor burden (32). Also in patients with B-cell

lymphoma, metastatic colorectal cancer and hepatocellular carcinoma, anti-tumor activity of ipilimumab has been established. Further, combination of ipilimumab with cytotoxic agents such as carboplatin, etoposide, and paclitaxel led to better clinical outcomes (33).

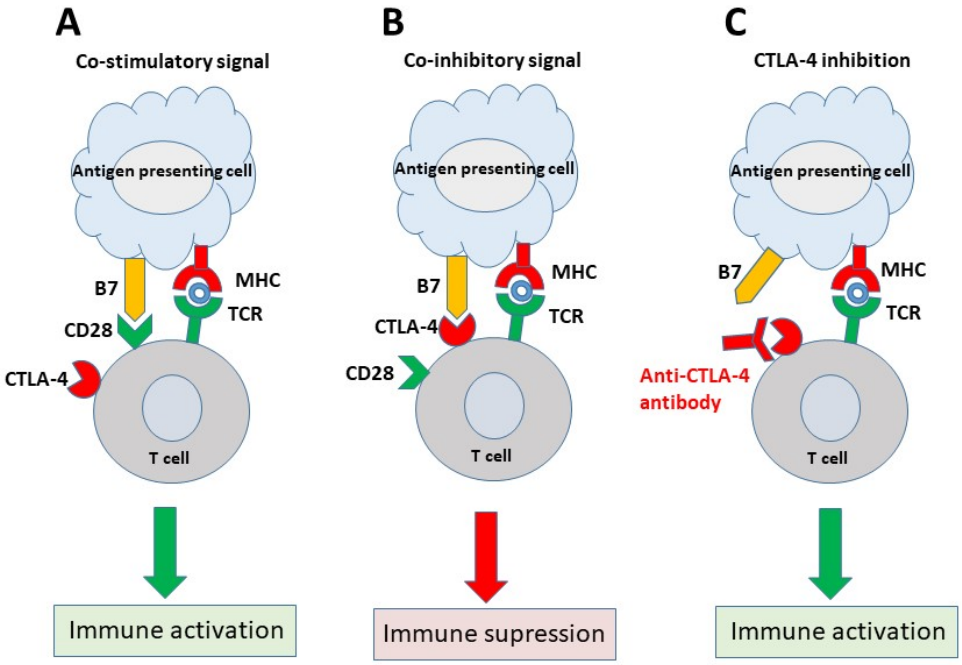


Figure 1: CTLA-4 as a regulatory checkpoint in the immune regulation. A, Binding of CD28 receptor on T cells with B7 on antigen presenting cell is a stimulatory signal for amtigen-specific activation of $CD8^+$ T cells. B, Binding of CTLA-4 to B7 molecule produces a inhibitory signal and abolishes T cell activation. C, Blockade of CTLA-4 by specific antibody restores T cell function. MHC, Major histocompatibility complex; TCR, T cell receptor.

3.3.2. PD-1/PD-L1 Pathway

Accumulating evidence indicates that inhibition of the PD-1/PD-L1 axis is the most successful checkpoint blockade (34). PD-1, discovered in 1992, is a 55-kDa transmembrane protein and expressed on many immune cells such as activated T cells, NK cells and B lymphocytes, macrophages, DCs and monocytes, it plays a key role in the maintaining immune tolerance by preventing overactivation of T cells and avoding autoimmunity (35). This is the benefical role of PD-1 it plays in the immune regulation. However, PD-1 has also deleterous effect in immune escape as the PD-L1 pathway is upregulated in cancer cells with a reduction in T cell activation which then results in T cell exhaustion, thereby immune destruction (36). The ligand of PD-1 (PD-L1) is a B7-series 33-kDa glycoprotein and usually expressed on many cell types such as activated T cells and B cells, macrophages, and DCs. More importantly, PD-L1 is expressed by tumor cells as a mechanism of immune escape as it acts as

a pro-tumorigenic factor via binding to PD-1 and activating proliferative cell signaling (37).

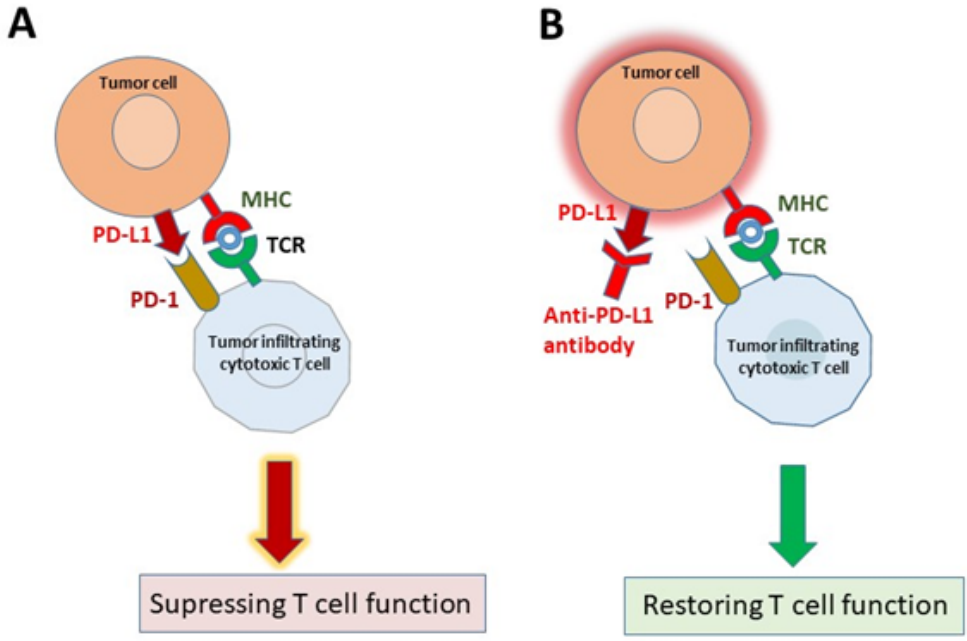


Figure 2: PD-1/PD-L1 axis as a regulatory chekcpoint in the immune regulation. This pathway plays a key role in the maintaining immune tolerance by preventing overactivation of T cells and avoding autoimmunity. A, As cancer cells overexpres PD-L1, this leads to the suppression of T cell function and thereby immunu escape of cancer cells. B, Blockade of PD-1 or PD-L1 by monoclonal antibodies restore the anti-tumor immune respoınse by activating cytotoxic T cells. MHC, Major histocompatibility complex; TCR, T cell receptor.

Anti-PD-1/PD-L1 therapies have been approved to treat patients with various cancer types, such lung, bladder, liver and kidney cancers and lymphoma and, melanoma and (30). Antibody-therapy against both PD-1 (Pembrolizumab, Nivolumab) and PD-L1 (Atezolizumab, Durvalumab) have been developed and approved and it has been shown that anti-PD-L1 antibodies are superior to anti-PD-1 antibodies in reverting PD-1 signaling (38). As the PD-1/PD-L1 axis is a critical regulatory point between tumor cells and immune and stromal cells (Figure 2), the PD-L1 expression in tumor cells as a predictive marker for the effectiveness has been shown to be associated with response to anti-PD-1 therapies in various cancers (39). Thus, immunohistochemical detection of PD-L1 on tumor sections is an approved companion test in some cancers including lung, urothelial carcinoma, cervical cancer, and triple-negative breast cancer so far. However, some subsequent studies did not confirm the use of PD-L1 as a predictive biomarker of chekpoint blockade (39). Here, an important variable is the PD-L1 positivity threshold which is different across cancer types. In lung cancer for instance, patients with a PD-L1 expression score higher than 50% are treated

with the anti-PD-1 antibody pembrolizumab as monotherapy whereas in those with PD-L1 expression <50%, pembrolizumab is combined with chemotherapy.

Although targeting the PD-1/PD-L1 axis is applicable to many cancer types, the overall response rates is variable among patients. A meta analysis from Chen S et al. (40) revelaed that PD-1/PD-L1 inhibitors had an overall response rate (ORR) of 19.56% (ranging from 11% to 32%) in all cancers. Melanoma and lung cancer are two malignant diseases with highest ORR of about 30%. The median duration of response (DOR) was 10.65 months overall and was shorther in lung cancer (8.41 months) than in breast cancer and melanoma (about 15 months). These findings reveal that the blockade of PD-1/PD-L1 axis by specific inhibitors is effective in various tumor types, even if with varying efficacy. Furthermore, the immunotherapy using PD-1/PD-L1 inhibitors has been found to be more effective in first-line treatment than secondary treatment and the combination of immunotherapy with chemotherapy increases the response rates compared to monotherapy (40).

3.4. Factors Modulating the Response to Cancer Immunotherapy by Checkpoint Inhibitors

The low response rate to checkpoint inhibitors in most cancers indicates that their efficacy is modulated by many factors and circumstances. Research is intensively ongoing to clarify the effects of different markers on the checkpoint blockade-based immunotherapy. As stated above, anti-PD-1/PD-L1 or anti-CTLA-4 therapies are not equally effective in all types of cancer or in all cancer patients (41). In some cancer such as lung cancer cancer and melanoma highest reponse rates are reported whereas in some other cancers such colon cancer immune checkpoint inhibitors are less effective. Mechanisms underlying this variable efficay are not fully understood. Intriguingly, in microsatellite stabile cancers such as colorectal cancer anti-PD-1/PD-L1 therapy has been found to have nearly no effect (42). Extensive study has revelaed that many factors such as tumor mutation load, PD-L1 expression, the composition of tumor tumor microenvironment are relevant predictive biomarkers for the efficacy of checkpoint inhibitors (43). However, biomarkers that are helpful in predicting the fully efficacy are still to be determined. Therefore, a deeper understanding of checkpoint biology will enable to develop more effective therapies and/or to enhance the efficacy of available drugs.

PD-L1 expression is the basic predictive marker of the response to anti-PD-1/PD-L1 therapies in some cancers such as lung cancer, as it has been found that the higher the PD-L1 expression is, the better the response to anti-PD-1 inhibitors (44). However, the findings of a meta-analysis including about 4000 cancer patients with advanced or metastatic cancer from eight randomised controlled trials suggest that PD-L1 expression score alone is not sufficient to predict which patients will benefit from PD-1 or PD-L1 blockade therapy in most cancers (45). This differential effect of PD-L1 expression on outcomes of checkpoint blockade therapy

in different cancers is still be determined.

As cancer cells are evolving in an immune-deficent microenvironment, the tumor microenvironment has a profound effect on the response to checkpoint blockade. It has been shown that a higher density of tumor infiltrating $CD8^+$ T cells correlates with an improved response to PD-1/PD-L1 blockade (46). In line with this, the loss of T cells' function to destroy tumor cells upon chronic antigen exposure has been shown to contribute to resistance to checkpoint blockade therapy. Beside, many cell types such such tumor-associated macrophages, regulatory T cells, fibroblasts, myeloid-derived suppressor cells, and stromal cells also contribute to a immune-defient microenvironmen and to the inhibition of the anti-tumor T cell response that attenuate the effectiveness of checkpoint inhibitors (47). Regulatory T cells release many immunosuppressive cytokines such as transforming growth factor-β and interleukin-10, which suppress the activation and proliferation of effector T cells (48). They also upregulate the expression of immune checkpoints such as CTLA-4 and PD-1 (48). Tumor-associated macrophages promote tumor growth through the expression of PD-L1 ligand and the production of immune supressive cytokines and vascular growth factor-A implicated in tumor evasion (49). Myeloid-derived suppressor cells alter the function of $CD8^+$ T cells through numerous mechanisms including blocking receptor function and IL-2 signaling.

Primary resistance of tumors to checkpoint blockade is also closely linked to genetic and epigenetic alterations that can shape the repertoire of tumor neoantigens (50). Neoantigens are peptides derived from somatic mutations in cancer cells and crucial for the recruitment of effector $CD8^+$ T cells to the tumor. Generally, tumors with high mutation rates respond better to checkpoint inhibition therapy compared to tumors with a low mutational burden (51) as the potency of anti-PD-1 inhibitors is dependent on the existence of antigen-specific T cells and the upregulation of PD-1/PD-L1 axis in tumor cells. It is supposed that without neo-antigens the blockade of immune checkpoints is weak. Therefore, as one of immune escape mechanisms, cancer cells downregulate the generation of neo-antigens and their presentation.

In recent years, the association between gut microbiota and response to immune checkpoint blockade has also been one of the aspects that gained interest. Several studies have provided evidence for the correlation between higher bacterial diversity and improved response to checkpoint inhibitors (52, 41). It has been shown that germ-free mice that have been transplated with fecal microbiota from responders to checkpoint inhibitors had improved outcomes with anti–PD-L1 treatment (53). Currently, it is not full understood how gut microbiota modulates immunotherapy. It is supposed that cross-reactivity between tumor neo-antigens and gut microbes; induced antigen presentation and the production of inflammatory cytokines mediated by microbiota could improve the response to checkpoint blockade (54).

4. Conclusion

The increasing trend in global cancer burden requires novel and more effective therapies, and in this sense, immuno-oncology is a relatively new discipline that involves the use of immunterapeutic approaches and agents to treat cancer patients. Development and use of tumor–targeting monoclonal antibodies in many cancers has been the first successful example of cancer immunotherapy. Noteworty examples of monoclonal antibody therapy include trastuzumab against HER2 which is amplified mainly in breast tumors, cetuximab directed to EGFR in advanced colon cancer and bevacizumab against VEGF approved in multiple cancers. Another major advance in cancer immunotherapy has been observed in cellular immunotherapies: T cells derived from patient are genetically manipulated to generate CAR-T cells that express bioengineered synthetic receptors consisting of the antigen binding site of a monoclonal antibody and a signaling domain of T cell receptor. CAR-T-based cellular treatments hold great potential with the primary use in hematological malignancies, and the research on their use and efficacy in solid tumors is ongoing. Another breaktrhrough in cancer immuotherapy is the development of immune checkpoint inhibitors that transformed the treatment landscape for patients with many advanced malignancies. Of the many immune checkpoints under investigation, CTLA-4 and PD-1/PD-L1 pathways are most studied immune regulatory points whose blocking by specific inhibitory antibodies results in the reactivation and proliferation of T cells, with clinically effective and durable immune responses in cancer patients. Despite this revolutionary effectiveness, however, only a proportion of patients (15-60% depending on the type of cancer) responds to immune checkpoint inhibitors, so that many patients do not benefit from immunotherapy. A key factor underlying the limited response seen in various trials is the complexity of tumor-host immune interactions and the existence of multiple additional mechanisms for tumor-mediated immune suppression. Thus, identifying biomarkers to optimally select patients who will benefit from the immune checkpoint blockade is an important research topic in the field of immuno-oncology. With the intention of determining predictive biomarkers, current research has mainly focused on the PD-L1 expression status in tumors, tumor microenvirontment, microsatellite instability, tumor mutational burden, and gut microbiota. A better understanding of checkpoint biology will make it possible to develop more effective immunotherapies in the future.

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CHAPTER 2

OFF-THE-SHELF ALLOGENEIC CELL PRODUCTS FOR CANCER IMMUNOTHERAPY

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ABSTRACT

Cancer immunotherapy uses the own defense mechanisms of the human body to target and eliminate cancer cells. Despite autologous cellular therapies have shown success in the treatment of hematological malignancies, they have limitations such as high costs, long manufacturing times, and potential quality issues. To overcome these challenges, the focus has shifted to allogeneic cell therapies. Derived from healthy donors or stem cell banks, these off-the-shelf products offer advantages such as mass production, reduced costs, and immediate availability. However, strict regulations and considerations must be addressed to ensure the safety and efficacy of allogeneic off-the-shelf therapies. This chapter provides an overview of the starting point of cellular cancer immunotherapies, the potential of allogeneic off-the-shelf products, and ongoing research and regulatory considerations in this field.

Keywords: Cancer immunotherapy, cellular immunotherapies, off-the-shelf cell products, allogeneic cell therapy

1. Introduction

According to the World Health Organization (WHO), cancer is the second leading cause of death worldwide and is expected to become the first in the near future (1). Conventional treatment modalities are inadequate in relapsed and refractory cases, and the use of targeted therapies becomes inevitable. Cancer immunotherapy is a group of treatments aimed to destroy cancer cells by utilizing the human body's own combat mechanisms against cancer. Cellular immunotherapies have gained a prominent role in the treatment of hematological malignancies owing largely to the success of engineering autologous T cells with chimeric antigen receptors (CARs) to target specific tumor-associated antigens (TAAs). So far, several autologous CAR-T cell therapies directed against CD19 or B-cell maturation antigen (BCMA) for the treatment of relapsed/refractory hematological cancers have been approved by the FDA and entered clinical use in the last decade (2-7). Autologous cellular therapies are leading the field of cellular immunotherapy primarily owing to the absence of immunogenicity and the risk of graft-versus-host disease (GvHD). However, these products have particular drawbacks such as low quality or leukemic contamination risk of the cells derived from patients (8, 9), high cost due to personalized production, and delayed patient treatment due to manufacturing time (10). These limitations have led cancer researchers to look for different solutions such as allogeneic cell therapies.

The raw material for generating allogeneic cell therapies includes various cell types derived from healthy donors and stem cell banks. Allogeneic cell therapies generated using different cell sources or through various gene engineering strategies, allowing mass production by processing cells obtained from healthy volunteers with well-characterized immunological profiles, can enable off-the-shelf use, facilitate easier scale-up, reduce costs, and prevent treatment delays by providing a readily available product. To achieve these attractive goals in the field of cellular therapies, a rising count of preclinical and clinical studies on allogeneic cellular products are being conducted. As a remarkable development, the first allogeneic off-the-shelf T cell therapy in cancer treatment, Ebvallo [Epstein-Barr virus (EBV) targeted cytotoxic T lymphocytes (CTL)], to treat EBV-associated post-transplant lymphoproliferative disorder (PTLD) received European Medicines Agency (EMA) approval in late 2022 (11). However, many points need to be optimized in the manufacturing of allogeneic off-the-shelf products where a potential defect can affect numerous patients. This situation also emphasizes the need for stricter regulations regarding the manufacturing of allogeneic off-the-shelf therapies.

In this chapter, the limitations of engineered autologous cell therapy and promises, limitations, types, preclinical and clinical studies, and regulatory considerations of off-the-shelf allogeneic cellular therapies will be discussed concisely.

2. Current Limitations of Autologous Cell Therapies in Cancer Immunotherapy

The breakthrough engineered autologous cell therapies for hematological malignancies are individualized products of a costly and labor-intensive process. Cells collected from patients by leukapheresis should be purified in the laboratory, activated, genetically modified by various viral transduction methods, expanded, concentrated, and prepared for infusion back to the patient after quality control (12, 13). Due to the individualized nature of autologous cell therapy, these steps require a considerable amount of manual handling despite the emerging automated closed-system cell processing devices (14). This situation leads autologous engineered cell production to be error-prone, non-standardized, and unable to be produced on a large-scale. Providing a safe, effective, autologous, engineered cellular product requires a group of highly specialized scientists and large quantities of advanced and expensive materials and reagents with adequate facility and infrastructure. The customized and labor-intensive production also increases the cost of these therapies, making them the most expensive form of cancer treatment. Moreover, these treatments must be administered in specialized hospital settings with the necessary expertise and infrastructure to ensure the safe and effective delivery of the therapy. A single infusion of the first two FDA-approved commercially available autologous CD19-directed CAR-T cell therapies, Tisagenlecleucel (Kymriah) and Axicabtagene ciloleucel (Yescarta), cost US$475,000 and US$373,000, respectively (15). This cost can exceed US$500,000 per patient with the treatment of major side effects (16).

Cancer patients often have inadequate numbers and quality of cells due to intensive immunosuppressive treatments such as chemotherapies or underlying congenital immunodeficiencies (9, 17). According to the reports from major CAR-T cell clinical trials, cell products of leukapheresis or further processing were of inadequate quality or insufficient content for around 7.5% of the enrolled patients. This led to repeated leukapheresis sessions, suboptimal cellular product administration, or no administration at all, leading to study exclusion (5, 18, 19). The phenotype and expansion capacity of the starting material obtained from patients plays a crucial role in determining the clinical response to the final product of the engineered cell therapy. In a study evaluating determinants of response to CD19 CAR-T cell therapy in chronic lymphocytic leukemia (CLL) patients, the frequency of $CD27^{+}CD45RO^{-}$ early memory CD8 (cytotoxic) T cell phenotype within apheresis samples of patients was associated with better proliferation of T cells before and after infusion and durable responses to therapy (20). Furthermore, studies evaluating baseline characteristics of collected T cells from myeloma patients undergoing anti-BCMA CAR-T cell therapy have found that higher CD4/CD8 ratio and early memory CD8 T cell ($CD45RO^{-}CD27^{+}CCR7^{+}$) enrichment with low or negative exhaustion marker expressions (including CTLA4, TIM3, LAG3, and PD1) is correlated with good clinical response, while exhausted effector T cell phenotype (with TIM3, LAG3, TIGIT and PD1 expressions) correlates with unresponsiveness (21-24). Similar

findings were also confirmed for non-Hodgkin's lymphoma patients undergoing CD19 CAR-T cell therapy (25). The detection of these positive predictive features in patients with earlier stages of disease reflects the effect of chemotherapy load on the quality and composition of T cells of cancer patients (17, 26). Taken as a whole, it is evident that even after stimulation and genetic modification, autologous T cell products of some patients, especially those who have received intensive treatments, are not effective enough, leading to treatment failure.

Another limitation of autologous engineered cell therapy is the time required between leukapheresis and administration of the final product, which leads to significant treatment delays. In clinical trials for CAR-T cells that received FDA approval, a substantial percentage of patients were unable to receive their autologous product due to disease progression or death. These were 1.98% of 101 patients in the Axicabtagene ciloleucel trial for refractory large B-cell lymphoma (7), 7.6% of 92 patients in the Tisagenlecleucel trial for children and young adults with B-cell lymphoblastic leukemia (5), 20.6% of 165 patients in the Tisagenlecleucel trial for adult relapsed or refractory diffuse large B-cell lymphoma (18), and 13.9% of 344 patients in Lisocabtagene maraleucel trial for patients with relapsed or refractory large B-cell lymphomas (19).

Cancer patients undergoing adoptive cellular therapies are mostly resistant cases. Especially in hematologic cancers, there is a possibility that malignant cells may be present in apheresis material and contaminate the expansion culture. Unfortunately, transduction of these cells with CAR may have undesirable consequences. It was reported that a patient relapsed after CD19 CAR-T cell therapy due to a single leukemic clone with an abnormal expression of CAR protein. CAR expression on leukemic cells resulted in masking the CD19 epitope, leading to the inability to detect CD19 by flow cytometry and treatment failure. The study also showed that a higher frequency of leukemic B cells in apheresis products was associated with a greater presence of leukemic cells in the final CAR-T cell products (8).

3. The Role of Allogeneic Cell Products in Cancer Immunotherapy

Due to all these limitations of autologous cellular therapies, research efforts have shifted toward discovering strategies and alternative cell sources for generating allogeneic, off-the-shelf products. These standardized, readily available products, which can be used for many patients, can be produced with one leukapheresis by processing pre-characterized, high-quality cells with optimal phenotypic characteristics from healthy donors. Using off-the-shelf products can significantly reduce the time between the decision to undergo engineered cell therapy and its administration. Off-the-shelf products can be immediately infused into the patient without the need for a patient's cells, or additional laboratory processes like leukapheresis, *in vitro* modification, expansion, or quality control testing, which may potentially prevent disease progression and death. Furthermore, moving from personalized to mass production could reduce costs and labor, and ensure that more patients can access

living drugs. Off-the-shelf engineered cell products with better cell fitness, established manufacturing characteristics, and minimized donor-to-donor variations may also guide the way to optimal results in the field of cell therapy. A comparison of autologous and allogeneic off-the-shelf cell therapies is presented in Table 1.

Table 1: Comparison of autologous and allogeneic off-the-shelf cell therapies for cancer immunotherapy

	Autologous	**Allogeneic off-the-shelf**
Cell source	Patients (themselves)	Healthy donors with optimal immunological features
Gene modification requirement for conventional T cells to avoid GvHD and rejection	No	Yes
Manufacturing	Small scale Personalized	Large-scale Standardized
Cost	High	Potentially lower
Ready-to-use	No	Yes
Limitations	Diminished number and quality of cells obtained from patients Risk of leukemic contamination Treatment delay	GvHD Reduced *in vivo* expansion and persistence due to rejection Toxicity of more potent lymphodepletion Potential risks of gene editing tools (off-target mutagenesis, etc.) Affected cell quality due to multiple genetic modifications Risk of contamination with donor-derived infections or inappropriate residual biological material

GvHD: graft-versus-host disease

The major obstacle of using donor lymphocytes in cellular therapies is the risk of serious alloimmune reactions due to major histocompatibility complex (MHC) mismatch between donor and recipient. Alloimmune reactions cause GvHD, a fatal medical condition in which the T cells from the graft recognize the host's healthy cells as foreign and systemically attack them through T cell receptor (TCR). On the other hand, alloreactivity also works in the opposite direction at the same time. The host generates a response against the allogeneic donor cells, limiting the persistence of engineered cells in the body and thus their clinical efficacy (27). This innate incompatibility can cause significant problems for allogeneic cell potency by preventing *in vivo* expansion and persistence, features that are associated with the durability of response and remission (20).

Therefore, the main challenge of allogeneic cell therapies is to develop strategies to prevent GvHD and the rejection of allogeneic cells. This can be addressed by modifying the TCR expression on conventional T cells or by utilizing immune cells that possess specificity or innate immune cells that do not respond to peptide-MHC mismatches. Prominent examples are virus-specific T cells, tumor antigen-specific T cells, innate-like T cells such as $\gamma\delta$ T cells, natural killer (NK) T (NKT) cells, and mucosa-associated invariant T (MAIT) cells, as well as NK cells and macrophages (28-30). Although they undergo similar manufacturing processes, allogeneic engineered cells have distinctive manufacturing difficulties, costs, feasibility, and safety concerns compared to autologous engineered cell therapies. For instance, extra regulations and controls must be applied to all stages of development, including starting material selection, cell phenotype modification, cell expansion, purification, and cryopreservation because of the scaled-up production, which has the potential to affect more people.

To produce allogeneic therapies, a reliable source of starting material is essential. Peripheral blood mononuclear cells (PBMCs) from healthy donors or various stem cells such as induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs) derived from stem cell banks can be used as starting material (31). Establishing a consistent relationship with potential donors who possess the desired cell characteristics and composition is required to secure cell sources for donor-derived cell engineering. Taking samples from a large potential donor pool and analyzing these extensive data to identify optimal donors are time consuming and labor-intensive. Stem cell-derived therapies theoretically originate from a single cell and do not display variations. Whereas, processing from healthy donor cells can still vary from batch-to-batch and should be minimized. This requires stricter policies due to the potential effect on thousands of patients (32).

Many types of immune cells with diverse properties are being investigated for allogeneic cell therapy, such as $\alpha\beta$ T cells, NK cells, $\gamma\delta$ T cells, NK T cells (NKTs), Cytotoxic T cells (CTLs), Dendritic Cells (DCs) and donor lymphocytes (Figure 1). According to the Beacon Adoptive Cell data module, currently, $\alpha\beta$ T cells and NK cells dominate clinical and preclinical research (32). Cell sources under investigation for allogeneic cell therapies are discussed in the following sections.

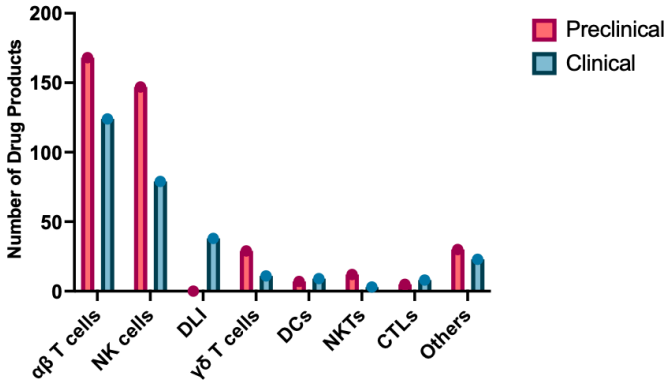


Figure 1: Cell types under investigation for allogeneic cell therapy (Modified from reference # 32, data was updated on 29.04.2022.) CTLs: Cytotoxic T cells, DCs: Dendritic cells, DLI: Donor lymphocyte infusion, NK cells: Natural killer cells, NKTs: Natural killer T cells

4. Cell Sources for Off-the-Shelf Allogeneic Cell Therapy

4.1. $\alpha\beta$ T Cells

$\alpha\beta$ T cells, which make up around 95% of peripheral blood T lymphocytes, are a vital component of the adaptive immune system (33). They express a TCR composed of an alpha and beta chain and play a critical role in recognizing and responding to foreign antigens. $\alpha\beta$ T cells undergo a process of positive and negative selection in the thymus to ensure that they can recognize and respond to foreign antigens presented by MHC molecules while avoiding reactivity to self-antigens (34). Depending on the type of antigen encountered and the local cytokine milieu, they can differentiate into various subsets of effector T cells, including cytotoxic T cells, helper T cells, and regulatory T cells (35). $\alpha\beta$ T cells are crucial for mounting effective immune responses against pathogens and cancer cells, in addition to their role in allogeneic response.

Successful *ex vivo* expansion of cells is a fundamental basis of adoptive cell therapy. Stimulation and expansion of $\alpha\beta$ T cells can be achieved easily through various methods, including using OKT3 (anti-CD3 antibody), irradiated feeder cells, or magnetic beads coated with anti-CD3/CD28 antibodies, and interleukin 2 (IL-2) replacement after PBMC isolation (36). However, the use of allogeneic $\alpha\beta$ T cells in adoptive cell therapy is complicated by the risk of GvHD. Normally, host self-antigens are tolerated and do not elicit an immune response. However, when host antigen-presenting cells (APCs) present these self-antigens, which are generally own MHC, to donor T cells, an MHC-mediated response is triggered, and the donor T cells recognize the host cells and tissues as foreign, leading to their destruction (37, 38). At the same time, host immune cells can also recognize and eliminate donor T cells in an MHC-dependent manner (38). To use $\alpha\beta$ T cells in allogeneic therapy, complex engineering strategies are needed to prevent the development of GvHD and rejection of allogeneic T cells, which are the main barriers to their use. Several methods have been reported in preclinical and

clinical trials to overcome these challenges and enable the use of $\alpha\beta$ T cells in the allogeneic context.

One of these methods is to disrupt the TCRα/β constant genes in donor cells to be engineered. With this method, donor T cells cannot target host cell antigens in an MHC-mediated manner due to the absence of TCR but can target TAA with CAR activity. In this method, CAR delivery is usually achieved by lentiviral or retroviral transduction, while gene editing methods such as Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9 (CRISPR-Cas9) are used for TCR disruption (39, 40). Simultaneously, disruption of β-2 microglobulin (B2M) and class II MHC transactivator (CIITA), which are essential for the expression of HLA class I and class II, respectively, with the CRISPR-Cas9 system can also reduce the allogeneic immune response against gene-edited T cells (39, 41). On the other hand, by targeting the CAR gene to the TCRα constant region with the CRISPR-Cas9 system, it is possible to knock-out the TCRα constant gene by simultaneous knock-in of the CAR gene. In this case, since the CAR gene is inserted into the TCR locus, intrinsic regulatory elements control CAR expression, which can reduce tonic signaling and exhaustion in modified cells (42).

An example of a similar method is the disruption of TCRα and CD52 genes simultaneously with a transcription activator-like effector nuclease (TALEN) system. This approach not only prevents GvHD but also provides resistance to Alemtuzumab, an anti-CD52 antibody, in engineered cells. Alemtuzumab administration prior to allogeneic cell therapy can selectively destroy host T cells, prevent rejection, and facilitate *in vivo* expansion (43). This approach has shown promising results in clinical trials, such as the use of allogeneic anti-CD19 CAR-T cells in pediatric and adult relapsed/refractory B-cell acute lymphoblastic leukemia (ALL) (44-46) and adult non-Hodgkin's Lymphoma (NHL) patients (47-49). Lentiviral transduction was used to deliver the CAR gene to the HLA-mismatched donor T cells, while TALEN was employed to disrupt the TCRα and CD52 genes to prevent GvHD and increase resistance to Alemtuzumab. These studies have demonstrated enhanced efficacy, persistence, and a limited toxicity profile with a low incidence of GvHD. The same strategy was tested with anti-BCMA CAR-T cells in relapsed/refractory multiple myeloma, and promising phase 1 interim results were recently published. The ongoing phase 1 UNIVERSAL study (Clinical Trial Identifier: NCT04093596) evaluated escalating doses of ALLO-715 in 43 patients with relapsed/refractory multiple myeloma. Grade $\geq$ 3 adverse events were reported in 88.0% of patients, with cytokine release syndrome observed in 55.8% and neurotoxicity in 14.0%. Infections were observed in 53.5% of patients. Overall, 55.8% of patients responded, and the median duration of response was 8.3 months. The response rate among those treated with the specific dosage and lymphodepletion regimen was 70.8% (50). Additionally, studies investigating similar products targeting solid tumors are currently underway (51).

Similar technologies can also be used to develop dual-targeted CAR-T cell products. The TCRα constant region and CD52 gene were disrupted using CRISPR/Cas9 technology to develop a universal CD19/CD22 dual-targeted CAR-T cell and preclinical and clinical efficacy has been demonstrated. The phase I study evaluating the safety, efficacy, and cellular kinetics of this product showed highly efficient gene editing without any genotoxicity or adverse events related to gene editing. The universal CD19/CD22 dual-targeted CAR-T cell therapy demonstrated a manageable safety profile, with cytokine release syndrome being the main side effect. The complete remission rate was 83.3% on day 28 of infusion, and some patients remained minimal residual disease negative (52).

Other engineering strategies to confer resistance to donor T cells include approaches such as providing resistance to purine and pyrimidine analog chemotherapy agents used for lymphodepletion through deoxycytidine kinase (dCK) knock-out in engineered cells (53), protecting CD7 targeted CAR-T cells for T cell malignancies with CD7 knock-out (54), and protecting resting lymphocytes while preventing alloreactivity by targeting activated host cells through alloimmune defense receptor (ADR) that recognize 4-1BB, which is overexpressed on activated lymphocytes (55). In addition, overexpression of NK cell inhibitor receptor ligands such as HLA-E (56) and knock-out of CD155 (57), an NK cell activator receptor ligand, can provide protection against NK cell-mediated alloresponse and can be considered along with B2M and CIITA disruption to reduce the immunogenicity of allogeneic T cells.

Table 2: Tools and strategies applied to engineer conventional T cells to prevent GvHD and allorejection

Tools			**Strategies**			
Gene delivery	**Gene editing tools**	**Non-gene editing methods**	**To avoid GvHD**	**To avoid host rejection of allogeneic T cells**	**To avoid the effects of lymphodepletion or other drugs on allogeneic T cells**	**To avoid fratricide**
Retrovirus (55) Lentivirus (57-59) AAV (42, 56, 60)	ZFN (61-63)	siRNA (64, 65)	TRAC disruption (40-43, 49, 52-54, 56, 58, 61, 66, 67)	B2M disruption (the main component of MHC-I) (39, 41, 56, 57, 68, 69) CIITA disruption (41, 57, 69)	CD52 knock-out (43, 49, 52, 58, 67, 70) (Provides resistance to alemtuzumab)	Target antigen knock- out CD7 (40, 54, 71), MICA/MICB (72) SLAMF7 (CS1) (66)
	TALEN (43, 49, 53, 56, 58, 66, 67)	shRNA (68, 72, 73)	TRBC1 and TRBC2 disruption (39, 61)	HLA-E (56, 57, 62), HLA-G (62, 74), siglec7/siglec9 ligands (75) (NK inhibitory molecules) overexpression CD47 (69) (phagocytosis inhibitory molecule) overexpression	dCK knock-out (53) (Provides resistance to purine and pyrimidine analog chemotherapeutics)	
Transposon (61)	CRISPR/Cas9 (39 42, 52)	TIM peptide (76) Protein expression blockers (77)	CD3 disruption (71, 73, 77)	Anti-4-1BB receptor expression (55) (to target alloreactive host T cells and NK cells specifically)	Glucocorticoid receptor knock-out (63) (provides resistance to glucocorticoids)	
Electroporation Mechanoporation Nanoparticles (78, 79)	MegaTAL nucleases (80) Engineered homing nucleases (60)	This CAR-T Intracellular retention of membrane proteins (TCR and/orHLA-I) (59,		CD155 (57), SLAMF7 (CS1) (NK cell activator ligands) knock-out		
		LUCAR platform (82)		PD-L1 overexpression (74) (suppresses PD1 expressing host T cells)		

AAV: Adeno-associated virus, B2M: Ş -2 microglobulin, CIITA: Class IIMHC transactivator, CRISPR/Cas9: ClusteredRegularly Interspaced ShortPalindromic Repeats, dCK: deoxycytidine kinase, shRNA: short hairpin RNA, siRNA: Small interfering RNA, TALEN: Transcription Activator-like Nucleases, TIM: TCR inhibiting molecule, TRAC: TCR-a constant chain, TRBC1 and TRBC2: TCR-β constant 1 and 2 chains, ZFN: Zinc-finger Nucleases

In summary, various tools and strategies have been proposed to modify conventional T cells to generate allogeneic off-the-shelf products avoiding GvHD and allorejection (Table 2). These tools used in cell modification include conventional gene delivery methods, emerging gene editing tools, and non-gene editing methods. Using these tools, sophisticated genetic modification strategies have been developed to prevent GvHD, host rejection of allogeneic T cells, the effects of lymphodepletion or other drugs on allogeneic T cells, and fratricide (CAR-mediated cytotoxicity between CAR-T cells carrying target antigen).

4.2. Natural Killer Cells

NK cells are a type of lymphocyte that share a common origin and functional similarities with cytotoxic T cells, as they can recognize and kill cancer cells and virus-infected cells. But they do not need prior sensitization. Morphologically, they resemble normal lymphocytes in their resting state, but activated NK cells carry large granules in their cytoplasm. In contrast to $\alpha\beta$ T cells, NK cells do not cause GvHD but preserve the graft-versus-tumor effect (83). NK cells mainly mature in the bone marrow and peripheral tissues and express a set of cell surface receptors, which enables them to interact with a variety of ligands and co-receptors that modulate their activity. While NK cells typically express CD56, they lack T cell markers such as CD3 or TCR. Subtypes of NK cells have been identified regarding their dominant effector functions, such as cytokine production or cytotoxicity and the strength of CD56/CD16 expression. Compared to $CD8^+$ cytotoxic T cells, NK cells are devoid of an antigen-specific TCR but can be activated by a broad range of stimuli. While tumor cells usually manage to hide from cytotoxic T cells by downregulating their MHC class I expression, they become susceptible to NK cell cytotoxicity through the activation of activator receptors such as NKG2D and abolition of inhibition by inhibitory receptors such as killer cell immunoglobulin-like receptors (KIRs). Activated NK cells can eliminate target cells using perforin and granzyme secretion, Fas-Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) pathways. They are also mediators of antibody-dependent cellular cytotoxicity (ADCC) through the CD16 receptor (84, 85). In addition, recent studies have shown that NK cells also have memory functions that are thought to be specific to adaptive immunity (86). As an innate lymphoid cell subset with extensive features, NK cells have multiple cytotoxic mechanisms against cancer cells, a low risk of GvHD, a safer toxicity profile, and high feasibility for off-the-shelf production, making them a potential candidate for allogeneic cell therapy.

Initially, autologous NK cells were tested in adoptive cell therapy. However, it was observed that self-MHC expressed in cancer cells could reduce the effectiveness of autologous NK cells (87). Additionally, KIR ligand mismatch between patients and donors was correlated with favorable outcomes in the context of hematopoietic stem cell transplantation (HSCT) (88). KIRs recognize MHC class I molecules encoded by different HLA allotypes. The

mismatch between KIR expression of donors' NK cells with their ligands on patients' cancer cells causes enhanced NK cell function. Therefore, research has shifted toward investigating allogeneic NK cell therapy (89, 90).

NK cells can be obtained from various sources such as peripheral blood, umbilical cord blood (UCB), human embryonic stem cells (hESCs), or iPSC (91), with peripheral blood and UCB containing NK cells around 10% (92) and 30% (93), respectively. Additionally, NK cell lines such as NK-92 can also serve as potential sources (94) but should be irradiated before administration as they are of malignant origin. Among these, peripheral blood-derived NK cells from healthy donors are considered to be more functional despite the high risk of T cell contamination causing GvHD. UCB is a promising source of NK cells for allogeneic adoptive therapy due to several advantages. These include ease of collection and a lower proportion of T cells, which reduces the risk of GvHD (95). NK cells can be expanded from PBMCs and UCB using different methods such as CD3/CD19 depletion or CD3 depletion and CD56 enrichment with cytokine support (94). Alternatively, feeder cells with cytokine support can also be used. The safety and efficacy of NK cells expanded *ex vivo* have been demonstrated in preclinical studies (96, 97) and clinical trials with both hematological and solid tumor patients (98-100). One of these studies evaluated the safety and efficacy of *ex vivo* expanded and activated NK cells (MG4101) derived from random healthy donors in patients with malignant lymphoma or advanced, recurrent solid tumors. The results demonstrate that the repeated administration of MG4101 was tolerable without significant side effects, with 47.1% of patients showing stable disease and 52.9% showing progressive disease. Furthermore, MG4101 administration increased NKG2D expression on $CD8^+$ T cells and upregulated T cell recruiting chemokines, while decreasing regulatory T cells and myeloid-derived suppressor cells and suppressing TGF-β production, indicating its potential to support the effector arm of the host immunity (99).

The NK-92 cell line is the most extensively studied NK cell line for allogeneic cell therapy. Although it does not naturally express the CD16 receptor required for ADCC, it is easy to expand and engineer, which makes it a highly attractive option for off-the-shelf therapy. Clinical trials have confirmed the safety and tolerability of NK-92 cells (92, 101, 102), and current research is focusing on the use of genetically engineered NK-92 cells to target a range of different cancers (103, 104). Another cell line, haNK, an engineered NK-92 cell line to express the high-affinity CD16 receptor, enables efficient lysis of many tumor cell lines and shows promise in clinical trials (91).

HESCs and iPSCs are also promising sources of allogeneic NK cells for cancer immunotherapy. Well-defined clinical scale differentiation protocols have been established to generate homogeneous and reproducible NK cells that lack the donor heterogeneity characteristic of peripheral blood and UCB NK cells (105). These stem cell-derived NK cells

exhibit functional and phenotypic features close to mature NK cells, including cytotoxicity against various cancer targets (106). Furthermore, NK cells derived from hESCs and iPSCs can be genetically modified to express CARs, offering a potential source for the development of standardized and homogeneous, allogeneic CAR-NK cell products to improve the efficiency of adoptive cell therapies (107).

Major challenges associated with using NK cells for adoptive transfer are their limited *in vivo* efficacy and persistence. These can be addressed with various strategies to improve NK cell functions while mitigating toxicity, such as armoring NK cells with cytokines (108) or using NK cells as a CAR vector (109) in allogeneic settings. An example of these approaches is UCB-derived HLA-mismatched NK cells that express a CD19 CAR in combination with the cytokine IL-15 and a caspase-9-based suicide gene (iCasp9). This product has shown significant efficacy and long-term *in vivo* persistence in preclinical studies (110), and a clinical trial involving 11 patients with relapsed or refractory NHL or CLL has shown that the administration of this product after lymphodepleting chemotherapy did not cause significant toxic effects. Additionally, a majority of patients had a positive response to the treatment with a 73% response rate and seven patients achieving complete remission. The infused CAR-NK cells sustained at low levels for at least 12 months (111). These findings indicate that engineered NK cells may be a promising treatment option for CD19-positive cancers with less potential for toxic effects compared to their conventional T cell counterparts. Genetic engineering and the clinical applications of adoptive NK cell therapies in the allogeneic context should be investigated further.

4.3. $\gamma\delta$ T Cells

$\gamma\delta$ T cells have a distinct position at the interface of innate and adaptive immunity, exhibiting characteristics of both the adaptive immune system with a TCR constituted by gamma and delta chains and innate immunity with the ability to activate effector functions without antigen processing. In contrast to $\alpha\beta$ T cells, the majority of $\gamma\delta$ T cells can recognize tumor cells based on their high phosphoantigen load without MHC restriction (112). Therefore, $\gamma\delta$ T cells can exhibit natural antitumor reactivity by displaying cytotoxic activity and producing various cytokines upon activation (113-115). These cells are also capable of ADCC. $\gamma\delta$ T cells have been shown to cause less GvHD than $\alpha\beta$ T cells while maintaining the graft-versus-leukemia effect in HSCT settings (116). Infiltration of tumors by $\gamma\delta$ T cells is the most important positive immunological criterion for good clinical outcomes in various types of cancer (117). Due to these promising functions, $\gamma\delta$ T cells represent a potential candidate for cancer immunotherapy, especially in the allogeneic context.

$\gamma\delta$ T cell-based immunotherapy research centers on the two main $\gamma\delta$ T cell subsets, which are Vδ1 and Vδ2. The Vδ1 T cell subset is abundant in tissues and involves effector and regulatory cells with antitumor and protumor properties, respectively. Their TCRs consist of a

Vδ1 chain coupled with one of several Vγ chains and detect multiple self and non-self peptides and lipids presented by CD1d. On the other hand, Vδ2 T cells constitute the predominant subset in peripheral blood (95%), and their TCRs typically contain the Vγ9 chain in addition to the Vδ2 chain. They are composed of rather uniform effector T cells that are reactive to phosphoantigens. Similar to NK cells, $\gamma\delta$ T cells express receptors like CD16, NKG2D, and DNAM-1, which are associated with antitumor functions. When activated, $\gamma\delta$ T cells show potent cytotoxic activity with the expression of TRAIL and Fas (CD95) ligands as well as secretion of granzyme B and perforin. In addition, $\gamma\delta$ T cells can produce cytokines such as IFN-γ and TNF-α to enhance the immune response, ultimately helping to counteract tumor development (118).

$\gamma\delta$ T cells constitute a lower proportion as ~5% of $CD3^+$ T lymphocytes in adult peripheral blood (119) and the production of adequate numbers of cells with optimal functionality *in vitro* should be addressed in order to use allogeneic $\gamma\delta$ T cells in adoptive therapy. Vγ9Vδ2 cells, which represent the most abundant subset of $\gamma\delta$ T cells in peripheral blood, recognize phosphoantigens derived from the mevalonate pathway of mammalian cells that increase during infection or malignant transformation. Aminobisphosphonates such as zoledronate inhibit farnesyl pyrophosphate synthase, the downstream enzyme of the mevalonate pathway, leading to the elevation of phosphoantigens on cancer cells and monocytes, which promotes Vγ9Vδ2 $\gamma\delta$ T cell activation *in vivo* and *in vitro*. With the *ex vivo* expansion protocol using the combination of zoledronate and IL-2 supplementation, a significant number of high-purity Vγ9Vδ2 $\gamma\delta$ T cells can be generated from PBMCs (120).

Due to the promising properties of $\gamma\delta$ T cells, preclinical (121) and growing clinical studies are ongoing to harness their natural antitumor functions and they show early signs of safety and efficacy. For instance, patients with locally advanced pancreatic cancer, which is typically resistant to immunotherapy due to its immunosuppressive microenvironment, were treated with combined irreversible electroporation (IRE) and healthy donor-derived *ex vivo* expanded Vγ9Vδ2 T cells with zoledronate and IL-2. The study enrolled 62 patients who received IRE therapy and were randomized into two groups: one group received at least two cycles of $\gamma\delta$ T cell infusion after IRE, while the other group did not receive any additional treatment. The group that received $\gamma\delta$ T cell infusion had better overall survival and progression-free survival compared to the other group. Additionally, patients who received multiple courses of $\gamma\delta$ T cell infusion had longer overall survival than those who received only one course (122).

Another study showed that $\gamma\delta$ T cells, isolated from healthy donor PBMCs and expanded with zoledronate, IL-2, and IL-15, demonstrated enhanced immune effector functions such as proliferation, and cytotoxicity *in vitro* and *in vivo*. A phase I clinical trial in 132 cancer patients with a total of 414 Vγ9Vδ2 cell infusions demonstrated that among patients with late

stage liver and lung cancer who received multiple infusions (5≤), some showed prolonged survival, suggesting the potential efficacy of allogeneic Vγ9Vδ2 T cell therapy in late stage cancer (123).

The less prominent in the blood and difficult-to-expand subset of $\gamma\delta$ T cells are Vδ1 cells. Due to the absence of an established consistent expansion protocol, researchers have been working on the effective expansion of Vδ1 cells from peripheral blood. A two-step protocol to expand and differentiate Vδ1 T cells using sorted $\gamma\delta$ T cells with clinical grade anti-CD3 mAb and multiple cytokines is referred to as the DOT protocol. Generated cells expressed natural cytotoxicity receptors and were able to target leukemia cells *in vitro* and inhibit tumor growth and prevent invasion in xenograft models of CLL *in vivo* (124). A one-step protocol for Vδ1 T cell expansion from PBMCs with current good manufacturing practice (cGMP) compatible reagents was also reported recently, using anti-CD3 mAb, and IL-15 support with depletion of $\alpha\beta$ T cells, CD56$^+$ cells, and Vδ2 cells. These Vδ1 cells can be modified further with viral vectors delivering CARs, and exhibit antigen-specific persistence, cytotoxicity, and cytokine production (125).

Another efficient expansion strategy for $\gamma\delta$ T cells is the use of feeder cells (126-128). For instance, polyclonal $\gamma\delta$ T cells with a dominant memory-Vδ1 phenotype were generated with irradiated engineered artificial antigen-presenting cells (APCs) expressing CD86/4-1BBL/CD40L and cytomegalovirus (CMV)-antigen-pp65 with cytokine support using Clinimacs Prodigy closed-system cell processing device. Expanded $\gamma\delta$ T cells expressed high activation and memory markers while lacking markers of exhaustion and alloreactivity and showed potent antitumor efficacy both *in vitro* and *in vivo*. These cells were also engineered effectively to enhance antitumor capacity. This study highlights the feasibility of establishing an off-the-shelf and easy-to-engineer, allogeneic, polyclonal $\gamma\delta$ T cell production compliant with cGMP (127).

Furthermore, $\gamma\delta$ T cells are a potential allogeneic CAR backbone to combine their natural antitumor cytotoxicity with CAR-mediated specific killing (125, 129-134). CD19-directed CD28/ CD3/ CAR-$\gamma\delta$ T cells were effective against CD19 antigen-negative leukemia cells after priming with zoledronate in addition to CD19-positive leukemia cells. It is suggested that CAR-$\gamma\delta$ T cells may target leukemic cells even after target antigen loss (130). In another study, allogeneic Vδ1 $\gamma\delta$ T cells engineered to express 4-1BB/CD3/ CAR against glypican-3 (GPC-3), a protein highly expressed in hepatocellular carcinoma, and secretable form of IL-15 (sIL-15). Constitutively secreted sIL-15 sustains *in vivo* proliferation, survival, and antitumor activity of engineered cells. GPC-3CAR/sIL-15 Vδ1 T cells effectively controlled tumor growth without evidence of GvHD in a subcutaneous HepG2 (a human liver cancer cell line) mouse model (131). An active phase 1 trial evaluated the safety and efficacy of an allogeneic $\gamma\delta$ CAR-T cell therapy targeting CD20 (ADI-001) in adults with relapsed/refractory B-cell

lymphoma (Clinical Trial Identifier: NCT04735471). Patients received ADI-001 at three dose levels following conditioning therapy. Most adverse events were grade 1-2 and there were no reported GvHD or dose-limiting toxicities. The overall response rate at day 28 was 67% and the complete response rate was 67% in the six patients enrolled in the study. Patients who achieved a complete response on day 28 remained disease-free at the 3-month follow-up (135).

Alternative engineering strategies to CARs in allogeneic $\gamma\delta$ T cells, including chimeric costimulatory receptors (CCRs) (136), secreted TAA-targeting opsonins (137), and secreted or membrane-bound cytokines (131) or more potent cytokine analogs (137) have also been reported. CCRs are chimeric receptors that lack CD3ζ but contain DAP10 stimulatory domain, a signaling molecule that is widely expressed in innate cells. These CCR-$\gamma\delta$ T cells did not display tonic signaling which is associated with exhaustion in CAR-T cells but were effectively activated and induced cytotoxic responses in the presence of the target antigen (136). Additionally, the opsonin-secreting Vγ9Vδ2 $\gamma\delta$ T cells armored with secreted potent IL-15 analog (stIL15-OPS-$\gamma\delta$ T) displayed enhanced cytotoxicity and also activated bystander lymphoid and myeloid cells and the untransduced portion of the cell product. stIL15 secretion also eliminated the need for exogenous cytokine supplementation during cell expansion. The efficacy of stIL15-OPS-$\gamma\delta$ T cells was also shown on 3D patient-derived osteosarcoma models and it was enhanced with zoledronate (137).

In the rapidly developing field of cellular therapies, $\gamma\delta$ T cells are promising candidates for allogeneic therapy. Various clinical trials with allogeneic *ex vivo* expanded unmodified or genetically engineered $\gamma\delta$ T cells for cancer immunotherapy are ongoing (118). It is most recently announced that the first patient was dosed in a phase 1 study (Clinical Trial Identifier: NCT05653271) investigating ACE1831, an allogeneic $\gamma\delta$ T cell therapy targeting CD20 for the treatment of NHL, developed using antibody-cell conjugation (ACC) technology derived from 2022 Nobel Prize laureate Dr. Carolyn Bertozzi's work.

4.4. Others

Virus-specific T cells have been in the spotlight as an alternative cell type for allogeneic cancer therapy, and a notable example is Tabelecleucel (Ebvallo). Developed under license from Memorial Sloan-Kettering Cancer Centre and approved by the EMA, Tabelecleucel is designed to target and destroy EBV-infected cells in an HLA-restricted manner. It is primarily used for the treatment of lymphoproliferative disorders such as EBV$^+$ PTLD and other EBV$^+$ hematological and solid tumors. T cells from 3rd party donors are stimulated and expanded *in vitro* using EBV reagent and B lymphoblastoid cell line (BLCL) antigen-presenting cells (BLCL APC) from the same donor. There are different clones designed to recognize a specific EBV peptide in complex with a specific HLA molecule on the surface of target cells via the TCR. This enables Tabelecleucel to exert its cytotoxic activity specifically against

EBV-infected cells and protect uninfected cells and exhibits reduced alloreactivity. For each patient, an appropriate Tabelecleucel clone should be selected from the product library according to the appropriate HLA restriction. Patients may be switched to a different lot, in case of suboptimal response. Tabelecleucel is the first officially approved off-the-shelf allogeneic T cell therapy that harnesses antitumor functions of EBV-specific cytotoxic T cells and provides a potential treatment option for EBV^+ malignancies (11).

Preclinical and early-phase clinical studies to generate allogeneic cell therapies are ongoing involving various less prevalent innate and innate-like immune cells in human blood (28). NKT cells are a unique subset of T cells that express the $\alpha\beta$ TCR for antigen recognition and display characteristics of NK cells such as CD56 and CD16 expression and granzyme production. NKT cells are being investigated as a promising tool for allogeneic cancer immunotherapy. Strategies include the application of α-galactosylceramide (α-GalCer)-loaded DCs to enhance antitumor immune responses by manipulating NKT cells. However, even in healthy human blood the proportion of these cells is very low (0.1%), and stem cell-based strategies come to the forefront for the generation of NKT cells (138). Allogeneic NKT cells are particularly attractive for adoptive cell therapy due to their non-alloreactive nature. Clinical trials are ongoing to evaluate the use of unmodified (Clinical Trial Identifier: NCT04754100) and modified (Clinical Trial Identifier: NCT00840853) allogeneic NKT cells for multiple myeloma and $CD19^+$ malignancies, respectively (139). To overcome the potential rejection of allogeneic NKT cells, strategies such as reducing or eliminating the expression of HLA class I and II molecules, as well as using NK cell inhibitory molecules can be utilized (139, 140).

Double negative ($CD3^+CD4^-CD8^-$) T cells (DNTs), another rare subset of mature T cells, offer an alternative cell source for allogeneic off-the-shelf therapy. It was shown that allogeneic DNTs were successfully modified with a second generation anti-CD19 CAR (CAR19) and demonstrated antigen-specific cytotoxicity against B-cell ALL and effective tumor control in CD19-expressing lung cancer models. The efficacy of CAR19-DNTs was comparable to conventional CAR19-T cells, but there was no alloreactivity or mortality associated with xenogeneic GvHD (141).

Allogeneic cell therapy can also be applied as donor lymphocyte infusion (DLI) or by modifying donor cells with CARs in relapse after hematopoietic stem cell transplantation (142-144), but this is beyond the scope of this review due to its personalized nature.

Allogeneic dendritic cells with off-the-shelf potential have also shown promising results in cancer immunotherapy, including long-term survival and induction of immune responses in patients with AML and metastatic renal cell carcinoma, and their use as immune enhancers in cancer vaccines has the promise of wider application in cancer therapy (145-148).

4.5. Stem Cell-Based Generation of Off-the-Shelf Allogeneic Cell Therapy

Among the major problems in the production of allogeneic off-the-shelf cellular therapies are that these products require additional genetic modifications compared to autologous therapies or require the use of infrequent cell subtypes in the human blood. Lentiviral and retroviral vectors continue to be widely used in cellular therapies for gene engineering purposes. However, due to their limited gene payload capacity, the number of genes that can be introduced into mature immune cells simultaneously is limited. It is also important to note that viral transduction and gene editing techniques can impact the yield and nature of the cells. Moreover, beneficial innate immune cell subsets are present at low frequencies in peripheral blood and necessitate extensive expansion for clinical application. This expansion process poses challenges in terms of scalability and standardization.

Stem cells are an attractive source for cellular therapies due to their unique abilities, such as unlimited replication, self-renewal, and the potential to differentiate into diverse cell types found within the human body. The use of stem cells as a cell source for the production of allogeneic off-the-shelf cellular therapies against cancer offers several distinct advantages. By performing gene modification on a small number of initial stem cells, the reliance on expensive gene engineering materials can be minimized. Moreover, the replication of these modified cells allows for maximum gene engineering/editing efficiency, which can be inherited by the final cell products. In addition, utilizing stem cell engineering in the development of cellular therapies provides opportunities including the generation of pure and highly potent cellular products, as well as large-scale production and stocking of these products (149).

Considering the stem cell sources, hESCs are obtained from embryos, whereas MSCs, UCB stem cells (UCBSCs), and HSCs are obtained from adult tissues. Pluripotent stem cells (PSCs), which include both hESCs and iPSCs, are considered the second generation of stem cell-based therapies (150). HSCs and PSCs are the primary sources of stem cells utilized in the development of therapeutic immune cells. Multipotent HSCs can be harvested from UCB and bone marrow or by apheresis of donor peripheral blood previously treated with G-CSF. The *in vitro* generation of different cell types from HSCs involves transducing HSCs with specific TCRs followed by differentiation in a supportive culture system or differentiating HSCs into NK cells using cytokines (149). These cells can then be further engineered and expanded *in vitro*. PSCs offer the advantage of an unlimited cell source to generate immune cells. However, ethical concerns have restricted the use of hESCs in cellular therapies. iPSCs are generated through the process of reprogramming somatic cells. Immune cells derived from healthy donor PBMCs can be reprogrammed to pluripotency and subsequently re-differentiated into young immune cells (151). These iPSCs derived from PBMCs can be further genetically modified to enhance their antitumor efficacy. It is important to note that the extended culture time required for the differentiation of PSCs carries the risk of genetic mutations and causes

safety concerns (152), which should be minimized through advanced screening methods.

There are two main methods for reprogramming mature somatic cells or PBMCs into pluripotent iPSCs; chemical reprogramming and genetic reprogramming. Chemical reprogramming involves using transcription factors (Oct4, Sox2, Klf4, and c-Myc) and small molecules (e.g. valproic acid) to induce pluripotency in mature cells (153-155). These factors work together to reset the cellular state and promote the development of iPSCs. On the other hand, genetic reprogramming utilizes viral vectors, such as lentivirus and Sendai virus, to convey important pluripotency-related genes (Oct4, Sox2, Klf4, and c-Myc) into PBMCs (156, 157). The viral vectors act as carriers to deliver these genes, facilitating the reprogramming process and the generation of iPSCs. iPSCs can then be differentiated into a variety of different immune cells, including $\alpha\beta$ T cells, NK cells, invariant NKT (iNKT) cells, MAIT cells, $\gamma\delta$ T cells, dendritic cells, myeloid cells, and macrophages (149, 158, 159).

Several culture systems have been used to support stem cell differentiation. The most common of these are humanized mouse models (e.g. bone marrow-liver-thymus (BLT) mouse model), *in vitro* feeder-dependent [e.g. OP9-DL1 and artificial thymic organoid (ATO)] and feeder-free culture systems (158). The *in vitro* generation of human cells for clinical purposes has been hindered by the mouse origins of current culture systems. To address this, strategies have been developed including designing feeder cells of human origin and creating feeder-free culture systems. Among *in vitro* feeder-free expansion methods, studies using serum-free media that enable iNKT production from HSCs or expansion of $CD34^+$ cells using StemSpan (Stemcell Technologies) and T cell production from iPSCs using StemFit (Ajinomoto) have shown promising results (160-162). Examples of human-sourced feeder cells include foreskin fibroblasts, bone marrow-derived MSCs, and adipose-derived stromal cells (163). Another approach is to reduce the contamination of stem cells with the mouse feeder cells by using a porous membrane (164).

To incorporate allogeneic stem cell products into adoptive cell therapy without triggering alloimmune reactions and to enhance their antitumor properties, retroviral or lentiviral transductions and gene editing techniques such as CRISPR-Cas9, TALEN and zinc finger nucleases (ZFN) can be employed stem cells before differentiation, similar to their application in mature cells. This approach offers the advantage of high proliferation of modified stem cells and effective expression of gene modifications at the final mature cell product, reducing the need for costly genetic engineering materials (165).

Safety concerns arise with stem cell-derived therapies due to the potential for tumorigenicity caused by genetic alterations during *in vitro* culture or the risk of residual undifferentiated cells in the final product forming teratomas. In addition, reprogramming factors used in iPSC therapies carry the risk of tumorigenesis. Various methods are being investigated to reduce the risk of tumorigenesis in allogeneic stem cell-derived products,

including more efficient differentiation protocols and purification methods, careful selection of iPSC lines, elimination of reprogramming factors, and comprehensive monitoring of genetic abnormalities (166, 167). Monitoring methods such as karyotyping and next-generation sequencing can be employed to detect chromosomal abnormalities and genomic alterations (167). Flow cytometry and RT-PCR can be utilized to identify stem cells in the final product (158, 168). Additionally, the inclusion of suicide switches can serve as a safety measure in stem cell-based therapies (169).

In a study utilizing iPSC and CAR technologies, iPSC-derived T cells, expressing CD19 CAR, displayed a similar phenotype with innate $\gamma\delta$ T cells and effectively suppressed tumor growth in a xenograft model (170). In a recent study, researchers showed that they effectively produced iPSC CD19 CAR-T cells from iPSCs derived from $CD62L^+$ naive and memory T cells. These iPSC CD19 CAR-T cells, created using CAR engineering and a 3D-organoid system differentiation method displayed similar characteristics to conventional CD8 $\alpha\beta$ CAR-T cells. They demonstrated antigen-specific activation, degranulation, cytotoxicity, and cytokine secretion comparable to standard CD19 CAR-T cells, while also maintaining consistent expression of the TCR adopted from the starting clone. Moreover, iPSC CD19 CAR-T cells showed potent antitumor activity *in vivo* and significantly prolonged the survival of mice with $CD19^+$ human tumor xenografts (171). To avoid immune rejection, hypoimmunogenic cancer-antigen-specific T cells were generated from iPSCs with knock-out of B2M, CIITA, and CD155 with CRISPR-Cas9 system, and lentiviral transduction with HLA-E. In mouse models of leukemia and lymphoma, these iPSC-derived T cells avoid recognition by various immune cells and maintained their antitumor effectiveness (57). iPSC-derived CAR-T cells have unlimited potential as a source of standardized and effective CAR-T cells for off-the-shelf cancer therapy. However, compared to iPSC-NK cells, iPSC-derived T cells are hard to generate and require a pre-existing TCR that drives T cell differentiation from iPSCs *in vitro* (159, 172).

Ongoing research explores iPSC-derived NK cells as a potential alternative to conventional CAR-T cells in cancer immunotherapy, offering favorable characteristics such as ease of engineering, large-scale expansion, and improved safety profile (158). An off-the-shelf NK cell product derived from the human clonal master iPSC line (FT500), is being evaluated in a phase I clinical trial for advanced solid tumors and lymphomas. The treatment regimen, involving lympho-conditioning followed by cycles of FT500 monotherapy or combination with immune checkpoint inhibitors, demonstrated a favorable safety profile without any dose-limiting toxicities or severe adverse events. Encouragingly, preliminary results showed stable disease or tumor reduction in some patients, and there were no significant instances of immune rejection, highlighting the potential of FT500 as a promising immunotherapy option (173). Off-the-shelf triple gene-modified iPSC-derived NK cells (iDuo NK cells) have

been created to target multiple antigens and enhance immune cell function. These iDuo NK cells armored with high-affinity CD16a, CD19 CAR optimized for NK cell signaling, and a membrane-bound IL-15/IL-15 receptor fusion, showed effective killing of various tumor cells *in vitro*, including leukemia, lymphoma, and solid tumors. They exhibited prolonged persistence in the body and demonstrated improved survival in mice with B-cell lymphoma, as well as reduced tumor burden in mice with leukemia and solid tumors (174). Promising outcomes have been observed in B-cell lymphoma treatment, with an objective response achieved in 13 out of 19 patients following a single dose of the therapy (158).

Developing stem cell technology has a remarkable potential to revolutionize anti-cancer cell therapy, offering standardized and widely accessible treatments. However, extensive research is required to address optimization and safety concerns associated with stem cell-derived cell therapies.

5. Manufacturing Challenges and Regulatory Considerations for Allogeneic Off-the-Shelf Cell Products

The development of allogeneic off-the-shelf cell products for cancer therapy presents numerous manufacturing challenges that differ from the production of autologous cellular therapies. Appropriate donor selection is an important consideration in the manufacturing of allogeneic cell products. To minimize adverse events and maximize product efficiency, establishing optimal donor selection criteria that account for donor-to-donor variations is crucial. Donor fitness assessment should include a comprehensive evaluation of the donor's medical history, infectious disease serology, and immunological phenotyping. An essential aspect is determining immunological criteria to identify optimal donors for allogeneic off-the-shelf cellular therapies. Decisive factors may include donor-derived cell composition characteristics such as exhaustion and naive-memory phenotypes or high productivity during expansion. Furthermore, it remains to be seen whether the high-quality markers known for $\alpha\beta$ T cell products also have counterparts in other cell types as clinical trials, which are still in their infancy, progress (125, 175).

While screening can minimize donor-acquired infections, regular biological or microbial contamination screening is essential during *in vitro* culture to reduce the risk of infections. Implementing automated systems can reduce manual manipulations and the risk of contamination (14). The complex genetic engineering process applied to allogeneic raw material to prevent GvHD risk and allorejection, enhance antitumor efficacy, and limit toxicity can also impact cell viability, yield, and expansion. Innovations in genome editing technology can enhance the efficacy of allogeneic off-the-shelf cellular products in cancer treatment while simplifying the manufacturing process and reducing costs (31, 158, 176).

Standardization is a crucial aspect of manufacturing allogeneic cell products. In addition to standardized features of the starting cell material, establishing standardized protocols

and implementing automated closed systems are essential to ensure consistent quality and safety across different batches and production facilities (14, 32, 177). All materials used from the beginning to the generation of the end product must be cGMP compliant and free of animal-derived substances and antibiotics (176). For large-scale production, the use of validated automated viable cell counting methods can improve accuracy (178). Cryopreservation plays a crucial role in ensuring that allogeneic cell products can be stored and used off-the-shelf. Long-term cryopreservation of allogeneic cell products could affect viability and phenotype, potentially compromising yield and safety. Early assessment of post-thaw cell performance helps researchers optimize the process by developing strategies to ensure the safety of the freeze-thaw process (32).

Traceability plays a critical role in ensuring the safety and quality of human cell products. Donors and products should be assigned unique identification numbers, and essential information such as the donor's identification number, manufacturing date, storage conditions, and shelf-life should be included on the product label (179). Digitally tracking the entire process, from production to patient administration and beyond, enables the identification and resolution of potential issues in a specific production batch before they affect other patients (32).

Transportation and storage pose additional challenges. As off-the-shelf cell products are cryopreserved and stored, establishing optimal storage conditions is essential to ensure their safety (180). Storage areas should be designed to protect against contamination and other hazards, while transport containers must maintain optimal conditions to protect the products during transportation.

Given the variety of cell types in the field of allogeneic therapies, significant resources must be allocated to develop compatible process and quality control strategies. These strategies are necessary to demonstrate important factors such as the detection of mutagenic cells, potency, batch-to-batch comparability, product viability, purity, and stability (32). Quality management systems (QMS) are essential for the safe and effective production of allogeneic off-the-shelf cell products. These QMS should comply with cGMP and current good tissue practice (cGTP) guidelines issued by national and regional competent authorities (175). The QMS should be designed to ensure the quality of starting materials, the safety of production processes, and the traceability of products (179). Regular monitoring and updates are required to meet evolving standards and regulations.

In summary, the production of allogeneic off-the-shelf cell products for cancer therapy requires addressing a variety of challenges ranging from finding suitable donors to maintaining product quality during transport and storage. In addition, further progress is required in advanced manufacturing technologies that enable large-scale, cost-efficient, and replicable production of high-quality cells. Standardized processes should be subject to frequent and

regular inspections by competent authorities.

6. Conclusion

Cancer immunotherapy is a rapidly developing field with promising potential for cancer treatment. However, the limitations of current autologous cellular therapies have led researchers to explore allogeneic cell therapies as an attractive alternative. Allogeneic cell therapies offer advantages such as off-the-shelf availability, mass production, and lower cost. Once personalized engineered cellular therapy evolves to allogeneic off-the-shelf products, many problems that hinder cell therapy efficacy and accessibility can be easily eliminated. Nonetheless, they require strict regulatory oversight to ensure safety of patients. With ongoing research and improvement, these therapies have the potential to dramatically change the outcomes of cancer treatment for a wider patient population.

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CHAPTER 3

CHIMERIC ANTIGEN RECEPTOR (CAR) T CELL THERAPY

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ABSTRACT

Cancer is one of the most common causes of death in children. More than 300.000 children are diagnosed with cancer all over the world in a year. Chemotherapy, radiotherapy, surgery, smart drugs, stem cell transplants and immunotherapies are among the standard treatment options. When a tumor is confined, tiny, and non-metastatic, surgery is beneficial. If the resection is insufficient, there is a chance that the cancer will return. Despite its efficiency, radiation and chemotherapy damage healthy tissues and can have serious side effects. As a result, new therapeutic modalities, like immunotherapy using CAR-T cells, are being used. Although it has shown great success in treating pediatric hematological malignancies, there is still scant information on solid tumors. Lately, Chimeric Antigen Receptor (CAR) T cell therapy has come to the agenda as a one of confidence and effective alternative treatment in cancer. Instead of classical treatment CAR-T has been promising more effective and a durable treatment in some of childhood cancers. CAR-T cell and immunotherapy are seen as the ”fifth basic pole” of cancer treatment in the scientific community.

Keywords: CAR-T cell, childhood cancers, treatment

1. CAR-T Cell Therapy

Surgery, chemotherapy, and radiation therapy have long been the three pillars of cancer treatment. Despite the fact that new types of treatment have lately modified the therapeutic environment for cancer patients, they remain critical therapy pillars. Immunotherapy, or medicines that boost a patient's immune system's ability to fight cancer, has quickly emerged as the "fifth pillar" of cancer treatment in the last ten years. This is because immune-stimulating medications have shown promise in shrinking or even eradicating tumors in certain people with advanced cancer. Chimeric antigen receptor (CAR) T cell therapy, a kind of immunotherapy, has also piqued the curiosity of oncologists and researchers. Despite being less common than immune checkpoint inhibitors, CAR-T cell therapies have proven the same ability to clear highly advanced leukemias and lymphomas and to keep the cancer at bay for many years. As of 2017, the Food and Drug Administration (FDA) had approved six CAR-T cell therapies. All of them have received approval for the treatment of blood malignancies, such as lymphomas, several varieties of leukemia, and most recently, multiple myeloma. The CAR-T cell treatments that are now on the market are unique for every patient. They are created by taking T cells from the patient and genetically modifying them so that they produce chimeric antigen receptors, or CARs, on their surface. Specific proteins, or antigens, on the surface of cancer cells are recognized by the CARs and bound to them (1, 2).

Immune cellular therapies with a specific target and demonstrated clinical efficacy include CAR-T. Three different domains, an antigen binding domain, a transmembrane domain, and a cytoplasmic domain make up a conventional CAR design. Due to their small size, ideal stability, high affinity, and manufacturing feasibility, nanobodies have been used extensively as the antigen binding domain of CAR-T (1, 2).

The use of CAR-T cells in cancer treatment is a recent and promising development. CARs are fusion proteins created as artificial receptors that instruct lymphocytes most frequently T cells to detect tumor antigens without the need for the HLA. As a result, they are better equipped to recognize a wider variety of target antigens than natural T-cell surface receptors (TCRs). Most currently available CAR-T therapies are manufactured using lentiviruses. However, researchers are actively seeking alternative methods due to the oncogenic nature of lentiviruses. So CAR-T cell therapy is based on reprogramming a patient's own T-cells with a CAR construct and returning them into the patient's blood, where they start to attack cancer cells. The cancer cell learns to be invisible to the immune system. With the help of CAR-T cells, genetically modified T cells are enabled to recognize and attack the cancer cell. The construction of a new immune system begins.

According to Renier J. Brentjens from the Memorial Sloan Kettering Cancer Center in New York, whose clinical procedures are among the best in the world, CAR-T therapy is "equivalent to giving patients a living drug." It was first tested on patients, both children and

adults, who had ceased receiving all therapies and had little chance of recovery, and it had incredibly positive effects (3-5).

Clinical studies on pediatric acute lymphoblastic leukemia (ALL) and adult diffuse large B-cell lymphoma subtypes (DLBCL) have proven that CAR-T cell therapy can be an alternative treatment option. The FDA approved anti-CD19 CAR-T cell therapy against B-cell malignancies in 2017, due to its unprecedented success. CAR technology offers significant potential, and there is plenty room for improvement. CAR-T cell therapies are also being developed for multiple myeloma, with clear evidence of clinical activity. CAR-T cell therapy still has significant drawbacks that require attention. These include the immunosuppressive milieu, tumor infiltration, immunological escape, limited durability, life-threatening toxicity linked to CAR-T cells, and B-cell malignancies with suppression and resistance that cause relapse (2-4).

2. CAR Technology and Basic Compounds of CAR Module

CARs are modular synthetic receptors consisting of four main components: an extracellular target antigen-binding domain, a hinge region, a transmembrane domain, and one or more intracellular signaling domains.

The antigen-binding domain is the portion of the CAR that provides target antigen specificity. Traditionally, CARs target extracellular surface cancer antigens, leading to major histocompatibility complex (MHC)-independent T-cell activation. However, there have been descriptions of CARs that recognize intracellular tumor-associated antigens using MHC-dependent mechanisms, as well as peptide-centric CARs and T-cell receptor (TCR)-mimic CARs (1-3).

The so-called hinge region, an extracellular structural area, extends the binding units from the transmembrane domain. It increases the antigen-binding domain's length and flexibility, allowing it to get closer to the desired epitope. It is significant to note that the selected hinge appears to affect CAR functionality since differences in the length and composition of the hinge region can affect flexibility, CAR expression, signaling, epitope recognition, strength of activation outputs, and epitope recognition. The transmembrane domain of CARs is arguably the least well-studied part of the structure. Although there is evidence to suggest that the transmembrane domain may potentially be important for CAR-T cell function, its primary purpose is to bind the CAR to the T-cell membrane.

In order to build CAR structures with the best endo domains, understanding the effects of CAR co-stimulation has attracted a lot of attention in the field of CAR engineering. The significance of co-stimulation for CD19-targeted CAR-T cell persistence was first demonstrated in early *in vivo* models of B-cell malignancies. When exposed to an antigen repeatedly, IL-2 production and proliferation were enhanced by the addition of a co-stimulatory domain. Once it was recognized how important co-stimulation was for the success of CAR-T

cell therapy, second-generation CARs were developed using one co-stimulatory domain in succession with the CD3 intracellular signaling domain. CD28 and CD137 had the best patient response rates among intracellular domains (4-6).

3. Controlling CAR-T Cell Activation and Elimination

CAR-T cell therapy's effectiveness hinges on striking a compromise between maximizing beneficial side effects and reducing effective anti-tumor activity. Using the most up-to-date instruments and technologies in molecular cell biology and synthetic biology, scientists from many domains are working together to develop next-generation goods. New techniques and tactics are being published at a rapid rate, furthering the area of immunotherapy. Controlling the activation and removal of CAR-T cells following treatment provides difficulties in addition to the negative effects of CAR-T cell therapy (2, 3).

Due to unpredictable CAR-T cell proliferation and patient toxicity, CAR-T cells must be removed during complete remission. There aren't many techniques for that. The earliest and still most common rational strategies include suicide genes, molecular safety switches, and inhibitory corticosteroids. The key components of the elimination process are the activation of natural cell death mechanisms such intrinsic and extrinsic apoptotic pathways, antibody-dependent cell-mediated cytotoxicity (ADCC), and the subsequent removal of cellular remnants by phagocytic cells.

Several on/off switches have been reported using different methods, such as lenalidomide, UV, ultrasound, and asunaprevir light-inducible nuclear translocation and dimerization (LINTAD) photothermal switch CAR hypoxia-induced CAR (HiCAR). Essentially, CAR-T cells are initially in an "off" state, resembling normal T lymphocytes in the bloodstream. Through the use of these switch methods, CAR-T cells can be altered to an "on" state. So they can bind the specifically target antigen and interact with it (4, 5).

4. Insights from Clinical Trials

Complete remission rates of 70-90% have been demonstrated in clinical trials on recurrent B cell acute lymphoblastic leukemia in both pediatric and adult patients receiving CD19 directed CAR T cells. However, 30-60% of patients relapse following CAR therapy, with CD19 negative relapses accounting for 10-20% of those instances. There are few available treatments for post-CAR recurrence (5-7).

With CD19-targeted CAR-T cell therapy, patients with refractory large B-cell lymphomas experienced a complete response rate of 58% and a partial response rate of 25% in the ZUMA study. Additionally, studies involving Multiple Myeloma patients exist. CAR-T cells that target the B cell maturation antigen were examined in the phase 1 CRB-402 clinical study, with an 86% response rate being seen. Additional research recommends investigating additional targets, such as CD138 or Syndecan-1, as they are only expressed on MM cells (8).

Limited data is available regarding patients treated with CAR-T cell therapy. CAR-T cell therapy has evident long-lasting effects on the immune system. Long-lasting B cell depletion and hypogammaglobulinemia are common occurrences following CD19-targeted CAR-T cell therapy.

Long-term follow-up studies have shown persistent B cell depletion in 25-38% of patients and IgG depletion in 18-74% of patients who received CD19-targeted CAR T-cells (7, 8).

5. CAR-T Treatment in Childhood Tumors

Indications for clinical use of CAR-T cells are lymphoid malignancies (acute lymphoblastic leukemia, lymphomas, DLBCL, chronic lymphocytic leukemia), solid tumors, multiple myeloma and myeloid malignancies. Especially hematological malignancies constitute a favourable target for CAR-T cells. Involvement of areas such as the bone marrow and lymph nodes, where lymphocytes are predominant, creates a better target than solid tumors (9-10). CAR-T is preferred in hematological malignancies, especially in relapsed refractory ALL or in bridging treatment before allogeneic transplantation. In these indications, CAR-T cell therapy has a more permanent effect than monoclonal antibody treatments. Good results have been reported with donor-derived allogeneic CD19 CAR-T cells in relapse after HSCT. Before CAR-T treatment, the patient is given chemotherapy for lymphodepletion (Flu/cy). Genetically modified CAR-T cells are given to the patient following this lymphodepletion. FDA approved anti-CD19 CAR-T cell therapies are Tisagenlecleucel (CTL019, Kymriah) and Axicabtagene ciloleucel (KTE-19, Yescarta). Tisagenlecleucel (Kymriah), axicabtagene ciloleucel (Yescarta), and brexucabtagene autoleucel (Tecartus), three CAR-T cell-based therapies, have so far received major regulatory agency approval for the treatment of refractory or in second or later relapse B-cell precursor acute lymphoblastic leukemia (B-ALL). Clinical research on CAR-T cell therapy has increased rapidly in 2016. About 100 clinical trials are added each year. In the USA alone, over 1000 patients have been given CAR-T cells to date. More than 350 patients in Europe have been given CAR-T cells (93% clinical trials) since 2018. CAR-T treatment response rates are 68-93% in ALL, 64-86% in B-cell lymphoma, 57-71% in CLL, and 9-35% in solid tumors, respectively (11). The main distinction between AML and B cell malignancies is that AML only partially responds to the immune system. As a result, AML has worse immunotherapy results than B cell malignancies. In AML, it is particularly challenging to identify a single target antigen. The discovery and advancement of immunological therapy against AML is hopeful thanks to novel approaches such the use of biodegradable modified T cells, anti-EGFR antibodies, and suicide genes (12). B cells make up Hodgkin lymphoma, although Reed Sternberg cells have CD30 rather than B-cell surface antigens. An existing medication called Brentuximab vedotin targets CD30. Modified T-cell therapy can be performed in patients with Hodgkin lymphoma who have relapsed after brentixumab therapy. CD30 can be targeted in CAR-T cell therapy for Hodgkin Lymphoma.

There are ongoing studies of modified T cell therapies against Hodgkin lymphoma (13). Although clinical trials have demonstrated that CAR-T continues to be effective in treating hematologic malignancies, treating solid tumors has proven to be quite difficult. The solid tumor's fibrotic stroma or the immunosuppressive nature of its tumor microenvironment could reduce the efficacy of CAR-T therapy. In other words, solid tumors limit the effective cytotoxicity from CAR-T to the target tumor cells because they prevent T cells from penetrating them effectively and exhibit a hostile microenvironment. In comparison to studies on hematological cancers, there were fewer and fewer clinical investigations on CAR-T cell treatment in solid tumors. Up till October 2018, CAR-T cell treatment had a 9% response rate in all solid tumor studies. Neuroblastoma had the best response rate, with an ORR of 35%. Neuroblastoma which develops in nerve tissues is the most common extracranial cancer in children. Recurrence after first-line treatment is common, at which point further options are limited and prognosis is extremely poor. Antigens that are intensely expressed in neuroblastoma are expressed in peripheral nerves and other neural tissues, albeit at a lower level. GD2 is the most studied tumor associated antigen in neuroblastoma and is a widely and highly expressed disialoganglioside (14). Prior studies have validated the tumor antigen GD2 as a relevant therapeutic target and have suggested sensitivity of neuroblastoma to immunotherapy. The most frequent form of kidney cancer in children is Wilms tumor (WT), commonly known as nephroblastoma. Three immunological targets are taken into account in the context of CAR-T cell therapy: glypican 3 (GPC3), epidermal growth factor receptor (EGFR), and B7-H3 (CD276). A lipid molecule anchors the heparan sulfate proteoglycan glypican 3 (GPC3) to the cell surface. It has been demonstrated that GPC3 stimulates Wnt signalling, which aids in the formation of hepatocellular carcinomas (HCCs). Numerous studies have demonstrated high GPC3 expression in both WT and hepatoblastoma. This is why young patients with WT are being enrolled in a phase I clinical trial that uses GPC-3 CAR-T cells along with the IL-15 gene to treat GPC3+ solid tumors. The epidermal growth factor receptor (EGFR), a protein that aids in the growth and division of some cells by attaching to the signaling chemical epidermal growth factor (EGF), is thought to be another target of CAR-T cell treatment. The study utilising EGFR806 CAR-T cells as a treatment of recurrent/refractory pediatric solid tumors, including WT, is a phase-I clinical trial, with two arms receiving various treatment regimens. B7-H3 is a third suggested target for CAR-T cell treatment of WT since it is a transmembrane protein of B7 superfamily molecules. Among the 388 pediatric solid tumor specimens examined, 325 (84%) tested positive for B7-H3, with 70% exhibiting high intensity staining of 2+ or 3+. All 12 Wilms tumor samples displayed intense staining of 2+ or 3+. For children and young adults with recurrent or resistant solid tumors, a second generation of B7-H3 CAR-T cells was created which is currently being examined in a phase-I clinical trial (14-16).

One of the most prevalent primary bone cancers in kids and teenagers is osteosarcoma (OS), also known as osteogenic sarcoma. The precise identification and targeting of the tumor cells is essential for the success of CAR-T cell therapy. The most frequently used CAR-cell therapy targets for OS today include HER-2, GD2 , B7-H3, IL-11 Ra, IGF1R , and EphA2 (17-20).

There aren't many choices for treating metastatic disease that is resistant to conventional chemotherapy in young patients with rhabdomyosarcoma (RMS), the most prevalent kind of malignant soft-tissue tumor. New treatment strategies, including CAR-T cells, are therefore necessary. The effectiveness of CAR-T cell therapy in the treatment of RMS was demonstrated in the instance of a 2-years-old male child from Beijing Children's Hospital. Middle-risk embryonal RMS (IIa, TNM stage 2) of the bladder cavity without distant metastases was the boy's diagnosis. The patient underwent three procedures, 25 cycles of chemotherapy, and one cycle of radiotherapy, but the disease nevertheless relapsed twice while the patient was receiving treatment. The fourth generation of CAR-T cells therapy was chosen due to the tumor's propensity for recurrence. Results from immunohistochemistry supported the CD56+ phenotype of the tumor's tissue, a frequently observed hallmark of RMS. The patient has been under regular observation for 3, 5 years and finally achieved complete remission with no evidence of metastases or thickening of the bladder wall.

The most frequent childhood eye cancer is retinoblastoma, which is brought on by a mutation in the RB1 suppressor gene. It makes up 2% of all pediatric malignancies and can be divided into two categories: unilateral and bilateral (21-23). According to preclinical research, CAR-T cell treatment is very successful *in vitro* against retinoblastoma and both CD171 and GD2 are useful targets on human retinoblastoma cell lines. Two phase-I clinical trials are now recruiting children and young people with, among other conditions, retinoblastoma who are between the ages of 26 and 30. The first evaluates the feasibility, safety, acceptability, and toxicity of B7H3-specific CAR-T cell infusion and B7H3 and CD19-specific CAR-T cells (24-26).

The ineffectiveness of current treatments for metastatic and recurrent Ewing's sarcoma prompted researchers to look for new, more potent treatments that would also be highly specific to tumor lesions while minimising harm to normal tissues, commonly known as a targeted therapy. CAR-T cell therapy meets these criteria and is being used in research that is aimed at identifying the ES tumor-specific antigens. Pediatric solid tumor CAR treatments have lagged behind those for hematological malignancies. This method's quick progress has presented scientists with significant problems to be resolved. It is important to briefly discuss the main CAR-T cell therapy limitations that prevent the treatment of pediatric solid tumors, including antigen escape and an immunosuppressive tumor microenvironment, CAR-T cell trafficking and tumor infiltration, on-target off-tumor effects, and CAR-T cell-associated

toxicities. Although the majority of the study to date has focused on adults, hematological malignancies, and preclinical data, it nonetheless serves as a guide for future research in this area (27). In studies on brain tumors, there was no significant benefit in studies involving mostly adults in humans. Modifications in animal studies brought fatal results. Penetration of CAR-T cells was found insufficient in brain tumors due to the blood-brain-barrier. At the same time, treatment monitoring difficulties and high neurotoxicity have limited its use in this field (28, 29). CAR-T cell therapy can cause significant side effects in the neurological system. The frequency of neurological events is between 21-64%. Encephalopathy, seizure, delirium can be seen. Neurotoxicity was reversible in most cases, and symptoms resolved within a few days without intervention or with long-lasting significant effects. However, life-threatening adverse neurological events may occur. The underlying causes of neurological side effects have not yet been explained. Systemic corticosteroids, anticonvulsants, antipyretics, anti-cytokine drugs can be used in treatment. Because CAR-related toxicities frequently manifest suddenly, optimal control methods would enable the clinician to quickly regulate CAR-T cell activity. The many study designs make it difficult to compare the on/off kinetics directly, however regulation at the protein level, as well as the use of suicide genes or elimination markers, are anticipated to act more quickly than regulation at the transcriptional level. However, complete removal of CAR-T cells nullifies the long-term anti-leukemic efficacy, therefore many approaches focus on reversible control, enabling the doctor to turn off the CAR-T cells when side effects appear (29). Normal CAR-T cell therapy destroys not only cancerous B cells but also normal B cells. CAR-T cell therapies against CD19 terminate normal B cell clones. Therefore, hypogammaglobulinemia occurs. This finding is actually indicative of treatment response. To avoid infection, intravenous immunoglobulin replacement treatment might be used. Tumor lysis syndrome is another well-known side effect of CAR-T cell treatment. Acute anaphylaxis has been reported on numerous occasions. Rarely, it has been demonstrated that cytokines generated following CAR-T cell therapy mimic hemophagocytic lymphohistiocytosis. Tocilizumab, an IL-6 inhibitor, can lessen CAR-T cell treatment side effects while maintaining therapeutic efficacy (12-17). In addition, the promising drugs sarilumab and olokizumab can be used to stop cytokine relasing syndrome. To avoid cytokine relasing syndrome, steroid therapy can be undertaken, however corticosteroid use may compromise the effectiveness of the therapy. The antitumoral efficacy of CAR-T cell therapy is a dynamic process dependent on the characteristics of the product and the host. Investigating target tumor-associated antigens by considering the balance of effect and toxicity is a serious problem in the application of CAR-T. By identifying only pairs of antigens that are expressed on tumor cells, producing bispecific CAR-T cells that need both antigens to be activated will be efficient and reliable.

6. Conclusion

CAR-T cells offer a potent treatment option for hematologic malignancies. However, the potential of CARs extends beyond their current applications. There are numerous potential targets that CARs can exploit. Although CAR-T cell-related side effects can be detrimental, there are methods available to mitigate these effects. There are several promising areas of research aimed at enhancing the durability of remission following CAR-T cell therapy. Pediatric solid tumors have responded well to CAR-T cell therapy, but further studies are required to completely understand its safety and effectiveness. According to studies, CAR-T cells may find and kill cancer cells in pediatric solid tumors, significantly shrinking the tumor and increasing survival rates. In order to create novel strategies to accurately target CAR-T cells to the surface of solid tumors, it is crucial to understand the variety of parameters influencing the effectiveness of these cells. Overall, we are currently in an exciting phase of CAR-T cell development, witnessing improved responses and the emergence of new treatment indications.

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CHAPTER 4

IMMUNOMODULATORY EFFECTS OF MESENCHYMAL STEM CELLS

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ABSTRACT

Stem cells are a rather unique group of cells among all eukaryotic cells. They have self-renewal capacity, asymmetric division, and regenerative properties. Due to these features, stem cells play critical roles in many biological processes such as regeneration, disease, cancer. Recently, the researchers have been focused on a subgroup of stem cells; mesenchymal stem cells (MSCs). They can be distinguished by three important characteristics: multipotency, adherence to plastics and the presence of specific surface antigens. MSCs have recently gained prominence for their immunomodulatory effects. In particular, MSCs with immunosuppressive activity have a major role in the regulation and suppression of the immune response. Unfortunately, the underlying mechanisms for this resistance have been poorly understood, yet. Studying these mechanisms in detail will enable the development of treatments such as immunotherapy and personalized therapy for autoimmune diseases, and tissue damage. Here, the current state of knowledge for this field of MSCs in the context of the immune system will be discussed.

Keywords: Mesenchymal stem cells, immunomodulation, innate immunity, adaptive immunity, autoimmune disease

1. History and Definition of Stem Cell

The first stem cell term originated from two questions of 1890's: the continuity of the germplasms and origin of hematopoietic cells. The researchers, Boveri and Häcker, used the 'stem cell' term to describe cells committed to germline in animal embryos (1). In their studies Valentin et al. have worked on cyclops, in the meantime Bovari et al. have worked on nematodes, in conclusion they defined the cells formed in the germline lineage process in fertilized eggs as stem cells. In parallel with these studies, it is known that Pappenheim, Maximov, and Neumann had continued similar studies in blood cells with their own groups (1, 2).

In our country, it is known that the first stem cell studies were carried out with fetal and cord blood grafts by Sürreyya Tahsin Aygün, who had studies on virus culture and anthrax vaccines in the 1950's. It has suggested that it is possible to extract stem cells from discarded placenta and umbilical cord cells after birth, and to use these cells to treat some conditions. (3).

As we approach today, we see that Capecchi and Smithies won the Nobel in 2007 for their studies using mouse embryo stem cells (4, 5). The mouse embryonic stem cells for specific gene modifications have opened a new perspective in medicine for research and therapy. Thus, the first state and private bone marrow stem cell banks in our country and world were established in the early 1990s. In 1998, Thomson et al. discovered "human embryonic stem cells" (6). Finally, in 2012, Yamanaka et Gurdon won the Nobel with the study of induced pluripotent stem cells (7, 8). Using retroviral gene transfer of transcription factors, they have discovered a method to convert mature epithelial cells into pluripotent stem cells.

Stem cells are a special type of cell. They can be self-renew and divide symmetrically or asymmetrically. While the number of stem cells is conserved constant by asymmetric division, on the other hand, the other cells compose the cells of the tissue from which they originated. It can also transform into different cell types upon stimulation by different signaling pathways (9, 10). In the differentiation process, signaling pathways, cell cycle regulators, and intracellular mechanisms such as microRNAs (miRNA) and transcription factors are known to play key roles. Stem cells generally remain in a dormant state in tissues. However, when tissues are injured, asymmetric division accelerates. Stem cells begin to differentiate.

2. Stem Cell Classification

Based on their differentiation properties, stem cells can be classified into 5 groups: "Totipotent, pluripotent, multipotent, oligopotent, and unipotent stem cells". Cells with the ability to form a complete embryo are called "totipotent stem cells" (11). It consists of the fusion of egg and sperm, which can be the source of all tissues and organs. "Pluripotent stem cells"' have less differentiation ability than totipotent stem cells. They are called with a prefix 'pluri' because they originated from triple germ layers; "endoderm, mesoderm, and

ectoderm" (12). Embryonic stem cells are considered as pluripotent stem cells. In Table 1, the tissues composed of embryonic germ layers are shown. "Multipotent stem cells" can differentiate into cell types within one lineage but are more limited than pluripotent stem cells (13). Also, they play a key role for tissue repair, development. The stem cells which reside in a tissue are known as "oligopotent stem cells" and they are able to differentiate to cells of only a specific tissue. "Hematopoietic stem cells" are considered as oligopotent stem cells and they are able to differentiate into only one lineage (14). "Unipotent stem cells" are able to differentiate into one specific cell group.

Table 1: Tissues from embryonic germ layers

Germ line	***Tissues***
Endoderm	Thymus, pancreas, liver, pulmonary, gastrointestinal system tissues
Mesoderm	Heart, bone, bone marrow, neural, vein
Ectoderm	Hair, nail, retinal, dental, epidermal

In addition to stem cell plasticity classification, they are also categorized into two groups according to being "embryonic stem cells" (ESCs) and "non-embryonic stem cells". ESCs are derived from a blastocyst. ESCs are derived from the embryo's inner cell mass. In Figure 1, characteristics of ESCs are shown.

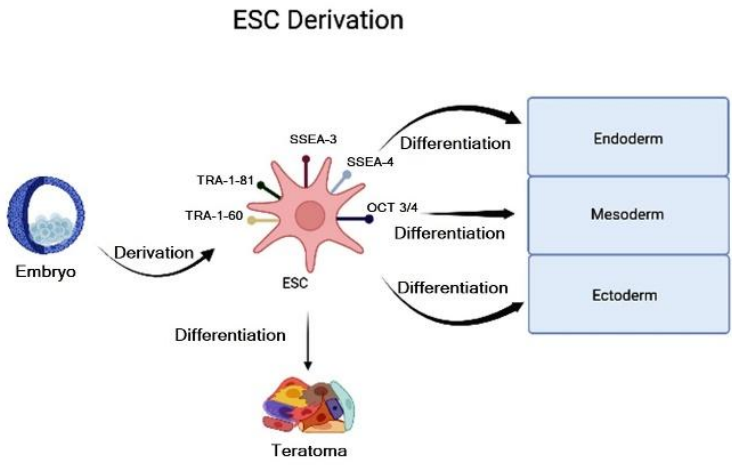


Figure 1: ESCs derivation and characteristics

Despite the ESC using, the limitations have emerged as a result of ethical, political, economic, and religious debates on embryonic stem cells (15). Besides debates, ESCs can result in teratoma and cancer. After the limitation debate, non-embryonic stem cells gained prominence.

3. Mesenchymal Stem Cells

The discovery and characterisation of mesenchymal stem cells (MSCs) was in the 1970s. (16). MSCs are distinguished from other stem cells by plastic adherence, specific surface antigens, and multipotent differentiation properties (13). MSCs have a spindle shaped appearance. It may be derived from a variety of biosources including umbilical cord tissue, bone marrow, dental pulp, and others. In addition, MSCs can differentiate into adipocyte, chondrocyte, osteocytes, astrocyte, and epithelial cells due to their multipotential property (17). Figure 2 has shown main sources of MSCs and differentiation (18). Because of its differentiation potential, it is being used as a powerful tool in regenerative medicine.

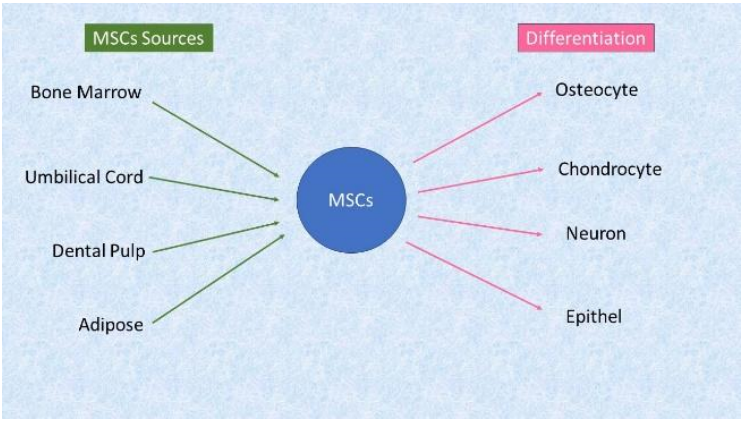


Figure 2: MSC sources and its differentiation (18).

MSCs have advantages, it can be obtained easily from different allogeneic sources or autologous individuals. Although obtained from many tissues, all MSCs have specific surface markers "[$CD44^+CD29^+CD105^+CD90^+CD73^+$ and lack of $CD45^-CD34^-CD14^-$ and HLA (human leukocyte antigen)-DR]" and are characterized (13).

4. Immunomodulation of Mesenchymal Stem Cells

The other important feature of MSC is tropism. Firstly, MSCs were used for regeneration and tissue repair and later, its tropism capacity has generated interest for many researchers. MSC can reach the inflammation, tumor, and wounding microenvironment due to its tropism property. It plays a key role for innate and/or adaptive immunity. MSCs can exert their immunomodulating functions through intercellular interactions and paracrine effects with T, B, macrophage, monocyte, natural killer (NK), and dendritic (DCs) cells.

MSCs enforce their immunomodulatory effects by secretomes such as chemokines and growth factors by paracrine mechanisms (19). These secretomes are enclosed in cell-secreted extracellular vesicles (EVs). They are classified as microvesicles (MVs), exosomes, and apoptotic bodies (20). Extracellular vesicles by MSCs (MSC-EVs) have similar immunoregulatory functions to their parent MSCs (21). However, the paracrine effects depend on the MSC source, the target cells, and the environment. (22).

4.1. The Effect of Mesenchymal Stem Cells in the Innate Immunity

The innate immunity is the defense system with which individuals were born. It can protect them against all antigens and involves barriers that prevent detrimental substances from entering their bodies. The barriers are the first line of immune defence. MSCs are known to have interactions with NK cells for innate immunity. Secretion of indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) by MSC inhibits interleukin-2 (IL-2) induced NK cell proliferation and induces cytotoxic activity (21). MSCs also enhance the interferon-gamma (IFN-γ) secretion capacity of IL-12/18 stimulated NK cells. This situation affects the infection defence in the injury environment and also tissue regeneration (21, 23). MSC-derived exosomes have been reported to increase neutrophil, while MSC-conditioned media increases neutrophil functions, indicating that MSC-conditon media are useful for enhancing immunity through neutrophil modulation (24).

PGE2 from MSCs allows MSCs to inhibit the differentiation of monocytes to mature DCs, while IL-6 secreted MSCs can inhibit monocyte differentiation through IL-10 anti-inflammatory properties (25, 26). It has been shown that MSC-EV inhibits the uptake of antigen by immature DCs and attenuates the maturation of DCs. (21, 27). MSC-EVs also play a critical role in polarizing macrophages by promoting “M2 macrophages” rather than “M1-like macrophages”, by down-regulating IL-22/23 (28, 29).

4.2. The Effect of Mesenchymal Stem Cells in the Adaptive Immunity

Adaptive immunity is the process by which specialised immune cells and antibodies attack and destroy foreign invaders. MSCs possess immunomodulatory effects and this effect can be useful for adaptive immunity. In the adaptive immune system, the main immune cell target of MSC is T cells. MSC can inhibit T helper 17 (Th17) cell differentiation by inducing PGE2 and IL-10. They also inhibit IL-17 and IFN-γ (30). It is thought to be that MSC inhibitory effect for Th17 cells is mediated by cell-cell contact (31). The mechanisms underlying these MSC-Th17/IFN-γ interactions are not fully understood. Through regulation of the IL-25/signal transducer and activator of transcription 3 (STAT3)/programmed death-1 ligand (PD-L1) axis, MSCs are reported to suppress Th17 responses (32).

In addition, programmed death ligands, PD-L1, and PD-L2, are also secreted from MSCs (21). MSCs have a direct immunosuppressive effect on T-cell behavior by suppressing activation of CD4 T cells (33). MSCs can suppress CD4 T cell activation by secreting PD-1 ligands. They downregulate IL-2 and induce cell death (34).

5. Mesenchymal Stem Cell Application for Autoimmune Diseases

The body’s immune system recognises healthy cells as foreign in autoimmune diseases. There are more than 90 types recognised of autoimmune disease. Clinical trials for treating autoimmune diseases are currently based on alleviating symptoms and averting disability (35).

In other words, at the moment there is no effective treatment available. MSCs are known to be immunomodulatory and could be a new kind of tool for treatment of autoimmune diseases.

MSCs-derived cytokines play a key role in the immunsuppression of MSCs. IFN-γ is the one of these cytokines and usually exerts immunomodulation effects with other cytokines such as IL-1/17 and tumor necrosis factor-α (TNF-α). IFN-γ can induce regulatory molecules in the immune response. These include "human leukocyte antigen G1 (HLAG1), vascular cell adhesion molecule-1 (VCAM-I), intercellular adhesion molecule-1 (ICMA-1), Jagged-1 and 2, and PD-L1" (35, 36). In addition, MSCs secrete several immunomodulatory molecules such as "IL-10, hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), IDO, IL-6, galectin (Gal)-1, and nitric oxide (NO)", which modulate innate and adaptive immune responses (35-37). MSCs suppress cytokine secretion and T cell proliferation, promote "regulatory T cells" (Tregs), regulate balance of Th1/Th2 cells, B cell activity and antigen presentation of DC (38, 39). Also, MSC-derived soluble molecules such as PGE2, and TGF-β act as Treg inducers (40). Specifically, MSCs can reduce pro-inflammatory cytokines and they can inhibit inflammation. TNF can cause an imbalance between Treg and Th1/Th17 in rheumatoid arthritis (RA) patients and is the major mediator of autoimmune disease. TNF debilitate Tregs in multiple sclerosis (MS) and RA patients (41). Anti-TNF property of MSCs may be used as therapeutic tool for autoimmune diseases. Besides of TNF, IFN-γ and IL-1 may also used for exerting MSC immunosuppressive functions. There have been reports that the immunosuppressive chemokine, molecules were produced by TNF-primed MSCs and these MSCs can attenuate the symptoms of myocardial infarction, cutaneous wound by TNFR1 pathway (42).

The activity of macrophages, NK, and T cells is regulated by MSC-derived TGF-β1 (43). MSC-derived TGF-β1 plays a key role for differentiation of Th cells. The overexpression of TGF-β1 in bone marrow-derived-MSCs (BM-MSCs) have improved a therapeutic potential with an increased Th2 response in type 1 diabetes (44). MSCs can inhibit B cells and antibody production when IL-40, CD-40L, IL-2, and IL-4 cytokines are presented. When stimulated with "T-cell-depleted peripheral blood mononuclear cells" (PBMCs), they have no effect on B cells (38). However, it remains unclear and controversial whether MSCs immunomodulate B cells. In addition, MSCs have an effect on DC maturation. DCs can mature by the down-regulation of "major histocompatibility complex-II (MHC class-II)" and co-stimulatory molecules release (35, 45, 46). Also, it has been reported that MSCs can prevent neutrophil apoptosis and infection (47, 48).

5.1. Rheumatoid Arthritis

RA, characterised by joint inflammation, synovial hyperplasia and progressive joint damage, is the most common autoimmune disease (49). The disease involves a disturbance in the balance of the immune system, leading to autoreactive immune cells that attack the collagen

dominated area the articular cartilage. It is recommended conventional treatments such as "methotrexate (MTX), nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, slow-acting anti-rheumatic drugs (SAARDs), and disease-modifying antirheumatic drugs (DMARDs)" (50).

Wang et al. have used umbilical cord derived-MSCs (UC-MSCs) along with DMARDs to compare the efficacy of MSC (51). In the study, they added 64 patients with RA and one or three year/s after UC-MSC treatment, they observed that liver, kidney and routine blood monitoring examinations were in the normal range. In conclusion, they declared that UC-MSC application for RA could be a feasible, safe and effective option. In a phase I/II anon-randomized study, single dose adipose derived-MSCs (AD-MSCs) were used to 15 patients with RA and TNF-α, IL-6 levels were monitored (52). After 52 weeks of MSC treatment, they suggested that AD-MSCs might be efficacious for joint function of RA patients.

5.2. Multiple Sclerosis

MS is a chronic autoimmune central nervous system disease, however its pathology is still unknown. But there is a consensus that autoreactive T-cells are responsible for CNS demyelination (53). Conventional treatments, such as medications, can reduce MS, but they have strong side effects and do not have an effect on MS symptoms.

Petrou et al. have demonstrated that autologous bone marrow derived-MSCs (BM-MSCs) were applied to progressive and active multiple sclerosis patients and found that MSC treatment ameliorates the symptoms and is well-tolerated (54). In another clinical trial by the same group carried out by the same group, neurofilament light chains were reduced for nine out 15 patients with MS which intrathecal MSC treatment (55). Contrary, Berard et al. have demonstrated that MSC application for MS patients showed no detectable effect in the short term for cognition but they declared that MSC application is safe for the patients (56). A recent study showed that neurotrophic secreting cells by BM-MSCs have increased cerebrospinal fluid markers and inflammatory markers decreased in progressive MS patients (57).

5.3. Type 1 Diabetes

"Type 1 diabetes mellitus" (T1DM) is a family of autoimmune diseases in which T cells attack pancreas-β cells, causing a total lack of insulin (58). The pancreas makes little or no insulin in T1DM. Although T1DM generally begins in childhood or adolescence, it may occur in the adult stage of life. There is no cure for type 1 diabetes. The aim of treatment is to control the amount of sugar in the blood with the use of insulin, diet and lifestyle, and to prevent complications.

A placebo-controlled trial showed that autologous BM-MSCs shifted cytokine profiles from "pro-inflammatory to anti-inflammatory" effects (59). Cai et al. have demonstrated that UC-MSC added to the autologous BM-mononuclear cells treatments were safe and ameliorate

symptoms of T1DM patients (60). An eight year follow-up clinical trial indicated that co-treatment of UC-MSC and BM-mononuclear cells reduced chronic diabetes complications in T1DM patients (61). Lu et al. have demonstrated that UC-MSC treatment may lead to healthier β cells during the first year after T1DM diagnosis than the T1DM patients with standard therapy alone (62).

5.4. Systemic Lupus Erythematosus

"Systemic lupus erythematosus", or SLE, is a chronic autoimmune disease. It is characterized by high levels of autoantibody production, resulting in the deposition of antibody-antigen immune complexes in various organs (63). Regulatory B cells (Bregs) are important for SLE pathophysiology, in addition to a Th1/Th17/Tregs imbalance.

In a study of Li et al., they have reported that BM-MSCs might ameliorate hematological abnormalities and clinical remission (64). Similarly, Chen et al have proposed that UC-MSC treatment increases Treg levels and alleviates SLE (65). Wang et al. have shown that allogeneic UC-MSCs mediate immune suppression in SLE patients by suppressing the proliferation of T cells through the release of high levels of IDO (66).

6. Conclusion

Due to their tropism towards other cells and their immunomodulatory functions, MSC has become a promising therapeutic strategy. MSC immunomodulation is regulated by several inflammatory cytokines, and immune cell-MSC interaction may contribute to regeneration and progression of several inflammatory diseases. However, there are still several issues that need to be clarified. MSCs have heterogeneous properties and alter the response to pro-/anti-inflammatory stimuli. Understanding how the variability of MSCs affects their induced immunomodulatory effect is therefore not straightforward. The influence of other factors on autoimmune diseases through MSCs should be investigated in future studies.

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CHAPTER 5

THE IMMUNOSUPPRESSIVE BONE MARROW MICROENVIRONMENT IN PEDIATRIC B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Acute lymphoblastic leukemia (ALL), a hematological malignant disease of abnormal lymphoid precursor cells with uncontrolled proliferation in bone marrow, is the most common childhood cancer. B-ALL, generating from B lymphoid progenitor cells, is the most common form of the disease. Although the survival rate is high in patients treated with chemotherapeutic agents, side effects and drug resistance might occur. Thus, immunotherapy is gaining importance as a treatment option. Due to their importance in anti-cancer immunity, studies on natural killer (NK) cells in immunotherapy have increased. However, the efficiency of this treatment is lower than expected, which may be due to the immunosuppressive microenvironment in bone marrow of B-ALL patients. There are some evidences for the immunosuppressive microenvironment within the bone marrow of leukemia patients. In our recent study, bone marrow plasmas of pediatric B-ALL patients were cultured with peripheral blood mononuclear cells of healthy donors to investigate the effects of humoral components in bone marrow on NK cell functions. Conditions with fetal bovine serum and bone marrow plasmas of patients without leukemia were used as control groups. Plasma cytokine levels of both patient groups were also measured. In the conditions with B-ALL plasmas, PD-1 and intracellular IL-10 levels were found to be increased, while the proliferative capacities of NK cells were decreased compared to the control groups. High IL-10 versus low IL-18 and IFN-γ levels were also detected in B-ALL bone marrow plasmas. These findings indicate that humoral components in the bone marrow microenvironment of B-ALL patients exert a suppressive effect on NK cells.

Keywords: Bone marrow microenvironment, acute lymphoblastic leukemia, immunotherapy

1. Lymphocyte Development

B and T lymphocytes, which are components of the acquired immune system, develop from hematopoietic stem cells (HSCs) by a process called "hematopoiesis" throughout life, and mature in the bone marrow or thymus, respectively (1). Hematopoiesis, which refers to the process of formation of cellular components in the blood, takes place mainly during embryonic development and continues into adulthood (2). This process produces large quantities of lymphocytes during the fetal and neonatal period, with a significant decrease after puberty (3).

Hematopoiesis which begins in the yolk sac, continues in the liver during the fetal period when the bone marrow has not yet developed, and finally settles in the bone marrow for B cells and the thymus for T cells (2). Although the production of new B cells continues in the bone marrow in adults, the development of new T cells in the thymus slows down in adulthood. However, T cell numbers are kept in balance by the proliferation of mature T cells outside the central lymphoid organs (4).

In the fetal period, hematopoiesis first begins with the production of red blood cells to facilitate oxygenation of embryonic tissues (5), followed by the development of multipotent HSCs from which all blood lineage cells originate (6). HSCs basically differentiate into two lineage cell groups: lymphoid precursor (common lymphoid progenitor - CLP) and myeloid precursor (common myeloid progenitor - CMP) cells. The lymphoid lineage gives rise to B and T lymphocytes and natural killer (NK) cells, while the myeloid lineage generates erythrocytes, megakaryocytes, granulocytes and monocytes/macrophages.

Stem cells that receive differentiation signals first gradually lose the ability to self-renew and form the next precursor cells (7). Molecules such as cytokines, chemokines and growth factors as well as transcription factors are critical for differentiation of HSCs into lymphoid precursor cells. Major cytokines involved in the differentiation of stem cells into lymphoid precursors include interleukin (IL)-1, IL-2, IL-3, IL-6 and IL-7. CLPs formed as a result of differentiation can produce all lymphocyte types depending on the stimulus they receive (8).

The developmental stages of T and B lymphocytes from lymphoid precursor cells are discussed below, with particular emphasis on B cells, which is the main topic of this chapter.

1.1. Development of T Lymphocytes

T lymphocyte development begins in the bone marrow in response to stimulation of CLPs. IL-7 produced by stromal cells in the bone marrow and epithelial cells in the thymus, plays a crucial role in T cell development and proliferation as well as ensuring the survival of lymphoid precursor cells (9). Gene rearrangement is a critical step in T lymphocyte development to establish the antigen receptor repertoire of T cells. As a result of gene rearrangement and recombination, approximately 10^7 different T lymphocyte clones are formed, each carrying a unique T cell receptor. However, some of them are eliminated during selection in the thymus

(10).

Unlike B lymphocytes, T lymphocyte precursor cells leave the bone marrow and migrate to the thymus before completing their development. T lymphocyte precursor cells in the thymus, also called thymocytes, complete their maturation in the thymus and enter the circulation after positive/negative selection processes. T cell maturation in the thymus has a critical importance, as most of the lymphocyte receptor repertoire formed in the bone marrow is eradicated at this stage, and only 1-3% of thymocytes enter the circulation passing through the elimination in the processes of positive/negative selection (11).

1.2. Development of B Lymphocytes

B lymphocyte development from CLPs occurs as a result of stimulation with specific cytokines and contact with the appropriate microenvironment in the bone marrow (12). Various factors such as E2A, EBF1, Ikaros, PAX5, FOXO1 transcription factors and CXCL12 chemokine play role in B cell differentiation. These factors arise as a result of the interaction between IL-7 and its receptor IL-7Rα expressed on B cells, and with the support of stromal cells, all together initiate the process of differentiation into B cells (13).

The first stage of differentiation from lymphoid precursor cells to B cells is the ”pro-B cell” phase. Pro-B cells are characterized by the expression of CD22, CD34, high CD38, and low CD45 on their surface, and the presence of intracellular terminal deoxynucleotidyl transferase (TdT) (14). CD19 molecule, one of the earliest phase B lymphocyte antigens, is expressed on the cell surface by the influence of PAX5 transcription factor. In the next phase, cells which express CD34, CD38, CD79a, and cytoplasmic (cy)TdT as well as high CD10 are called ”pre-B-I” cells. In this phase, gene recombination occurs at the immunoglobulin (Ig) heavy chain locus with the participation of recombination-activating gene (RAG)1 and RAG2 proteins.

While gene rearrangement continues, the cell enters “pre-B-II” phase with the emergence of the μ chain which will form the IgM molecule within the cell. At this stage, CD20 molecule is expressed on the cell surface while CD34 and intracellular TdT disappear (15). Pre-B-II cell expresses CD79a and CD79b molecules on the membrane surface and pre-B cell receptor (pre-BHR) complex formed by Igμ (16). B cell maturation stages are shown in Figure 1.

When pre-BHR binds self-antigens in the bone marrow microenvironment, pre-B cell undergoes positive selection as a result of the signals it received and is saved from apoptosis. Igμ chain within pre-B cell is confirmed to be functional by the positive selection, otherwise the cell is eliminated (15). Pre-B cell differentiates and proliferates for the next phase with the positive signals received from pre-BHR, while at the same time, heavy chain recombination stops and light chain recombination begins (17). As a result, the cell in which the completed IgM molecule is expressed on the membrane surface is defined as an “immature B cell”. In this phase, cells which express CD5, CD20, low CD10, low CD21, high CD38, CD45, and

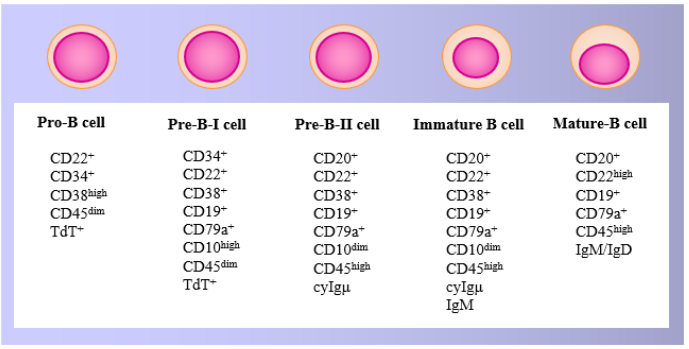


Figure 1: Stages of B cell maturation in the bone marrow (18)

high CD81 molecules on the membrane surface undergo positive/negative selection (18).

Immature B cells in which the gene rearrangement is successful are selected positively and continue the maturation process as a result of the survival signals they receive (19). If immature B cell recognizes self-antigens, a rearrangement occurs again in the BHR complex, allowing the cell to develop a non-self-reactive BHR (20). Failure of this rearrangement results in negative selection, leading to apoptosis of the cell, while positively selected cells leave the bone marrow and move to secondary lymphoid organs to complete their maturation. The cells which lose their CD10 and CD38 expression during maturation and carry IgD and IgM molecules on their membrane surfaces are named "mature B lymphocytes".

Figure 2 shows the stages of B cell maturation in the bone marrow by immunophenotyping with flow cytometry.

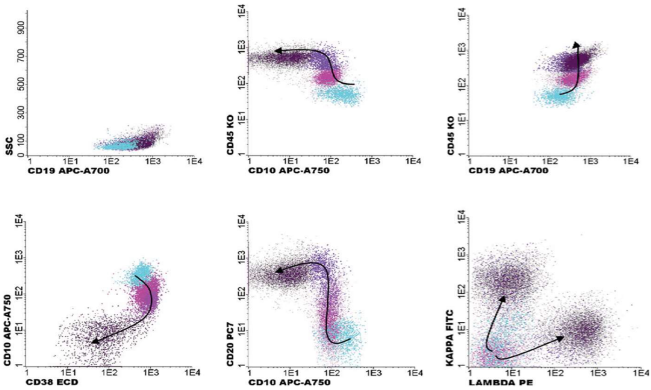


Figure 2: Demonstration of B cell maturation stages with a flow cytometry (15)

1.3. Bone Marrow Microenvironment

Hematopoietic cells in the bone marrow reside in an organized and unique microenvironment composed of stromal cells and an extracellular matrix rich in collagens, fibronectin and proteoglycans. In addition to supporting and organizing the tissue

structure, this environment has functions such as promoting self-renewal of stem cells and multipotent progenitor cells and regulating the differentiation of each cell lineage in bone marrow. Specialized microenvironments in different regions of the bone marrow regulate hematopoiesis and organize precursor cells, cell types and cell lineages (21).

Hematopoiesis is a complex process in which HSCs are expanded and differentiated into progenitor cells to give certain progeny. This process takes place in close association with the bone marrow microenvironment. Humoral components produced by bone marrow stromal cells stimulate differentiation of hematopoietic cells and contribute to the maintenance of lineage precursor cells (22). Adhesion molecules such as E-selectin, P-selectin and vascular cell adhesion molecule (VCAM)-1, which are expressed by stromal cells, bind to their receptors on HSCs and control the maintenance and functions of them (23). Therefore, stromal cells are used as a supporting cell layer for *in vitro* studies with HSCs. The use of stromal cells has an important function in the production of the cytokines which promote the differentiation and proliferation of hematopoietic progenitor cells in the cell culture (24). In the absence of a stromal layer, long-term cultures of hematopoietic precursor cells can be established by repeated addition of defined cytokines (25). These cytokines include granulocyte colony-stimulating factor (G-CSF), IL-1, IL-3, IL-6, leukemia inhibitory factor (LIF), stem cell factor (SCF) and basic fibroblast growth factor (bFGF) (26).

In addition to cytokines and growth factors, chemokines and chemokine receptors within the bone marrow microenvironment are also involved in hematopoiesis. These chemokines and their receptors regulate proliferation and ensure the survival and maintenance of hematopoietic precursor cells. In particular, the interaction between CXCL12 and chemokine receptor CXCR4 is fundamental for the homeostasis of HSCs (27).

The production of large numbers of lymphocytes in a short time to equip the immune system through hematopoiesis, which takes place predominantly in the fetal and neonatal period, is considered to be one of the underlying reasons for the more frequent occurrence of acute lymphoblastic leukemia in childhood, which is characterized by abnormal proliferation of lymphocyte precursors in the bone marrow (28).

2. Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) is a hematologic malignant disease characterized by the uncontrolled proliferation of immature lymphocytes and lymphocyte precursor cells in the bone marrow (29). Hematopoietic cells which develop abnormally in bone marrow fail to complete their maturation by being stuck in the lymphoblast stage of the normal maturation pathway, resulting in a reduced number of mature lymphocytes, erythrocytes and platelets in the peripheral blood. The clinical symptoms of ALL are associated with anemia, thrombocytopenia, neutropenia, or infiltration of leukemia cells into tissues (30).

Although ALL affects many organs, involvement of extramedullary sites is common, and

leukemia cells infiltrate mainly lymph nodes, spleen, liver and skin tissue after joining the peripheral blood. Central nervous system (CNS) involvement is detected in 5-8% of patients at the time of diagnosis (31). Clinical symptoms of ALL include fever, weight loss, fatigue, shortness of breath, easy bleeding or bruising and infections (32).

Although there are similarities in clinical presentation, ALL is a heterogeneous disease with different biological and prognostic classifications. According to the immunological classification of World Health Organization (WHO) updated in 2016, ALL is examined in two main groups as T- or B-ALL depending on the type of lymphocyte precursor cells from which it originates (33).

ALL is the most common disease of all leukemias and all childhood malignancies (34), accounting for 25% of cancers occurring in children under 15 years of age (35). Although ALL is also seen in adults, approximately 80% of patients are children, especially those aged 2 to 5 years (36).

Although the factors which cause ALL are clearly unknown, the disease is thought to be multifactorial. There are evidence that genetic and environmental factors take place in disease development. In addition to environmental factors such as irradiated radiation, pesticides, and some chemical solvents, there are findings that viral infections are also involved in the development of the disease. Genetic factors include multiple mutations which follow each other rather than a single mutation, and the disease is thought to emerge as a result of the contribution of environmental factors to these mutations (30, 37-39).

For the preliminary diagnosis of ALL, hemogram values and laboratory findings obtained from peripheral smear, supporting clinical symptoms are required. Subsequently, the methods such as the morphologic evaluation of bone marrow specimens, cytogenetic diagnosis, reverse transcriptase-polymerase chain reaction (RT-PCR) test, and immunophenotyping with flow cytometry are used for definitive diagnosis.

2.1. Immunophenotyping in ALL

Immunophenotyping with flow cytometry is currently one of the main methods used for the diagnosis and subclassification of ALL. A bone marrow sample obtained from a patient with a preliminary diagnosis of ALL based on clinical evaluation and laboratory findings is processed for immunophenotyping by flow cytometry, so that a definitive diagnosis is made with a clear ALL/AML distinction. As a result of the immunophenotypic analysis, the disease subtype is determined as B- or T-ALL, and subclassification can be made according to the immunophenotypic characteristics of the blast population (40).

The markers which can be used for T-ALL immunophenotyping include particularly CD2, CD3 and CD7, besides CD1a, CD4, CD5 and CD8 molecules. The stem cell marker CD34 and myeloid antigens CD13 and/or CD33 can also be detected in some cases. Evaluation of surface/cytoplasmic CD3 expression levels is important for the definitive diagnosis of T-ALL

(41, 42).

The most important markers for diagnosis and subclassification in B-ALL are CD10, CD19, CD20, CD22, CD24, CD38 and CD79a. CD34 and TdT markers can also be detected at high levels in different stages of B-ALL. The cytoplasmic CD22 molecule, which can be considered specific to the B cell lineage, is one of the markers that can be used to clarify the diagnosis. Definitive diagnosis of B-ALL requires the presence of at least two of CD19, CD79a or intracellular CD22 molecule expressions, but the lack of T or myeloid cell antigens on blast cells (43, 44).

2.2. B-ALL

B-ALL, which is the most common ALL type with 80-85% of all cases, is caused by abnormal proliferation of malignant B precursor cells in the bone marrow (34). Being the most common childhood malignant disease, B-ALL is the leading cause of cancer-related death in children and young adults (45). Although the 5-year survival rate of pediatric B-ALL patients is around 90% with current treatment protocols, relapse occurs in 15-20% of the patients, and the 5-year survival rate decreases to 50% after relapse. On the other hand, destructive side effects of the treatment are observed even in children who have survived the disease (46, 47).

Molecular abnormalities such as gene mutations, abnormal protein interactions, continuous cell cycle and increased autophagy are known to involve in the pathogenesis of B-ALL. It is known that factors such as viral infections, high birth weight, exposure to radiation and environmental toxins including various chemicals are among the predisposing risk factors for disease development (48, 49).

2.2.1. B-ALL Pathophysiology

In the differentiation and maturation process, cells passing through pro-B and pre-B stages transform into mature lymphocytes and become functional by the successful fulfillment of signal transduction, activation of transcription factors, and positive/negative selection mechanisms. However, in the case of B-ALL, lymphoid cell differentiation is inhibited, resulting in abnormal cell proliferation and survival (50). Dysregulated cell cycles, uncontrolled proliferation of HSCs and the formation of immature lymphoblastic cells lead to leukemia development (51).

B-ALL formation is associated with a series of gene mutations, usually beginning at the multipotent stem cell stage and replacing normal lymphoid cells with malignant cells as a result of clonal expansion, differentiation, cell proliferation, and dysregulated cell apoptosis (52).

In addition to gene mutations and dysfunctions in B-ALL, there is an imbalance in the concentrations of humoral immune components, including chemokines and cytokines,

which is thought to promote the survival and proliferation of leukemia cells while creating a suppressive environment for normal cells in bone marrow (53, 54).

2.3. Treatment Approaches in B-ALL

2.3.1. Classical Treatment

The main treatment for B-ALL is chemotherapy, which aims to exterminate leukemia cells in the bone marrow and peripheral blood. In cases which CNS involvement is detected at the time of diagnosis, radiotherapy is also used in addition to chemotherapy. The chemotherapy applied in first stage lasts approximately 2-3 years and consists of 4 phases which are remission induction, consolidation, intensification/reinduction and maintenance treatment (55).

In addition to the conventional therapy, HSC transplantation is a highly effective treatment option for patients with high-risk disease at first complete remission, drug-resistance or relapsed disease (56). Recent advances in transplantation have reduced morbidity and mortality. However, transplantation therapy has some major complications such as infertility, growth retardation, metabolic diseases and secondary malignancies that affect even the adulthood of the patients, which are the consequences of the radiation therapy applied before the transplantation (57).

Today, conventional treatment modalities have improved the survival rate of pediatric ALL patients to over 90% especially in developed countries (58). However, there are still a significant number of relapses and/or deaths despite the treatment. In addition, both chemotherapeutic agents and radiation therapy appear to have genotoxic potential and might cause secondary malignancies (59). Long-term problems, such as chronic health problems and neurobehavioral disorders, might be seen even in patients who have a full recovery of ALL with treatment (60). Increasing the dose of treatment to increase survival rates is therefore not feasible, and different treatment strategies are being studied to improve the life quality of patients and to eliminate the additional health problems associated with applied therapies.

The treatment approaches which are clinically applied or are being studied as alternative to classical treatment protocols for B-ALL are discussed below.

2.3.2. Immunotherapeutic Applications in B-ALL Treatment

In cases with high-risk and/or relapsed disease in which cannot be achieved full recovery with the conventional methods applied in the treatment of B-ALL, and who are resistant to chemotherapy drugs, there is a need for different treatment approaches to increase the survival rate and to eliminate the toxic side effects of treatment. Among these approaches, immunotherapy is of great interest, especially for pediatric B-ALL cases with appropriate target antigens. Immunotherapeutic approaches for B-ALL treatment consist of monoclonal antibodies and chimeric antigen receptor (CAR)-cell applications.

2.3.2.1. Monoclonal Antibodies in Immunotherapy

One of the main immunotherapy methods being studied for the treatment of B-ALL is application of monoclonal antibodies against specific antigens. Most of these therapeutic antibodies target CD19, CD20 and CD22 molecules which are highly expressed on B-ALL blast cells. While CD20 antigen is expressed in 30-50% of cases, the blast cells of more than 90% of B-ALL patients express CD19 and CD22 molecules (61).

Rituximab, an anti-CD20 monoclonal antibody-based drug, is the first immunotherapeutic to be developed for B-ALL treatment (61) and is used in addition to the conventional treatment protocol in $CD20^+$ B-ALL patients. Ofatumumab, a second-generation anti-CD20 antibody, provides stronger cellular cytotoxicity responses than Rituximab (62). However, it is not clear whether it will provide better results in the long run.

Epratuzumab is a drug targeting CD22 molecule, the efficacy of which has not yet been clarified in pediatric patients (61). The most promising immunotherapy drugs for the treatment of B-ALL are Inotuzumab Ozogamicin, an antibody-drug conjugate targeting CD22, and Blinatumomab which is a bispecific drug against CD19. Inotuzumab Ozogamicin binds to CD22 and allows calicheamicin, which has a cytotoxic effect on DNA, to enter the cell (63).

Loncastuximab Tesirine, a conjugate-drug targeting CD19 molecule, has demonstrated a potent effect against $CD19^+$ B cell malignancies in experimental studies, but further studies are needed to evaluate the drug's efficacy (64). Blinatumomab, designed as a bispecific antibody-based drug, contains variable regions which bind both CD3 on T cells and CD19 on B cells. The working principle of Blinutumomab, which provides $CD8^+$ cytotoxic T cell proliferation as a result of binding, is based on treating B cell malignancies by increasing the association of T cells with CD19-expressing tumor cells (65).

2.3.2.2. CAR-T Cell Therapy

CAR-T cell therapy is a form of adoptive immunotherapy and is based on transplanting effector cells which are genetically modified, to the patients to induce an immune response against leukemia. CAR-T cells are generated by modifying T cells from the patient to contain a monoclonal antibody recognition site specific to certain antigen on the targeted cell (66). Although CD19 is the main targeted antigen, CAR-T cells targeting CD22 have also been developed (67). After *ex vivo* expansion in culture media, CAR-T cells are made ready for re-infusion. Before the administration, lymphodepletion is generated in patients via chemotherapy (66).

After the application, CAR-T cells are distributed throughout the body, including the CNS (68). When CAR-T cells bind to the target cell through an antigen-antibody interaction, their cytotoxic function to kill the target cell is activated as a result of the signals they receive (69). CAR-T cells have the potential to be permanent after transplantation, suggesting the opportunity of long-term control of the disease (70).

CAR-T cells have been shown to provide clinical benefit in some hematologic cancers such as B-cell lymphomas and ALL. However, CAR-T cell therapy has some side effects despite all the advantages. Although self-limiting in activity, serious and life-threatening toxicities from CAR-T cells have been reported. The most common and severe complications are cytokine release syndrome (71), and immune effector cell-associated neurotoxicity syndrome (72). Chronic B cell aplasia, which occurs in B-ALL patients treated with CAR-T cells, also remains a problem (68).

While most CAR-T products use patients' own T cells, autologous CAR-T cell therapy presents two major challenges. One of these problems that it takes a significant amount of time to generate a therapeutic dose of CAR-T cells, which is a limiting factor in cases where the disease progresses rapidly. Another problem is the difficulty of obtaining sufficient amounts of autologous T cells for CAR-T cell production from patients who have received intensive drug therapy and are therefore mostly lymphopenic (73). Due to the existence of all these disadvantages, the search for alternative treatment methods continues. Among these approaches being studied are adoptive transplantation of natural killer (NK) cells and CAR-NK cell therapy.

2.3.2.3. Adoptive NK Cell Therapy

NK cells, components of innate immune cells, involve in the first line of defense against infection and cancer, and play a critical role in recognizing and destroying rapidly dividing and abnormally developing cell populations. In addition to the natural anti-tumor properties of NK cells, studies on the use of NK cells in immunotherapy have increased in recent years, as they seem to have a less toxic potential on patients.

Although NK cells have the ability to kill cancer cells, NK cell function is known to be impaired in leukemia patients. This deficiency in NK cell function has been shown to be associated with both the severity and progression of the disease (74). Furthermore, the presence of NK cells in the bone marrow of ALL patients is known to significantly improve survival (75). Therefore, NK cell immunotherapies based on the administration of adoptive NK cells and immune checkpoint inhibitors to overcome the deficiency in NK cell function in leukemia patients, show promise for the leukemia treatment.

Based on the fact that NK cells can directly kill abnormal immature leukocytes in the peripheral blood of ALL patients (75), NK cell transplantation from healthy individuals to patients is thought to be beneficial in terms of disease prognosis, and studies on adoptive NK cell transplantation and CAR-NK cell therapy are being conducted.

Adoptive NK cell therapy is the process of administering NK cells isolated from peripheral blood, bone marrow or cord blood obtained allogeneically from a healthy donor or autologously from the patients themselves, after undergoing certain processes (76). The isolated NK cells can be genetically modified and expanded with cytokine stimulations such

as IL-2, IL-15 or IL-21, prior to the administration (77). Autologous application seems to be more useful than the allogeneic transfer, which might cause HLA incompatibility (78).

Clinical trials have shown that adoptive NK cell therapy is effective for the treatment of hematologic cancers as well as some solid tumors (79). While T cell transplants can cause "graft versus host disease" (GvHD), which causes significant damage to non-target tissues, NK cells have been shown to suppress this interaction by releasing IL-10 and destroying antigen-presenting cells of the patient (80).

The use of NK cells in immunotherapy is still being studied, because of not having serious harmful effects on normal cells other than the targeted cells. On the other hand, NK cells derived from peripheral blood of a donor have limited proliferative capacity and functional competence. CAR-NK cell therapy is one of the approaches to overcome these limitations.

2.3.2.4. CAR-NK Cell Therapy

Due to the limitations of CAR-T and adoptive NK cell therapies, CAR-NK cells have emerged as an alternative treatment option. Although experimental studies on the use of CAR-NK cells in leukemia treatment are still ongoing, positive results have been obtained so far with low side effects (81).

CAR-NK cell therapy has some advantages such as shorter production time, unlimited administration of allogeneic NK cells without GvDH risk, production of ready-to-use products with NK cell lines, recognition and killing of tumor cells by NK cell receptors independent of CAR (82). Similar to CAR-T cells, CAR-NK cells are generated by genetically modifying NK cells to express CARs recognizing an antigen that is specific for or abundant on target cells and CD19 is the main target antigen of CAR-NK cells for B-ALL (83).

Many different cellular sources have been used for the production of CAR-NK cells, including peripheral blood, cord blood, induced pluripotent stem cell (iPSC)-derived NK cells and NK92 cell line (84). Cord blood and iPSC-derived NK cells appear to be functionally more useful than peripheral blood-derived NK cells (85).

For optimal function, CAR-NK cells must be proliferated and activated with cytokines such as IL-2 or IL-15 prior to the application. Otherwise, the killing function of resting NK cells has been shown to be decreased (86). Factors such as whether NK cells are activated or proliferated, the choice of stimulatory cytokines used, and the type of supporting cells cause NK cells to differ phenotypically and functionally. In addition, there are some challenges specific to NK cells including loss of viability and/or activity after the freeze-thaw phase and lack of persistence *in vivo* (87).

Compared to the use of monoclonal antibodies for ALL treatment, CAR-NK cell therapy has been shown to eliminate tumor cells more effectively by exerting a higher cytolytic effect (88). On the other hand, the conflicts about the amplification of NK cells in appropriate amounts for clinical application and from which source they will be obtained are the problems

to be overcome in CAR-NK cell therapy. In addition to the acquisition, production and storage methods of NK cells, the immunosuppressive environment in the peripheral blood and bone marrow of the patients is thought to have an effect on having short lifespan and low functional capabilities of NK cells administered for immunotherapy.

2.4. The Immunosuppressive Bone Marrow Microenvironment in B-ALL

Bone marrow is a bone-limited, vascularized, and complex organ which contains several cell types, including hematopoietic and non-hematopoietic cells. These cells are involved in the regulation of hematopoiesis by secreting soluble factors such as cytokines, growth factors and hormones, as well as expressing receptors and adhesion molecules (89). The soluble factors form the extracellular matrix (ECM), which contains more than 200 proteins (collagens, fibronectin, proteoglycans, laminin, enzymes, growth factors, and cytokines), and regulate the functions of the cells in which they are secreted (90). The supportive cells in the niches of bone marrow produce soluble molecules and ligands which bind the ligands and molecules on HSCs and progenitor cells in bone marrow, leading their differentiation, proliferation and migration (91). In the case of leukemia, malign stem cells are also present within the bone marrow microenvironment as well as stromal cells, HSCs, and soluble factors (92).

It has been demonstrated that the microenvironment in bone marrow has effects on leukemia phenotypes and protect leukemic cells from apoptosis induced by chemotherapy (93, 94). Vice versa, leukemia cells lead alterations in the leukemic niche in order to facilitate the disease development (95, 96). There are studies demonstrating the roles of soluble components in bone marrow microenvironment CXCL12, a chemokine produced by bone marrow stromal and endothelial cells and mediates the growth, differentiation and migration of HSCs by binding to its own receptor CXCR4, has been suggested to play an important role in hematological malignancies (97, 98).

There are evidences suggest that inflammatory factors in bone marrow are involved in development and progression of leukemia. A study proved that the inhibition of inflammatory signaling in PAX5 mutant cells reduces B cell leukemogenesis (99). Some reports demonstrated the concentration of inflammatory cytokines in blood samples of neonatal children, who subsequently become leukemia patients, differs in comparison with the control group (100, 101). In addition, pro-inflammatory cytokines such as IL-1 beta (β), IL-6 and tumor necrosis factor-alfa (TNF-α) contribute to creating a suitable environment for ETV6-RUNX1-expressing cells and their transformation by increasing DNA damage (102).

Immune cells together with inflammatory mediators create a favorable microenvironment for tumor growth, contributing an immunosuppressive tumor microenvironment for normally developing cells (103). The class I major histocompability complex (MHC-I) molecules expressed by self-cells in the body suppress NK cell activation by acting as inhibitory ligands

for NK cells under normal conditions (104). In cancer cases, as a result of ligands expressed on tumor cell membranes binding to NK cell activating receptors, or tumor cells stop MHC-I expression to escape from cytotoxic T cells, NK cells are activated and destroy malign cells (105). In addition to ligands expressed on tumor cell membranes, tumor-derived soluble ligands have also been shown to activate NK cells via NKG2D and NKp44 (106). However, the failure of NK cells to kill tumor cells that do not carry MHC-I or have high NKG2D ligand expression (107) suggests the presence of immunosuppressive factors in the tumor microenvironment.

Under normal conditions, NK cells develop and mature from lymphoid precursor cells in the bone marrow and migrate to peripheral blood circulation and other tissues (108). However, there are evidences that NK cell maturation is suppressed in the bone marrow as a result of disruption of this process is in case of tumor formation (109, 110). Humoral components in the bone marrow microenvironment are among the factors which cause this suppression. One of these soluble factors is tumor growth factor (TGF)-β, which suppresses proliferation and cytotoxic functions of NK cells and downregulates their NKG2D expression (111). Type I interferon (IFN) signals required for NK cell maturation were also found to be suppressed in the bone marrow environment (112).

In addition to solid tumors, there are findings that the leukemia microenvironment also affects NK cell activation and functions, and also has important effects on the prognosis of leukemia (113). Like other cancer cells, leukemia cells escape immune surveillance by altering the phenotype and functions of immune cells. Although NK cells have a role in the immune surveillance of ALL (114), especially in cases of treatment-resistant or relapsed leukemia, the anti-leukemia functions of immune components are impaired, and leukemia cells cause alterations in the bone marrow microenvironment to survive and protect against treatment (115).

NK cells have been shown to be insufficient to kill ALL blast cells *in vitro*, and progression of leukemia is thought to have adverse effects on NK cell maturation. NK cells are known to have numerical, phenotypic and functional differences in ALL patients compared to healthy individuals (116). In a study conducted in B-ALL patients, TGF-β released from blast cells was shown to have a role in the impairment of NK cell functions (117).

As a result of all the findings obtained from the studies in the literature, it is concluded that the changes in the bone marrow microenvironment in leukemia patients have suppressive effects on NK cell maturation, phenotype and functions, which also negatively affect the anti-leukemia functions of NK cells. However, the effects of leukemia microenvironment on NK cell phenotype and functional properties, especially in B-ALL patients, are not clearly known.

In our recent study based on available data, it was aimed to examine the effects of

humoral components in the bone marrow microenvironment of B-ALL patients on healthy NK cell functions. For this purpose, peripheral blood mononuclear cells (PBMCs) from healthy pediatric donors were cultured with the addition of bone marrow plasma samples of pediatric B-ALL patients. Bone marrow plasmas of pediatric patients without pathology in terms of leukemia, and fetal bovine serum (FBS) alone were used in the culture for control conditions. After cell culture, the levels of PD-1 and intracellular IL-10, which have suppressive effects on the anti-leukemic functions of NK cells, were found to be higher in the presence of B-ALL plasma samples than in the control groups. Conversely, the proliferation capacities of NK cells were demonstrated to be decreased in conditions with B-ALL plasmas in comparison with control groups in this study. On the other hand, IL-10 levels were increased but IL-18 and IFN-γ levels were decreased in plasma samples of B-ALL patients compared to patient control group.

Overall, the increase of PD-1 and intracellular IL-10 levels in contrast to the decrease of proliferative capacity of NK cells in the presence of B-ALL plasma samples suggest that an immune-suppressive microenvironment exists for non-malignant cells in the bone marrow of B-ALL patients. Elevated IL-10 but decreased IL-18 and IFN-γ levels detected in bone marrow plasma samples might be among the causes of suppression on NK cells. However, it is clear that this immunosuppressive microenvironment would have an effect not only on NK cells, but also on other cells developing or completing their development in the bone marrow.

In further studies, it will be possible to clearly define the content of the microenvironment which suppresses anti-leukemia immunity in the bone marrow, by examining other humoral components such as anti-inflammatory cytokines, chemokines and complement proteins. The results from these studies might increase the success of immunotherapeutic agents used in B-ALL treatment in the future.

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CHAPTER 6

CHRONIC LYMPHOCYTIC LEUKEMIA CYTOKINE CONTENT: RELATIONSHIP WITH ZAP70 EXPRESSION

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ABSTRACT

T cell activation requires many intracellular and extracellular processes. One of them is intracellular pathways, in which protein-tyrosine kinases play a role. T-cell antigen receptor (TCR) activation leads to tyrosine phosphorylation of many cellular proteins including Zeta (ζ)-chain associated protein kinase with a molecular weight of 70 kDa (ZAP-70), which co-precipitates with zeta upon TCR stimulation. Activated ZAP70 regulates immune system cell motility, adhesion and cytokine expression, including gamma delta T, memory $CD8^+$ T, natural killer (NK), mucosal associated invariant T (MAIT), naive $CD8^+$ T, regulatory T, memory $CD4^+$ T, and naive $CD4^+$ T cells. ZAP70 circuitously participates in B cell development and activation. ZAP70 is identified as a biological marker for chronic lymphocytic leukemia (CLL), which independently of its kinase activity, enhances B cell receptor signaling of CLL cells. It also enhances migration to chemokines and the response to survival stimuli from the microenvironment. The cytokines secreted in the CLL cells, and the cytokine reserve of the T cells, including interleukin (IL)-2, IL-4, IL-6, IL-9, IL-10, IL-15, IL-21, IL-1 beta, tumor growth factor beta (TGF-beta), tumor necrosis factor alpha (TNF-alpha), etc, affect the development and life span of the CLL cells.

Keywords: B neoplasms, chronic lymphocytic leukemia, cytokine, zeta (ζ)-chain associated protein kinase 70

1. B Lymphocyte Neoplasms

B lymphocyte neoplasms are clonal diseases from different stages in the development process of B cells, these stages are important to classify B cell-derived lymphoproliferative diseases. B cell development occurs antigen-dependent and independent, and antigen-independent B cell development in bone marrow (Figure 1) (1).

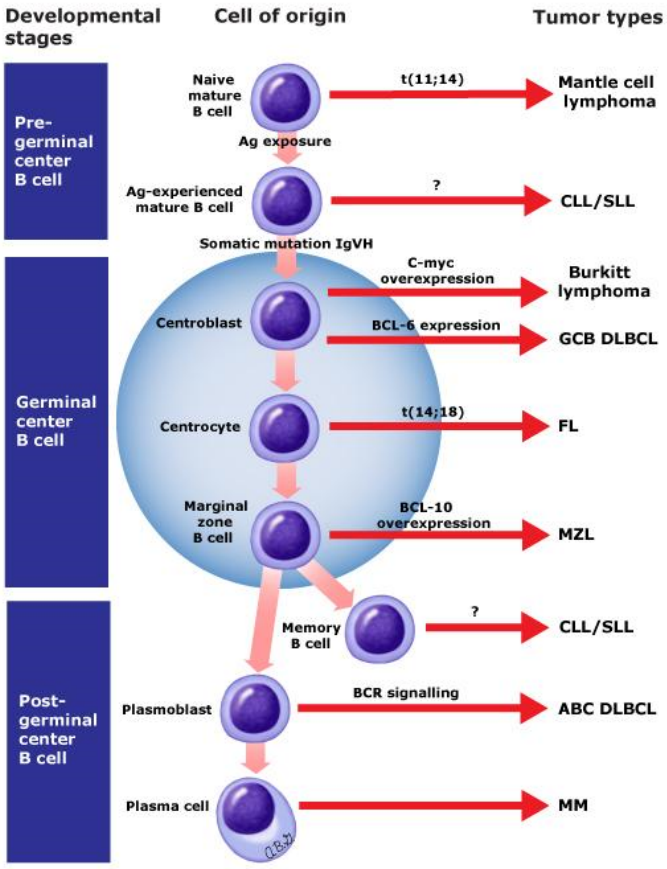


Figure 1: Developmental stages of B cell and neoplasm relationships (1)
Ag: antigen; CLL/SLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; GCB DLBCL: germinal center B cell diffuse large B cell lymphoma; FL: follicular lymphoma; MZL: marginal zone lymphoma; ABC DLBCL: activated B cell diffuse large B cell lymphoma; MM: multiple myeloma

After the transformation of proB lymphocytes from bone marrow stem cells, they express CD19, CD79a, CD34, CD10 and Tdt on their surface, without recombination of immunoglobulin genes. Recombination begins when the diversity (D) segment in the heavy chain merges the splicing (J) segment, then the variable (V) merges with DJ. Cells with cytoplasmic μ chain production are called immature stage B cells. Lymphomas and leukemias consisting of lymphocytes at these stages are called pre-B-cell lymphoblastic lymphoma/leukemia. In the next step, recombination takes place in the immunoglobulin

light chain genes. Thus, cells begin to carry IgM molecules on their membrane surfaces and pass to peripheral tissues. Tdt and CD10 expression has been lost in these cells, which are called mature B cells and carry CD20 and CD22 (2).

In the antigen-dependent phase, surface IgM and IgD expressed cells circulate in the blood and form primary follicles in lymphoid tissues. Some of the mantle cell lymphomas, Chronic lymphocytic leukemia (CLL), and small B-cell lymphoma/leukemias are suggested to origin from cells at this stage. After antigen interaction, these cells are activated to blast phenotypic large B cells in the paracortex, where T cells are located in lymphoid tissues, or they transformed into short-lived plasma cells with IgM production and form germinal center B cells derived from centrocytes or larger centroblasts in the follicle centers. After this stage, somatic mutations begin in immunoglobulin genes and Burkitt lymphoma, follicular lymphoma, Hodgkin lymphoma, and large cell lymphomas develop (1).

Activated B lymphocytes then transform into memory cells and settle in the marginal region around the follicles in the lymphoid tissue. Marginal zone lymphomas occur at this stage. When the antigen is re-encountered, B cells in the splenic marginal zone migrate to the germinal center and then rapidly emerge as antigen-specific plasma cells in the T cell zone. Tumors derived from plasma cells are called plasmacytoma or myeloma (2).

2. Chronic Lymphocytic Leukemia

CLL is a mostly B lymphocyte origin disease with a low proliferation rate and a different clinical course (3, 4). Patients diagnosed with CLL are affected by cytopenias due to bone marrow filtration, cellular immune disorders related to autoimmune diseases and neoplasms, or infectious complications caused by hypogammaglobulinemia.

Rai and Binet's classifications are useful in predicting treatment requirements and survival of patients with CLL, but these classifications have limited prognostic value in early-stage cases, whereas approximately 40-60% of cases presenting as an early stage progress to advanced stages and die due to the disease. Identifying these cases in the early period may help to select the appropriate treatment. The inadequacy of the existing classification systems in distinguishing these cases has led to various studies to find the prognostic factors in the early period of CLL. Many molecules related to CLL and their genetic and biological properties have been investigated in this direction (5, 6, 7).

CD5 and CD19 double positivity, also CD23, CD20, and cytoplasmic or surface light chains (Lambda and Kappa) are considered the main B cell markers in the diagnosis of CLL. Additional markers (CD10, CD43, CD79b, CD81, CD200, and ROR1) are also considered useful in for a differential diagnosis from other B-cell lymphomas/leukemias. In addition to del (11q), del (13q), del (17p), and trisomy 12 assessment, TP53 mutation analysis, Immunoglobulin gene heavy chain variable (IGHV) region somatic hypermutation analysis, and B-cell receptor (BCR) stereotype subset analysis are required for a full prognostic

assessment of CLL. Detection of karyotypic complexity and BTK, PLCG2, and BCL2 mutations remain desirable for therapy. TP53 aberration and IGHV mutation are a part of the CLL-international prognostic index, just like clinical stage, age and beta 2-microglobulin level. Palpable lymph nodes, absolute lymphocyte count $>15 \times 10^9$/L and IGHV mutation status are components of early-stage CLL international prognostic scoring (8).

Somatic mutations in the (IGHV) region as a prognostic factor have been found very valuable (9, 10). CLL cases with *IGHV* mutations have a good clinical outcome, whereas patients without mutations response partially to chemotherapy. However, *IGHV* gene sequencing and mutation analysis are available to a limited number of laboratories. Molecular genotypic analyses are expensive and cannot be performed for routine monitoring or follow-up of patients. Therefore, new studies have focused on different methods for determining mutations.

3. Zeta-chain Associated Protein Kinase 70 (ZAP70)

Zeta (ζ)-chain associated protein kinase 70 (ZAP70) is a cytoplasmic, 70 kDa protein tyrosine kinase (PTK). It acts as initial phases of T cell activation. It binds to phosphorylated tyrosines, ζ chain in the cytoplasmic tail of the T cell receptor (TCR), thereby phosphorylating adapter proteins involved in other components of the signal cascade. As a result of the combination of TCR with the antigen, the signal transduction system is activated. In the first step of T cell activation, Lck, a PTK belonging to the Src family, is activated. Activated Lck phosphorylates the immunoreceptor tyrosine activation motifs (ITAM) located in the subunits CD3 and ζ of the TCR complex. Src-phosphorylated ITAM motifs interact with ZAP70 and Syk to activate them. Activated ZAP70 and Syk kinases phosphorylate proteins such as Vav, Lat, and SLP76 that will provide signaling, and play a role in T cell proliferation, cytokine release, and T cell maturation (11-15) (Figure 2).

T cell activation and the role of activation for ZAP70 are schematically as follows (15): The ZAP70 is involved in TCR signaling initiation and subsequent T cell activation (16). This protein kinase has two SH2 domains, which are responsible for ZAP70 dependent signal transduction (17). ZAP70 gene methylation and ZAP70 expression are associated (18), whereas abnormal methylation is frequent in malignancy. ZAP70 enhances the BCR signaling of CLL cells independently of its kinase activity. Chemokine migration and the response to survival stimuli from the microenvironment are enhanced by ZAP70 (19).

This tyrosine kinase increases malignant B cells by reshaping the microenvironment and by enhancing survival, protein synthase capacity increment CLL progression effective studies are published (20).

Typically it is a T cell antigen receptor signal protein. A methylation process in the promoter zone of this protein is published to be a mechanism for malign transformation, which has an association with unmutated IGHV gen status and poor prognosis. Early studies

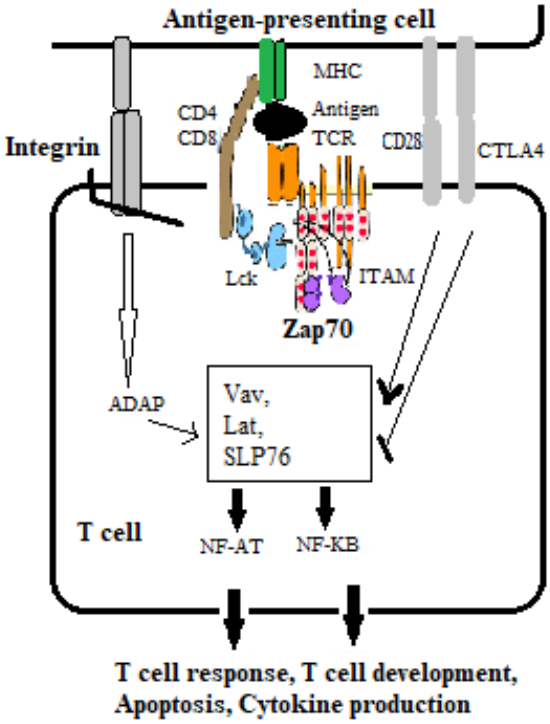


Figure 2: T cell activation and the role of activation for ZAP70 (13)
TCR, T cell receptor; MHC, major histocompatibility complex; Lck, lymphocyte-specific kinase; ZAP-70, ζ-chain associated protein of 70 kilodaltons; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for the activation of T cells; SLP-76, SH2-containing leukocyte protein of 76 kilodaltons; CD, cluster of differentiation; ADAP, degranulation-promoting adaptor protein; NFAT, nuclear factor for the activation of T cells; NFKB, nuclear factor kappa-light-chain-enhancer of activated B cells

indicate ZAP70 action on development of B cell receptor independently from kinase activity (21), whereas current studies focused on migration of CLL cell and increment of ability to form supportive microenvironment (22).

ZAP70 is identified as a biological marker for CLL in the last two decades. CD38 and ZAP70 expression on B lymphocytes are associated with aggresive feature, poor reponsive to conservative therapy, and rapid progression of CLL. CLL patients exhibit 36 to 57% positivity of ZAP70 expression in many studies. To determine ZAP70 in an early diagnostic phase of CLL accurately is important to select patients with an aggressive course and to maintain their therapy (23).

T lymphocytes, natural killer (NK) cells, and a subgroup of B lymphocytes express ZAP70. Due to its tyrosine kinase activity, ZAP70 primarily has a crucial role in TCR activation. ZAP70 is expressed aberrant especially highly in CLL patients (24), but also on different B cell malignant cells, which expression is associated with unfavorable disease

outcome (25). Mature B cells do not express ZAP70 under normal conditions, the expression of ZAP70 on CLL and other mature B malignancies may point to different cellular origins (26, 27).

Soluble IgM expression showed that ZAP70 has an important role on CLL pathogenesis and progression, which found to have an association with the aberrant expression (28). On the other hand, hypomethylation of ZAP70 genes is reported to have an association with overexpressed ZAP70 and poor outcome (18, 29, 30). ZAP-70 is found mostly expressed in unmutated CLL (31). Therefore, ZAP70 is considered as an independent prognostic marker (32).

Studies focus now on the intrinsic mechanisms of tumors, including activation of BCR signaling. Current studies indicate that ZAP70 can modulate crosstalk between immune environment and malignant B cells, suggesting a major complex role (19, 22, 24-26). By the way, the irrevocable roles of ZAP70 in NK cell and T lymphocytes activation in addition suggest that ZAP70 in immune environment may be a main objective in tumor immunity modulation. ZAP70 and tumor immunology association, as well as immunotherapies in the treatment of these conditions may help us to understand the different molecular functions.

Normally, mature B lymphocytes do not express ZAP70, this expression points to its different cellular origins in B-cell neoplasms (26, 27). ZAP70 point mutations which cause to lack of ZAP70 protein expression on human T lymphocytes were unidentifiable in normal human B lymphocytes and ZAP70 negative malignant B lymphocytes (26). Thus, downregulation of ZAP70 may be physiological for B-cell maturation.

Overexpressed ZAP70 in B cell neoplasias may be due to clonal evolution and epigenetic modulation. Hypomethylation of ZAP-70 gene is related to ZAP70 overexpression and poor disease course in CLL (18, 30). Abnormal ZAP70 expression via alternative mechanisms is related to tumor microenvironment-mediated induction of ZAP70: in peripheral blood-derived B lymphocytes with consistently low levels of ZAP70, stimuli such as sCD40L, anti-IgM, interleukin (IL)-4, IL-6 and IL-10 which activate the BCR, upregulate the expression of ZAP70 (33). Through TLR9 activation, unmethylated CpG oligodeoxynucleotides aggregate an innate immune response, generation in a subgroup of CLL cells with ZAP70 expression is promoted (34, 35).

A ZAP70 deficient mouse model showed both pro-B and pre-B lymphocytes express protein during pre-BCR complex formation, which plays a role in transform of pro-B cells to pre-B lymphocytes in bone marrow (36). SYK and ZAP70 were dynamically unnecessary in B-lymphocyte development, only in both SYK and ZAP70 deficient mice model development of B lymphocytes is completely blocked (36, 37). Another study supported this finding analyzed B lymphocytes, pro-B and pre-B lymphocytes, originated from peripheral blood, tonsils and human bone marrow, express ZAP70, in contrast to most normal mature B

lymphocytes (26). In malignant and normal B lymphocytes, ZAP70 is similarly modulated by phosphorylation after BCR activation (33, 38).

4. Cytokines in Relationship with CLL and ZAP70

IL-17 is a cytokine family, whose members 17A, 17B, 17C, 17D, 17E (IL-25), and 17F share similar protein structure. This family regulates many immune signaling processes. IL-17 induces and mediates proinflammatory responses and is often related to allergy. Many cytokines including IL-1beta, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), transforming growth factor (TGF) beta, tumor necrosis factor (TNF) alpha, and chemokines synthesis is also induced. Cellular expression pattern of this family members is unique. As an example activated T cells express IL-17A and IL-17F, these cytokines are upregulated in inflammatory responses. Immune tissues express IL-17B, and inflammation upregulates IL-17C. Following discovery of IL-17 cytokines and the Th17 pathway discovered, treatment of human diseases changed (39).

Activated T lymphocytes secrete IL-22, which has a relation to IL-10. IL-10 suppresses the synthesis of proinflammatory cytokines by monocytes in response to lipopolysaccharide. IL-10 functions on monocytes are not affected by IL-22. IL-22 inhibits the synthesis of IL-4 by Th2 T lymphocytes (39).

Plasma levels of IL-17A (40, 41) and IL-22 (40) were found significantly higher in CLL patients when compared to healthy controls. ZAP70 was in associaton with higher IL-22, but in contrast with lower IL-17A levels (40). Controversial studies exist about the relationship between increased levels of IL-17A and the stage of diseases. Studies with presence (41) and absence (40) of inversely relation is published.

IL-22 levels are increased and associated with ZAP70 expression in CLL patients. Negative expression of ZAP70 showed a significant association with elevated IL-17A levels (40). Increased number of Th17 cells are suggested to be in association with suitable prognosis in patients with CLL (42), but conversely, CLL progression is found to be correlated with downregulated IL-17 secreting T and expansion of T regulatory (Treg) cells (43). Therefore the function of IL-17 in CLL should be studied further.

$CD4^+$ Th2 lymphocytes produce IL-10, which blocks activation and effector roles of T lymphocytes, monocytes, and macrophages. This cytokine diversely affects hematopoietic cells and terminates inflammatory responses. B lymphocytes, NK cells, T lymphocytes (helper and cytotoxic) growth and/or differentiation is regulated. It is important for differentiation and function of Treg cells. Tregs control immune responses and tolerance (39).

The association between Treg, Th17, IL-10 and IL-17 ratios with the prognosis of CLL were investigated by Pang et al. and showed that Treg and Th17 cell proportion in blood IL-10 and IL-17 levels are elevated in CLL patients in comparison to healthy subjects. In addition,

Treg/Th17 and IL-10/IL-17 ratios were also higher in CLL patients, but lower in CLL patients in remission in comparison to healthy individuals (44).

Studies indicate the increment of Treg cells in CLL, along with the Rai staging, which may affect the prognosis of the disease (45-48). Also, this finding was similar to a study at late Rai stage (49), however in contrast to a study with no significant association (50), therefore the effect of Th17 cells in CLL pathogenesis is controversial.

T lymphocytes and specifically $CD4^+$ Th cells produce IL9. It is a facilitator for the growth of some Th cells but this facilitation is not eligible for cytolytic T lymphocytes. When erythropoietin is present, erythroid colony formation is supported by IL-9. With erythropoietin, IL-2, IL-3, IL-4 and IL-9 may enhance hematopoiesis, which is a glycoprotein structured hematopoietic growth factor derived from a megakaryoblastic leukemia (39). A strong correlation between IL-9 overexpression and clinical staging of CLL was also reported (51). IL-9 is reported to show an association with CLL, it activates lymphoma cell proliferation (52), and prevent dexamethasone–induced apoptosis (53). The pro-proliferative effect and anti-apoptotic effect of IL-9 are related to IL9 and its receptor IL-9R bond. There are several factors such as CD40L, IL-2, IL-4, IL-10, IL-15 and IL-21, which play role at proliferation or survival of CLL cells (54-56).

IL-2 is produced by $CD4^+$ Th lymphocytes during an immune response, which is important against microbial infections and in self and nonself discrimination. After binding of antigen to T cell receptor, IL-2 is secreted and IL-2 receptor expression is activated. IL-2 and IL-2 receptor binding activates differentiation, growth, and survival of cytotoxic T lymphocytes. IL-2 is required for T lymphocytes' immunologic memory development, which has an autocrine effect. $CD4^+$ T lymphocytes produced it and then IL-2 is acting on them (39).

IL-4 transforms naïve helper T (Th0) cells to Th2 cells, that secrete mainly this cytokine. Activated mast cells may also produce it. This cytokine stimulates activated B cells and T cell proliferation and the differentiation of $CD4^+$ T cells into Th2 cells. IL-4 activates B cell class switching to IgE and also upregulates MHC Class II synthesis. It acts on resting B lymphocytes to enlarge (39).

IL-15, as a T lymphocyte growth factor, is secreted by mononuclear phagocytes and other cells against viral infections. IL-15 mainly stimulates NK cell and T lymphocyte proliferation. *In vitro* studies indicate that it induces cytotoxic T lymphocytes. IL-15 and IL-12 share many biological properties. Both cytokines bind to common hematopoietin receptor subunits, and therefore negatively affect each others function (39).

IL-21 regulates immune system cells NK and cytotoxic T lymphocytes that act on virally infected or tumor cells. IL-21 activates its target cell to division and/or proliferation. Its acts on its receptor, IL-21R, is expressed on lymphocytes and bone marrow cells. *In vitro* assays indicate on its role in costimulating with anti-CD40 of proliferation and maturation of NK

cell and mature B lymphocyte, whereas in costimulating with anti-CD3 of T lymphocytes proliferation (39).

CD40L is an antigen presented on stimulated $CD4^+$ T lymphocytes, which is the ligand for CD40 and it interacts with CD40 on B cell proliferation (39). CD40 antigen is expressed on tonsillar B cells and peripheral blood from pre-B cell stage until plasmacyte stage, where it is absent. Also, this antigen is expressed on B cell lymphomas and leukemias, in addition to some carcinomas, and weakly on monocytes. CD40 antigen is important in B cell proliferation, which serves as a receptor for B cell costimulatory signals. It interacts with CD40 ligand on T lymphocytes and induces B lymphocyte proliferation (39, 57).

A leukemogenic process in CLL, just like in plenty of lymphoproliferative neoplasias, is assumed to be driven by an antigenic stimulation (58-61). Actors like auto-antigens or different microbiological antigens are suspected to take part at chronic stimulation (61), when a limited use of *IGHV* genes and the presence of stereotypic BCR on CLL cells (62-64) are used to provide evidence.

IL-4 protects CLL cells from apoptosis or killing related to DNA damaging agents (65-68). Scleiss et al. studied BCR co-factors to stimulate B cells due to limited functional CLL cell studies. It is reported that concomitant to CD40L, after IL-4 and IL-21 stimulation *ex vivo* CLL cell proliferation was successful (69).

BCR activation is known to be crucial for lymphocyte development of, also very important for the proliferation and survival of CLL cells *in vivo* (59). It is also believed that when the stimulation withdraws, *ex vivo* apoptosis will occur spontaneously (70). Increased BCR signaling in CLL is linked with ZAP70 expression (24). ZAP70 also might release CLL cell BCR signaling by lowering ligand-mediated receptor internalization, therefore inhibites the BCR signaling termination (21, 71, 72). In addition, ZAP70 may have pro-survival roles in CLL independently of BCR signaling (73).

Monserrat et al. investigated naive, central memory, terminated memory and non-terminated memory cells. According to their study, interferon (IFN) gamma, TNF-alpha, and IL-2 expressed activated T cell percentage is decreased, $CD4^+$ T cell percentage, which express IL-4 or IL-10 is increased, whereas naive cells are decreased but non-terminated memory and terminated memory cells are expanded in ZAP70 positive CLL patients in comparison to ZAP70 negative (74).

IFN-gamma is a glycoprotein, which is synthesized by activated CD4+ Th1 lymphocytes, $CD8^+$ T lymphocytes, and NK cells. It has antiproliferative and antiviral functions. IFN-gamma activates mononuclear phagocytes strongly, with increased destroying ability of tumor cells and intracellular microorganisms. IFN-gamma facilitates B and T lymphocyte differentiation. It activates NK cells in addition to neutrophils and vascular endothelial cells. Patients with CLL, lymphoma, and virus infections have low levels of IFN-gamma (39).

TNF-alpha, a cytotoxic monokine, is released by macrophages, which are activated with bacteria endotoxins. TNF-alpha plays role in remodeling of tissues, wound healing and inflammation. TNF-alpha, also called cachectin, induces cachexia and septic shock. It is released by majority of cells such as NK cells, monocytes, macrophages, B and T cells, activated by endotoxins or other microbial products (39).

Monserrat et al. also showed CLL cells have ZAP70 overexpression, which helps to distinguish activation/differentiation CD4 cell subsets and act on switching of cytokine secretion pattern (74).

Monoclonal neoplastic $CD5^+$ B cells that remain in the G0 phase. CLL cells slowly but progressively accumulate in peripheral blood, spleen, lymph nodes and bone marrow. As a cause for the accumulation of leukemic cells, failure of apoptosis plays a major role when compared to cell proliferation. $CD5^+CD19^+$ CLL cells are strongly affected by T cell-derived cytokines (IL-1 alpha, IL-1 beta, IFN-gamma, IL-6, IL-7, IL-8, IL-10, IL-13, and TGF-beta, which are mediators for B and T cell interactions (75, 76).

IL-1 alpha and beta proteins comprise IL-1 molecule, which are synthesized from dendritic cells, monocytes and macrophages. IL-1, an endogenous pyrogen, acts on inflammatory response to infection. In addition to infection, it also regulates hematopoiesis. IL-1 alpha and beta have both the same biological features and they use same receptors on cells. IL-1 alpha is membrane-related, whereas beta is free in circulation (39).

Activated T lymphocytes, fibroblasts, mononuclear phagocytes, vascular endothelial cells, and various neoplasms produce IL-6, which act as a acute phase response and fever mediator. IL-6 increases body temperature and is synthesized in response to IL-1 and/or TNF. This cytokine mainly acts on B lymphocytes and hepatocytes. Significantly it causes a differentiation of B lymphocytes into cells that produce antibodies. IL-6 is an important growth factor for active B cells in late stages of B lymphocyte differentiation (39).

IL-7 induces lymphoid stem cell transformation into progenitor B lymphocytes. These bone marrow stromal cells product is a growth factor for T cells. It activates lymphopoiesis, macrophages and T lymphocytes. In addition, IL7 rules on stem cell transformation into early pre-T and -B lymphocytes. It activates the proliferation of fetal and adult thymocytes. Thus IL-7 is crucial for NK cell and T lymphocyte survival and development and also for B cell maturation. (39).

Macrophages and other cells like epithelial or endothelial cells secrete IL-8. IL-8 secretion is led by a cascade of biochemical reactions and innate immune system responses. All innate immune system cells expressing Toll-like receptors are able to produce IL-8. It induces antigen phagocytosis of neutrophils, which cause to Toll-like receptor activation. After processing of antigen by macrophages, these cells produce IL-8, which signals immune cells to an inflammation site (39).

Especially Th2 lymphocytes, but also other numerous cells, secrete IL-13. It mediates allergic inflammation and allergic disease. It acts on immune cells, which is similar to IL-4 cytokine. It acts through the alpha chain of the IL-4 receptor. IL-13 functions resemble IL-4, especially the induction on hematopoietic cells. Activated human B cells secrete Ig, which is induced by IL-13 (39).

The ability of TGF-beta to growth regulation, varies according to the types of cells or the presence of other growth factors in the environment. Bone marrow cell proliferation is inhibited by TGF-beta and in addition interferon alpha induced stimulation of NK cells is blocked. It decreases cytokine-induced proliferation of thymocytes, and decreases IL2-induced activation and proliferation of T lymphocytes. It inhibits cytotoxic T cell differentiation from T cell precursor. TGF-beta is released by mononuclear phagocytes, active Th2 cells, and other cells. It mainly affects on inhibiting proliferation and differentiation of T cells, macrophage activation, and counteract pro-inflammatory cytokines (39).

Programmed cell death, apoptosis, may be activated as a response to many of physiologic stimuli in different cell types. $CD5^+$ B cells in CLL are arrested in G0 and display prolonged survival *in vivo*, which spontaneously go to apoptosis *in vitro* (77). Cytokine network changes in T cells and malignant cells may lead to expansion of the B cell clones caused by various reasons. A new formation of cytokine network may manage apoptosis and cell proliferation in CLL patients.

CLL cells are capable of secreting various cytokines (IL-1 alpha, IL-1 beta, IL-6, IL-7, IL-8, IL-10, IL-13, IFN-gamma, TNF-alpha). Granulocyte cells were found to secrete macrophage colony stimulating factor and TGF-beta. The cytokines secreted in the CLL cell and the cytokine reserve of the T cell affect the development and life span of the CLL cell (78).

In the advanced stages of CLL, the altered cell cytokine network with the insufficiency of immune system cell functions may be the cause of secondary neoplasia, autoimmune diseases or infection (79,80). When the interaction between the CLL cell and T cells changes, leukemic B cells can expand uncontrollably. The newly formed cytokine network may inhibit apoptosis while increasing cell proliferation in CLL patients. The rapid death of CLL cells *in vitro*, but their long survival *in vivo* proves this hypothesis. Studies have shown that IL-2 and TNF-alpha stimulates the proliferation of leukemic cells *in vitro*; whereas IL-4 increases cell viability. IL-5 and IL-10 lead cells to apoptosis (78). Also, high levels of IFN-gamma synthesized by T and B cells in CLL patients increase the production of TNF-alpha and cause the CLL cell to proliferate in number and have a long life (78).

Expressions of ZAP70, CD38, and CD23, which are accepted as prognostic markers, and intracellular IL-4 and IFN-gamma levels were measured in 28 CLL and 10 healthy controls by Isiksacan et al. (81). Cases with 20% or more CD23 and ZAP70 expression, and cases with

30% or more CD38 expression were considered positive. ZAP70 expression was positive in 15 of 28 cases. However, no significant correlation was found between the expression of ZAP70 and the age and gender of the patients. Since only one case was found to be positive for ZAP70 and CD38 at the same time, no significant relationship was found. When the relationship between ZAP70 and CD23 expression was examined, it was seen that CD23 was positive in all cases without any difference between ZAP70 negative and positive cases. Since ZAP70 expression can't be associated with prognosis due to the long course of the disease, it is thought that longer-term follow-up studies are needed to clearly reveal the relationship between ZAP70 and CLL prognosis (81).

When intracellular IL-4 and IFN-gamma levels were compared in CLL cases and healthy volunteers, it was shown that IL-4 was decreased in T cells of CLL cases and IFN gamma was significantly higher in B cells. It was concluded that IFN-gamma, whose antiapoptotic role is known, may be responsible for the accumulation of malignant cells in the bone marrow in CLL and it may cause the progression of the disease (81).

The ratio of $CD3^+IFN\text{-}gamma^+/CD3^+IL\text{-}4^+$ in CLL cases and healthy volunteers were compared. This ratio was found to be lower in CLL cases than in healthy subjects. Due to the cytokines (high IL-4, low IFN-gamma) secreted by T cells in CLL cases, T cell type 2 was seen to be at the forefront of this effect. These results show damage to the body's immune system as one of the causes of contamination of cells and autoimmunity (81).

The cytokine pool around the tumor environment has a crucial role in the disease pathogenesis. Released cytokines can act as a suppressor of tumor development or progression, and on the other hand, they may promote the growth or encourage the invasion. Identification of the cytokines with multi evaluations is important for disease progression and therapeutic approaches. In light of all knowledge, ZAP70 has a central role in regulating T cell functionality. This role extends from development in the thymus to regulation of T-cell responses in the periphery. When the CLL background and the cytokine pool in the disease process are evaluated, it is suggested by studies to reduce (IL-15, IL-12, IFN, CXCL9, CXCL10, CXCL11, CCL3, CCL4, etc.) or increase (IL-6, IL-8, GM-CSF, and TNF, etc.) associated poorer outcome cytokines.

Biological basis of heterogeneous behaviour in CLL remains unclear and needs further studies. CLL patients have heterogeneous clinical outcome due to different biological features of leukemic cells like genomic alterations and role playing factors in B cell interaction with genetic and somatic environment (Figure 3) (82, 83).

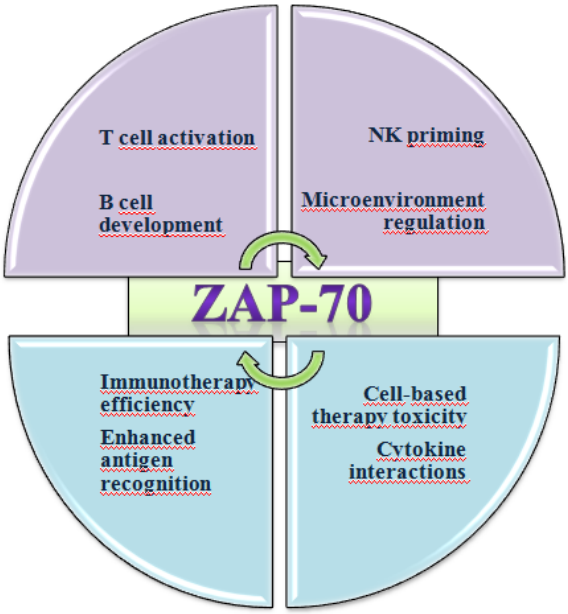


Figure 3: Physiological roles of the ZAP70 (83)

As a prognostic factor in CLL, ZAP70 has a proven reliability in the last two decades. Although functional CLL cell studies are limited, it is predicted that studies will develop further in parallel with technological developments in the future, and it is thought that the importance of this marker will be better understood in different diseases other than B-cell neoplasms.

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CHAPTER 7

THE ROLE OF CD8$^+$CXCR5$^+$ FOLLICULAR CYTOTOXIC T CELL AND THEIR SUBSETS IN B CELL RESPONSES AND DISEASES

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ABSTRACT

CXCR5 expressing CD8$^+$ T cells called follicular cytotoxic CD8$^+$ T (T_{FC}) cells differ phenotypically and transcriptionally from other CD8$^+$ T cells. T_{FC} migrates to B cell follicles and eliminates infected or malignant B and follicular helper T (T_{FH}) cells secreting perforin and granzyme. Recent studies showed that there is a subset of T_{FC} that support antibody responses in B cells by expressing CD40L or ICOS. The studies showed that there is a correlation between the frequency and cytolytic function of T_{FC} cells and the viral load in chronic infections. T_{FC} have been reported to infiltrate the tumor and nearby lymph nodes in colorectal cancer patients, and levels of TNF-α, IFN-γ, granzyme-B, and T_{FC} frequency are positively correlated with disease prognosis. The cytotoxic effect of T_{FC} in CLL has been analyzed, but the CD40L-mediated stimulatory effect of T_{FC} has not been examined yet. Based on the literature, whether T_{FC} cells and subsets in CLL patients stimulate malignant B cells and increase proliferation, like the T_{FH} phenotype, or whether they affect malignant B cell lysis via perforin and granzyme were investigated. It provides information about the functional properties, similarities, and differences of ICOS$^+$ T_{FC}, CD40L$^+$ T_{FC}, and ICOS$^-$CD40L$^-$(Double Negative-DN) T_{FC} subsets defined. In addition, it has been shown that CD40L$^+$ T_{FC} have the effect of enhancing IgG responses of B cells. However, ICOS$^+$ T_{FC} express high amount of perforin and granzyme. Further studies are needed to understand the role of T_{FC} subset in the pathogenesis of diseases such as B cell malignancy.

Keywords: Chronic lymphocytic leukemia, CLL, follicular cytotoxic CD8$^+$ T cell, T_{FC}, follicular helper CD4$^+$ T cell, T_{FH}, cytotoxicity

1. $CD8^+$ Cytotoxic T Cell

Cytotoxic T cells, which are one of the effector cells of the adaptive immune system, are the lymphocytes that express CD3 and CD8 (1). $CD8^+$ T lymphocytes are involved in the immune response against intracellular pathogen-infected cells and tumor cells (2). Transcription factors such as B-lymphocyte-induced maturation protein 1 (Blimp1), Eomesodermin (Eomes) and T-box expressed in T cells (T-bet) and various cytokines such as Interleukin-2 (IL-2), IL-15, type I interferon (IFN)-α, and IFN-β are involved in the differentiation of $CD8^+$ T lymphocytes (1, 3). The cytoplasm of $CD8^+$ T lymphocytes contains numerous granules involving the enzymes called perforin and granzymes to ensure the lysis of the target cells (4, 5).

$CD8^+$ T lymphocytes that have not encountered specific antigenic peptides are called "naive $CD8^+$ T lymphocytes" (6). Naive $CD8^+$ T lymphocytes migrate from peripheral blood to lymphoid tissues to recognize their specific antigen (7). $CD8^+$ T lymphocytes are activated by binding the antigen within major histocompatibility complex-I (MHC-class I) presented by antigen-presenting cells (APC) via the T cell receptor (TCR). After recognition, $CD8^+$ T lymphocytes rapidly proliferate and differentiate into effector cells (8, 9), and subsequently migrating to the inflammation site and fulfilling their functions, effector $CD8^+$ T cells either undergo apoptosis or differentiate into memory phenotype (6, 10). Memory $CD8^+$ T lymphocytes are an important component of T cell immunity and perform effector functions by rapidly activating upon re-encounter with the same pathogen (10). If naive $CD8^+$ T lymphocytes do not encounter specific antigen, they leave the lymphoid tissues and return the circulation (11, 12).

$CD8^+$ T lymphocytes have different cytotoxic mechanisms for target cell lysis, the first of which is mediated by the release of the cytolytic molecules like perforin and granzyme (4). Perforin has a role in pore formation in the target cell surface that facilitate the passage of small molecules such as ions and water. These pores disrupt the cell membrane integrity and structure, causing the osmotic pressure change, leading the lysis of the target cell and entry of the granzyme into the cell (4, 13). Granzyme activates endonucleases by increasing the concentration of calcium (Ca^{++}) inside the cell and activate the caspase pathway, to induce apoptosis (4, 5). Another cytotoxic mechanism is that FAS Ligand (FASL) on the surface of activated $CD8^+$ T lymphocytes binds to the FAS receptor on the surface of the target cell, and directs the target cell to apoptosis (9, 14). Finally, cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) secreting by $CD8^+$ T cells can indirectly lead to apoptosis by triggering the caspase in the target cells (14).

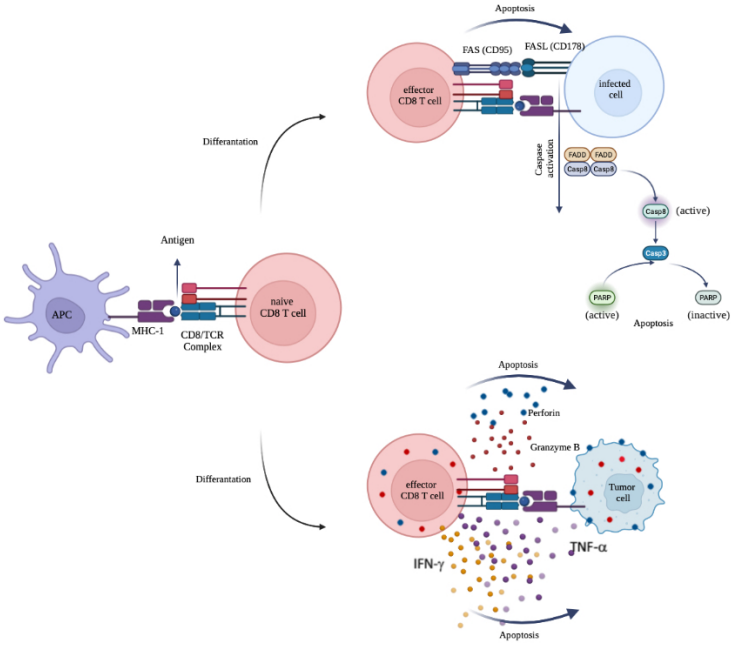


Figure 1: Cytotoxic mechanism of CD8⁺ T cells. APC: Antigen Presented Cell, Cas8: Caspase 8, FADD: FAS associated death domain, FASL: Fas Ligand, IFN-γ: Interferon-gamma, MHC-I: Major histocompatibility complex-I, PARP: Poly ADP ribose polymerase, TNF-α: Tumor necrosis factor-α

CD8⁺ T lymphocytes are known to play a role in various diseases such as cancer, infection and autoimmunity. It has been reported that the density of CD8⁺ T lymphocytes infiltrating the tumor and CD8⁺ T lymphocytes with CD103 expression localized in the epithelium increase survival in various cancer types such as breast, ovarian and lung (15-17). CD8⁺ T lymphocytes, an important cell of the adaptive immune system, are powerful effector cells of anti-cancer responses and form the basis of cancer immunotherapy (18).

Continuous exposure to pathogen or tumor-derived antigens leads CD8⁺ T lymphocytes to transition from functional to dysfunctional state, called 'exhaustion CD8⁺ T lymphocyte' (19). Exhausted CD8⁺ T lymphocytes have a phenotype associated with reduced antigen-driven stimulation of effector cytokines and inhibitory receptors with high expression, such as programmed cell death protein-1 (PD-1) (20). In contrast to effector cells, exhausted CD8⁺ T lymphocytes have a decreased capacity to proliferation and secrete cytokines due to chronic TCR stimulation. (20, 21). Studies have shown that CD8⁺ T lymphocytes have decreased IL-2 production, cytolytic function and proliferation capacity in patients with chronic viral infections and this situation is related to the exhausted CD8⁺ T lymphocyte phenotype (22).

2. Follicular Cytotoxic CD8⁺ T Cell (T_{FC})

CXC Chemokine receptor-5 (CXCR5) is a G-protein-coupled 7 transmembrane protein receptor and a member of the CXC chemokine receptor family (23). CXCR5 is a surface

molecule which strongly expressed on a subset of $CD4^+$ helper T lymphocytes, follicular helper T cells (T_{FH}), and on B cells (24). It is specific for the chemokine CXC chemokine ligand-13 (CXCL13) expressed by follicular dendritic cells (FDC) in the lymph nodes (25). In response to CXCL13 chemokine, B and T_{FH} cells migrate to follicular zone of secondary lymphoid organs (25, 26). The interaction of B and T_{FH} cells results in affinity maturation, isotype transformation, plasmablast differentiation, and memory B cell development in B cells (27).

Recent studies have indicated that some $CD8^+$ T cell subgroups have CXCR5 expression like T_{FH} cells (28). For the first time, CXCR5 expression on $CD8^+$ T cells were identified in tonsils and is called follicular cytotoxic $CD8^+$ T cell (T_{FC}) (28, 29). T_{FC} cells having lower CCR7 and higher CXCR5 expression than naive $CD8^+$ T lymphocytes are different from other $CD8^+$ T lymphocyte subsets (30). Because of CXCR5 expression, T_{FC} cells migrate to the B cell zone in the lymph node (30).

2.1. Phenotype

T_{FC} cells exhibit a $CD3^+CD8^+CXCR5^+$ phenotype, showing both similarities and distinctions in phenotype compared to other $CD8^+$ T lymphocyte subsets. Like $CD8^+$ T lymphocytes, T_{FC} cells express cytolytic molecules and possess the capacity to produce cytokines such as IFN-γ and TNF-α. Transcription factors such as T cell factor 1 (Tcf1), B cell lymphoma-6 (Bcl6), B lymphocyte-induced maturation protein-1 (Blimp1), DNA-binding proteins 2 and 3 (Id2 and Id3) are involved in the differentiation of T_{FC} cells (22, 31-33).

T_{FC} cells express inhibitory receptors like PD-1, cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene 3 (LAG3), T cell immunoglobulin and mucin protein-3 (Tim-3) (29). On the other hand, it has been shown that inhibitor receptor expressions were decreased but activator receptor expressions were increased in T_{FC} cells compared to $CD8^+$ T lymphocytes (34).

In a mouse model infected with lymphocytic choriomeningitis virus (LCMV), it was reported that T_{FC} cells have high levels of the cytolytic molecules granzyme-A, granzyme-B and than naive $CD8^+$ T lymphocytes but lower than $CXCR5^-CD8^+$ T lymphocytes (22). It has been reported that T_{FC} cells have low levels of stimulatory molecules like inducible costimulatory (ICOS) and CD28 than T_{FH} cells but higher levels than naive $CD8^+$ and $CXCR5^-CD8^+$ T cells (22, 29, 32, 33).

2.2. Functions

The function of T_{FC} cells has been extensively studied in the context of cancer and viral infections (22, 32, 33, 35). These cells demonstrate cytotoxic properties like $CD8^+$ T lymphocytes in various cancers and infectious diseases. In comparison to $CD8^+$ T lymphocytes, T_{FC} cells have exhibited increased expression of PD-1, IL-2, IFN-γ, and TNF-α, as well as elevated levels of cytotoxic molecules such as granzyme-A, granzyme-B,

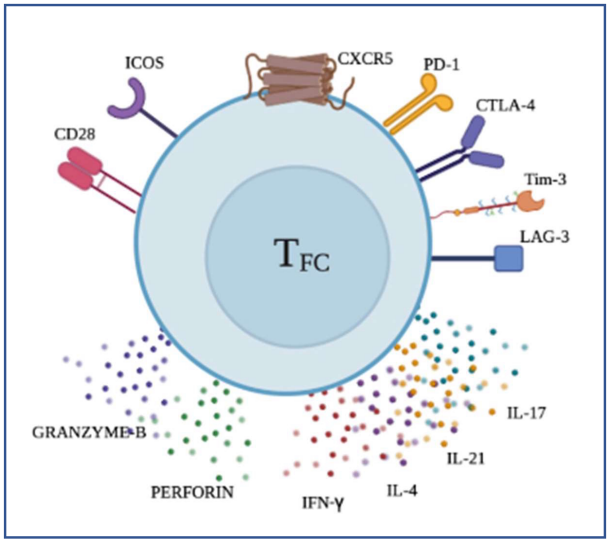


Figure 2: Surface molecules and cytokines of T_{FC} cells. CXCR5: CXC Chemokine receptor-5, ICOS: Inducible T-cell costimulatory, CTLA-4: Cytotoxic T lymphocyte antigen-4, IL: Interleukin, LAG-3: Lymphocyte activation gene 3, Tim-3: T cell immunoglobulin and mucin protein-3, PD-1: Programmed cell death-1

and perforin. Additionally, they tend to localize in proximity to B and T_{FH} cells within follicles (33, 34). These studies have provided evidence that T_{FC} cells eliminate infected or malignant B and T_{FH} cells in the germinal center by secreting perforin and granzyme-B (33, 36-38).

It has been reported that T_{FC} cells can lyse B cells, as well as to regulate antibody responses (39). The studies revealed that cells can express surface molecules such as ICOS or CD40L and various cytokines such as IL-21, IL-4 and IFN-γ at high levels in human tonsils. In co-culture experiments indicated that B cells were differentiated to plasmablast as a result of T_{FC} cells and B interaction but adding anti-CD40L and anti-IL-21 in culture medium B cells did not differentiate to plasmablast. As a result, it was suggested that T_{FC} cells may have a role in the regulation of antibody responses through CD40L and ICOS expression (39). Contrary to this, some studies have been demonstrated that T_{FC} cells localize in B cell follicles and suppress T_{FH} cells and inhibit antibody responses via inducing apoptosis in T_{FH} cells and suppressing IL-21 expression (40, 41).

It is known that decreased cytokine expression, cytolytic function, proliferation capacity and increased expression of Tim-3 and PD-1-like inhibitory receptors are associated with a 'exhausted' phenotype in $CD8^+$ T lymphocytes in cancer and chronic viral infections (19). In contrast, T_{FC} cells display distinct features compared to $CD8^+$ T lymphocytes, showing low levels of Tim-3 expression, while secreting elevated levels of IFN-γ, IL-2, TNF-α, and the degranulation marker CD107a (22, 24, 42).

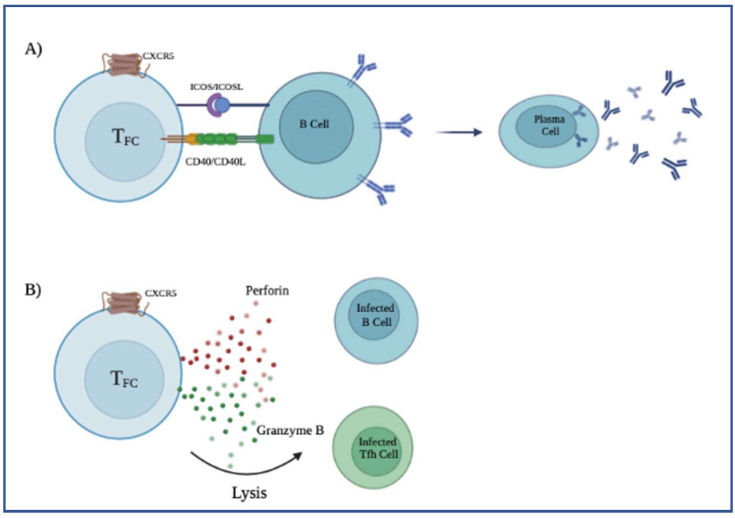


Figure 3: The functions of T_{FC} cells A) interaction with B cells, B) Cytotoxic mechanism. CXCR5: CXC Chemokine receptor-5, ICOS: Inducible T-cell costimulatory, ICOSL: Inducible T-cell costimulatory ligand.

2.3. The Role of T_{FC} Cells in Diseases

T_{FC} cells have primarily been studied in the context of cancer and chronic viral infections (22, 33, 43, 44). Research findings have indicated a significant correlation between the frequency and cytolytic function of T_{FC} cells and the viral load in chronic infections (22). Recent data has revealed that if lymphocytic choriomeningitis virus (LCMV)-specific T_{FC} cells of mice with chronic LCMV infection were transferred LCMV-infected another mice, viral load was significantly reduced in recipient mice (22). Additionally, the studies focused on HIV has shown that T_{FC} cell was observed to increase in B cell follicles and germinal center and have stronger cytotoxic capacity than the $CXCR5^{-}CD8^{+}$ T cell subset and are more effective in reducing viral load in HIV-infected patients (37, 45, 46). Reports indicate an elevated number of T_{FC} cells in the lymph nodes of primates infected with chronic monkey immunodeficiency virus (SIV), suggesting a potential role in infection control (34). Moreover, in patients with hepatocellular carcinoma infected with hepatitis B virus (HBV) and individuals with hepatitis B, the peripheral blood T_{FC} cell ratios have been observed to increase compared to healthy individuals. Additionally, T_{FC} cells express higher IFN-γ, IL-10, and IL-21 compared to $CXCR5^{-}CD8^{+}$ T cells and T_{FH} cell are negatively correlated with in patients with chronic hepatitis B (38).

Recent studies have indicated that T_{FC} cells have important roles in various cancers like colorectal, pancreatic, and thyroid cancer (31, 35, 47). T_{FC} cells have been reported to infiltrate the tumor and nearby lymph nodes in colorectal cancer patients, and levels of IFN-γ, TNF-α, granzyme-B, and T_{FC} cell frequency is positively correlated with disease prognosis (31). Similarly, T_{FC} cells have been shown to strongly infiltrate the tumor site and express

lower PD-1 and TIM-3, and have higher cytotoxic activity compared to $CXCR5^{-}CD8^{+}$ T lymphocytes in pancreatic tumors (35). It was found that anti-PD-1 or anti-TIM-3 treatment increased the T_{FC} cells functions and the survival of patients after tumor rejection was positively correlated with the frequency of T_{FC} cells (35). Furthermore, PD-1, IL-2, IFN-γ, TNF-α, granzyme-A, granzyme-B, and perforin expression of T_{FC} cells were reported to be increased compared to $CD8^{+}$ T lymphocytes in patients with thyroid cancer (47). The studies shown that T_{FC} cells infiltrate the tumor tissues in patients with non-small cell lung cancer (NSCLC), and express IFN-γ, IL-2 and TNF-α and degranulation marker CD107a at higher levels than $CXCR5^{-}CD8^{+}$ T lymphocytes (24).

A current study has indicated that PD-1 expressing T_{FC} cells are found in various hematologic malignancies such as Chronic Lymphocytic Leukemia (CLL), Diffuse Large B Cell Lymphoma (DLBCL), Multiple Myeloma (MM), and Follicular Lymphoma (FL). They have memory-like cells phenotype and express Tcf1 transcription factor. These cells secrete IFN-γ, IL-2, TNF-α, but have limited proliferative capacity (48). However, it was observed that proinflammatory cytokines increased after anti-CD3/CD28 stimulation in CLL, while granzyme-B decreased and memory-like T_{FC} cells were transformed into effector T_{FC} cells in CLL (48). Additionally, increased T_{FC} cells in patients with CLL and high level of activator molecules such as OX-40 and ICOS expression have been reported (49).

It was found that there was an increase in the cytotoxic molecule expression of T_{FC} cells compared to $CD8^{+}$ T lymphocytes in DLBCL, whereas there was no significant difference in degranulation level (36). As a result of blocking IL-10 which is highly expressed by tumor cells in DLBCL, by anti-human IL-10/IL-10R antibodies in *in vitro* experiments, it was detected that granzyme-A, granzyme-B and perforin expressions of T_{FC} cells was increased. (36). Studies have demonstrated that T_{FC} cells have an inhibitory effects on T_{FH}-mediated B cell differentiation and display potent cytotoxic activity in patients with follicular lymphoma (FL). Furthermore, when T_{FC} cells were adoptively transferred into a lymphoma animal model, they exhibited significantly greater antitumor activity compared to $CXCR5^{-}CD8^{+}$ T cells (50). Recent studies suggesred that T_{FC} cells can express CD40L and have a role in the regulation of antibody responses by interacting with B cells like T_{FH} cells (39). In a study examining the interaction of T_{FC} cells with B cells, it was observed that the addition of anti-CD40L and anti-IL-21 to the T_{FC}-B co-culture reduced antibody responses in B cells (39). In the study conducted by Xing et al. emphasized that there was a negative correlation between CD40L expression in T_{FC} cells and perforin and granzyme-B expressions in patients with colorectal cancer. Le et al. analyzed ICOS expressing T_{FC} cells, and it was found that $ICOS^{+}$ T_{FC} cells increased antibody responses in B cells compared to $CXCR5^{-}ICOS^{+}CD8^{+}$ T cells, but no information was given about the $ICOS^{-}$ T_{FC} group in this study (51).

It has been emphasized that T_{FC} cells have a cytotoxic effect on malignant cells in cancer

and infectious diseases. It has also been suggested that T_{FC} cells may have a stimulatory effect on malignant B cells via CD40L expression. CLL is a B-cell malignancy and the cytotoxic effect of T_{FC} cells in CLL has been analyzed, but the CD40L-mediated stimulatory effect of T_{FC} cells has not been examined yet. Based on the literature, whether T_{FC} cells and subsets in CLL patients stimulate malignant B cells and increase proliferation, like the T_{FH} cell phenotype, or whether they affect malignant B cell lysis via perforin and granzyme were investigated in our study. The relationship between T_{FC} subsets and the clinical course of the disease was tried to be revealed.

Firstly, to determine the differences of cytotoxic enzymes and cytokine profile of T_{FC} subsets in healthy subjects, T_{FC} cells were divided $ICOS^+$, $CD40L^+$ and $ICOS^-CD40L^-$ (double negative- DN) T_{FC} cells according to ICOS and CD40L expressions. It was determined that the majority of T_{FC} cells consisted of the DN T_{FC} subset. Various cytokines and enzyme contents were compared to show the similarities and differences of the identified subsets with each other. $ICOS^+$ T_{FC} and $CD40L^+$ T_{FC} subsets express the elevated level of IL-4, IL-17 IL-21 and IFN-γ than DN T_{FC} subset. $ICOS^+$ T_{FC} have also high level of perforin and granzyme-B compared to the other T_{FC} subsets. Additionally, DN T_{FC} subset had lower levels of cytokines, perforin and granzyme-B. To investigate the effect of T_{FC} subsets on plasmablast differantation of B cells, T_{FC}–B co-culture was performed by flow cytometry. All T_{FC} subsets had an effect of plasma cell and plasmablast differentiation of B cells. Increased plasma cell and plasmablast differentiation was detected $CD40L^+$ T_{FC} cells than, $ICOS^+$ T_{FC} and DN T_{FC} subsets. IgG responses were analyzed in the culture supernatant by the ELISA method. High level of IgG was detected in $CD40L^+$ T_{FC} subset than $ICOS^+$ T_{FC} susbset, but there were no differences on IgG responses in DN T_{FC} subset.

The total ratio of T_{FC} cell was increased in CLL patients compared to healthy controls. Similarly, increased $ICOS^+$ and $CD40L^+$ T_{FC} subsets were found in CLL patients compared to healthy controls, whereas significant difference was not observed in DN T_{FC} subset between patients and healthy controls. Elevated IL-4, IL-17, and IL-21 levels were found in $ICOS^+$ T_{FC}, $CD40L^+$ T_{FC}, and DN T_{FC} subsets of CLL cells compared to healthy cells. Similar to the cytokine profile of T_{FC} subsets in healthy controls, IL-4, IL-17, IL-21, and IFN-γ levels were higher in $ICOS^+$ and $CD40L^+$ T_{FC} cells compared to DN T_{FC} subsets in CLL patients. It was also observed that $ICOS^+$ T_{FC} cells expressed high perforin and granzyme-B than the other T_{FC} cell subsets in CLL patients. It was found that the $CD5^+CD19^+$ cell ratio was positively correlated with the total T_{FC} ratio. These results might indicate that $CD40L^+$ T_{FC} have a high cytokine content, $ICOS^+$ T_{FC} have a high cytolytic enzyme content, and DN T_{FC} have a low cytokine and enzyme content. Additionally, $CD40L^+$ T_{FC} cells are more effective in regulating antibody responses in B cells. The elevated $ICOS^+$ T_{FC} subset having high cytotoxic capacity, as well as the $CD40L^+$ T_{FC} subset which might stimulate malignant

B cells, in CLL patients might give the impression that malignant B cells might direct T_{FC} cells to different subsets (unpublished data).

T_{FC} cells are a subset of $CD8^+$ T cells that migrate with the follicle. It has been proposed that they have the ability to kill infected or malign B and T_{FH} cells located in the follicle. T_{FC} cells have been shown to have effective cytotoxic effects on malignant cells in various types of cancer such as colorectal, pancreatic, thyroid, and hepatocellular carcinoma. Similar cytotoxic effects have been shown in infectious diseases such as HIV, LCMV, and EBV. In infected mouse models, T_{FC} cells have been shown to be more potent cytotoxic than $CD8^+$ T cells and more effective in reducing viral load. Recent studies have suggested that T_{FC} cells also stimulate B cells via CD40L to enhance antibody responses.

In summary, this study defines previously undifferentiated subsets of T_{FC} cells. It provides information about the functional properties, similarities, and differences of $ICOS^+$ T_{FC}, $CD40L^+$ T_{FC}, and DN T_{FC} subsets defined according to ICOS and CD40L expression. In addition, our study shows that $CD40L^+$ T_{FC} cells have the effect of enhancing IgG responses of B cells like T_{FH} cells. However, $ICOS^+$ T_{FC} cells express a high amount of perforin and granzyme like cytotoxic $CD8^+$ T cells. Further studies are needed to understand the role of T_{FC} subset in the pathogenesis of diseases such as autoimmunity, B cell malignancy. However, the findings presented in this study propose that T_{FC} cells hold promise as potential candidates for future immunotherapy in B cell malignancies. Further investigation through animal experiments and studies involving B cell lymphoma tissues should be pursued to explore their therapeutic potential in greater depth.

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CHAPTER 8

BIOLOGICAL FUNCTION OF ACTIVATION-INDUCED CYTIDINE DEAMINASE (AID): POTENTIAL ROLE IN B-CELL MALIGNANCIES

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ABSTRACT

Activation-induced cytidine deaminase (AID) enzyme is discovered by Japanese scientist Tasuku Honjo and his team. AID, a member of the cytidine deaminase family, converts cytosine to uracil in DNA. AID play a role in the process of generating antibodies with higher affinity for antigens in B lymphocytes is called “affinity maturation”, and the conversion of antibodies from the IgM isotype to the IgG, IgA, or IgE isotypes is called “isotype switching”. Recent studies have shown that the AID can cause mutations and DNA breaks not only in the IgVH and Ig C regions but also in non-immunoglobulin genes. AID is known the first mutator enzyme and recent studies have indicated that AID is responsible for genomic instability like point mutation, translocation in hematologic malignancies and may affect the clinical course of the disease. AID ”knock-out” mice studies showed that AID plays a role in the development of Burkitt lymphoma by creating dsDNA breaks and causing translocations in the IgH/c-myc. It was shown that there is a correlation between AID expression and poor prognosis in various B-cell lymphomas and leukemias. Additionally, chronic lymphocytic leukemia (CLL) patients who do not have somatic hypermutation in the IgVH genes have a worse clinical course. In CLL patients with chromosomal deletion, the expression of the AID gene is higher than in patients without these deletions, and that the AID gene may be responsible for these deletions. According to common findings, AID is necessary for tumorigenesis and affects the prognosis in patients with B-cell lymphoma and leukemia.

Keywords: Activation-induced cytidine deaminase, AID, B-cell malignancies

1. Introduction

B cells responsible for humoral immune responses in the adaptive immune system play an important role in eliminating pathogens through the antibodies they secrete. Antibodies, in addition to being antigen receptors on the surface of B cells, are secreted by these cells to destroy pathogens. Antibody molecules, consisting of two heavy and two light chains, bind to antigens with their two antigen-binding fragments (Fab), while the fragment crystallizable region (Fc) mediates biological reactions such as antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis by binding to Fc receptors (FcRs) on the surface of immune system cells.

The interaction between an antibody and an antigen is a chemical reaction that occurs due to the effect of electrostatic forces, hydrogen bonds, hydrophobic bonds, and Van der Waals forces, causing the antigen and antibody molecules to come close to each other within 1-2 A. The strength of all non-covalent bonds between the antigen-binding site and the epitope determines the affinity of the antibody to that epitope. Therefore, the higher the ”affinity” of the antibody to an antigen, the stronger it binds to that antigen, indicating the antibody’s capacity to bind to the antigen.

There are 5 types of antibodies found in humans: Immunoglobulin (Ig) G, IgA, IgM, IgE, and IgD. Naive B lymphocytes, which have not encountered an antigen, have IgM as an antigen receptors on their surface. Additionally, the first antibodies secreted by B lymphocytes in response to pathogens are of the IgM isotype. As the infection progresses, the IgM isotype antibodies are replaced by IgG, IgA, or IgE isotype antibodies with higher affinity for the pathogen, depending on the type of pathogen.

The process of generating antibodies with higher affinity for antigens in B lymphocytes is called “**affinity maturation**”, while the conversion of antibodies from the IgM isotype to the IgG, IgA, or IgE isotypes is called “**isotype switching.**” Both affinity maturation and isotype switching rely on the activation-induced cytidine deaminase (AID) enzyme.

The *AID* gene, discovered by Nobel Prize-winning Japanese scientist Tasuku Honjo and his team in 1999, is located on the short arm of chromosome 12 (12p13) in humans (1). It consists of 5 exons and synthesizes a protein of 198 amino acids (2). AID, a member of the cytidine deaminase family, converts cytosine (C) to uracil (U) in the structure of DNA. It has been observed that in mice and humans lacking AID, class switch recombination (CSR) and somatic hypermutation (SHM) do not occur, leading to immunodeficiency and recurrent infections. In individuals lacking this enzyme, isotype class switching cannot occur and a type 2 hyper-IgM syndrome, which is an immunodeficiency disorder, develops (3).

2. AID Function

Naive B lymphocytes are activated by recognizing the specific antigen of them in the lymph nodes and rapidly undergo clonal expansion. Some of these cells differentiate into

short-lived plasma cells and germinal center (GC)-independent memory B cells, while others become GC B cells. GC B cells begin to express CCR7 by changing their surface chemokine receptor expression and start moving towards the T cell zone. Near the intersection of the T and B zones in the lymph node, B cells interact with follicular T (T_{FH}) cells, a subset of helper T cells that express CXCR5. After this interaction, GC B cells re-express CXCR5 on their surface and return to the B zone to form the germinal center. Firstly, SHM occurs in the dark zone of the germinal center, then followed by B cells moving to the light zone and undergoing CSR with re-interact of T_{FH} cells (4, 5).

Interaction with T cells is necessary for AID expression in B cells. CD40-CD40L (ligand) interaction and interleukin-4 (IL-4) play a pivotal role in AID activation. CD40L on the surface of T cells binds to CD40 on B cells and activates the nuclear factor kappa B (NF-KB) signaling pathway in B cells. IL-4 secreting from T cells binds to IL-4R on the surface of B cells and the Janus kinases (JAK) signal transducer and activator of transcription (STAT) pathway is activated inside the cell. Both the NF-KB and JAK-STAT pathways are the main transcription factor to stimulate *AID* expression in B cells (2).

Studies have shown that *AID* is a single-stranded DNA (ssDNA)-specific cytidine deaminase and has no effect on double-stranded DNA (dsDNA) or DNA-RNA hybrids (6). There are two different views on whether *AID* targets a specific C base in the target region or targets a C in a specific DNA sequence when both SHM and CSR. According to one view, it is hypothesized that *AID* acts on specific recognition sequences and targets the C base in the 5'-WRCY-3'/5'-RGYW DNA sequences in the target regions. Within these DNA sequences, it is thought to recognize the 5'-AGCT-3'/ 5'-AGCT-3' repeat sequences in particular and target the C base in these regions for deamination. The other view is that *AID* activity does not have any recognition sequence, as the Ig V or C regions do not contain any common DNA sequence. The specificity of AID binding has not been fully elucidated (7).

AID is mainly expressed in B cells. However, recent studies have shown that AID is expressed in cells other than B lymphocytes. Studies have shown low levels of *AID* mRNA expression in oocytes. Although the role of *AID* in oocytes has not been clearly explained, it has been suggested that it may play an epigenetic regulatory role in DNA demethylation (8). Additionally, low levels of *AID* mRNA have been found in embryonic stem cells, and it is suggested that *AID* may play an important role in preserving the totipotent effects of these stem cells. The transition from pre-B to immature B stage in B lymphocyte development is the first control point of central B cell tolerance. Recent studies have revealed the role of AID in B cell tolerance (9).

2.1. Intra-Celular Location

Both nuclear and cytoplasmic levels of *AID* expression are increased when B cells are stimulated. A significant amount of AID is found in the cytoplasm of B cells to restrict its

mutagenic activity. Immunohistochemistry studies show that B lymphocytes in the germinal center, where SHM occurs, have higher levels of AID enzyme in their nuclei. However, it is also known that subcellular localization plays an important role in regulating AID activity (10). Ito et al. reported that there is a nuclear localization signal and three nuclear export signal (NES) recognition regions in the C-terminal region of the AID protein, and these regions facilitate the transition between the nucleus and cytoplasm. It has also been shown that when nuclear export is inhibited and AID accumulates in the nucleus, it induces DNA damage. The first 5-50 amino acid region in the N-terminal region of the AID protein is the nuclear localization signal region. This region alone cannot keep the AID protein inside the cell, and it needs to function in conjunction with the nuclear localization signal region located between amino acids 181-198 in the C-terminal region to exert its effect (11).

2.2. AID Protein Stabilization

The stabilization of the AID protein is directly related to the compartment in which it is located within the cell. The stabilization of cytoplasmic AID is greater than that of nuclear AID. The half-life of the AID protein in the cell is approximately 8 hours, with a half-life of 2.5 hours in the nucleus and 18-20 hours in the cytoplasm (12). When there is a loss of function due to deletion or mutation in the NES regions that allow the transport of AID to the cytoplasm, the nuclear amount of AID increases, but its half-life decreases.

Heat shock proteins (HSP) play an important role in the degradation of the AID protein. In the cytoplasm, HSP-90 ubiquitinates the AID protein and initiates its proteasomal degradation. AID, which is directed to the chaperone pathway by ubiquitination with HSP-90, interacts with the HSP-40 and HSP-70 systems and these proteins also play a role in proteasomal degradation (13).

2.3. AID Phosphorylation

Studies have shown that the activity of AID can also be regulated by phosphorylation mechanisms following protein expression within the cell, in addition to CD40-CD40L interaction and IL-4 stimulation. These studies indicated that AID phosphorylation plays an important role in regulating deamination activity. The 198 amino acid AID protein has phosphorylation sites at serine 3, threonine 27, serine 38, threonine 140, and tyrosine 184 regions, which are phosphorylated by protein kinase A. To confirm this information, ”knock-out” mice were produced, in which alanine was substituted for serine 38 and threonine 140. The mutant mouse carrying alanine in place of serine 38 showed only 20-30% of the activity of wild type AID for both CSR and SHM, while the mutant mouse carrying alanine in place of threonine 140 showed a decrease in both CSR and SHM activity, although less than that seen in the mutant mouse carrying alanine in place of serine 38 (14). Suppression of threonine 27 phosphorylation significantly reduced CSR activity. However, suppression of phosphorylation in the serine 3 region did not have a significant effect on AID activity (15).

2.4. The Transcriptional Regulation of AID

Many transcription factors play a role in the transcriptional regulation of AID. These transcription factors bind to four well-defined regions on the *AID* gene locus. The first region is the *AID* promoter region, the second region is the region where regulatory elements in the first intron bind, the third region is near the fifth exon, and the fourth region is the transcription initiation site.

NF-KB, Pax5, STAT6, Interferon regulatory factor 8 (IRF8), and E-protein are transcription factors that induce *AID* gene expression, while IRF4, B lymphocyte-induced maturation protein-1 (BLIMP1), Inhibitor of DNA Binding 3 (ID3), and ID2 are negative regulators. However, the transcriptional regulation of *AID* has not been fully elucidated yet. Recent studies have shown that when HoxC4 binds to a specific region of the *AID* promoter, this binding site has a synergistic effect on *AID* with the binding sites of Sp1, Sp3, and NF-KB transcription factors (16).

2.5. N-Terminal ve C-Terminal Site of AID

The N-terminal and C-terminal sites of the AID protein not only determine its cellular localization but also have an impact on its functions. Recent studies on AID activity and function have revealed that SHM and CSR are independent processes and not prerequisites for each other. Studies have suggested that different domains of the AID protein may be responsible for SHM and CSR. Specifically, the N-terminal region of AID is thought to play a role in SHM, while the C-terminal region is believed to be involved in CSR (14).

2.6. Splice Variants of AID

It has been shown that both the natural transcript and several splice variant forms of *AID* are expressed in B cells. However, the exact functions of these splice variants have not yet been fully understood. *AID* mRNA lacking exon 4 (AID-Δex4) leads to loss of CSR activity, but causes an increase in SHM mechanism (17). Studies have shown that *AID*-Δex4 mRNA is present in inflammatory tissues such as nasal polyps. Some studies have also found that different splice variants of *AID* are present in the IgVH regions of patients with CLL who do not have mutations, suggesting a possible relationship between this and the disease pathogenesis (18).

2.7. Somatic Hypermutation (SHM)

The spontaneous mutation rate during each cell division in somatic cells is 10^{-9}, but the point mutation rate in the V region of Ig variable (V) genes is about 10^{-3}-10^{-4} in SHM mechanism. The frequency of mutations in SHM is about a million times higher and therefore, mutations occurring in the Ig variable (V) regions are defined as hypermutations (7).

During Ig affinity maturation, *AID*, which performs point mutations in the Ig variable (V) regions of B lymphocytes, enables the production of antibodies that bind more strongly to

antigens, thus providing stronger protection against the same pathogen when the organism encounters it again

AID converts cytosine (C) residues to uracil (U) in the IgV regions of single-strand DNA. An adenine (A) nucleotide is inserted opposite the U nucleotide during the synthesis of the complementary chain. dU:dA mismatched base is recognised by the mismatch repair mechanisms (MMR). U nucleotide is removed from the DNA structure and replaced with an adenine nucleotide in this region. As a result, somatic hypermutation is performed by creating an A:T change instead of G:C in the IgV regions. Another mutation type in SHM is as follows: *AID* deaminate C and convert C to U. Then uracil DNA glycosylase (UNG) enzyme removes U base from the DNA structure. The UNG enzyme does not affect the phosphate bonds in the DNA structure. Therefore, it does not create breaks in the DNA chain and preserves the integrity of the chain. ”Error-prone” repair mechanism randomly adds one of the four bases in the DNA structure to the location where uracil was removed, and SHM occurs (19). The role of *AID* in SHM was shown in Figure 1.

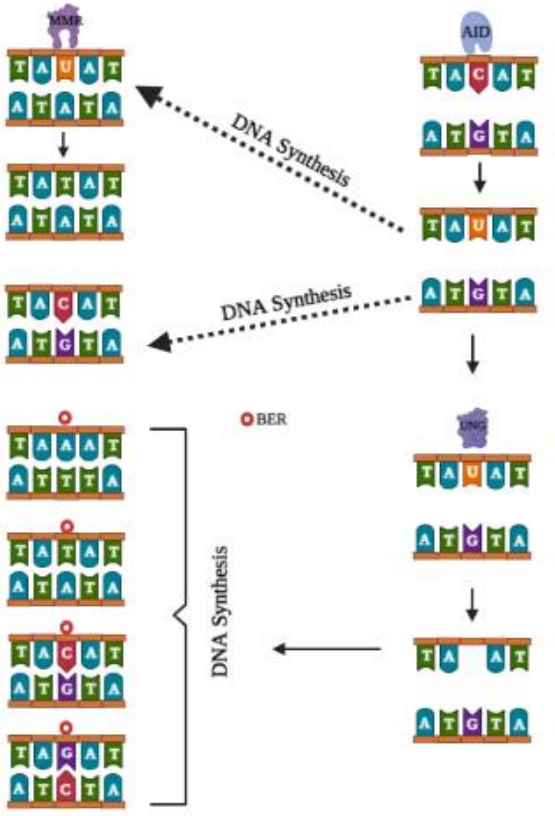


Figure 1: The role of *AID* in SHM mechanism. AID: Activation-induced cytidine deaminase, UNG: Uracil DNA glycosylase, MMR: DNA mismatch repair, A: Adenine, T: Thymine, C: Cytosine, G: Guanine, U: Uracile

As a result of somatic hypermutation, antibodies that bind to antigens more strongly or weakly can be produced. During the infection decline stage, the antigens that are bound to by B cells are decreased in the organism. If the B cells do not bind antigen, the apoptosis mechanisms are activated in B cells. The B cells having low affinity antibodies die, but the B cells which have undergone affinity maturation and have antibodies that bind to antigens more strongly are transformed into memory cells. If the same pathogen enters the organism, these memory cells quickly recognize this pathogen and generate a stronger response than the primary immune response.

2.8. Class Switch Recombination

AID is also involved in a process called CSR, which enables B cells to switch the class of the antibodies they produce without altering their antigen specificity. During CSR, *AID* deaminates cytosine residues in the switch (S) regions of the immunoglobulin heavy chain (IgH) gene, leading to the formation of DNA double-strand breaks (DSBs). The DSBs are then repaired by non-homologous end joining (NHEJ) or alternative end joining (a-EJ) pathways, resulting in the deletion of the intervening DNA between the two switch regions and the replacement of the constant (C) region of the antibody. This allows for the conversion of IgM class antibodies to other Ig class antibodies (IgM $\rightarrow$ IgG, IgA, IgE). Through Ig isotype switching, antibodies can be converted into Ig classes that can provide a faster and more effective response to different infectious agents (20).

AID is essential for CSR as it initiates the DNA cleavage necessary for the process. *AID* is regulated in a similar manner as in SHM and is primarily induced in activated B cells in response to signals from T cells and cytokines. The specificity of *AID* for the S regions of the IgH gene is thought to be regulated by the formation of RNA-DNA hybrids, or R-loops, which expose single-stranded DNA that can be targeted by AID. The R-loops are stabilized by factors such as the RNA-binding protein Spt5 and the histone chaperone FACT.

The AID enzyme, activated by both CD40-CD40L and cytokine stimulation, deaminates the cytosines in the Ig C regions (like Cμ, Cγ, Cα) during isotype switching and converts these cytosines to uracils. Subsequently, the UNG enzyme removes the uracils from the DNA region. The integrity of DNA is preserved because the phosphate bonds in the DNA structure are not affected by UNG enzyme. Then, the apurinic endonuclease (APE) enzyme cuts this region. DNA breaks are created in the Cμ and other C region loci. The regions between the C regions are removed by forming a ”loop” structure. Isotype switching is achieved by binding to another C gene region instead of Cμ (19,20). The role of *AID* in CSR is shown in Figure 2.

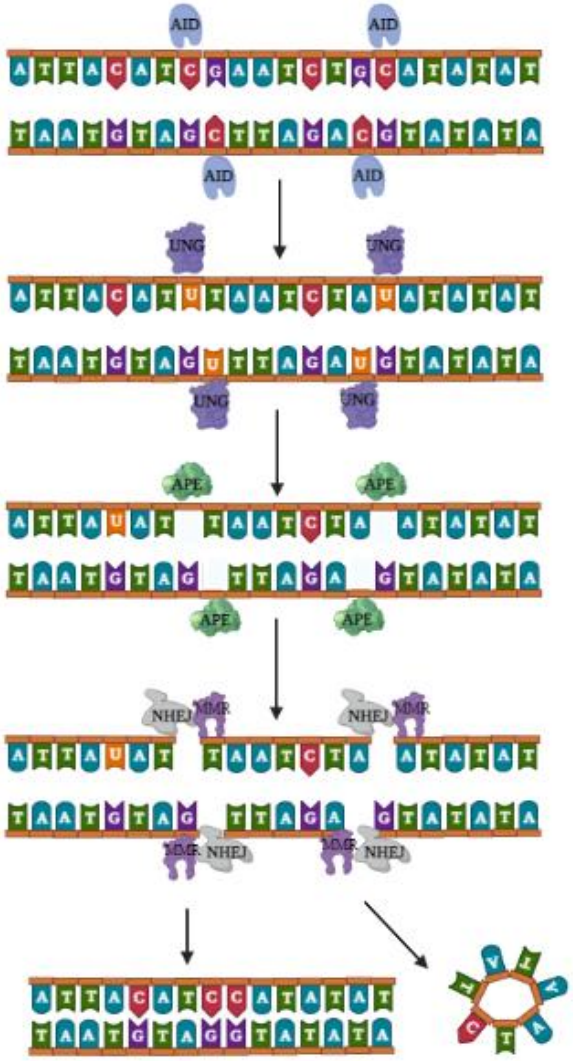


Figure 2: The role of *AID* in CSR mechanism. AID: Activation-induced cytidine deaminase, UNG: Uracil DNA glycosylase, APE: apyrimidinic endonuclease, MMR: DNA mismatch repair, NHEJ: Non-homologous end joining, A: Adenine, T: Thymine, C: Cytosine, G: Guanine, U: Uracile

3. The Potential Role of AID in B-Cell Malignancies

Lymphoid neoplasms associated with lymphocytes and plasma cells are called lymphoproliferative neoplasms (LPN) and present themselves with many different morphological features and clinical syndromes. Lymphoid neoplasms arise in the differentiation process of T lymphocytes, natural killer (NK) cells, or B lymphocytes. The most important factor in identifying different lymphoid neoplasms is which lymphocyte

subtype the uncontrolled lymphoid cell proliferation originates from and at which stage of differentiation it begins. For example, acute lymphoblastic leukemia (ALL) originates from early lymphoid progenitor cells and leads to pre-B or pre-T cell phenotypes, chronic lymphocytic leukemia (CLL) originates from more mature B lymphocyte progenitors, and myeloma originates from B lymphocyte progenitors in the advanced stages of maturation. The World Health Organization (WHO) revised the LPN classification in 2022, which covers a wide range of diseases, including B- or T-cell lymphomas, hairy cell leukemia, prolymphocytic leukemia, NK cell leukemia, large granular lymphocyte leukemia, myeloma, and plasmacytoma. Hodgkin lymphoma is also an LPN that originates from neoplastic B lymphocytes (21).

Clinical and laboratory findings associated with LPN include abnormal immunoglobulin production, infiltration of bone marrow and other tissues, and release of cytokines. Hematopoiesis is suppressed due to bone marrow infiltration, leading to anemia, neutropenia, and/or thrombocytopenia. Proliferation or infiltration of malignant lymphocytes leads to splenomegaly and superficial and deep lymphadenopathy. Hodgkin lymphoma and diffuse large B-cell lymphoma involve isolated lymph node groups, while low-grade lymphomas (follicular lymphoma) and chronic lymphocytic leukemia often present with widespread lymphadenopathy and splenic involvement (22). Prolymphocytic leukemia and hairy cell leukemia progress with bone marrow fibrosis and massive splenomegaly due to bone marrow and spleen infiltration (22).

The most common systemic symptoms observed during the course of LPN are fever, night sweats, weight loss, anorexia, and cachexia, which are characterized by the presence of widespread disease. Itching is a symptom particularly seen in Hodgkin lymphoma. Laboratory findings of tumor lysis syndrome, which may occur depending on tumor burden, include elevated creatinine, hyperuricemia, hypercalcemia, hyperkalemia, and hyperphosphatemia (23).

Genomic instability is a hallmark of hematologic malignancies, including leukemias, lymphomas, and myelomas. These diseases are characterized by the accumulation of genetic alterations, including mutations, deletions, amplifications, or translocations, which can lead to the dysregulation of key signaling pathways and the development of cancer. One of the main mechanisms underlying genomic instability in hematologic malignancies is DNA damage. DNA damage can occur through a variety of mechanisms, including exposure to environmental toxins, replication errors, and errors in DNA repair pathways. In addition, certain genetic mutations, such as those affecting DNA repair genes, can increase the likelihood of DNA damage and contribute to genomic instability.

AID is a DNA-editing enzyme that is normally expressed in B cells, where it plays a critical role in the process of antibody diversification. However, recent studies on the *AID* gene have

shown that the AID enzyme can cause mutations and DNA breaks not only in the IgVH and Ig C regions but also in other non-immunoglobulin genes. AID is known first mutator enzyme and recent studies have indicated that AID is responsible for genomic instability like point mutation or translocation in hematologic malignancies. Additionally, the studies have shown that the *AID* can cause chromosomal translocations in various lymphoproliferative diseases and may affect the clinical course of the disease (8).

The p53 gene is an important tumor suppressor gene and it is known to play a significant role in tumor development through undergoing point mutations or deletions in many types of cancer. When *AID* is overexpressed, it has been shown to cause point mutations, particularly on tumor suppressor genes such as p53, leading to the loss of their functions and contributing to cancer pathogenesis. In addition, it has been found that in B-cell tumors, proto-oncogenes create breakpoints, facilitating their translocation with the Ig gene region. Studies conducted particularly on *AID* "knock-out" mice have shown that this enzyme actively plays a role in the development of Burkitt lymphoma by creating double strand DNA breaks and causing translocations in the *IGH/cMYC* translocations. In animal models, the incidence of B-cell tumor development decreases under conditions where AID is absent. Interestingly, the bcr-abl1 kinase encoded by the Philadelphia chromosome can induce *AID* expression and lead to the development of worse prognosis of ALL. Studies on *AID* expression in various B-cell lymphomas and leukemias have shown a correlation between *AID* expression and poor prognosis. According to common findings, *AID* is necessary for tumorigenesis and affects the prognosis in patients with B-cell lymphoma and leukemia (24).

In chronic lymphocytic leukemia (CLL), the disease is divided into two subgroups based on the presence or absence of somatic hypermutation in the immunoglobulin heavy chain variable region (IgVH). It is known that CLL patients who do not have somatic hypermutation in the IgVH genes have a worse clinical course compared to those who do have somatic hypermutation in the IgVH region. In recent years, studies have been focused on the expression levels of *AID* gene in patients with CLL. As a result of these studies, significant increases in the expression of *AID* have been detected in CLL patients compared to healthy individuals. It has also been shown that there are significant increases in *AID* expression over time in these patients. Furthermore, it has been suggested that in CLL patients with chromosomal anomalies such as del13q, del11q, and del17p, which are associated with clinical outcomes, the expression of the *AID* gene is higher than in patients without these deletions, and that the *AID* gene may be responsible for these deletions (25). In our study, the *AID* mRNA and protein expression in CLL patients with and without deletions was investigated. Increased levels of *AID* mRNA and protein expression in CLL patients with chromosomal deletion like 17p13, 11q22.3 or 13q14 was found compared to to healthy subjects and CLL patients without deletions (26, 27). Our results might showed that *AID* expression may be associated with

deletions in CLL patients.

It has been reported that *AID* gene expression increases in the presence of chronic inflammation, and overexpression of *AID* is responsible for tumor formation in inflammatory areas. Marusawa et al. have shown that AID enzyme may be responsible for TP53 mutations in stomach cancers caused by Helicobacter pylori infection. Viral or bacterial infections can also induce *AID* gene expression by stimulating proinflammatory cytokines. However, some viruses can affect *AID* gene expression with proteins they acquire to escape the immune system response. EBV can induce *AID* expression through the LMP-1 viral protein, which mimics the cellular CD40. Abelson murine leukemia virus (Ab-LMV) can induce AID expression in bone marrow-infected B cells. There are also studies that showed some AID enzyme isoforms are expressed more in patients with bcr/abl translocation, and there is a relationship between these enzyme isoforms and the development of the disease (21).

4. Conclusion

The first mutator enzyme, AID, has a role in SHM and CSR in physiologic conditions, but the recent studies indicated that over-expression of AID can cause the point mutation or double strand DNA breaks in non-immunoglobulin genes. The fact that AID can cause mutations, especially translocations, in B-cell leukemia and lymphoma, and that there is a relationship between *AID* expression and the clinical course of these diseases, demonstrates the important role of *AID* in the pathogenesis of these diseases. In addition to overexpression of *AID*, it is very important to fully elucidate the control mechanisms of *AID*. *In vitro* and animal studies aimed at suppressing *AID* expression with small inhibitor molecules, which have begun to be used in current treatment approaches, may provide data that could enable *AID* to be used as a therapeutic target in the future.

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CHAPTER 9

CURRENT APPROACH TO THE PROPERTIES, USES AND IMMUNOLOGY OF CORD BLOOD CELLS

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ABSTRACT

Umbilical cord blood (UCB) is an important source of life, providing the nutrients and oxygen necessary for the development of the fetus. In addition, immune cells in the UCB, which connects the mother and the fetus and helps the development of the fetus, have a critical role in this development process. Cord blood stem cells (CBSCs) were also included in therapeutic applications for the first time in 1972 for the treatment of a case with lymphoblastic leukemia. Fetal umbilical CBSCs are used in the treatment of blood, immune system and some metabolic storage diseases in children and young adults today, from the classical bone marrow in the treatment of blood, immune system and some metabolic storage diseases, because they are easier to obtain, do not impose an additional burden on the donor, do not require full tissue compatibility for successful transplantation and have a low tissue rejection rate. It has become an alternative to stem cell transplants. Apart from these, clinical studies for the use of CBSCs in the treatment of hereditary biochemical and metabolic diseases, type 1 diabetes and immune system diseases, and experimental studies for the use of neurological diseases and regenerative medicine are ongoing.

Keywords: Cord blood stem cell, immunotherapy, cellular therapy, microbiota

Introduction

The umbilical cord is the tissue that connects the fetus to the placenta and is the source of nutrients, originating from the allantois and yolk sac at 5 weeks of fetal development (1). Umblical cord is known to reach to an average of 50-60 cm in length and 2 cm in diameter at the end of pregnancy (2). The umbilical cord is comprised of a vein and 2 arteries that spiral around it, and a Wharton's jelly, which is a jelly-like, lamellar, proteoglycan-rich matrix that protects them and prevents their compression (3). While the vein delivers nutrient-rich oxygenated blood from the placenta to the fetus, the arteries return the deoxygenated blood back to the placenta, whose nutrients have been depleted by the fetus. The umbilical cord is not directly connected to the mother's circulatory system, but to the placenta. The placenta provides the transfer of nutrients and other substances without mixing the blood of the mother and fetus (4).

The blood remaining in the umbilical cord after birth is called the cord blood (CB). CB contains all the elements found in peripheral blood, but contains a high percentage of natural killer cells (NK) and immature T cells, and a low percentage of absolute T cells (5, 6).

CB contains higher numbers of hematopoietic progenitor stem cells compared to the levels in bone marrow and peripheral blood. The cord blood stem cells (CBSCs) have longer telomeres and higher proliferative capacity. CBSCs, which adapt more easily to the environment, play an important role in the formation of engraftment and the functioning of the bone marrow after allogeneic stem cell transplantation (7, 8). In addition, CB contains a unique lymphocyte profile. The regulatory T cells (Treg) constitute the majority of these lymphocytes. The anti-inflammatory characteristics of CB cells and the fact that they contain fetal hemoglobin as a natural oxygen carrier allow the use of CB in other areas in addition to transplantation (9).

Researchers in a study in the literature reported that the count and proliferation rates of granulocyte macrophage colony-forming unit (CFU-GM) and multipotent progenitor cells (CFU-GEMM) contained in CB obtained from term babies were much higher than the counts in adult blood (10). Although the umbilical cord blood (UCB) is an important source of life that provides the nutrients and oxygen necessary for the development of the fetus, it was also included in therapeutic applications in 1972 for the first time in treatment of a case with lymphoblastic leukemia (11).

1. Cord Blood Immunology

1.1. The Immune Profile of the Cord Blood

The UCB is highly rich in immune cell profile and contains a greater proportion of NK and immature T-lymphocytes compared to the levels in adult peripheral blood. CB immune cells are generally immature and therefore the T cells they contain respond with less intensity to alloantigens immunologically. CB also contains Tregs which have strong effects

in suppressing allogeneic immune responses and the mesenchymal stem cells (MSCs) (12). CB contains a small number and more immature immune cells compared to bone marrow, therefore researchers suggested that it may prevent the occurrence of Graft versus leukemia (GvL) effect after transplantation however studies showed that the T and NK cells in the CB became active after allogeneic transplant, and could develop GvL without causing any severe graft versus host disease (GvHD) (13, 14).

Publications in the literature report significantly deranged dysregulation of hematopoietic cytokines and lymphokines in UCB cell sources compared to adults. Cairo et al. in a series of studies comparing CB and adult mononuclear cells, reported a major decrease in mRNA expression and protein production of granulocyte colony-stimulating factor (G-CSF) in activated CB mononuclear cells, protein expression of interleukin-3 (IL-3), granulocyte macrophage colony-stimulating factor (GM-CSF), beta-1 transforming growth factor (TGF-beta-1) and macrophage inflammatory protein-1 alpha (MIP-1α) and protein production of cytokines such as IL-12, IL-15 and IL-18. However, comparison of adult fibroblasts and endothelial cells reported that the cell expression and protein secretion of IL-11, stem cell factor (SCF) and thrombopoietin were significantly increased in CB (15, 16).

The human CB contains a lower number of $CD4^+$, $CD8^+$ and $CD3^+$ T cells and a higher CD4/CD8 ratio compared to adult peripheral blood, and the ratio of $CD4^+/CD45^+$ T cells is also lower compared to adult peripheral blood (17-19). This decrease in T cells also causes a decrease in the activation of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (17). The CB lymphocytes were reported to have produced adequate count of IFN-γ, IL-4 and IL-10, however, it generated small amounts of mRNA for IL-2, which has an crucial role in the functions of NK and T lymphocytes in mononuclear cells. Furthermore, the polyclonal T cell repertoire is highly developed and they can be protected from apoptosis due to low CD95 expression. Studies in the literature have reported that the activity of NK cells in CB is lower than that of NK cells in adult peripheral blood (20).

Dendritic cells (DCs) are the antigen-presenting cells that play an important role in the initiation of the immune response. Three types of DCs were described as myeloid, lymphoid and immature, and the CB dendritic cell profile is comprised of lymphoid and immature phenotype (21). Myeloid DCs participate to Th1 effector and lymphoid DCs participate to Th2 effector cell development and are instrumental in immune regulation of T cell function (22). The immature DCs in the CB stimulate Tregs and cause T cell energy in the environment (14). Thus, the mature DCs regulate the inflammatory response, and the immature DCs induce the tolerance.

1.2. The Immunologic Interaction of Maternal Microbiota with the Cord Blood

The human body has been colonised by thousands of different microbial species that are key to survival (23). The human microbiota is the environmental community of symbiotic,

commensal and pathogenic microorganisms that live on the surfaces and specific niches of organisms (vagina, gut, skin, mouth, etc.) (24). The concept of microbiome refers to the communication of all these microorganisms with the microenvironment and has important roles related to human physiology. The microbiome is known to help food digestion, prevents the colonisation of pathogens by preserving the structural integrity of the gut mucosal barrier, participates in the communication of gut-brain axis, supports the function of immune system cells, helps to maintain a healthy state and to be alert against possible dangers. In addition, it contributes to the production of ATP owing to the short-chain fatty acids (SCFA), produces B and K vitamins (B5, B12), contributes to the fight against pathogens by regulating cells such as macrophages, and takes part in the recognition and modulation of pathogens (25). The early life plays a key role for microbial development (26). Food taken by the mother has an important role for the fetal microbiota. The nutritional status of the mother during pregnancy can affect the health of the baby from early life to adulthood (allergy, tolerance, diabetes, heart diseases, etc.) (27). It is estimated that the first steps of microbiota formation for the fetus are taken while in the placenta, and based on this, it is suggested that bacteria can be found in the placenta and amniotic fluid (28). It is known that in the maternal process, the gut microbiota colonisation of the fetus begins in the in utero period, and its composition constantly changes in the first few years of life, which is a process dependent both on ecological and nutritional factors. Around the age of three, the microbial composition becomes stable (29). Molecules such as immunoglobulin (Ig), cytokine and adipokines play an important role in fetal development during pregnancy (30). Previous studies have reported that the dietary intake of omega-3 fatty acids (EPA and DHA) decreased the levels of the proinflammatory cytokines and have a positive interaction with the IL-10 cytokine group in maternal plasma, however this is not the same in the CB (31). A recent study revealed that the maternal enterotype highly affects the immune and adipokine composition of umbilical cord plasma compared with the maternal plasma. Cytokines and Igs are the molecules that are mostly affected by the composition of the microbiota. The mother's diet type can also affect the baby's microbiota and therefore the immune system. Research has shown that high levels of Lachnospiraceae family or Ruminococcus from Ruminococcacae family, pro-inflammatory cytokines and IgA, and IgM are found in high levels in the gut flora of babies whose mothers had a western type and Mediterranean type diet during pregnancy, however the counts of types such as Peptoniphilus, Porphyromonas, Anaerococcus and Prevotella were higher, and the IgG and proinflammatory cytokine levels were lower in babies whose mothers had a diet mainly composed of animal foods and carbonhydrates (Figure 1) (32).

The umbilical cord connects the mother and the fetus and helps the growth of the fetus, and the immune cells in the CB have critical roles during this entire developmental process. Bacteria was detected in 45% of the CB of newborns in a study conducted with the samples

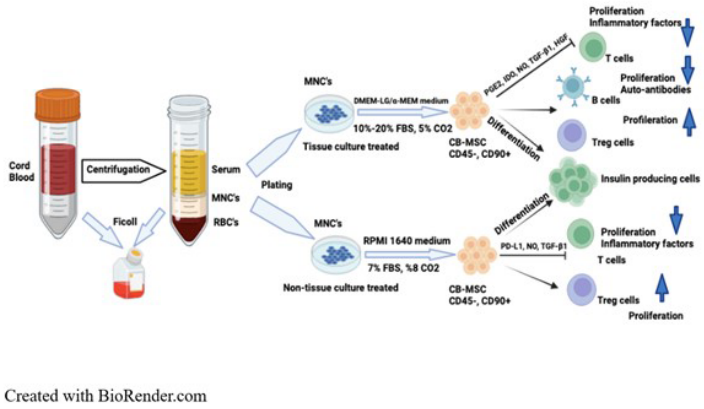


Figure 1: The effect of diet on the enterotype, umbilical cord composition and birth outcomes (32)

taken from the CB of 20 healthy newborns (33). Various factors can affect the microbiota, which could be counted as stress, geography, diet, alcohol consumption, and medicine use. During pregnancy, significant changes are known to develop in the gut flora of the mother and the baby who are exposed to one or more of these factors. A higher ratio of *Proteobacteria*, and lower levels of *Bifidobacteria* were found in the microbiota of the baby whose mother was under high stress during pregnancy in a study (34).

There is a need for communication between the mother and the fetus in order to have a healthy pregnancy, and birth. The mother offers passive and natural immunity to her offspring through placenta during pregnancy and breastfeeding process (35). The gut microbiota studies in infants have begun to suggest that the newborn gut flora is formed by the interaction with the vaginal microenvironment of the mother during the transition from the normal delivery bed. To date, the fetus was known to be in a sterile state in the uterus, however, today the concept of maternal microbiota fecal microorganisms has emerged through cross-contamination during childbirth (36). A new showed that the vaginal Lactobacillus species in newborns taken from the mother make up about 1/4 of the microbiota in babies but are found in a race with breast milk Lactobacilli therefore they literally cannot take part in the baby's gut microbiota (37). In this sense, it would be more accurate to suggest that the baby's microbiota is shaped depending on the breast milk. The estimations suggest that a baby who consumes 800 ml of breast milk takes as much as $8x10^4$-$8x10^6$ commensal bacteria. Differences were found in the examination of the gut flora of babies of mothers who had cesarean section and normal delivery. 'The *Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium catenulatum, Bifidobacterium longum subsp. Longum*, and *Bifidobacterium pseudocatenulatum*' monophylatic bacteria were commonly found in the microbiota of mother and baby. While 11 out of 12 babies born with normal delivery had a monophyletic strain, no monophyletic strain was found in babies born with caesarean section (38). The mother provides passive and natural immunity to her offspring through lactation during pregnancy or

by transplacental route during breastfeeding (35). The human milk oligosaccharide (HMO) found in breast milk has antibacterial properties. The main food source of the *Bifidobacteria* found in the gout population of infants is the HMOs. *Bifidobacteria* are among the beneficial bacteria and fight with pathogens (39). The placental dysfunction the exact cause of which is unknown, and the number of which has currently been increasing usually results with abortion (40). Current studies indicate that microorganisms in the placenta, that is named the "placental microbiome", show different colonisation between healthy and miscarried pregnancies based on a physiological relationship. However, these results contradict with the data that the placental environment is sterile. The baby's first stool is called meconium, and meconium consists of mucus, blood, bile acids, amniotic fluid, epithelial cells and a high amount of water. One study showed that the newborn meconium, amniotic fluid and placenta have similar microenvironments, which again confirms the data that microbial colonisation starts in the uterus (41).

2. Cord Blood Stem Cells (CBSC)

There are high-density stem cells in CB in addition to blood cells such as erythrocytes, lymphocytes, leukocytes and thrombocytes, and CBSCs are included in the group of fetal stem cells. CBSC has longer telomeres and higher proliferation potential compared to bone marrow and peripheral blood stem cells. In addition, the CB $CD34^+$ cells have a higher migration capacity compared to adult progenitor cells. The CBSC are known to possibly differentiate into MSCs, hematopoietic stem cells (HSCs) and endothelial progenitor stem cells (EPCs) (42).

The CB contains immature stem cells and a large amount of regulatory T cells (42). These properties have been reported to provide advantages to CBSC such as a reduced risk of infection transmission, the development of tolerance to human leukocyte antigen (HLA) incompatibility and a lower risk of inducing GvHD compared to embryonic stem cells (ESCs) and stem cells originating from adult bone marrow (43).

2.1. Mesenchymal Stem Cells

MSCs have a high proliferation ability and the potential to differentiate into a wide variety of cell strains. They can be isolated from various tissues such as bone marrow, UCB and adipose tissue, and most of the studies in the literature have focused on bone marrow as the source of MSCs. However, with aging, the resulting decreases in the differentiation and proliferation capacity of MSCs originating from the bone marrow have drawn attention to an alternative source of MSCs (44). Currently, the CB MSCs are accepted as a highly suitable source for the transplantation process. MSCs exhibit the potential to differentiate into mesodermal cell strains such as bone, cartilage, adipose, tendon, muscle and bone marrow stroma, as well as endodermal cell strains such as hepatocyte-like cells and

ectodermal cell strains such as neuronal, and neuroglial cells. In addition to this remarkable wide differentiation capacity, MSCs have immunosuppressive properties and can support hematopoiesis. The characterisation studies reported that CB MSCs show the ability to differentiate into chondrocytes, osteocytes and adipocytes such as nerve cells and visceral mesoderm (45). In addition, studies in the literature have reported that when isolated and cultured, the CB MSCs lose their surface markers such as CD14, CD34 and CD45 and express CD106, CD54, SH2, SH3 and SH4. The light and electron microscopy studies showed that the MSCs derived from CB are positive for mesenchymal markers such as smooth muscle actin (SMA), vimentin, nestin and desmin (46,47).

2.2. Hematopoietic Stem Cells

The CB HSCs were reported as the most suitable source for transplantation, a successful method used in the treatment of hematologic diseases (48). These stem cells have the potential to differentiate into bone marrow tissues, as well as into the blood components. Researchers in studies reported that CB HSCs may be closely associated with the progenitors of primitive hematopoiesis. A study in the literature reported that the CB HSCs have a higher potential for proliferation and expansion compared to cells obtained from adults, as well as their self-renewal capacity is superior to adult cells (49). The expression of CD34 antigen is a characteristic feature of HSCs. Researchers in the studies reported that this molecule regulates the hematopoietic cell adhesion. HSCs with $CD34^+$ also express HLA-DR and CD38 antigens to a large extent. In addition, it has been reported that they are characterised as the colony forming cells which express the fetal liver kinase 1 (flk-1), vascular endothelial growth factor receptor 2 (VEGFR2), Brachyury (mesodermal marker), Oct-4, and CD41 (50).

2.3. Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are the endothelial precursors involved in the repair of damaged tissues and in vascular recovery. Studies in the literature reported that CB can be a useful source for the isolation of endothelial progenitor cells and that they express cell surface molecules such as CD34, Flk-1/KDR, Tie-2 and VE-cadherin at early stages of differentiation (51). CB EPCs were characterised as $CD133^+/CD34^+$ or $KDR^+/CD34^+$ or VEGFR2 and $CD14^-$ using magnetic cell separation in a study. In another characterisation study conducted using a real-time reverse transcriptase polymerase chain reaction, it was reported that they also express STAT3, c-kit, CXCR4 and Oct-4 (52). One study reported that the human CB endothelial progenitor cells differentiate in a shorter time compared to those derived from peripheral blood, and this result was interpreted as indicating that EPCs derived from CB may have more proliferative activity due to having higher cell cycle rates and longer telomers than those derived from adult peripheral blood. It has also been reported that CB EPCs help in the regeneration of human hematopoietic bone marrow (53).

3. The Area of the use of Cord Blood Stem Cells - Applications of Cord Blood Cells?

3.1. Clinical Studies

3.1.1. Type 1 Diabetes

Type 1 diabetes (T1DM) is caused by the irreversible damage or loss of the function of insulin-producing beta cells in the islets of Langerhans in the pancreas. This picture, which occurs as a result of organ-specific immune system destruction, is an autoimmune condition. Beta cells sense the blood glucose levels and play an essential role in the progress of diabetes. Under physiological conditions, insulin release is necessary to maintain glucose levels at normal levels. Therefore, insulin plays an important role in ensuring glucose homeostasis. Due to autoimmune destruction in T1DM patients, the blood glucose level cannot be controlled and patients have to use external insulin supplements throughout their lives. However, the insulin used cannot have any effect on autoimmunity, which is the mechanism of the disease, and therefore cannot be effective on the definitive treatment of the disease. In recent studies, the approach of controlling the autoimmune dimension of diabetes with CBSCs has gained importance. In addition, the idea that stem cells can provide the restructuring of beta cells due to their ability to transform and differentiate into many cells that make up the body has become an approach that stem cell and cellular therapy research focuses on (54). In recent years, studies in the literature have focused on the use of CB cells in the treatment of diabetes. CB-derived cells were thought both as an important biological source for beta cell production and an immunomodulatory cell source for T1DM therapy. Among CB cells, it has been reported that $CD45^+$ multipotent stem cells and $CD90^+$ MSCs originating from CB have similar properties in terms of differentiation and immune system regulatory potentials, and the efficacy of these cells in the treatment of T1DM has been investigated (55).

In a double-blinded clinical study, researchers reported that the function of beta cells could be restored after infusion of CB MSCs in newly diagnosed T1DM patients. Moderate improvement in metabolic function was reported in another pilot randomised controlled clinical trial with T1DM patients treated concomitantly with CB MSCs and autologous bone marrow mononuclear cell transplantation (56).

It was also shown that CB multipotent cells can exert immunosuppressive effects on induced $CD4^+$ T cells and $CD8^+$ T cells. In a study using an autoimmune mediated non-obese diabetic (NOD) mouse model, it was reported that the multipotent stem cells derived from CB can reverse the functional defects of $CD4^+CD62L^+$ Tregs, leading to the prevention of the onset of diabetes and reversal of diabetes (57).

3.1.2. The Hereditary Biochemical and Metabolic Diseases

The hereditary biochemical diseases are the inherited disorders that occur especially during the synthesis and catabolism of proteins, carbohydrates and lipids. In the clinical studies in the literature, improvements in the quality of life, survival and improvement in comorbidity of recipients have been demonstrated with the use of CBSCs in hereditary biochemical diseases such as mucopolysaccharidosis, glycoproteinosis and sphingolipidosis. Lysosomal storage diseases are the inherited diseases characterised by the accumulation of glycolipids, sphingolipids, mucopolysaccharides or oligosaccharides in lysosomes, which usually occur as a result of deficiency of lysosomal hydrolase enzymes (58). Treatment modalities for the disease have focused on replacing the defective enzyme, and enzyme replacement therapies have been administered to these patients. Studies using animal models in the literature have shown that HSC transplantation has a healing effect on the prognosis of the disease and alleviates the symptoms. One clinical study comparing the results of HSC transplant and enzyme replacement therapy reported that the HSC transplant is more effective than the enzyme replacement therapy in terms of metabolic healing in lysosomal storage diseases (59).

3.1.3. Onco-hematologic Diseases and Allogeneic Stem Cell Transplant

The transplantation results using CBSCs and bone marrow stem cells were reported to be similar in oncohematological diseases. Studies with children with onco-hematological malignancies have shown that the rate of engraftment is over 90%, although the number of $CD34^+$ cells is lower than the adult donor grafts. In addition, although most of the transplanted CB grafts do not have full HLA compatibility, the incidence of GvHD development after transplantation was reported as much lower than that of adult HSC grafts (60).

3.1.4. Immune System Diseases

Systemic lupus erythematosus is a disease that occurs as a result of immune system disorder and causes inflammation in many tissues and organs, and it has been reported that mesenchymal stem cell transplantation originating from CB is a safe and effective method in the treatment of this disease. In addition, it has been shown in clinical studies that CB mesenchymal stem cell infusion has a positive effect on the course of the disease by reducing the inflammation in rheumatoid arthritis patients (61).

3.2. Experimental Studies

3.2.1. Neurologic Diseases

The potential importance of CBSC therapy has been demonstrated mostly for stroke among neurological diseases. A study with a medial carotid artery occlusion (MCAO) stroke model in rats showed that the infusion of CBSCs can ameliorate many of the physical and behavioral defects associated with this disease. In addition, various studies using animal models have reported that the CBSC infusion results in observable behavioral improvement in diseases

such as amyotrophic lateral sclerosis, Parkinson's disease, cerebral palsy and spinal cord injury (63).

MSCs are preferred more than other stem cell types especially in incurable nervous system and neurodegenerative diseases due to their ability to differentiate into neural cells, secrete neurotrophic factors and secrete anti-inflammatory and angiogenic cytokines to repair damaged tissues (64). The most important pathological marker of Alzheimer's disease is the accumulation of extracellular amyloid beta (Aβ) plaques in the brain. Many animal model studies in the literature have reported that the CB mesenchymal stem cell transplantation reduces Aβ plaques. The increase and accumulation of Aβ levels in Alzheimer's disease also causes inflammation. One study with mice has shown that the CB mesenchymal stem cell therapy significantly increased IL-10 expression and decreased the proinflammatory microglial activation and proinflammatory cytokines such as IL-1β and TNF-α (65). In addition, another study showed that the anti-inflammatory effects of Tregs increased after CB mesenchymal stem cell therapy, while IFN-γ levels decreased, IL-10 and TGF-β1 levels increased (66). It has been reported that locomotor recovery and repair is provided in the damaged area in many studies examining the effect of CB mesenchymal stem cell therapy in patients with cerebral palsy (67).

3.2.2. Regenerative Medicine

Regenerative medicine is the science that investigates the ability of an organism to repair damaged cells, tissues and organs on its own. Currently the regenerative medicine is used in the form of separatiation, concentrating and giving the cellular products such as blood, bone marrow and adipose tissue to the same person without being cultured.

CB exhibits highly totipotent properties owing to their high percentage of Oct-4^+ and $CD105^+$ cells involvement, and has the potential to differentiate into adipogenic, chondrogenic and osteogenic strains with this feature (68). One study showed that CBSCs differentiate into chondrogenic cell strain with 3 weeks incubation in chondrogenic medium (69). Another study showed that the MSCs derived from CB differentiate into adipogenic cell types after 21 days of incubation in adipogenic medium (70). It was shown in this study on patients with knee articular cartilage defect or osteoarthritis who had undergone cartilage repair surgery that the CB MSCs may be effective in cartilage regeneration. Another study investigated the potential of $CD34^+$ cells in human CB to improve cardiac function following myocardial infarction and reported a decrease in left ventricular dilatation and an improvement in cardiac function 4 weeks after infusion of CBSCs (71). In a study on the corneal endothelium, researchers reported that CB MSCs can settle in areas of damaged corneal endothelium and that the phenotype of established MSCs can be changed to cells similar to human corneal endothelial cells (72). In addition, there are also studies in the literature showing that CBSCs or cells that arise as a result of its differentiation are also effective in the regenerative treatment

of other tissues such as neural tissue damage (73), skin damage (74) and liver tissue damage (75).

3.2.3. Cord Blood Derived Pluripotent Stem Cells

The induced pluripotent cells (IPSCs) are the cells that are intended to be used in the treatment of diseases, new drug screenings, tissue engineering studies and individualised treatments, and are obtained by genetic reprogramming of a wide variety of cells such as dermal fibroblast, peripheral blood cells, keratinocyte, urinary epithelial cell, liver, gastric epithelial cells, mesenchymal cells, B and T cells, neural stem cells, pancreatic cells, progenitor blood cells, and CB cells (76). Currently, stem cell studies have focused on IPSCs due to both the difficulty of obtaining ESCs and the problems arising from ethical issues.

The characterisation studies have reported that the IPSCs originating from CB have a higher proliferation ability than other cell sources due to the high amount of $CD34^+$ cells in the CB (77) and also, IPSCs originating from CB have less immune rejection because CB contains immature cells (78). In a clinical study to compare normal and pathological tissue formation and to evaluate the *in vitro* recovering effects of drug therapy, the patient models were created using the IPSCs in patients with severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS) and Gaucher disease type III (79). In addition, there are studies to develop customised HLA compatible IPSCs in the literature. Thanks to these studies, it is possible to develop regenerative treatments against various diseases. The regenerative capacity of HLA compatible articular cartilage is very low and it is very difficult to obtain healthy cartilage for transplantation. Therefore, various adult stem cells and pluripotent stem cells are actively used to treat degenerative or damaged joint diseases (80). Due to their high regenerative capacity, particularly the MSCs and joint chondrocytes are widely used in the treatment of joint cartilage damage. However, the limited proliferation and differentiation potential of these cells has led researchers to work on pluripotent stem cells. In studies, chondrogenic pellets were produced using the IPSCs derived from CB, and it was shown that the expression of cartilage-specific genes and related differentiation in these pellets increased day by day. In addition, it has been reported that the expression of COL10A1, a hypertrophic cartilage marker, is lower in IPSCs originating from CB compared to the IPSCs derived using the MSCs (81). Another study in the literature evaluated the potential of IPSCs to treat ischemic retinopathies and reported that vascular progenitors produced using CB-derived IPSCs were successful in repairing ischemic retinal vasculature (82).

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CHAPTER 10

EPIGENETIC REGULATION OF HLA-G EXPRESSION IN MESENCHYMAL STEM CELLS

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stem cells morphologically similar to fibroblasts. Thanks to their ability to transform into different cell types and immune-regulatory properties, they make important contributions to regenerative medicine and immunotherapy treatment protocols. One of the most unique molecules involved in the immunomodulation of human MSCs is the nonclassical MHC-I molecule, human leukocyte antigen G (HLA-G). Unlike classical MHC I molecules, HLA-G shows a limited tissue distribution. This limited expression pattern may be explained by the fact that its transcriptional regulation differs from that of classical MHC I molecules. Here, the regulation of HLA-G expression in MSCs is discussed specifically through epigenetic mechanisms.

Keywords: Epigenetic regulation, HLA-G, mesenchymal stem cells, transcriptional regulation

1. Mesenchymal Stem Cells Biological Characteristics

Mesenchymal Stem Cells (MSCs) are multipotent stem cells with a fibroblastoid phenotype that can be isolated from various tissues throughout the body and do not display hematopoietic properties (1). Examples of tissues from which they are isolated include peripheral blood, fatty tissue, dental tissues, thymus, endometrium, amniotic fluid, umbilical cord and Wharton's Jelly (2, 3, 4).

To identify MSCs, the International Society for Cellular Therapy (ISCT) has established the following criteria:

- MSCs are culture conditions have the ability to adhere to plastic surfaces,
- They express the cell surface antigens CD73, CD90 and CD105,
- On the other hand, they do not express surface antigens of hematopoietic lineage (CD14, CD34, CD45 etc.) and human leukocyte antigen (HLA-DR),
- They have the ability to differentiate into chondroblasts, osteoblasts and adipocytes *in vitro* (5).

In addition to these characteristics, MSCs have become an important alternative in regenerative medicine and immunotherapy because they can secrete factors that accelerate tissue regeneration and have immune regulatory properties (6, 7).

The precise mechanisms responsible for the immunosuppressive characteristics of MSCs have not been completely understood as of yet; however, various molecules such as HLA-G (8), indoleamine 2,3-dioxygenase (IDO) (9), and prostaglandin E2 (PGE2) (10) have been proposed.

1.1. The Immune Modulatory Effect of MSCs and their Relationship with HLA-G

In specific contexts, there is extensive documentation supporting the capacity of MSCs to effectively regulate the immune responses of both adaptive immune components, such as T and B cells, and innate immune elements, including natural killer (NK) cells, antigen-presenting cells (monocytes, macrophages, dendritic cells), and polymorphonuclear cells (11).

Various mechanisms have been proposed to explain MSC-mediated immunomodulation, with a consensus emerging that the secretion of soluble factors plays a pivotal role (12). Among these factors, MSCs release HLA-G, TGF-β, PGE2, IL-10, hepatocyte growth factor (HGF), and IDO. It has been shown that all of these factors work in a coordinated manner and induce HLA-G expression (13). For example, MSCs express IL-6 and PGE2, which induce monocyte differentiation. Subsequently, IL-10 secreted from monocytes activates MSCs to upregulate HLA-G expression (soluble and membrane-bound). Figure 1 illustrates this process (14).

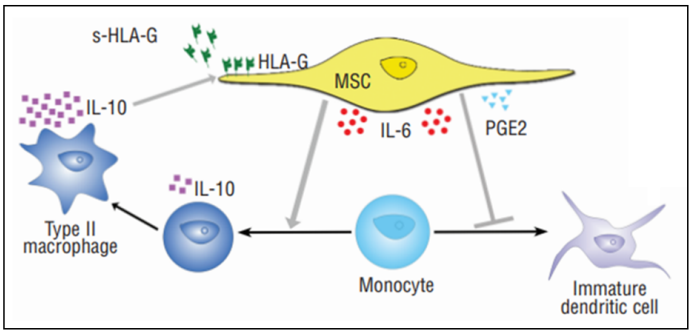


Figure 1: MSC-mediated immunomodulation (14).

One of the most crucial molecules that enables MSCs to control the functions of immune system cells are HLA-G, a non-classical major histocompatibility complex (MHC) class I molecule (15). Initially discovered in placental trophoblasts, HLA-G facilitates maternal acceptance of the developing fetus (16, 17). Its primary function lies in modulating immune responses, both in normal physiological states and during pathological conditions (18).

Studies have demonstrated that HLA-G inhibits T cell and NK cell cytolysis and suppresses $CD4^+$ T cell alloproliferation through interaction with inhibitory receptors expressed on immune cells irrespective of whether the conditions are normal or pathological (19, 20). Therefore, the constitutive expression of HLA-G in placental trophoblasts serves to maintain immune tolerance between the fetus and mother during pregnancy (16) (Figure 2) (21).

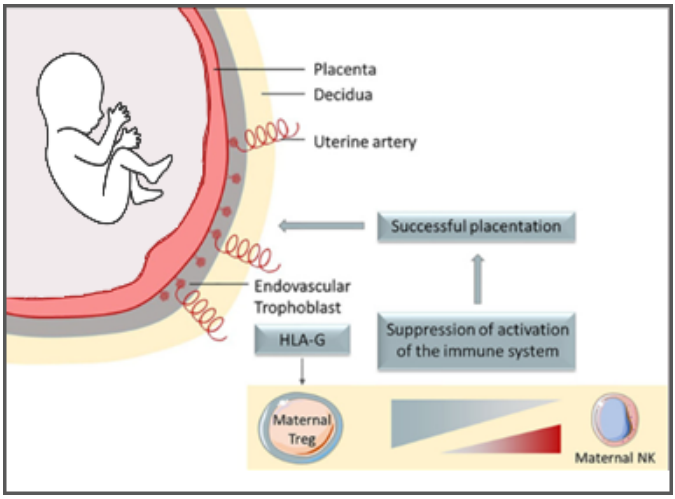


Figure 2: HLA-G serves to maintain immune tolerance between the fetus and mother during pregnancy (21).

HLA-G expression has also been associated with reduced incidence of rejection after transplantation (22). In this study conducted by Lila N. and colleagues, it was revealed that the number of acute and chronic graft rejections in the heart post-transplantation was significantly lower in HLA-G-positive patients compared to HLA-G-negative patients.

Consequently, the immunomodulatory functions of MSCs, including maintenance of fetal-maternal immune tolerance, prevention of post-transplant rejection, and other immune modulation functions are thought to result from the effects of HLA-G expressed by MSCs. These effects include the inhibition of $CD8^+$ T and NK cells, an increase in cytokines secreted by Th1 and Th17 cells, stimulation of Tregs, and the activation of myeloid-derived suppressor cells with immunosuppressive activity (23, 24, 25).

2. HLA-G Gene Expression and Epigenetic Mechanisms

The term epigenetics was first introduced in 1940 by British embryologist, geneticist, and philosopher of science Conrad Hal Waddington, to reveal the connection between environmental factors, genes, and phenotypic outcomes.

Today, epigenetics is defined as non-DNA sequence-related alterations in the regulation of gene expression and gene product activity (26). Epigenetic modifications are critical for transcriptional regulation. These modifications involve DNA methylation and histone tail modifications such as acetylation, phosphorylation, methylation, ubiquitylation, and sumoylation (27).

Epigenetic mechanisms play a significant role in the regulation of implantation, placentation, organ development, and fetal growth. There is a gradual decrease in DNA methylation from zygote formation to the morula stage, coinciding with the onset of HLA-G gene expression during this period (29, 30). Alterations in epigenetic patterns may be implicated in conditions such as pre-eclampsia (31) and are widely acknowledged as contributors to the development of tumors (32).

2.1. DNA Methylation and HLA-G Expression

One of the primary epigenetic processes that regulates the expression of tissue-specific genes is DNA methylation (33). The enzymes responsible for DNA methylation are referred to as DNA methyltransferases (DNMTs) (28).

The addition of a methyl group to the 5'-position of cytosine residues represents a reversible covalent alteration of DNA, leading to the formation of 5-methylcytosine (34). These methyl groups can prevent DNA recognition by certain proteins while promoting the binding of other proteins to DNA (35). Specifically, methylation occurring in CpG islands within promoter regions is linked to the suppression of transcription, whereas the removal of methyl groups, or demethylation, leads to the activation of transcription (Figure 3) (36, 37).

The DNA methylation within the promoter region is recognized by a family of proteins called the methyl-CpG binding domain (MBD) proteins. These proteins specifically recognize methylated CpG dinucleotides. MBD proteins, especially methyl-CpG binding protein 2 (MeCP2), can lead to chromatin remodeling and suppression of gene expression (38, 39, 40, 41).

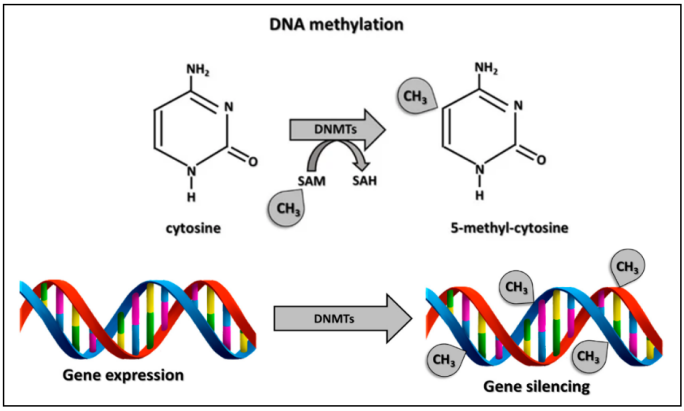


Figure 3: Diagram illustrating the process of DNA methylation (36, 37).

2.2. Histones Modifications and HLA-G Expression

DNA methylation and histone modifications are critical epigenetic control mechanisms in the regulation of gene expression, as they influence the accessibility of regulatory factors to DNA (15).

For instance, in a study examining chromatin remodeling at the HLA-G locus with histone deacetylase inhibitor treatments, it was observed that, although at lower levels compared to DNA demethylation agent 5-Aza-dC treatment, the HLA-G gene was activated in certain cell lines such as M8 (melanoma) (42, 43).

In another study focusing on histone acetylation at the promoter regions of the HLA-G gene, it was observed that histone acetylation was more pronounced in cells expressing HLA-G compared to cells lacking HLA-G expression (44).

2.3. MicroRNAs and HLA-G Expression

Some studies have drawn attention to the roles of microRNAs in the regulation of HLA-G gene expression. For instance, in a study by Manaster and colleagues, it was demonstrated that under physiological conditions, the expression levels of microRNA-148a, microRNA-148b, and microRNA-152 in the placenta were lower compared to other tissues, while the mRNA levels of HLA-G were higher (45).

However, in pathological conditions characterized by decreased HLA-G levels, such as preeclamptic placentas, it was observed that the levels of these microRNAs were elevated (46). The same holds for placentas of pregnant patients with intrahepatic cholestasis (47).

3. Levels of HLA-G and Epigenetic Mechanisms in MSCs

While MSCs express MHC class I surface markers, they do not express MHC class II and costimulatory molecules such as CD40, CD80, and CD86, which gives them an advantage in allogeneic and heterogeneous transplantation (48, 49, 50).

The restricted expression pattern of HLA-G can be explained by its transcriptional regulation being different from that of classical MHC class I molecules. For example, the interferon-stimulated regulatory element (ISRE), which is one of the strongest inducers of MHC class I proteins and is activated by interferon-gamma (IFN-gamma), is not highly functional on the proximal promoter of HLA-G (44, 51, 52) (Figure 4) (53).

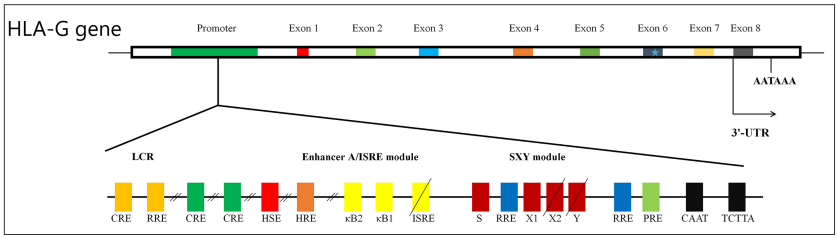


Figure 4: HLA-G gene region (53).

In addition, epigenetic regulations also play a critical role in tissue-specific gene expression (54). For example, in a study by Yen et al. on HLA-G gene expression, a general pattern of hypomethylation was observed in the HLA-G proximal promoter regions of human MSCs and placental tissue extracts (Figure 5) (55).

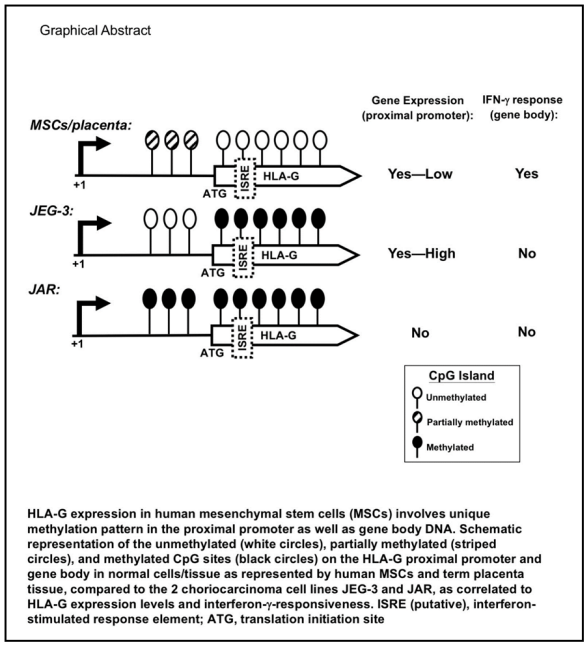


Figure 5: Hypomethylation pattern in the HLA-G proximal promoter regions (55).

In a study by A. Verloe et al. in 2017, low methylation was detected in the region of the HLA-G promoter in human embryonic stem cells. After the administration of DNA demethylation agent, an enhancement in HLA-G expression became evident (56).

However, HLA-G expression decreases during *in vitro* MSC culture serial passages (57).

In a study by Teklemariam et al. on MSCs obtained from bone marrow and adipose tissue, HLA-G upregulation was observed in both P1 and P5 passage MSCs following the addition of the DNA demethylation agent to MSC cultures (Figure 6) (58).

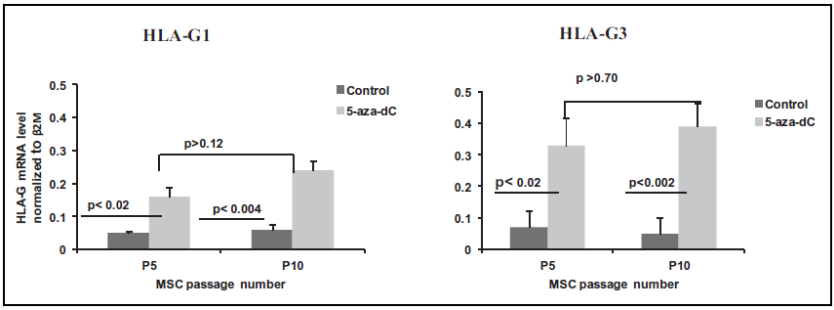


Figure 6: HLA-G1 (B) and HLA-G3 (C) mRNA levels on BMSCs (58).

These findings demonstrate that demethylation of the HLA-G promoter region significantly increases HLA-G expression in MSCs.

Conclusion

Understanding the role of epigenetic mechanisms in the transcriptional regulation of HLA-G is crucial for the development of new and effective options in MSC-based therapies, given the overall importance of HLA-G in immunomodulation and the clinical significance of human MSC immunobiology. Currently, studies in this field have mostly focused on DNA methylation. Investigating the impact of other epigenetic mechanisms on the expression of the HLA-G molecule in MSCs is an open area of research to further advance the clinical application of MSCs as an effective option for immunosuppressive therapy and to add new knowledge to the literature.

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CHAPTER 11

STEM CELLS IN REGENERATIVE MEDICINE

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ABSTRACT

The advancements in understanding stem cell characteristics and potential opened new opportunities for its application with various modalities in therapy. The regenerative capacity of stem cells has revealed great promise for eradicating many diseases by transplanting healthy or genetically engineered stem cells into patients. However, there is still a way to go with significant challenges; in the near future, the power of science and hard work will inevitably succeed.

Keywords: Stem cells, regenerative medicine, stem cell-based therapy, hematopoietic stem cells, mesenchymal stem cells

1. Introduction

The basis of stem cell therapy is the transplantation of healthy stem cells into a patient to repair diseased cells or reconstruct the functioning cells and tissues of the recipient's body. Transplanted cells preserve the donor genotype, and consequently, transplantation can be considered a form of gene transfer therapy as it leads to modification of the somatic genome. Studies in this area have made tremendous advancements within the last few decades and have more experience in adults. Stem cells are cells that can self-renew and potentially differentiate into many cells. Historically, the term "Stem Cell" was first used in the literature in 1868 by German zoologist Ernst Haeckel (1). In his studies, Prof. Haeckel created pedigree trees showing hierarchical order for the evolutionary relationship between primordial unicellular organisms from which more complex multi-cellular organisms originate by relating their morphological similarities. Later, Prof. Haeckel generated the idea of root-tree and stem cell terminology to describe the fertilized egg and then applied this concept on a cellular level (2). In the following years, the theory of hematopoiesis in the bone marrow and the cell production from a common progenitor cell was generated with the emergence of leukemic cells by histopathologist Arthur Papenheim. Named these precursor cells as "hematopoietic stem cells" (HSCs) (3). More advanced imaging techniques combined with the morphological features of blood cells were applied in these studies. Thus, the concept of the first tissue-specific stem cell was generated. From the very beginning, the concept of stem cells was emphasized based on a tree-like model on the idea that multipotent stem cells form a series of sequential branching cascades and give rise to their lineage (4).

2. Stem Cell Hierarchy

Today, a stem cell is defined as a unique single cell capable of self-renewal and differentiation into multiple cell lines (5). A single stem cell commits and gives rise to committed progenitor cells, which produce more restricted progeny in their differentiation potential and self-renewal capacity (Figure 1).

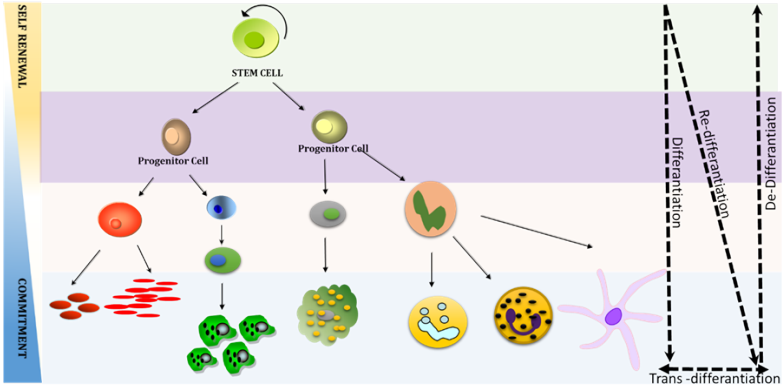


Figure 1: Stem cell hierarchy and root-tree model

All stem cells maintain a delicate balance between self-renewal and differentiation in homeostasis. Self-renewal without differentiation controls the stem cell number at a constant level, whereas stem cell differentiation allows the generation of enough cells belonging to multiple cell lineages (6). Stem cells are quiescent in the G0/G1 phase of the cell cycle and rarely divide to maintain the stem cell pool by residing in a niche they belong (7).

2.1. The Basis of Stem Cell Differentiation

In mammals, the most primitive and "totipotent" cell is created by the fertilization of an egg (oocyte) with a sperm generating a Zygote (Figure 2). By that time, Zygote enters into rapid cell cycles. It performs cell divisions, creating a multi-cellular embryo in that each generated cell carries the Zygote's exact cellular characteristics and potentials. By day 3-5, this fertilized egg now includes 16-32 cells, all with the same phenotypic and genotypic potential as a zygote called Morula (8). From this stage of embryogenesis, the Morula initiates the differentiation process and generates Blastocyst, which includes more differentiated cells; Trophoblasts will develop the placenta, and inner cell mass cells will form the fetus. By day 7, the formation of the inner cell mass will have 45 ± 10 cells (8). Each cell in the inner cell mass can create all types of cells in the human body, including germ cells. The inner cell mass cells, now called embryonic stem cells (ESCs), are pluripotent, having a restricted potential for differentiation compared to totipotent cells. Through embryogenesis, such cells proliferate, differentiate, and generate all embryonic tissues for fetal development. After eight weeks, the fetus has initiated organogenesis, generating more differentiated and committed cells. At this stage, cells residing in each organ system are multipotential, capable of developing organ systems, and called somatic stem cells. After this stage, through the adult life of humans, the most potent cell type of the human body will have these somatic stem cells.

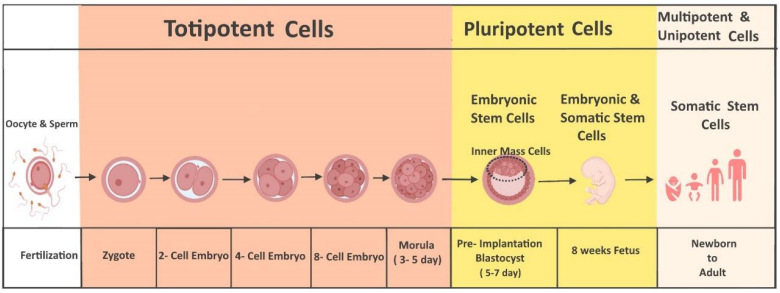


Figure 2: The basis and differentiation potentials of stem cells

Depending on stem cell localization, a somatic stem cell is expected to have a limited spectrum for differentiation. Their functions include maintaining tissue homeostasis and regeneration. Such as mesenchymal, hematopoietic, neural, and dermal stem cells (MSC, HSC, NSC, and DSC, respectively). i.e., if the stem cell resides in the central nervous system, that stem cell is expected to give rise to the three different cell types of neuronal tissue and

generate neurons, oligodendrocytes, and astrocytes (9). HSCs give rise to cells belonging exclusively to the hematopoietic system. However, through the differentiation process, the oligo-potent cells have a restricted capacity to differentiate only into specific cell types, such as lymphoid progenitor cells, which are expected to produce only various subgroups of lymphocytes. Unipotent cells give rise to only single cell types, such as endothelial progenitor cells, known to differentiate into endothelial cells (10). Adults have multipotent, oligopotent, and unipotent cells dispersed throughout the organs (11) (Table 1).

Table 1: Differentiation potentials of human stem cells

Terminology	Differentiation potential	Examples
Totipotent	All embryonic and extra-embryonic tissues	Zygote
Pluripotent	All embryonic tissues	ICM cells, ESC, iPS cells
Multipotent	All lineages of tissue/ organs	NSC, HSC, MSC
Oligopotent	Some lineages of tissue/organs	CMP, CLP
Unipotent	Single lineages of a tissue/organs	Endothelial Progenitor Cells

2.2. Stem Cell Heterogeneity

The recent advances in molecular and genetic techniques enabled the study of the cell at a single-cell level. Stem cell studies have revealed that the stem cell pool might get affected by various factors, which all create heterogeneity and richness in the differentiation capacity of stem cells and their plasticity. Still, our knowledge of the reasons that make this heterogeneity and generate its dynamics is limited. So far, it is conceivable to collect the results of all such studies that may cause stem cell heterogeneity and plasticity in four groups (12, 13) (Figure 3).

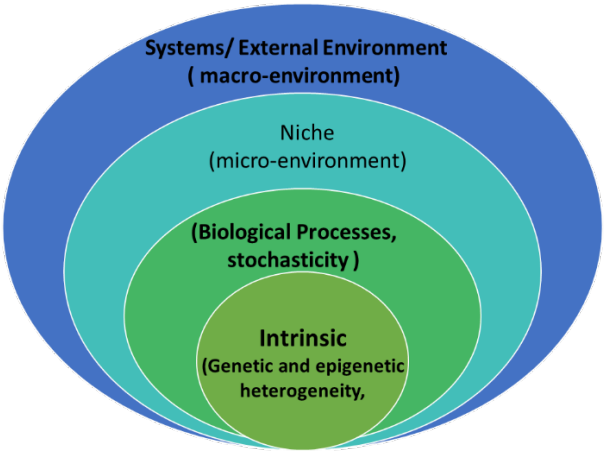


Figure 3: The factors that affect stem cell heterogeneity and plasticity

The highlight of these factors might include intrinsic factors, biological processes and stem cell stochasticity, and the micro and macro environment.

Intrinsic factors: Stem cells lack well-characterized immune and genetic features for cell phenotypes. The reasons for this heterogeneity might be initiated initially, particularly during the early embryonic development of totipotent cells. Genetic mosaicism due to variation in genomic DNA at each cell division has been identified in stem cells, which might lead to alterations in functional properties (14). Any genomic variation/ mutation and epigenetic changes that occur in the primitive cell during cell divisions are transmitted to all their progeny depending on the commitment of the stem cell, creating clonal heterogeneity (15). ESCs are enriched in epigenetic marks associated with elevated gene expression. Likewise, somatic stem cells are exposed to somatic mutations or epigenetic changes such as DNA methylation, histone modification, and chromatin accessibility, revealing that the epigenome is an essential factor contributing to stem cell heterogeneity (16, 17). A large-scale change in gene expressions occurs during differentiation, and expression profiles become more specific and stable in more differentiated cells, creating a more heterogeneous somatic stem cell pool.

The cell-fate decision of stem cells affects both the absolute number of the SC pool and the number of committed cells. Stem cells, theoretically, have multiple options for their fate decision, including; remaining in a quiescent stem cell state (18), undergoing symmetrical cell division producing precisely the same two stem cells resulting in stem cell expansion (19), undergoing asymmetrical division (20), producing one stem cell and one progenitor resulting in stem cell maintenance (21) committing to differentiation and producing two progenitor cells, leading to stem cell depletion or undergoing apoptosis (22). These processes of cell-fate decisions are critical for maintaining the numbers of normal functioning stem cells within their niches and generating progenitors for producing cells belonging to various lineages, all of which have limited life spans (23). Such a choice has to be made between alternative gene expression programs implemented by transcriptional and epigenetic regulators in response to extracellular signals during that moment. Multipotent cells are thought to exhibit multi-line preferences that require simultaneous, low-level activation in gene expressions to induce differentiation into alternative lines. When lineage selection is made this way, the ”winner” (activated) gene for the preferred lineage is expressed, and the alternative program is eliminated as the “loser” (downregulated). This antagonist mechanism and preference that determines the lineages are thought to be through auto-regulation. Furthermore, during these processes, stem cells or their progeny localize to various sites or niches depending on their tendency of lineage commitment or depending on their differentiation status. Research supports that lineage preference in the hierarchy occurs very early from immune-phenotypically defined HSC and that self-renewing and limited-capacity progenitors may arise directly from HSC.(24). For example, a healthy adult individual has an average of 3,000-10,000 HSCs. These cells divide once every three months or three years (25). Approximately $1.4x10^{14}$ mature blood cells are produced yearly depending on the body’s demand (26). This might indicate that an average

of 1-5% HSC / year enters the blood circulation from the bone marrow (27).

Biological Processes: Stem cells are also influenced by cellular states such as cell cycle or metabolism, resulting in bio-molecular heterogeneity. The functional properties of each differentiated stem cell will significantly impact the progeny. Progenitors have a large proliferation capacity and are highly active in metabolic events, dependent on oxidative metabolism and mitochondrial function. Stem cells are usually in the G0 phase of the cell cycle, called the quiescent phase, and do not divide or reproduce in phase (28). Damage to stem cells due to stress must be tightly controlled to prevent loss of function or oncogenic mutations that increase the risk of malignancy (29). Stem cells in the sleep phase are metabolically inactive due to high glycolysis and low mitochondrial activity, often showing anaerobic metabolism (30). Notably, when stem cells leave the G0 phase and re-enter the cell cycle during the dormant phase, transcriptional, biosynthetic, and translational activity and cell size were observed (31). HSCs are dependent on autophagy (32, 33). Events disrupting homeostasis drive stem cells out of the sleep phase and enter the cell cycle (34). Conditions that stress stem cells, such as DNA damage (35), protein disorder (36), metabolic stress, infection, blood loss, and chronic inflammatory process, protect the stem cell population by activating cellular stress response and quality control mechanisms. Increasing the number of progenitor cells by the interferon signal transduction pathway is one such mechanism (37). Surprisingly, data from clonal dynamics studies for HSCs suggest that long-lived progenitor populations successfully participate in blood cell formation rather than constant cell turnover of HSCs from the dormant phase to the cell cycle to sustained blood cell formation (38). However, other studies show that stem cells contribute to a steady state and commitment under stress. Still, there are many unknowns about the functional properties of stem cells due to the appropriate experiment models for stem cell testing, either *in vitro* or *in vivo*. Although each approach differs, and their common goal is to provide the most remarkable resemblance to reality in the *in vivo* situation, they still have restrictions.

Niche: The niche is defined by its structure and function (39). In particular, it regulates and assists the process of the creation, maintenance, and repair of the tissue in the microenvironment where the stem cells are located. In addition, it protects stem cells from excessive proliferation or self-destruction/depletion (40). It is believed to achieve all these by collecting the signals from stem cells for their requirements and reflecting these signals to the neighboring cells, and balancing the response for the tissue physiology (41). Such as, HSC coexists with endosteum and vessels in a highly specialized niche in the bone marrow and maintains tissue physiology (42). HSCs can be localized to different bone marrow niches, including femurs, hip bones, ribs, and the medulla of the sternum, and exposed to different biochemical and biophysical environments, forming the infrastructure of heterogeneity formation. In every location or situation HSC provides requirements of the

homeostasis by producing functioning cells to the periphery.

Distant Systems/External Environment (macro-environment): It is well received that the distant organ system and the external environment significantly impact the body's homeostasis and the heterogeneity of the stem cells. Even the unexpected processes in the body (i.e. accidents, external administration of substances, exposures) deliver the signals to the stem cell pool in an instant manner. Various signal trajectories might be applied to the stem cells for the generation of each response strategy. Regardless of the source of the event, as discussed earlier, the target cell gets affected through the signaling process, and the bio-molecules may have facilitated the triggering signals of stem cells. In addition, the activating signals might also include extracellular genomic materials (EGMs) (43, 44), localized in extracellular space as free (cell free DNA, miRNA, long non coding RNA ext.), protein bound (nucleosome, virtosome) or inside the vesicles (extracellular vesicles such as exosomes, microparticles ext.) and have ability to travel and transfer the genomic materials to the distant cells. The significance of EGMs to the stem cells might address many aspects of cancerogenesis with metastasis, and also, the generation of cancer stem cells in such processes. Still, further studies are necessary to highlight the mechanism of such effects.

2.3. Stem Cell Plasticity

Now, it is known that stem cells have broader functional potentials than had been previously recognized. If certain environmental stimuli are supplied to a tissue-specific stem cell, it can generate cell types belonging to other tissues. This process is termed stem cell plasticity. The plasticity of stem cells might be explained by three different processes: de-differentiation, re-differentiation, and trans-differentiation (45) (Figure1). De-differentiation refers to regaining the functional and immunophenotypic features of less differentiated progenitor cells while losing the differentiated cell characteristics. This process, the de-differentiation of the stable cell, can occur at any differentiation level, i.e., at the progenitor or mature cell level. De-differentiation might be combined with re-differentiation. For example, after differentiating into the progenitor cell, the original cell can descend from a different lineage and re-differentiate itself as a cell type from another tissue. Trans-differentiation is the direct differentiation of one cell type into another without differentiation into a more primitive intermediate. The stimuli and the activation of such commitment for stem cells are not limited to the previously reported processes for stem cell heterogeneity and require further studies.

3. Stem Cell Therapy

Stem cell therapy is a cell-based therapeutic approach that is assisted by regenerating the damaged cells and tissues in the human body by exogenous delivery of healthy stem cells (46). Therapy applies to the patient's cells, known as autologous cell therapy, or allogeneic sources that use cells from a healthy donor for treatment (47). The stem cell-based treatment

approaches started with the pursuit of treatment options for hematological diseases after the atomic bombings in Hiroshima and Nagasaki in 1945. The war and its consequences revealed that HSCs are radio-sensitive and radiation suppresses the bone marrow. Moreover, the bone marrow or spleen cells could be applied to save the lives of people exposed to radiation (48). Subsequent studies and *in vivo* experiments have focused on the effect of radiation on hematopoietic cells (48-50). With the knowledge, understanding, and expertise on stem cells and in parallel with the development of technology, stem cell therapy today blast in a versatile, intense, and rapid manner. The ideas that were believed to be fantasy before, are becoming a reality now. According to the Stem Cell Market Size Analysis (2019-2025), the number of diseases stem cells can treat increased by 300% between 2005 and 2013 (51). Clinical trial registry databases, ClinicalTrials.gov, and the International Clinical Trials Registry Platform of the World Health Organization (ICTRP, https://trialsearch.who.int/Default.aspx) provide information about clinical trials of stem cells for varying diseases (52).

3.1. Source of Stem Cells

Stem cell therapies have evolved and become the most popular therapeutic agent for regenerative medicine. The three primary sources supply the stem cells' therapy preferences today (1). Embryonic stem cells (ESCs) derived from the embryo, (2) somatic stem cells derived from tissues such as the brain, skin, liver, peripheral blood, umbilical cord, and bone marrow, and (3) induced pluripotent stem cells (iPS), which are adult stem cells that have been genetically engineered *in vitro* to have the potential of ESCs.

3.1.1. Embryonic Stem Cell

Human embryonic stem cells (ESCs) can differentiate into all cell lineages in the body. They can be used for various medical purposes, including tissue repair and regenerative medicine (53) (Figure 2). ESCs are often referred to as human pluripotent stem cells. Due to the ethical concerns for the ESCs isolation process, which destroys the human embryo at the blastocyst stage, ESCs are restricted or prohibited in many countries (54). Therefore, the application of ESCs for research and therapy is a controversial issue. In addition, ESCs, non-autologous in nature, might cause immunological rejection by the host (55). Despite the limited experience with ESCs, the researchers have a better understanding of the potential of stem cells and differentiation capacity. It is believed that ESCs have the massive potential to develop new treatments for various diseases and conditions (56) (Table 2).

Animal models applying ESCs are promising, insulin production for diabetes (57), production of healthy heart muscle cells after a heart attack (58), replacement of neurons damaged by Alzheimer's disease, Parkinson's disease, spinal cord injury, or stroke (59), treatment of bone-joint injuries, ligament-tendon injuries, osteoarthritis, cartilage defects (60), restoration of damaged organs or tissues (61). Still, there are challenges to overcome before

clinical application. Nonetheless, the restriction of ESCs application in research/clinical use opened a new era for induced iPS cells generation and application.

3.1.2. Somatic Stem Cell

Somatic stem cells, also called adult stem cells, are found in various tissues in the body and can differentiate into different cell types (62, 63). Somatic stem cells have more limited differentiation ability than ESCs (64). They are typically referred to as 'multipotent' rather than 'pluripotent' cells (65). Somatic stem cells include hematopoietic stem cells (HSCs), skin stem cells (SSCs), neural stem cells (NSCs), and mesenchymal stem cells (MSCs) (66). They are found in various tissues throughout the body and play an essential role in protecting the tissue they reside in, including fat cells, umbilical cord tissue, and bone marrow. They also have the potential to be used in tissue repair and regenerative medicine. HSCs are primarily found in the bone marrow and comprise all mature blood cells: red blood cells, white blood cells, and platelets (67). SSCs, such as epidermal stem cells and hair follicle stem cells maintain skin integrity (68). NSCs are self-renewing stem cells in the central nervous system and can give rise to nerve cells, oligodendrocytes, and astrocytes (69). MSCs and HSCs are widely used in regenerative applications, reprogramming damaged cells or regulating tissue homeostasis (67, 70). Most therapeutic applications for somatic stem cells were performed with the MSCs and HSCs. Therefore, due to the limited space in this chapter; we will focus on MSCs and HSCs.

3.1.2.1. Mesenchymal Stem Cells

Arnold Caplan first named MSCs in the 1990s (71). Unlike HSCs, MSCs can differentiate into several lineages, including mesodermal (adipocytes, osteocytes, and chondrocytes), ectodermal (neurocytes), and endodermal lineage (hepatocytes) (72). Therefore they reside in many tissues, including adipose tissue, peripheral blood, umbilical cord, and the placenta (73). MSCs are widely used in treating various diseases due to their self-renewal, differentiation, anti-inflammatory and immunomodulatory properties (74). MSCs can be easily extended from different sources and do not require immunosuppressive agents before transplantation (75).

In vitro and *in vivo* studies have supported the understanding of the mechanisms, safety, and efficacy of MSC therapy in clinical practice (76). And it opens up the possibility of MSC-based tissue regeneration and organ engineering. There are optimized criteria to characterize MSCs. These include; adherence to the cell plate on which it is cultured, express CD105 (endoglin-1), CD73 (ecto-5-prime-nucleotidase), and CD90 (thymocyte antigen-1) as surface markers, and CD45 (protein tyrosine phosphatase, receptor type C), must not express CD34, CD14 or CD11b (integrin-a M), CD79-alpha or CD19 and human leukocyte antigen-DR (HLA-DR) surface molecules on the cell surface; MSCs must differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. There are several studies in literature now.

Some studies have shown that MSCs can differentiate into cells with neural features under certain conditions (77). One study found that MSCs treated with specific growth factors and exposed to a neural induction medium could differentiate into cells with characteristics of both neurons and glial cells, which are cell types that support and protect neurons in the nervous system (78). However, to what extent MSCs can differentiate into fully functional neural cells remains unclear. More research is needed to fully understand the potential of MSCs to differentiate into neural cells and the potential use of MSCs in treating neural disorders. In immune system disorders, HSCs and MSCs, with their broad mechanisms of action, have often been used as investigational agents to attenuate disease activity and have shown varying success rates depending on the disease type (79).

MSCs have been investigated in cancer microenvironments for their anti-tumorigenic properties but with limited success. It has been shown that MSCs do not participate in tissue regeneration due to their implantation in the tissue-damaged area but in creating a favorable microenvironment that supports the proliferation and differentiation of endogenous cells. Therefore, in recent years, great attention has been paid to paracrine factors secreted by MSC (80). These factors form the MSC secretome, consisting of soluble and extracellular vesicles. MSC-derived exosomes can interfere with processes related to tissue regeneration, such as proliferation, differentiation, apoptosis, angiogenesis, migration, and immunomodulation, among others (81, 82). It is important to note that MSC culture and expansion for use in cell therapy have been performed outside of their native niche. Therefore, such conditions can affect their regenerative capacity. Studies have shown that cells obtained from the umbilical and adipose tissue multiply faster than cells derived from bone marrow (83). Other potential problems with MSCs in cell therapy include the heterogeneous differentiation abilities of different MSC populations. It is known that there are differences in the proliferative and differentiation potentials of the MSCs obtained according to their origin (84). Overall, MSCs have been the subject of much scientific research and have the potential to be used in the treatment of a wide variety of diseases and conditions, including diabetes, Parkinson's Disease, spinal cord injury, and chronic inflammation. MSCs even help slow down the overall aging process (Table 2).

3.1.2.2. Hematopoietic Stem Cells

Beginning with bone marrow transplantation, HSCs have long been the standard treatment for hematological malignancies due to their regenerative potential (85). They are still the most studied and applied stem cells in regenerative medicine. The most common source of HSC is bone marrow (BM), peripheral blood (PB), and umbilical cord blood (CB) cells (86).

The bone marrow (BM) is the first and most widely used source of HSCs and still remains the standard when comparing other sources of HSC for transplantation. The technique of aspiration of BM cells from the posterior iliac crest under general anesthesia has been used

for years. However, BM harvest of stem cells from healthy donors is invasive and painful and carries little risk of severe morbidity.

Peripheral Blood: The existence of HSCs in the peripheral circulation was suggested by animal studies conducted in 1951 (87), and the presence of HSC in human peripheral blood other than bone marrow was demonstrated by Goodman and Hodgson in 1962 (88). Typically, the concentration of HSCs in peripheral blood is very low and requires the processing of large volumes of blood to collect the number of HSCs equivalent to the amount that can be collected in a bone marrow harvest. The ability of recombinant hematopoietic cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-macrophage colony-stimulating factor (G-CSF), to increase the level of myeloid progenitor cells in the blood was reported by different groups in 1988 (89, 90). G-CSF produces myeloid hyperplasia in the bone marrow by expanding mature and immature myeloid cells. After 4-5 days, more $CD34^+$ cells are released into the circulation. Although the success of peripheral blood stem cell (PBSC) transplantation as a source of HSC varies depending on the effective mobilization of HSC obtained by cytokine or chemokine administration and the reliability of the same-day flow cytometer analysis to assess the quality of the collection, it is rapidly and widely adopted because it is advantageous in ease of collection compared to BM due to the no need for general anesthesia and the rapid engraftment of the cells. It is reported that, in adults, peripheral blood is the primary source of allogeneic stem cells used for transplantation in the United States between 2000-2019 (91).

Cord Blood (CB): Intensive studies on alternative sources of stem cells outside the BM, revealed that the most significant source of HSCs was CB in 1988. Then, in the 1990s, CB transplantations began first for children and later for adults (92). Especially with the increasing genetic heterogeneity of societies and shrinking family sizes, the necessity of such treatment options has increased dramatically. CB has become an alternative donor source for HSC transplants. Today, with this approach and with the availability of CB banking, using CB as a source of stem cells is possible for patients unable to match with a suitable donor or with no relatives.

T cell subsets facilitate graft-versus-host disease (GVHD), graft versus leukemia, and HSC homing and engraftment. When the composition of the stem cell is compared depending on its source (BM, PB, and CB), PB has the most T cells and may constitute approximately 30% of the total nucleated cell content. The composition of T cells in the bone marrow is about 10%. And this rate is around 20% for CB. Because T cells in CB tend to be more immature, the risk of developing Graft-versus-Host (GVHD) is lower than in both BM and PB-HSC transplants. The risk of chronic GVHD and graft rejection is also lower. Unlike PB or BM, CB transplantation requires less HLA matching. Unfortunately, engraft kinetics are slower following cord blood transplantation (93).

3.1.2.3. Induced Pluripotent Stem Cell (iPS Cells)

Induced Pluripotent Stem (iPS) Cells are produced by genetically reprogramming adult somatic cells with limited differentiation potential using transcription factors (94, 95). iPS genome editing was first demonstrated by Yamanaka et al. in 2006 by transferring a cocktail of four different transcription factors consisting of octamer-binding protein (*Oct4*), SRY-box containing gene 2 (*Sox2*), Kruppel-like factor 4 (*Klf4*), c-myelocytomatosis oncogene (*c-Myc*), for reprogramming the target cell, a mature fibroblast. And showed that the mature cell could be reprogrammed and reveal the stem cell characteristics that it had previously lost (96, 97). In this way, the idea is that natural stem cells and programmed cells can be used as a stem cell source and it is among the treatment options today (98).

iPS cell technology has been tested in phase I clinical trials since 2016 and has been successfully applied in treating retinal degeneration in 2018 (99). Now, iPS cells technology various application platforms: To obtain the pluripotent capacity of cells for treating diseases, testing of new drugs, tissue engineering studies, creating disease models and personalized applications are among the top of the list (100). iPS cells play an essential role in disease modeling and regenerative use for their pluripotency and self-renewal properties. Production of highly efficient iPS cells through patient-specific biochemical and epigenetic protocols increases their therapeutic potential. Disease models of tissue-resident macrophages, critical for immunity and derived from human iPSCs, immunologically based on iPS cells from conventional monocyte-derived macrophages: hereditary long QT syndrome (LQTS), dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), and arrhythmogenic has been studied for cardiac channelopathies, including right ventricular cardiomyopathy (ARVC) (101) iPS cells technology also appears to promote screening on animal models, primarily to overcome the differences in electrophysiological properties between human and animal cardiomyocytes in preclinical drug trials (102). In another study, the degenerative disease often goes through multiple stages of differentiation, and using iPSC models, these pathways can be easily identified to assess better the cause as well as the etiopathology (103); in studies, such as Type 1 diabetes (104, 105), myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), and myeloproliferative neoplasms (MPN) (106) have applied iPS technology for identifying varying aspects of diseases. One study attempted to generate iPSC clones from patients' bone marrow and blood by integrating mutation analysis with cell programming to create different iPS cell clones representing different disease stages and the disease spectrum, including susceptibility and low- and high-risk conditions (107). Human iPS cells are being considered a potential resource for generating organs that can overcome the risk of deficiency and rejection. Studies have explored the possibility of developing a three-dimensional vascularized and functional liver organ from human iPS cells (108). It has been reviewed that generating hepatocyte-like cells using iPS technology is fundamentally

beneficial in treating severe liver disease, screening for drug toxicities, liver transplantation, and facilitating basic research.

iPS is a new option for personalized gene therapy. It combines this technique with modern gene editing methods to provide a completely new subset of treatment, reducing the chance of rejection in transplant methods and techniques that do not require returns using the patient's cell. On the other hand, the primary use of iPS cells in regenerative medicine is still in debate; challenges to be studied include tumorigenicity, heterogeneity, and immunogenicity. Teratoma formation remains a severe problem and limiting factor in iPSC transplantation. And as a result of the use of integrating vectors that pose a risk for reactivation of commonly used reprogramming factors are also highly expressed in various types of cancer, iPS poses a cancer risk (109).

3.2. The Pre-Transplant Factors that Affect the Treatment Success

Although most studies of stem cells are satisfactory, varying pre-transplantation factors affect the success rate of stem cell transplantations. There are several factors that affect stem cell pretreatment. Many factors such as different sources of stem cells, routes of administration of stem cells, culturing and reproducing stem cells, characteristics of donor and recipient, *ex vivo* differentiation of stem cells, metabolic changes and physical stimuli affect the success of stem cell treatments. Among the various factors, the most problematic are schematized in Figure 4.

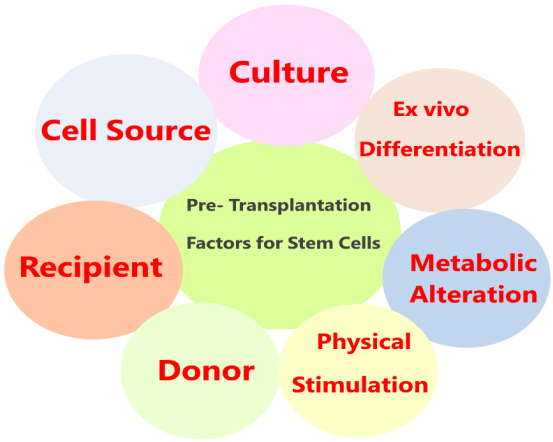


Figure 4: Schematic representation of the pre-transplant factors that affect the treatment success

The difficulties of finding a suitable donor are the most essential step in a successful treatment. Among the progress made in this period, searching for alternative sources generated new preferences. It has been revealed that CB can also be used reliably as a source of stem cells in stem cell transplantation therapy. There are, however, some factors that affect the CB transplantation success. Such as, single or pooled CB collection might also affect

the success rate of the transplant. In particular, with the recently developed pre-transplant preparation and immunosuppressive regimens, the immunological consequences of the most feared GvHD or host-versus-graft diseases after transplantation have been reduced and have allowed haploidentical transplantation to become a therapeutic option. As a result, such treatment has revealed that the body's immunological response to the therapy is a significant barrier that must be overcome. Allogeneic hematopoietic stem cell transplantation has been a life-saving approach in treating various diseases, from congenital disorders such as Fanconi's anemia to cancer. Although some protocols require cell culture to obtain as many cells as needed, cell culture conditions and different spectrums of cell differentiation protocols might affect the success rate. In addition, such production requires good manufacturing practice (GMP) conditions, which are clinically proven safe in patients.

The advancements in gene therapy allowed gene editing technology to apply to the diseased genome in HSCs and be used in stem cell transplantation therapy. The conditioning of such stem cells through the *ex vivo* culture has quite an impact on stem cell therapy. Successful treatment is not limited to these factors; these issues raise most obstacles.

3.3. Disorders Treatable with Stem Cell-based Therapies

Stem cells are accepted as the source of human growth and development. The ability and successful techniques in stem cell isolation, proliferation, and modification have a tremendous impact on the comprehensive application options of stem cells for therapy. Today, using stem cells to treat disease has enormous benefits, with more than 100 diseases treated with stem cell transplants. Numerous preclinical studies have shown that stem cell therapies can delay disease onset in many organs, such as the kidney, lung, heart, and liver, via immunomodulatory and paracrine mechanisms (110).

This chapter will overview the highlights of some examples of stem cell therapy applications (Table 2) (111-139).

Table 2: Application fields of stem cell therapy

Application Fields of Stem Cell Therapy	*Stem Cell Type*	*Delivery Method*	*Disease*	*References*
Cardiology	Mesenchymal Stem Cells, iPS cells	Prenatal Intracoronary, Intramyocardial	Vulvar Disease, Dilated Cardiomyopathy, Ischemic Heart Disease	(111-114)
Endocrinology	Hematopoietic Stem Cells, Mesenchymal Stem Cells, Embrionic Stem Cells, iPS cells	Intrathecal, Intrathymic & Hepatic	Insulin, Dependent Diabetes, Thyroid Disease, Cushing's Syndrome	(115-119)
Orthopedics	Mesenchymal Stem Cells, iPS cells	Bone Matrix, Subcutaneous Bone Sections	Osteogenesis Imperfecta, Lysosomal Storage Disease, Hypophosphatasia Cleft Lip and Palate	(120-123)
Hematology / Oncology	Hematopoietic Stem Cells, Mesenchymal Stem Cells, iPS cells	Intrathecal, Intravenous	Graft vs. Host Disease, Neuro- Blastoma & Glioma Multiple Myeloma Blood Disorder	(124-127)
Neurology	Mesenchymal Stem Cells, Hematopoietic Stem Cells, Embrionic Stem Cells, Neural Stem Cells, iPS cells	Intrathecal Subcutaneous, Intraparenchymal	Autism, Cerebral Palsy Spinal Muscular, Atrophy Multiple Sclerosis, Alzheimer's disease, Parkinson disease	(128-134)
Respiratory	Hematopoietic Stem Cells, Mesenchymal Stem Cells, iPS cells	Intrathecal	Bronchopulmonary Dysplasia, Pulmonary Complications, Lung Disease	(135-139)

3.3.1. Cardiovascular Diseases

Cardiovascular disease (CVD) is the most common disease in the population and is the leading cause of adult death worldwide. The number of deaths from CVD is expected to grow to more than 23 million by 2030 (140). Stem cell therapy is one of the promising therapeutic tools for CVD (114) (58). Many conducted studies applied varying sources of stem cells in ischemic heart disease as it provides a drug-free surgical treatment to patients and promotes a safer and more feasible cardiac repair (141). The main goal of stem cell-based therapies for heart disease is to rebuild the heart muscle by differentiating into cardiac cell types and/ or providing a source of cardioprotective paracrine growth factors and inevitably restoring the structure and function of the heart. When cardiomyocytes are damaged by ischemia or other factors, the dead cells are replaced by fibrous tissue. The remaining viable cells have limited proliferative abilities; such a condition leads to functional impairment and the progression of heart failure. Stem cell transplantation might produce new cardiac cells to have beneficial effects in reducing the occurrence and symptoms of heart failure, and improved heart function and repair of damaged myocardial cells might be achieved (142, 143). Theoretically, various stem and progenitor cell populations, including HSCs, MSCs, endothelial progenitor cells, $CD133^{+}$ cells, skeletal myoblasts, resident cardiac stem cells, and ESCs, might be used for cell-based therapies for a variety of cardiac diseases. Although stem cell therapies failed to support the regeneration of cardiovascular diseases in previous studies, the findings indicate that most clinical trials are generally small, and the results are inconclusive (144). Consequently, more work is required to find the correct answers to the challenges that have been faced so far, including finding suitable cell sources such as ESC and iPSC cells, optimizing conditioning regimens and protocols for the stem cell culture, and also application of the correct dose and route of transplant to develop better stem cell therapies in cardiovascular diseases. Meanwhile, applying advanced techniques for monitoring and diagnosing CVD will facilitate a positive outcome.

3.3.2. Diseases of the Digestive System

The gastrointestinal (GI), pancreatic, and hepatic systems work harmoniously to digest and absorb nutrients, maintain homeostatic glucose levels, and helps to remove harmful toxins from the body. The GI tract is protected from adverse effects in the intestinal environment by the epithelial cell layer, which has regenerative properties in response to any tissue damage and cell cycle (145). In case of damage, epithelial cells divide faster. In normal condition, epithelial cells in intestinal crypts self-renew themselves every 2-7 days, keeping the cell population stable (146). Most of the time, diseases develop as a result of tissue damage and inflammation of the GI tract. As a result of loss of function in intestinal stem cells, deterioration of intestinal functions occurs. Crohn's disease (CD) and ulcerative colitis (UC) are the two primary forms of inflammatory bowel disease (IBD). The

CD is a chronic, uncontrolled inflammatory condition of the intestinal mucosa characterized by segmental transmural mucosal inflammation and granulomatous changes (147). UC is a chronic inflammatory bowel disease that affects the colon and rectum, characterized by mucosal inflammation that begins in the rectum and extends continuously to the proximal part of the colon (148). The stem cell treatment option for IBD was applied with HSC and MSC-based therapy and cellular therapies.

The application of HSC in treating IBD (149) revealed that autologous HSC treatments in CD patient groups had better results than MSC-based treatments. As a result of stem cell therapy, it was observed that inflammation decreased and the patient's quality of life increased. However, it is still necessary to investigate these treatment methods and consider the side effects.

3.3.3. Liver Diseases

The liver is the largest vital organ in the human body. Its functions are to support digestion, metabolism, vitamin storage, detoxification of the organism, and other biological processes (150). Impairment of liver homeostasis and function can lead to the development of pathological conditions such as liver failure, cirrhosis, cancer, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), and autoimmune liver disease (ALD). The accepted treatment for liver diseases is orthotropic liver transplantation. However, the limitation of this treatment is the limited number of available organ donors and the costs of transplantation. In addition, it shows that there is a need for new treatment methods in the case of organ rejection of the donor (151). Stem cells (HSCs, MSCs, iPS cells, and liver progenitor cells) are currently used as an alternative treatment for liver disease (152). Liver cirrhosis is a disease that results in death when liver transplantation is not performed. Stem cell-based therapies are used to treat liver cirrhosis (153). Promising results have been obtained in these treatments using MSCs. However, to be used therapeutically, better results can be obtained by conducting trials in larger patient groups to confirm the effectiveness of the treatment. Autoimmune liver disease (ALD) occurs when a directed T-cell-mediated autoimmune response against liver autoantigens occurs. Haploidentical HSC and MSC-based therapies are potentially used in treating ALD (154). However, these studies should be supported by further studies with limitations such as small sample sizes and lack of control groups. Cell-free therapy can be used as another treatment method for liver fibrosis. It can be active in treatment through extracellular genetic materials secreted from stem cells (155).

As a result, the potential use of stem cell therapies for liver disease is possible. It is necessary to determine, prepare and standardize the most suitable stem cell for the disease types. To prove the effectiveness of the treatments, studies should be carried out by considering parameters such as the route of administration, source, dose, and intervention time of stem cells.

3.3.4. Arthritis

Arthritis is a disease that causes pain and inflammation in the joints, characterized by the breakdown of the articular cartilage at the ends of the bones. As the cartilage breaks down, the bone begins to rub against the bone, and movement becomes painful. This disease can also damage ligaments and muscles. Osteoarthritis (OA) is the most common type of arthritis (156). OA is a disease characterized by joint destruction and reduces patients' quality of life with the loss of joint function and increasing pain. There are two types of OA, idiopathic OA and secondary OA (157). The progression of OA is chronic, unlike rheumatoid arthritis. Currently, no disease-modifying drugs can effectively alter the natural history of OA and provide structural improvements in damaged articular cartilage (158). Although joint arthroplasty solves end-stage disease, these procedures are not recommended for young patients. Lack of modifying drugs in the treatment of OA, stem cell therapy has recently emerged as an alternative approach for OA and has attracted great interest in the regenerative field. Post-application analysis was performed to evaluate the stem cell efficacy in treating HSC in OA patients. It was observed that bone lesions decreased and bone regeneration increased (159). In another study, MSC treatments are used in the treatment of OA due to their immune regulatory functions and anti-inflammatory properties. In treating OA patients, the patient's adipose tissue, umbilical cord, and bone marrow are used to obtain MSC. Early experimental studies for MSC-based cartilage regeneration focused on chondrogenic induction from MSCs. Numerous studies have been conducted to induce chondrogenesis from MSCs by transforming cells through an appropriate combination of growth factors or transfer of chondrogenic genes (160). Stem cells undergo rapid cell death, surviving from three days to several weeks, depending on the intra-articular administration and microenvironment (161). Since the paracrine action of MSCs is mainly explained by extracellular vesicles (EVs) secreted by MSCs, EVs isolated from MSCs can be used as stand-alone substitutes for MSCs in regenerative medicine for OA (162). Injection of isolated EVs may offer a more straightforward and safer alternative when you expect only paracrine effects with intra-articular injection without considering cell survival and engraftment (163). In the studies conducted, there is no complete evidence of whether MSC is an effective treatment for OA. As a result of 14 clinical studies, the patients were followed up for one year. Better clinical results were obtained in 57% of patients compared to control (164). However, it could be more effective when compared with magnetic resonance imaging results. Studies show that the use of stem cell therapies for the treatment of OA is known to be safe and applicable, but it can be used as an alternative treatment in the treatment of OA. Current interest in stem cell therapy may shift to other substances, including EVs or small molecules, if well-controlled, quality trials show results that do not meet high expectations due to high cost and complex regulatory processes.

3.3.5. Cancer

The inadequate and nonspecific targeting of traditional therapeutic approaches in cancer treatment often leads to therapy resistance and cancer recurrence. The ability to treat cancer depends on its type and progression and, most importantly, on the treatment method. Surgery may be considered the first choice for solid tumors in a single region. Radiotherapy can kill the cancer cell by damaging its DNA. Tumor growth can be slowed by drugs used in chemotherapy. Recently, better clinical results have been achieved with immunotherapy, and it has taken an important place in cancer treatment (165). Stem cell therapy, which includes all procedures using stem cells, has offered a promising option in the fight against cancer (166). The expected outcome of using stem cells in cancer treatment is to reduce systemic side effects, protect therapeutic agents from rapid biodegradation, and increase local levels of therapeutics due to the intrinsic tumor-targeting impact of stem cells.

The anti-tumor efficacy of this system depends on the number of stem cells localized in the tumor microenvironment. There are many preclinical studies based on stem cells in cancer. While these studies show promise for cancer treatment, they pose some challenges. Many methods have been developed for the treatment of cancer. These are like stem cells for HSC transplantation (167), vaccine production, production of immune effector cells (168), MSC infusion (169), and therapeutic carriers (170). Stem cell therapy in cancer is performed using autologous HSC, a stromal vascular fraction (SVF) and MSCs. Allogeneic cell-based treatment suppresses and regresses hematological malignancy in treating allogeneic HSC from its graft versus effects. In another study, overall survival was improved in metastatic breast cancer patients with autologous HSC treatment after high-dose chemotherapy (171). However, in a survey conducted with a larger group of patients with breast cancer, there was no significant difference between patients who received HSC after high-dose chemotherapy and those who received conventional therapy. In line with recent preclinical studies, MSC-based therapies are considered to be potentially used in cancer treatment.

With the use of MSC, cytokines, chemokines, and growth factors provide the ability to migrate to damaged areas (70). Similarly, the chemokine promotes MSC migration in damaged tissue using cytokine signals. MSCs exhibit protumor and anti-tumor effects after migrating to cancerous tissue. MSCs are well known for their regenerative products that regulate tissue repair and healing. MSCs have been found to secrete growth factors to inhibit apoptosis in cancer cells (172). Although MSCs have protumor effects, they also have tumor suppression abilities in cancer therapy. It has been suggested that they exert tumor suppression by reducing the process of angiogenesis, arresting the tumor cell cycle, and inducing apoptosis. MSCs can be modified by gene therapy to express anticancer miRNAs. It is intended to be used as a treatment in cancer treatment studies with MSCs (173). In this direction, phase 1 and 2 studies are carried out. MSCs were combined with an oncolytic virus approach (174). Oncolytic viruses are specific types of viruses that confer the ability to selectively

infect naturally existing or genetically engineered cancer cells and kill them without harming surrounding healthy cells. A phase I/II study using oncolytic adenovirus-infected BM-MSCs can potentially treat metastatic and resistant solid tumors in children and adult patients (175). The same group showed in their studies three years later that MSC-based therapy eliminated all signs of cancer. In another study conducted in the following years, the MSC-based virus vaccine could destroy tumor cells. Despite all these results being successful in both preclinical and clinical models, many challenges of stem cell therapy still need to be overcome. Further experiments will be conducted to investigate the effects of stem cells on tumor growth and metastasis, and an appropriate route for stem cell therapy will be determined.

In summary, the results obtained with stem cell treatment methods are promising for tumor treatment. However, it is necessary to increase the safety and effectiveness of stem cells before they are used in clinical trials.

3.3.6. Neurological Diseases

Brain diseases, including brain tumors, neurodegenerative disorders, cerebrovascular diseases, and traumatic brain injuries, are among the significant conditions affecting human health for which there is no effective treatment (128). Irreversible loss of functional neurons and nerve tissue damage occurs due to low regeneration capacity of neurons, insufficient secretion of neurotrophic factors, and exacerbation of ischemia and hypoxia after nerve injury. Repairing this damage and regenerating the central nervous system after injury is difficult. Stem cell therapy will be essential in treating many diseases affecting the brain and spinal cord, both in regenerating the affected neurons and glia and in neuroprotection. ESCs, iPS cells, or neural stem cells (NSCs) are potential candidates for therapy.

Alzheimer's disease (AD) is a progressive neurodegenerative disease, and features of this disorder include difficulty performing daily tasks, confusion, and memory loss. Current AD treatments are not very effective, most likely due to the massive loss of neurons in the brain (176). Therefore, cell replacement therapies involving iPS cells and ESC-derived neural cells may be beneficial in treating individuals with AD, particularly those without typical pharmacological treatments (177). When autologous HSCs are transplanted to patients with an autoimmune disease of the central nervous system, multiple sclerosis (MS), a decrease in mortality rates has been detected. Because MS is an immune-mediated disease, it was designed to reset rather than suppress the immune system with HSC transplantation. The translational effects of NSCs in human patients are unknown due to the lack of *in vitro* and *in vivo* studies in neurodegenerative disease models. Therefore, further investigation is required to determine a more precise role for NSCs in various transplantation areas. Many studies have provided evidence of an association between an increase in synaptic activity and cognitive improvement, closely related to an increase in neuronal and glial cells (178). NSCs transplantations support behavioral and cognitive functions. Choosing the best time for stem

cell therapy is closely related to the clinical prognosis of the patients; however, no study has so far reported the best course of treatment.

3.3.7. Pediatric Diseases

Pediatric stem cell treatments involve challenges that the patient and their environment endure. However, more children benefit from stem cell treatments each year and achieve better treatments and survival rates than in previous trials.

In addition to early screening, diagnosis, and interventions in pediatric diseases, the search for alternative therapy options with the less toxic potential for immunosuppressive agents and advances in unique selection and conditioning regimens of transplanted cells, together with new vaccine manipulation techniques such as T-cell suppression, and of course genetic manipulations on the cell by correcting the diseased genome is also very promising for the use of stem cell transplantation therapy.

4. Future Directions

Stem cell therapies have now had significant success in treating diseases beyond traditional therapies and where other treatments have failed. Cell-based therapy is a valuable tool for drug screening, disease modeling, and their potential for organogenesis and regeneration (179). Organ bioengineering is a rapidly developing and exciting field of application for stem cells. For example, human pluripotent stem cells have been used to grow lung organoids *in vitro* with native lung-like tissue and cellular compartmentalization (180). Similarly, kidney organoids structurally equivalent to the human fetal kidney were derived from human pluripotent stem cells, demonstrating native tissue-specific functions (181). Stem cells also hold such potential for complex tissue engineering. Organ transplantation without suppressing the immune system by using the patient's cells is now possible with this technology. This technology is expected to eliminate the wait for organ donation. There is a high potential for developing new treatment regimens by growing organ-like tissues in laboratory environments and determining their activities at the cellular level without posing a risk to patients. Another area of use is to treat hereditary blood diseases (182). It offers promise in future regenerative medicine applications by providing *in vitro* three-dimensional structural and functional mimicry of organs in organoid models derived from adult and embryonic stem cells in translational stem cell studies.

The application of stem cell-based genetic therapy has gained significant momentum with the gene editing technology. The CRISPR-Cas system (183) used in recent years and has started to be applied to the developments in genetics (184). CRISPR-Cas systems can be applied to correct genetic mutations and have become valuable in various areas, such as understanding the role of genes in biological processes, preclinical uses for drug development, and genome surgery.

Stem cells represent a new treatment option in medicine and pharmacy and are increasingly used to treat many diseases (185). Stem cell drugs are products used in treatment, healing and prevention of disease. Stem cell drugs refer to live stem cell-based products used as drugs for certain conditions, and their use in disease treatments has recently been revolutionary (186). Unlike autologous stem cell transplantation, stem cell drugs are ready-to-use products without further manipulation (187). The difference from allogeneic stem cell transplantation is that the stem cell drug is a product. These products may be of HSC or MSC origin. These drugs, with reduced immunogenic effects and easy manipulation, are promising for clinical use.

Overall, the advantages of stem cell use in regenerative medicine include; (i) does not require surgical intervention (ii) Stem cells are multipotent. They can be applied in the treatment of many diseases. (iii) With the use of stem cells, faster recovery and shorter treatment time occur. However, for the time being, the limitations of these treatments are troublesome. More work is needed to fully understand stem cells' potential and develop safe and effective therapies.

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CHAPTER 12

MESENCHYMAL STEM CELLS RESEARCHES IN THE ANIMAL MODELS

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ABSTRACT

MSCs are multipotent stem cells, described by their capability to self-renewal, while continuing the ability to differentiate into various cell lineages from their own germ layer. Research in MSC therapy keep evolving, with hopeful results and increasing expectations in the medicine. MSCs therapy is attributed on its immunomodulatory, regenerative and anti-inflammatory potency. MSCs can differentiate into the necessary cell types, giving permission them to regenerate the damaged tissue. MSCs have a perfect immunomodulation capability through paracrine signaling with various growth factors and cytokines, leading to vascularization, reducing inflammation and proliferation. Recently, MSCs are predominantly researched for tissue engineering therapies, regenerative medicine practices and the correction of genetic diseases. The animal models are very important for the development of regenerative medicine practices and possible treatments of diseases for the purpose of application in human clinical experiment. This reason, the research orienting on examining the therapeutic potential of MSCs on the animal models in the cardiology, urology, orthopedy, pneumology, dermatology, ophthalmology and dentistry has greatly increased.

Keywords: Mesenchymal stem cells, animal models, laboratory animals, large animals, therapy

1. Overview of Mesenchymal Stem Cells in the Animal Models

1.1. The Importance of Animal Models

Mesenchymal stem cells (MSCs), first determined in bone marrow by a German pathologist, Julius Cohnheim in 1867 (1, 2) and described by Friedenstein and colleagues in 1968 (3, 4). MSCs have been the most investigated cells, with potential and reliable results in various medical areas. The treatments are based on the regenerative, immunosuppressive and anti-inflammatory effects of MSCs (5-9). Recently, various diseases of animals and humans have been already tested with cellular therapies, decreasing inflammation and regeneration of damaged tissue. However, there are still some worries about the application of the stem cell therapy, negative adverse effects because of the migration of cells and inadequate cell transplantation (10).

The resort of adverse effects may depend on the amelioration of animal models and using of suitable material and methods for transplantation, and may aid the use of stem cells in clinics (11, 12). Animal models are growing increasing important in medicine, to understanding the pathology of diseases and for the development of new treatments. The most commonly used animal models are rats, hamsters, mice and rabbits. The laboratory animal models are widely used and make a great contribution to pilot applications in medical research. However, the laboratory animal models have various limitations, since they are significantly different from the human organ, body, and resulting in physiological, behavioral and metabolic differences. In order to solve this problem, after the early research were applied on small laboratory animals and to supplement them, the larger animals have been used, such as pigs, dogs, goats, sheeps, and horses (13).

Gene sequencing now includes pigs, cattle, goats and sheep, so genetically modified animal models are also suitable for medical research. The use of large animals in studies provides important advantages related to their dimensions and anatomy. Large animals are bigger than traditional laboratory animals, being fairly similar to humans in their physiological systems (14). Small ruminants are known animal models in the preclinical studies for human medical research. The small ruminants have the important advantage of being peaceful, easy to obtain and manage and cost-effective. The care and feeding of small cattle does not require excessive interest. Their size and weight allow medical studies to be managed closer to the clinic.

The small ruminants are livestock species, so their use in experimental studies is accepted by society to some extent. Sheep models have been commonly used for medical research, resulting in various scientific reviews and articles. Goat model has been used largely for the research of human diseases such as orthopedic, reproduction, cancer research, congenital myotonia, chronic rheumatoid arthritis, cardiovascular diseases, antibody and protein producer for immunological and pharmaceutical studies and models for diseases and hosts for the development of organs. The small ruminants such as mainly goats and sheeps have been

lately used in the therapy of myocardial infarction and orthopedic research with stem cells (15). The large animals constitute very little of the animals used in stem cell research due to the lack of information about stem cells (16).

1.2. Obtainment of Mesenchymal Stem Cells in the Animal Models

MSCs are multipotential and undifferentiated cells with self-renewal capability, located in various extra-embryonic or adult tissues. MSCs are capable into differentiate into different cell lines, such as neuronal cells, cardiomyocytes, myocytes, osteoblasts, chondroblasts, hepatocytes and endothelial cells (5). MSCs can be to get from embryonic cells in the early stages before embryo implantation, and from adults. MSCs can be obtained from many tissues, such as placenta, umbilical cord, bone marrow, periosteum, adipose tissue, and dental pulp (Figure 1) (17). MSCs are obtained from adipose tissue or bone marrow, because these tissues are more easily obtained (18).

MSCs may be autologous, allogeneic, and xenogenic. MSCs can generally be administered intravenously or locally and systemically (17). MSCs have the capability to differentiate into the cell type required for regeneration of the damaged area and also have an important immunomodulatory property. Its immunomodulatory potential is due to the paracrine effects of various growth factors and cytokines (exosomes) and cell-cell contacts, thereby reducing inflammation and leading to cell proliferation and vascularization in damaged tissues (19). Various diseases in animals with cell therapy have been cured by reducing inflammation and regeneration of injured tissue (5, 20).

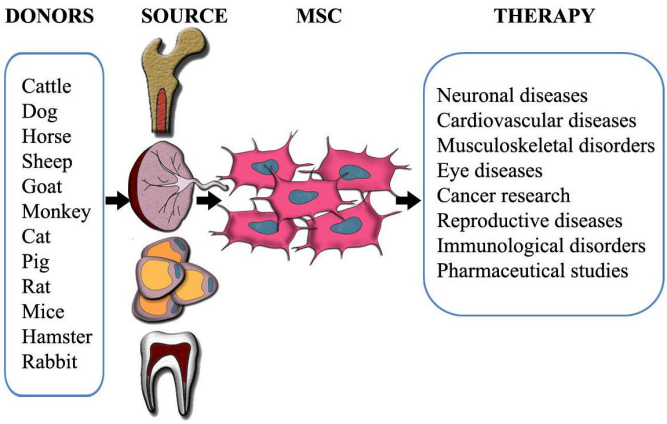


Figure 1: The MSCs sources for transplantation therapy in various diseases: MSCs isolated from the bone marrow, periosteum placenta, umbilical cord, adipose tissue, and dental pulp.

1.3. Utilization of MSCs in the Animal Models

MSCs can be used in clinics, such as neurology, orthopedics, traumatology, ophthalmology, immunopathology, and dermatology (13). MSCs can be used in tissue engineering to increase regenerative potential and therapeutic efficacy. The synthetic or

natural materials in the tissue engineering can inhibit or induce MSCs proliferation, adhesion, and differentiation, which plays an important role in therapeutic potential. Therefore, the various materials can be used to increase proliferation and differentiation and promote survival of MSCs.

The scaffolds widely used for tissue engineering include scaffolds, hydrogels, and microspheres. The scaffolds can be produced from natural polymers such as collagen, elastin, hyaluronic acid, gelatin, chitosan, and silk fibroin containing adhesion peptides. Synthetic polymers are also important molecules for cell adhesion, proliferation, and differentiation and are generally obtained by immersing and absorbing scaffolds in different cell culture media. (21, 22). Hydrogels are generally used in different fields such as neural regeneration, kidney repair, tendon repair and vascularization (21). Hydrogels are a good choice for stimulating to chondrogenic differentiation of MSCs in animal models and humans and also allow the receipt of cytokines and growth factors (8). The tissue engineering has developed in recent years, and scaffold can be adapted to better match and imitate the *in vivo* conditions (23).

2. Application Areas of MSCs in the Animal Models

Recently, stem cell-based therapies have become a new strategy for various diseases including digestive, cardiovascular and respiratory diseases. MSCs are an ideal cell source due to their various specifications, including easy isolation and growth, multigenerate differentiation and immunoregulatory features (24).

MSCs give rise to muscle, cartilage, bone, tendon, ligaments, skin, connective and adipose tissues. This reason, the MSCs received the great interest in regenerative medicine. The success of MSCs therapy depends on studies of stem cell sources from various animal species. Traditionally, MSCs are obtained from both adipose tissue and bone marrow. The bone marrow cells are aspirated from femur bone of cats and dogs and from sternum bone of horse for the collection of MSCs (25). The bone marrow derived MSCs are moved into the laboratory and continued with either direct applications into the recipients or culture for purification and growth. Since direct administration of bone marrow derived MSCs mixes with growth factors, hormones and other cell types, MSCs can produce faster and better results. On the other hand, the culture of MSCs in the laboratory provides necessary quantity cell mount for transplantation of cells into many recipients.

After a few passages, MSCs are usually ready use for clinic and can be harvested (26). In subsequent repetitive passages, MSCs lose their characteristic properties and undergoes to differentiate to other cells. The adipose-derived MSCs are an important option for the treatment of inflammation of mammary gland, mastitis. MSCs decreased somatic cell amount of the milk in mastitis, is a finding indicating that MSCs may be safe for therapeutic application in bovine (27). The MSCs of fetal bovine secretes antiproliferative and anti-bacterial factors. *In vitro* studies reported that MSCs reduced the bacterial population and supported

neovascularization by proangiogenic factors to treat in the goat mastitis (28, 29). Research on MSCs has demonstrated the effectiveness of stem cells in the differentiation of cells into mammary epithelial cells (30). Such studies demonstrate the promising versatility and immunomodulation properties of stem cell derived adults in the treatment of mastitis. (31).

In animal models, the immune response after xenogeneic MSC administration is not definitively known. In rodents, MSCs by allotransplantation and other animal MSCs trigger the immune response (32). The large animals such as baboons and pigs have also demonstrated an important immune response (33). When the high and repeated-doses were used, there was no relation between MSCs survival and the alloantibodies expression. The various comments can be made for these disagreements between datas and animal species, including the origin, naivety and characterization of the cell, and other conditions. The differences of MSC's in the different species may also contribute to variability of the outcomes. Although mouse MSCs have immunosuppressive properties *in vitro*, they have been found to be much weaker than human MSCs (34).

The further research in larger animals, such as primates, are needed to provide safety of patients in using MSCs in the clinic (35). There is a few research indicating some complications, such as fibrosis in the liver tissue and severe inflammation. The adipose tissue cells have become the preferential resource of MSCs due to their availability and purity. In order to ensure the safe use of such cells in the clinic, additional studies should be performed on suppression of immune response, cell survival, and any side effects.

3. Diseases in the Animal Models

3.1. Neuronal Diseases

There are important studies on the use of neural stem cells for the treatment of neurodegenerative diseases and brain and spinal cord injury. The primates given a neurotoxin to imitate Parkinson's disease, show symptoms such as frontal striatal cognitive deficits, resting tremor, and autonomic disturbances, which are not observed in rodent models. Additionally, primates are the most appropriate model for preclinical assessments using technologies that require intra-brain targeting. The method of administration in clinical settings significantly influences the effectiveness of treatment and should be tried in animal models. Because of the idiosyncratic features of rodents, which are widely used in Parkinson's disease treatment studies, different from humans, stereotaxic atlases of the brain can be trusted for the delivery of stem cells. The brain anatomy and complexity of primates is more similar to that of humans and for this reason, provides more reliable information in the clinic (36). The function and survival of human derived dopamine neurons in the monkey model have been reported, this provides very important data for clinical use (37).

An important advantage of using large and long lived animal species is the possibility to have a longer surveillance period for any side effects, together with the beneficial advantages. In addition, primates have also allowed reassessment of the dopaminergic pathway in the brain

by fetal tissue transplantation and growth factors into the human brain-like substantia nigra and striatum (38).

In spite of these advances in animal models, human treatments have shown highly variable improvement until now, suggesting that more improvement of techniques is needed to safety before clinical trials begin. Rodent models are widely used to study the beneficial effects of various stem cells for the stroke therapy, and functional improvement and positive results have been demonstrated. However, most studies have not demonstrated proliferation and differentiation of transplanted cells into neural cell type (39). Although newly produced neurons have been reported, their survival have been reported to be short-live. There are important differences in the sensory, vascular, and motor systems in the brains of humans and rodents. In addition, rodents spontaneously eliminate very dysfunctions caused by experimental operations within a few weeks. As a result, demonstrating the efficacy of the treatment in primates will provide further safety before clinical trials begin. The use of primates should address questions necessary for clinical practice about cell types, dosage, procedures and post-operative care, the use of appropriate techniques for follow up to migration of the stem cells transplants, and long-term investigations.

Recently, several studies have been conducted by Sasaki and colleagues using stroke models in primates. Researchers have developed a new stroke model using an infusion of human MSCs into monkeys (40). The operation was safe and results leading to functional recovery were detected. The researchers provided an important investigation that could affect next studies and possibly be observed in clinical trials due to the anatomical brain characteristics of primates. The variations observed in collateral circulation even among animals with similar lesions importantly affected the results. The experiments suggest that neurons can be preserved using stem cell therapies. Nevertheless, studies should be conducted to develop approaches targeting proliferation, migration and integration process of neurons in large animal models before clinical trials begin.

3.2. Cardiovascular Diseases

Much of the information on cardiovascular diseases has been obtained using rodents. Nevertheless, there are important differences between many cardiac properties in humans and mice, including capillary density, coronary structure, mechanical properties and heart rate, structure of the myocardium, limiting the evaluation of research for clinical treatment. In the larger animal species, sheep, pigs and dogs are advantageous models for cardiovascular diseases. The cardiac magnetic resonance imaging and echocardiography techniques used in humans are also practicable to the larger animal species. Improvements in cardiac functions have been reported in sheep, dogs, and pigs using various stem cells, containing bone marrow, myoblasts, and adipose derived stem cells, endothelial and cardiac stem cells (41). These studies can solve many important problems before clinical trials begin, including determining

the required cell types, methods of administration, timing of administration, and the type of disease in which a treatment may be helpful.

In cardiovascular diseases, significant heterogeneity and inconsistencies were observed in the results obtained. The inconsistency of results was not related to the cell type, animal species, or infarct model used. In the myocardial ischemia-reperfusion injury animal model, higher cell counts of MSCs and the use of more injections showed the most beneficial effects compared to the application of bone marrow mononuclear cells and the chronic infarction model. The results of research in large animals have also shown that MSCs given at high doses and repeated intramyocardial injections are safe and have no side effects, similar to the results in rodents. All studies have consistently proposed new strategies to optimize cell number, administration time and cell type. Although studies using mouse MSCs raise concerns about tumorigenesis, this issue was not observed in any of the large animal studies. Studies should continue longer for reliable methods and focus on developing the necessary protocols to increase treatment efficacy (42).

3.3. Musculoskeletal Disorders

In the treatment of musculoskeletal tissue pathologies, stem cell therapies include transplantation of cells to form tissue, application of biomolecules to repair and injury sites. The rodent models are also widely used in osteoarthritis studies. However, in rodents, the thin cartilage structure, insufficient dimension of defects, self healing ability, and inability to execute long-term research make them less useful than larger animal models for clinical research.

Bone marrow and fat-derived MSCs have been used for the osteoarthritis treatment in goats, dogs and sheep, and have been detected with successful results (43). Autologous MSCs have shown positive results for the repair of chondral defects and bone in rabbits and dogs. Pigs and horses have also recently attracted attention as the promising animal models for future research (44). Despite its positive effects, MSCs the lack of osteogenic potential in the desired amount suggests that it may be beneficial to enrich the osteogenic population of MSCs prior to administration.

The treatment of skeletal muscle cell loss in dogs with muscular dystrophy has been tested one of the best human-like models. The clinical symptoms of the Duchenne muscular dystrophy in dogs are heavier than in mice. Therefore, dogs are better suited to understand disease and test therapies. Human and canine umbilical cord MSCs were administered to dogs but did not differentiate into muscle cells. Whereas, MSCs derived from human adipose cells express the human dystrophin for months and were observed to be healthy after a few years. Studies using the dog model have also shown that multiple injections over months may be necessary to maintain the appropriate level of exogenous dystrophin (45).

3.4. Eye Diseases

The stem cell therapy has been used for therapy of blinding diseases in the macular degeneration and retinitis pigmentosa, and its clinical trials have already concluded. Recent research have shown that most of the results obtained from the stem cell grafts in rodents, can also be used in larger mammalian species (46).

The results showed that the transplanted cells are integrated to the retina, but photoreceptor cell differentiation is restricted. In the dogs and rabbits, retinal damage was treated with human adipose MSCs and obtained important advancements (47). The human bone marrow derived MSCs and dental pulp stem cells have been successfully used to therapy of ocular surface reconstruction in the rabbit model with chemical burn (48).

4. Animal Models for MSC Therapy

4.1. Pig

Pig MSCs derived from adipose tissue and bone marrow have been shown to differentiate into various lineage and cell phenotypes and express a variety of gene and cell surface markers *in vitro*. Pig MSCs show differential gene expression, multilineage differentiation and cell surface marker expression (49). Pig models are very similar to humans in anatomy, nutrition, and physiologic/pathophysiologic responses (50). Pig MSCs derived from many tissue sources such as adipose, umbilical cord, and bone marrow have been used as a treatment model for diseases such as chronic ischemic cardiomyopathy (51), cutaneous wound healing (52), cardiac repair (53), cartilage repair (54), osteoarthritis (55), and bone repair (56).

Pig skin is more similar to human skin than the rodent skin, heals physiologically similar, and is a perfect model for investigation of the local agents. In human wound healing, pigs instead of rodent skin with different dermal layers and wound healing stages, make an ideal animal model (57). Treatment of chronic and diabetic wounds, hypertrophic scars, and burns with MSCs has been patterned in pigs. There are thousands of publications in peer review journals to examine wound healing and the pathology of different wound types in pigs (52).

The pig heart is more similar to the human heart than the heart of rodents and therefore its a more suitable model for cardiac studies. Contrary to the mouse heart, the human heart does not beat 500 to 700 times per minute and requires the regeneration of several grams of tissue rather than a few milligrams to repair human myocardial infarctions, making it difficult to compare with humans in studies using mouse models (51). Pig models are much more suitable than rodents for myocardial infarction and subsequent cardiac repair. In pig models mimicking cardiac disease, administration of MSCs has shown to stimulate to endogenous repair by inducing stem cell regeneration in the heart (58). MSC-derived structures have been constructed for the regeneration of articular cartilage in pigs and showed that the repaired tissue has mechanical features similar to normal pig cartilage in friction and compression tests (55).

It is important to improve the distribution of MSCs in the body, and large animal models such as pigs have supplied a good animal model for testing MSC applications. To repair cartilage defects in pigs, intraarticular injection of MSCs was tried and improvement was observed both histologically and morphologically (54). In the pig models, bone regeneration with MSCs are suitable for bone research such as tissue rejection and limited tissue regeneration (56). Significant regeneration has been shown to occur with MSC treatment in the porcine model of perforating mandible for bone injury. In the pig model with mandible perforating for bone damage has been shown to occur of significant regeneration with MSC treatment (59).

MSCs injected into the damaged site and MSCs injected intravenously showed similar regeneration; intravenously injected MSCs migrated to the damaged site via the blood circulation. In addition, researchers also used fluorescein labelled MSCs in their study and reported to contributed tissue regeneration of injected MSCs. The use of pigs as a model for MSC applications helps us develop regenerative models and should be considered as a suitable model for testing therapies in future research (59).

4.2. Cattle

Gao et al. found that cattle MSCs are capable of multi-lineage differentiation and contain the CD105, CD90 and CD73 markers but do not express CD45 (60). Cattle are less similar to humans in anatomy and size than other animal models; but, stuations that affect both cattle and humans may be useful for testing MSCs treatments. Cattle model is an important factor for evaluating healing processes, for example, in bone injury. In establishing a model for the study of bovine bone damage, MSC treatment can be applied to transfer the genetics of valuable animals for breeding, shorten recovery time and save breeders from wasting time (31).

Another disease model is osteoarthritis, which affects both humans and cattle. MSCs treatment for affected animals will provide additional information, increase quality of life and protect genetically valuable animals from dying from disease. Mastitis in cattle is a condition that affects production in the dairy industry. MSCs therapy for mastitis in cattle provides a perfect model for the treatment of the disease. The time a cow recovers from mastitis also serves to produce and economic gain (61).

MSCs have a long life span in *in vitro* and can be used modified by genetic engineering methods. MSCs have been shown to migrate and homing capability to sites of injury and inflammation. Shortening the recovery time with the treatment of MSCs from osteoarthritis, bone injury, mastitis or any production disease is very important for profitability and growth in livestock. While the cattle industry is undisputed the largest economic contributor, it is increasing the importance of cattle as a research model because of increasing productivity and reducing losses with MSCs treatment (62).

4.3. Sheep

Sheep MSCs have been isolated and identified by Vivas et al. Sheep MSCs can express known current MSC cell surface markers and differentiate into multiple lines (63). MSCs therapy in the sheep models can be applied to test treatment and recovery in various diseases such as bone graft substitutes (63), osteoarthritis (64) spinal repair (65), and acute renal failure (66).

Large animal models better imitate the size and healing stages of human bones than rodent models. Bone grafts have been successfully researched in sheep models using osteoconductive material enriched with MSCs to increase spinal fusion rates. Sheeps are more advantageous for bone repair models along with size, breeding and experimental expenses. Sheeps can provide insight for both cattle and human research (67).

The effect of MSCs in sheep models of acute kidney injury was also investigated. MSCs delivered to the renal artery of sheep showed renal engraftment in both glomeruli and tubules, and podocyte and tubule epithelial cells expressing MSC cell markers were detected and, a significant correlation was also found between injury and injection time (66).

In patients exposed to prolonged ischemia, such as myocardial infarction, MSC treatment can have a significant effect in decreasing mortality and morbidity (68). Inflammation, which greatly contributes to the adverse effects of ischemia-reperfusion injury, appears to be regulated by secreted factors by MSC. Ischemia and reperfusion injury is associated with severe clinic demonstrations and it is worth investigating how MSCs in livestock can be used as a treatment option for such cases (69). Sheep fetal models using human MSCs have been used to study multipotential capacity and immunological properties after transplantation in cell and gene therapies (63).

In a sheep model of osteoarthritis, chondrogenically stimulated MSCs were applied into the knee joint of an animal with osteoarthritis and, there was a regression in disease progression. Therefore, sheeps should be considered as a studyable model for human comparison in the treatment of MSC (64).

4.4. Goat

It was shown by Mohamad-Fauzi et al. that goat MSCs differentiate into three-lineages and are capable of expressing cell markers CD73, CD90 and CD105 and, lack of expression of the hematopoietic marker CD45 (70). Goats like sheeps also, due to their physiological and anatomical resemblance to humans, have become one of the large animal models often used to treat various diseases such as mastitis healing (30), osteoarthritis (71), and lung injury with MSC cells (72).

Mastitis affects the dairy industry by decreasing the number of animals available and can cause irreversible changes in the mammary gland of the animal, reducing its capability to produce at the same capacity. For the treatment of mastitis in goats, improvement in

mammary gland functionality has been demonstrated after application of MSCs into the affected udders (30). MSC treatment can provide important economic gains by shortening an animal's recovery time and preserving the amount of milk produced.

Osteochondral situation between goats and humans, such as osteoarthritis or traumatic injury, are under investigation due to similarities in structure of knee joint (73). It was determined that the medial meniscus regeneration of osteoarthritic jointed goats with MSC treatment and, implanted cells were found in the regenerated tissue (74).

Disease conditions such as bronchopleural fistula that can occur between the pleural space and the bronchial tree as a complication from trauma, surgery, or lung damage have been examined using goat models because of an anatomically similar mediastinum and pleural space to humans. Examination of tissue regeneration and healing time of goat may give an idea about the treatment of MSC in humans. MSCs treatment may have positive effects on surgical cases or secondary complications that reason bronchopleural fistula (72).

Such experimental results are of great importance for efficacy and safety tests in the development of methods for applying cell therapy in clinical trials. The structure, size and function of the species used as models are important for researching disease damage. In addition, goats also may be a more viable option than larger animals due to their smaller size and lower care costs. The goat model in MSC treatment is very important for the animal industry and clinical therapies, making them a perfect choice as an animal model (75).

5. Conclusion

MSCs therapy has the potential to treat the various diseases with positive and safe results. MSCs have the capability to regulate the immune system *in vivo*. MSCs are an important candidate for the treatment of many diseases with their isolation, reproduce and ease of *in vitro* administration (13).

MSCs have been used to treat diseases in clinical trials (76, 77), however, the effects of MSCs on diseases are still controversial. The relationship of MSCs to tumors is still unclear. Analysis of clinical studies is required to better understand the relationship of MSCs to tumors, but reported datas are lacking. Therefore, as noted by Zhao et al., the effect of MSCs on tumors is difficult to explain by data analysis (32). Because of the lack of information and experience in MSCs treatment, preclinical and clinical trials are still ongoing.

In addition, other important issues related to MSCs, alleviation of ischemic heart disease (78, 79), development of tendinopathy model for MSCs therapy (80), discovery of new drugs to facilitate and expedite MSCs differentiation into osteoblast (81), role of hormones in differentiation of MSCs by Kulebyakin et al. (82), insulin sensitivity in telomerase-immortalized mesenchymal stem cells by Barachini et al. (83), single-cell transcriptome integration analysis of MSCs (84) and senescence state in MSCs (85). In the future, academics, clinicians, and industry institutions should continue to collaborate, sharing

practices and knowledge that will advance MSC treatment.

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CHAPTER 13

INDUCED PLURIPOTENT STEM (IPS) CELL RESEARCH IN ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's disease is an irreversible and progressive neurodegenerative disease-causing memory loss and cognitive decline in patients. There are more than 50 million Alzheimer's patients worldwide. Number of Alzheimer's disease patients is predicted to continue to increase over the years. However, except for a limited number of drugs only help to relieve the symptoms, there is no cure for Alzheimer's disease. Animal models or post-mortem brain samples of patients are frequently used to study the mechanism of Alzheimer's disease. Since animal models do not fully reflect the physiology of the human brain, they cannot accurately reveal the pathology that occurs in Alzheimer's disease, and accessibility to post-mortem brain samples is limited and free of healthy controls. Induced pluripotent stem (iPS) cell technology offers a great opportunity to overcome these problems. iPS cells, which can be easily produced by reprogramming from adult somatic cells can be differentiated into the desired cell types including the cells of central nervous system. Several groups have developed various methods to differentiate human iPS cells into neurons, astrocytes, oligodendrocytes, and microglia, enabling the investigation of Alzheimer's disease-related mechanisms in a human model. The fact that iPS cells have no ethical concerns, can be easily obtained, reproduced, and can be transformed into almost any desired cell type makes iPS cells very valuable and highlights them as an unlimited resource that can be used in Alzheimer's disease modeling, drug trials and stem cell therapies.

Keywords: Alzheimer's disease, human model, iPS cells, differentiation

1. Alzheimer's Disease

Alzheimer's disease is an irreversible and progressive neurodegenerative disease first described by the German psychiatrist and neuropathologist Alois Alzheimer in 1906. Alzheimer's patients experience a decline in cognitive abilities such as memory, reasoning, logic, and adaptation. The most obvious symptoms of the disease are memory loss affecting daily life, difficulty in planning and problem solving, confusion in space and time, difficulty in perceiving spatial relationships, experiencing new problems with words while speaking or writing, decrease in reasoning ability, withdrawal from work or social activities, changes in mood and personality (1).

There are more than 50 million Alzheimer's patients worldwide (2). According to the data of Alzheimer's Association (1); in 2022, there are approximately 6.5 million Alzheimer's patients which are 65 years old or over in the United States. Additionally, 200,000 people under 65 years old are estimated to have younger-onset dementia. 4 million of the patients over 65 are women and 2.5 million are men. It is predicted that the number of Alzheimer's patients over the age of 65, which was 6.1 million in 2020, will rise with the increase in population and reach 8.5 million in 2030. It is thought that there are approximately 600,000 Alzheimer's patients in Turkey (3). According to the data of TURKSTAT (4), the number of elderly people in Turkey who died from Alzheimer's disease was 12,239 in 2021 and 2.2% of the elderly men died as a result of Alzheimer's disease, while this rate increased to 3.8% in elderly women. Number of Alzheimer's disease patients is predicted to continue to increase over the years. However, except for a limited number of drugs only help to relieve the symptoms, there is no cure for Alzheimer's disease (5).

There are two types of Alzheimer's disease: early-onset (EOAD) or familial (fAD) and late-onset (LOAD) or sporadic (sAD) (6). Familial Alzheimer's disease accounts for approximately 10% of the cases, occurs before the age of 65, and mutations in either the genes encoding amyloid precursor protein (APP) or presenilin isoforms (PSEN1 and PSEN2) ensure the onset of the disease (6,7). Sporadic Alzheimer's disease, which accounts for most of the cases (about 90%), begins after the age of 65 (8). The major risk factor for sporadic Alzheimer's disease is apolipoprotein E (APOE) ε4 allele which is one of the three polymorphic alleles of the (APOE) gene, encoding the main cholesterol transporter protein in the brain (9). People with the APOE ε4 allele are 3 to 8 times more prone to develop the disease, and each APOE ε4 allele brings the average age of symptom onset about ten years earlier (8,9). In recent years, whole genome analyses have revealed that rare variants of the trigger receptor 2 expressed in myeloid cells (TREM2) gene, which is expressed in microglia cells in the brain, increase the risk of sporadic Alzheimer's disease by about 3 times. This reinforced the idea that immune and inflammatory pathways may also be involved in the pathogenesis of the Alzheimer's disease (10).

The most important molecular mechanisms of Alzheimer's disease are after APP

proteolysis, accumulation of amyloid β peptides forming extracellular amyloid β plaques, and upon hyperphosphorylation, aggregation of tau proteins forming intracellular neurofibrillary tangles (11). APP is a protein that is highly expressed in the brain, but its physiological function is not fully understood. It is a single-pass transmembrane protein cleaved by α, β, and γ-secretases and splits into multiple fragments, including amyloid β. Fragmentation of APP occurs by two pathways: amyloidogenic and non-amyloidogenic pathways (Figure 1). In the non-amyloidogenic pathway, APP is firstly cleaved by α-secretase and splits into two parts, soluble amyloid precursor protein α (APPsα) and C terminal fragment α (α-CTF). Then α-CTF is cleaved by γ-secretase to form smaller fragments called 3-kDa peptide (p3) and amyloid precursor protein intracellular domain (AICD). In the amyloidogenic pathway, APP is primarily cleaved by β-secretase and two parts are formed, soluble amyloid precursor protein β (APPsβ) and C terminal fragment β (β-CTF). Afterwards, γ-secretase cleaves β-CTF, producing AICD and amyloid β (12). PSEN1 and PSEN2 proteins which are risk factors in familial Alzheimer's disease, are the catalytic components of γ-secretases (13).

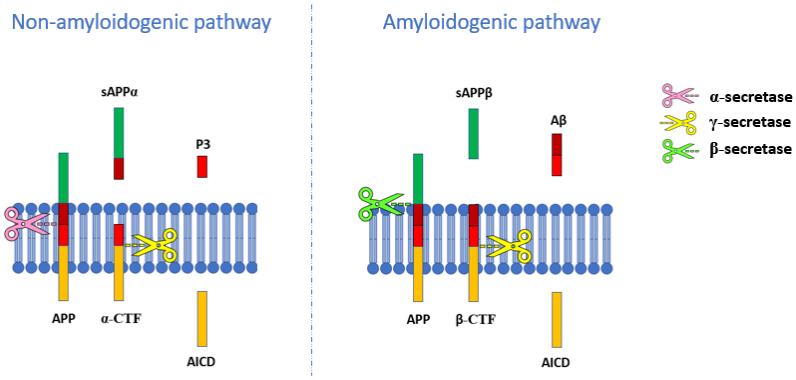


Figure 1: Cleavage of amyloid precursor protein (APP) by different combination of secretases produce different fragments. In non-amyloidogenic pathway, APP is cleaved by α-secretase and γ-secretase, respectively, producing P3 and AICD. In amyloidogenic pathway, cleavage of APP by β-secretase and γ-secretase, respectively, results in amyloid β and AICD formation. AICD: amyloid precursor protein intracellular domain, α-CTF: C terminal fragment α.

Amyloid β monomers, which are formed as a result of the amyloidogenic pathway, accumulate to form toxic amyloid β oligomers, which later form amyloid v protofibrils. When these protofibrils come together, amyloid β fibrils and then amyloid β plaques are formed (Figure 2) (14).

Tau is a microtubule-associated protein found mainly in neurons and much lower levels in glia cells. Tau is also released out of the cell, physiologically. Although the concentration of tau in the interstitial fluid is one-thousandth that in neurons, it is ten times the concentration in the cerebrospinal fluid. Interstitial tau is taken up by the cells, destroyed locally or spilled into the perivascular space (15). Normally, the function of tau protein is to stabilize microtubules

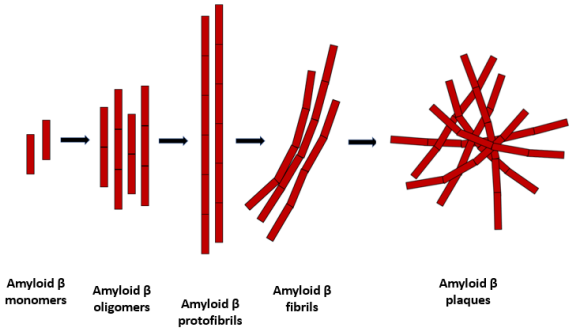


Figure 2: Amyloid β plaque formation steps from amyloid β monomers. Aggregation of amyloid β monomers as oligomers, protofibrils and fibrils eventually leads to amyloid β plaque formation.

formed by tubulin subunits. The ability of tau to maintain this microtubule-supportive function depends on its level of phosphorylation. As a result of hyperphosphorylation, tau becomes a neurotoxic protein. Hyperphosphorylated tau in Alzheimer's disease causes destabilization of microtubules (Figure 3). This leads to disruption of the axonal transport system, in which microtubules play important role (14). Meanwhile, tau monomers accumulate first as oligomers and then as paired helical filaments (PHF), forming neurofibrillary tangles in the neuronal cell body (16). Eventually, cargoes cannot be transported within the cell and synaptic communication between neurons is disrupted (14).

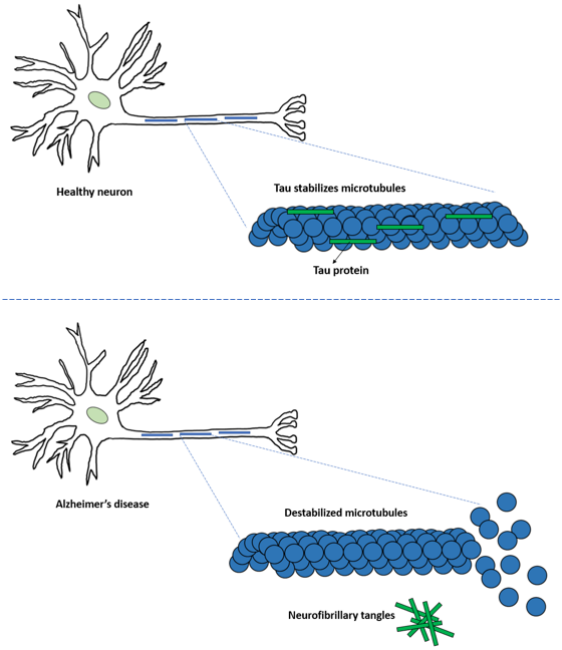


Figure 3: In the axons of healthy neurons, tau binds microtubules, assisting their stabilization. In Alzheimer's disease, hyperphosphorylation of tau prevents its binding to microtubules, causing neurofibrillary tangle formation together with microtubule destabilization.

Using advanced imaging systems, recent studies revealed that amyloid plaques and neurofibrillary tangles can also be found in healthy elderly besides Alzheimer's patients. Additionally, attempts to destroy amyloid β plaques in animal models and humans could not prevent the progression of the disease although helped to reduce the amyloid β burden. These results showed that the "amyloid cascade hypothesis" cannot fully explain the mechanism of Alzheimer's disease and the view that neuroinflammation may be a component that actively contributes to the pathogenesis of Alzheimer's disease rather than a secondary outcome has been strengthened (17).

Recent studies indicate that neuroinflammation, especially near amyloid deposits and neurofibrillary tangles, contributes to the pathogenesis of Alzheimer's disease. Neuroinflammation is defined as the inflammatory response to various pathological conditions in the central nervous system (CNS) that may result from infection, trauma, ischemia, neurodegeneration, aging, or toxins (18). During neuroinflammation, substances such as proinflammatory cytokines, chemokines, secondary messenger small molecules and reactive oxygen species (ROS) are produced in the relevant brain region (19). Microglia, which are the phagocytes of the CNS, are among the main cells involved in neuroinflammation (20). Other cells that contribute to neuroinflammation are astrocytes, capillary endothelium, and peripheral immune cells that infiltrate from the blood to inflammation region, especially in cases where the blood-brain barrier is destroyed (18). Although neuroinflammation is a beneficial process providing protection of CNS tissue and restoration of homeostasis after pathological threats, chronic persistence and spread of neuroinflammation over years may cause damage in healthy tissues, too (21). There are many studies showing increased levels of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in brain tissues, cerebrospinal fluids and serum samples of Alzheimer's patients (22-24). Also, microglia cells and astrocytes were shown to cluster around amyloid β plaques in brain sections from post-mortem brain tissues of Alzheimer's patients and from transgenic mice in which an experimental Alzheimer's model was created (20). Various epidemiological studies showing that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing Alzheimer's disease has increased the importance of neuroinflammation in the mechanism of Alzheimer's disease (25, 26). Studies on mouse models of Alzheimer's disease also support this view that NSAIDs reduce the production and accumulation of amyloid β and improve cognitive functions (27, 28). Although most of the NSAIDs did not show the expected curative effect in clinical studies, which raises questions, genome-wide association studies (GWAS) in recent years again have underlined the importance of neuroinflammation in Alzheimer's disease (29, 30). GWAS studies have identified more than 20 gene variants (including TREM2, complement receptor 1 (CR1), the cluster of differentiation 33 (CD33), B3 domain-containing transcription factor (ABI3), ATP-binding cassette transporter A7 (ABCA7) that may be risk

factors for Alzheimer's disease. Common features of the identified genes are that they play roles in the immune response and are highly expressed in microglia cells (8). In line with these data, the role played by neuroinflammation and microglia in Alzheimer's disease has become one of the main focal points of Alzheimer's disease studies.

In preclinical studies of Alzheimer's disease, streptozotocin-induced animal models or transgenic animal models are often used. Streptozotocin is a diabetogenic agent which causes damage of pancreatic β cells and induce diabetes when administered peripherally in animal models. Since intracerebroventricular streptozotocin injection disrupts cerebral glucose uptake, it is also used to generate an animal model of sporadic Alzheimer's disease in which brain glucose/energy metabolism decreases (31, 32). Transgenic animal models of Alzheimer's disease represent familial Alzheimer's disease and are generated by overexpression of human mutant APP, PS1 and tau genes. Both streptozotocin-induced and transgenic animal models of Alzheimer's disease mimic certain aspects of the disease such as formation of amyloid plaques and neurofibrillary tangles, learning and memory deficits (31).

Animal models has been brought very important contribution to the literature. However, humans and rodents (as mostly used and highly eligible animal model) diverged from a common ancestor about 60 million years ago which explain main developmental and structural differences at both molecular and cellular levels between human and rodent brains (33). Meta-analysis of transcriptomic studies comparing gene expressions in post-mortem specimens of Alzheimer's patients with different mouse models of Alzheimer's disease revealed that mouse models show poor correspondence to human disease (34). Additionally, studies comparing gene expressions of human and mouse microglia stated that there are several differentially expressed genes in human microglia cells, which highlights the inability of animal models to represent humans (35, 36). These differences may explain why drug candidates do not exert expected beneficial effects in clinical trials while they seem effective in preclinical studies (33).

The use of human-derived brain cells instead of the cells obtained from animal models is not always possible due to the difficulty of obtaining samples from the CNS. Usage of human CNS material in studies is limited to post-mortem tissue samples or biopsies that must be taken because of a particular disease. Moreover, these samples carry certain problems such as issues regarding post-mortem artifacts, lack of healthy control cells and presence of only limited number of cells (37). The discovery of induced pluripotent stem cells overcomes the limited possibilities of animal models and offers a unique opportunity to investigate neurodegenerative disease mechanisms using human-derived brain cells.

2. Induced Pluripotent Stem (iPS) Cells

Stem cells are cells that have the capacity to self-renew and differentiate into specialized cells of the body. Several types of stem cells are present in the body in the embryonic and

adult stages. Differentiation of stem cells into specific cell types occurs step by step, and their differentiation potential decreases with each step. Stem cells are divided into five types according to their differentiation potential: totipotent, pluripotent, multipotent, oligopotent and unipotent. Totipotent stem cells (eg, zygote) have the capacity to form cells of the whole organism, including embryonic and extra-embryonic structures. Pluripotent stem cells (eg, embryonic stem cells derived from the inner cell mass of the embryo, induced pluripotent stem cells) can differentiate into all three germ layers. Multipotent stem cells (eg, hematopoietic stem cells) have narrower differentiation capacity but still can differentiate into different cell lineages. Oligopotent stem cells (eg, myeloid stem cells) can differentiate into different cell types belonging to a particular cell lineage. Unipotent stem cells have the capacity to differentiate into only one cell type (38).

In 2006, Takahashi and Yamanaka reprogrammed mouse fibroblasts with only four factors (Yamanaka factors: Oct3/4, Sox2, c-Myc, Klf4) to obtain undifferentiated cells. These undifferentiated cells, which were called iPS cells, showed characteristics similar to embryonic stem cells (ESCs) and could differentiate into three germ layers (Figure 4) (39). One year later, reprogramming of human fibroblasts into iPS cells were successfully accomplished, too (40, 41). Studies on iPS cells gained momentum after this groundbreaking discovery in the scientific world, and it was shown that iPS cells could be obtained from various cell types, such as hepatocytes, keratinocytes, melanocytes, pancreatic β cells, neural progenitors, and blood cells (42).

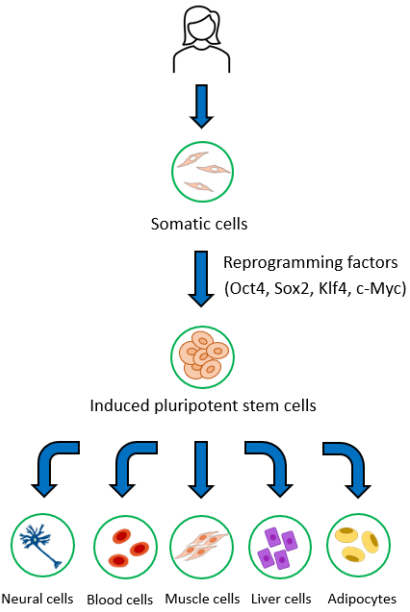


Figure 4: Induced pluripotent stem cells (iPS) cells are produced from somatic cells using reprogramming factors. iPS cells have the capacity to differentiate into all cell types originating from three germ layers.

iPS cells, which can be easily produced by reprogramming from adult somatic cells (most commonly from fibroblasts), can be differentiated into the desired cell type. Since iPS cells can also be differentiated into cells belonging to the CNS, they constitute an unlimited resource to study diseases related to the CNS, for which it is very challenging to obtain samples of human origin (43). The most significant advantage of iPS cells over embryonic stem cells is that an embryo is not required for production. Thus, their usage does not cause ethical concerns in therapeutic studies. As iPS cells allow for autologous transplantation, the risk of immune rejection is also eliminated (44). iPS cells keep the genetic background of the patient which is pivotal to study Alzheimer's disease mechanisms and cannot be replicated in animal models. Transgenic animals are usually created with a single or combination of a few gene mutations. However, especially in the case of sporadic Alzheimer's disease, which constitute more than 90% of the Alzheimer's cases, genetic factors cannot explain the whole mechanism of the disease. Genetic, epigenetic, or environmental factors contribute to the disease and this complicate to represent it with an animal model. Carrying the genetic information of the patient makes iPS cells favorable over animal models (33). iPS cells also give a great promise for personalized treatments. They can be obtained from the patient's own cells, which can be easily sampled (such as fibroblast, keratinocyte or blood cells), and after differentiation into the desired cell type, personalized drug trials, toxicity tests can be performed, or treatment methods can be developed (Figure 5). In addition, by culturing brain cells differentiated from iPS cells together, three-dimensional organoids can be formed, and physiologically similar brain organoids (brain in a dish model) can be produced. The fact that iPS cells are of human origin, can be easily obtained, reproduced, have no ethical concerns, and can be transformed into almost any desired cell type makes iPS cells very valuable and highlights them as an unlimited resource that can be used in disease modeling, drug trials and stem cell therapies (45).

2.1. iPS-Neurons

Besides pivotal advantages of iPS cells over ESCs, as both of them are pluripotent and have the unlimited proliferation and differentiation capacity to all somatic cells, similar methods are used to differentiate them into neural cells. In these methods, *in vivo* neural differentiation is mimicked (46). Neural system development begins with neural tube formation during embryonic development. At the third week of embryonic development, signals from notochord induce ectoderm to thicken and form neural plate. By bending and folding, neural plate takes tubular shape and becomes neural tube. Central nervous system elements originate from the neural tube (47, 48). Therefore, neural differentiation methods recapitulate neural differentiation steps: neuroepithelium formation, neural stem cell (NSC) production, neural progenitor cell (NPC) generation and mature neural cell differentiation (46). Early attempts to differentiate pluripotent cells into neural cells include induction with retinoic acid, co-culture with stromal cell lines and treatment with serum-containing or serum-free medium (49).

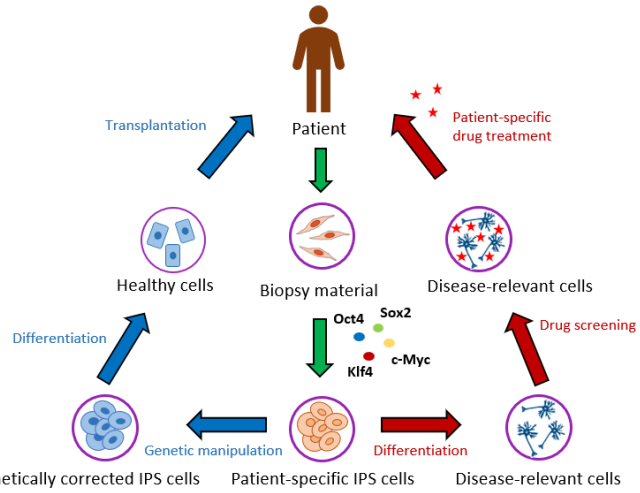


Figure 5: iPS technology provides to produce pluripotent cells carrying genetic background of the patients from biopsy materials by overexpression of Yamanaka factors. Patient-specific iPS cells can be differentiated into disease relevant cells to screen the effects of drugs thus allowing patient-specific drug treatment. Also, patient-specific iPS cells can be corrected via genetic manipulation and disease-free iPS cells can be differentiated into desired healthy cells to be used in transplantation.

Retinoic acid is the active form of vitamin A and known to be an important inductive signal in embryonic development, especially in the early stages of the CNS development (50). Hence, *in vitro* studies used retinoic acid to drive pluripotent cells into neuronal differentiation (50, 51). Bain et al. (52) reported that retinoic acid shows its effect by upregulating neural genes and downregulating mesodermal genes. However, retinoic acid also upregulates apoptotic genes, reducing efficiency of neural differentiation and cell yield (53). The method termed stromal-derived inducing activity includes co-culturing pluripotent cells with stromal cells to induce neural differentiation. Usage of PA6 cells (stromal cells derived from skull bone) as feeder layer provided neural differentiation of pluripotent cells without the use of retinoic acid (54, 55). Alternatively, some studies used another stromal cell line, MS5 (murine bone marrow–derived stromal cells) as feeder layer together with several morphogens in differentiation medium to get neuronal cells (56, 57). Serum-containing mediums are also used by some researchers (58, 59). Although both stromal cell co-culture and serum-containing medium induction methods successfully induced neural differentiation of pluripotent cells, they are found inadequate since their mediums contain large numbers of undefined factors which might complicate the study of the mechanism of differentiation (49).

Embryoid body-based methods including neural rosette formation are widely used in differentiation of pluripotent stem cells into neural cells. In these methods, firstly, embryoid bodies which are three dimensional aggregates of the pluripotent stem cells are formed (60). Embryoid bodies maintain the ability to differentiate into derivatives of three germ layers

(61). With the initiation of neural differentiation, embryoid bodies generate radially arranged cellular structures named neural rosettes. Neural rosettes represent the neural tube and contain neural progenitor cells which are then isolated, expanded and induced to differentiate into neuronal or glial lineages (46, 62).

Dual SMAD (suppressor of mothers against decapentaplegic) inhibition is another well-established method to derive neural cells from pluripotent stem cells. Dual SMAD inhibition method is based on blocking of BMP (bone morphogenetic protein) and TGF-β (transforming growth factor-β) signaling pathways. BMP protein family belongs to TGF-β superfamily and plays a major role in embryonic development. Activation of the BMP pathway led to expression of the genes driving epidermal differentiation, whereas its inhibition induced neural differentiation (46). Binding of BMPs (BMP2, BMP4, BMP7, GDF6, and BMP3) to BMP receptors cause phosphorylation of the signal transducer SMAD proteins (Smad1, Smad5, Smad8, and Smad9). These proteins form a complex with Smad 4 or Smad 10 and translocate to the nucleus. In nucleus, they associate with several transcription factors and drive epidermal gene expression. However, activation of TGF-β receptors induces cells into mesodermal fates, and inhibition of TGF-β receptors leads to neural differentiation. Binding of TGF-β proteins (activins, nodal, nodal-related Vg-1, TGF-β1, TGF-β2, and TGF-β3) to TGF-β receptors lead to phosphorylation of Smad2 and Smad3. These proteins associate with Smad4, and then translocate to the nucleus where they induce expression of mesodermal genes. In dual SMAD inhibition method, both BMP and TGF-β pathways are inhibited, providing efficient induction of neural differentiation. Various inhibitors and small molecules are defined to inhibit BMP and TGF-β pathways. Noggin, chordin, cerberus, follistatin, Xnr3 (Xenopus nodal-related 3) and TSG (twisted gastrulation) are examples of BMP pathway inhibitors whereas follistatin, follistatin-related protein and cerberus are examples of TGF-β pathway inhibitors (63). Chambers et al. (64) used this strategy to induce neural differentiation of pluripotent cells. Using synergistic effect of two SMAD inhibitors, Noggin and SB431542, they successfully induced neural differentiation and yielded PAX6+ neural progenitor cells (with 80% efficiency) which are capable of neural rosette formation as early as 11 days. After obtaining neural progenitor cells, neuronal subtypes such as dopamine neurons and motor neurons were derived following exposure to different combinations of morphogenic factors (retinoic acid, FGF8, SHH, BDNF, ascorbic acid, GDNF, TGF-β3, and cyclic-AMP). This protocol allowed a much shorter differentiation period as 19 days to derive neuronal subtypes when compared to co-culture and embryoid body-based methods in which neuronal differentiation lasts 30-50 days (64). Subsequently, similar other protocols were suggested which improve the efficiency of the method by decreasing heterogeneity of neural progenitor cells using other inhibitory molecules or aim to obtain specific neurons using different morphogenetic factors (65-67).

In recent years, researchers have developed methods to directly differentiate induced pluripotent stem cells into neurons bypassing classical neural differentiation stages (embryoid body, neural rosetta, neural stem cells and neural progenitor cells). Transient overexpression of specific transcription factors provides direct reprogramming of induced pluripotent stem cells into specific neuron types (46). Pang et al. (68) showed that forced expression of three lineage-determining transcription factors Brn2, Ascl1 and Myt1l convert human iPS cells into so-called "induced-neurons" (iNs). iNs were shown to be functional (could generate action potentials) and could be obtained from iPS cells as early as 6 days after transgene activation. Subsequently, Zhang et al. (69) revealed that overexpression of only one transcription factor either Neurogenin-2 (Ngn2) or NeuroD1 alone is sufficient to generate neurons from iPS cells. Less than 1 week neuron-like cells, and less than 2 weeks iNs which show mature neuron morphology were obtained. With this protocol, researchers successfully obtained excitatory neurons with almost 100% yield and neuronal purity. Later, Yang et al. (70) found that combination of transcription factors Ascl1 and Dlx2 convert human iPS cells into GABAergic neurons. With this method, cells reached synaptic maturity after 5 weeks and pure GABAergic neurons were obtained. Afterward, several protocols were published based on direct reprogramming of iPS cells into neurons developing the method using other transcription factors aiming specific neuron subtypes in need or combining it with SMAD inhibition method (71, 72).

2.2. iPS-Astrocytes

Astrocytes are one of the three main supportive neuroglia cells (together with oligodendrocytes and microglia cells) found in the central nervous system. They are the most abundant cells of the brain; their number is five times greater than the number of neurons. Also, they are the largest neuroglia cells. They have star-shaped morphology with many extensions. Their extensions extend to synapses and envelope them. Main function of astrocytes is maintaining homeostasis by transporting nutrients, waste material and metabolites between neurons and capillaries. They constitute a component of the blood-brain barrier with their perivascular foot processes. Also, they help to regulate the extracellular ion concentration of the neuronal environment (73).

Since the origin of astrocytes and neurons are similar, astrocyte differentiation methods include differentiation from neural progenitor cells, too. Ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), FGF2, BMP are morphogens that are known to induce glial fibrillary acidic protein (GFAP) expression which is an intermediate filament protein expressed exclusively in astrocytes. CNTF, LIF and FGF2 induce Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways whereas BMPs activate SMAD pathways. Both of these pathways regulate GFAP expression by converging at some point and induce astrogliogenesis (46, 74). Various methods have been proposed to differentiate iPS cells

into astrocytes. However, these methods have prolonged culture time or need cell sorting (75-77). To overcome these problems, Tcw et al. developed a fast and robust method in which astrocytes were obtained from iPS cells after 30 days of differentiation process. Firstly, NPCs were differentiated from iPS cells using either dual SMAD inhibition or neural induction medium. Then, astrocyte differentiation was induced from NPCs using an astrocyte medium containing an astrocyte growth supplement. iPS cells-derived astrocytes were shown to be functional as they responded to inflammatory stimulants, phagocytosed myelin and displayed spontaneous calcium transient activity (78).

2.3. iPS-Oligodendrocytes

Oligodendrocytes are the myelin-producing cells of the central nervous system. They form myelin sheath around axons by spirally wrapping their cell membranes several times. Myelin sheath allows saltatory impulse propagation to be quick and efficient. Demyelination of axons is present in several neurodegenerative diseases. Additionally, oligodendrocytes provide neurons metabolic support and axonal protection (79, 80).

Various methods have been developed to induce oligodendrocyte differentiation from iPS cells (81-84). Since origin of oligodendrocytes is similar to astrocytes and neurons, oligodendrocyte differentiation methods include differentiation from neural progenitor cells, or oligodendrocyte progenitor cells (OPCs) which are known as common progenitors of oligodendrocytes and astrocytes (85, 86). As the first step of oligodendrocyte differentiation, OPCs were differentiated from iPS cells. Main approach at this step was to activate SHH signaling or inhibit WNT/β-catenin signaling by using several mitogens such as FGF2, PDGF, and EGF or morphogens like retinoic acid. At the maturation step of oligodendrocytes from OPCs, insulin-like growth factor (IGF1) and triiodothyronine (T3) were used frequently. IGF1 provides inhibition of the BMP signaling pathway, which is known to induce differentiation of OPCs into astrocytes when activated. T3 induces expression of transcription factor KLF9, thus leading to oligodendrocyte maturation. However, the majority of these methods remained time-consuming or with low-efficiency (46). Douvaras et al. proposed a differentiation method lasts 75 days (short version lasts 55 days) which was much shorter than previous protocols. They differentiated iPS cells into NSCs which give rise to OPCs and finally to oligodendrocytes using chemically defined and growth factor–rich medium (87).

2.4. iPS-Microglia

The name microglia is a combination of the words *micro* meaning small and *glia* meaning glue. as they together with other neuroglia. This nomenclature indicates that microglia cells are the smallest of the neuroglial cells, as other two main neuroglial cells (oligodendrocytes and astrocytes) are named as macroglia and serve as a nerve glue keeping neurons together in the organism (88). Microglia cells are the innate immune cells of the central nervous system,

constituting the first line of defense in the brain and spinal cord (89). They are a member of the mononuclear phagocytic system and comprise approximately 10% of the cells in the brain (90-92).

Microglia cells are the only cell type of myeloid origin resident in the healthy brain parenchyma (93). While neurons and macroglia cells of the central nervous system originate from neuroectoderm, microglia cells are of mesodermal origin (94).

During the embryonic period, hematopoiesis occurs as a two stage process: primitive hematopoiesis and definitive hematopoiesis. As the first stage of the hematopoiesis, primitive hematopoiesis begins with the formation of blood islands in the extraembryonic yolk sac at E7 in mice. Primitive hematopoiesis gives rise to erythro-myeloid cells. Primitive macrophages originating from the yolk sac are distributed to different parts of the embryo after the circulatory system is formed between E8.5 and E10. Primitive macrophages differentiate into fetal macrophage populations when they reach to their target tissues. Yolk sac originated in the fetal macrophage population migrating to the brain before the blood-brain barrier is formed in the fetal period give rise to microglia cells (95). As the proliferation capacity of the fetal macrophage cells is quite high, after microglia population is established in the brain, their persistence is maintained by local proliferation in the adult period, as well. Under normal conditions, monocytes or perivascular macrophages in the blood do not contribute to the microglia population (96). As the second stage of the hematopoiesis, definitive hematopoiesis begins around E8.5 in mice. It begins as a new wave of hematopoiesis in the aorta, gonads, mesonephros (AGM) region of the embryonic mesoderm and forms the hematopoietic progenitors. Hematopoietic stem cells produced in the AGM region colonize the fetal liver at E10.5 (95). At E16.5, they colonize the bone marrow remaining as a self-renewing population throughout adult life (97). All hematopoietic lineages, including monocytes, originate from hematopoietic stem cells (95).

Lineage tracing studies have revealed that microglia originate from mesodermal primitive yolk sac progenitors and these cells form Myb-independent erythromyeloid progenitors (EMPs). On the contrary, hematopoietic stem cells develop from Myb-dependent EMPs (98). In humans, EMPs give rise to yolk sac macrophages at E17. Starting from E31, yolk sac macrophages colonize the brain until the blood-brain barrier is formed. After arriving in the brain, they start to mature through interaction with other brain cells. During the maturation step, microglia cells gain their typical branched structure and functional properties (99, 100).

Various methods have been developed to obtain microglia from iPS cells. Although the details of these methods differ from each other, they all use the same strategy: following the embryonic development stages of microglia and providing cells with a neuronal microenvironment (101). Differentiation of iPS cells into microglia lasted between 24 days to 74 days in these protocols (102, 103). Some of these protocols directly initiated differentiation

from iPS cells, while others firstly formed embryoid bodies which are three-dimensional clusters of iPS cells and then started differentiation. Mimicking the embryonic developmental steps, iPS cells were drived into mesodermal differentiation. For this purpose, mesodermal growth factors such as BMP4, FGF, VEGF, and SCF were added in the media and myeloid progenitor cells were formed. Subsequently, myeloid progenitor cells were cultured in the media containing specific growth factors and signaling molecules known to induce microglial differentiation, such as M-CSF, GM-CSF, IL-34, and TGF-β. In order to get mature microglia, these cells were co-cultured with neurons or astrocytes, or alternatively, some defined factors known to play role in microglia maturation like CD200 and CX3CL1 were added in the media (99, 104-106). Although the basic logic of the methods used to obtain microglia from iPS cells is the same, there are particular differences in each protocol. Some protocols differentiated iPS cells under low oxygen conditions at the first steps of differentiation to reproduce the hypoxic environment of developing embryo (102, 105-107). After obtaining myeloid progenitor cells, some protocols selectively separated these cells by fluorescence-activated cell sorting (FACS) according to carrying certain markers such as CD43 and used these specific marker-carrying progenitor cells in further microglia differentiation steps (105-108). The time spent for differentiation and the yield of the microglia cells obtained were also variable in the protocols.

3. Conclusion

Using iPS cells-derived brain cells which are generated from patients own cells may help to overcome the limitations of Alzheimer's disease studies related to animal models. Since human iPS cells-derived brain cells reflect human physiology more precisely and recapitulate disease pathology, iPS cells technology offers an eligible tool to understand Alzheimer's disease pathogenesis, identify therapeutic targets and allow large-scale drug screening of novel therapeutic candidates.

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CHAPTER 14

EMBRYONIC STEM CELLS DERIVATIVES: IMMUNOLOGY AND THERAPEUTIC BENEFITS

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ABSTRACT

Human embryonic stem cells (hESCs) have the capability to develop into various cell types including adult cells. This characteristic of hESCs has been very attractive to researchers due to their potential applications in regenerative medicine and therapeutics such as stem cell (SC) transplantation. On the other hand, the immunologic properties and the risk of teratoma formation after transplantation have been the major challenges in treatment. To overcome these challenges, several approaches were developed such as forming hESC derivates or establishing hESC banks comprised of a vast diversity of major histocompatibility antigen-presenting cells. Although the tremendous potential of hESCs has been tempting, the use of adult stem cells in therapeutic approaches has exceeded embryonic cells. In this review, first, we have briefly summarized the preimplantation embryo development, the isolation of hESCs, and the immunologic properties of these cells, and then presented different examples for ESCs derivatives.

Keywords: Human embryonic stem cells, human embryonic stem cell derivatives, immunogenicity, stem cell therapy

1. Introduction

Human embryonic stem cells (hESCs) were first established in 1998 following successful studies on other species such as mice. The totipotent and, later, pluripotent nature of these cells and their potential use in therapeutics and regenerative medicine were tempting for researchers. ESCs can be obtained from the inner cell mass (ICM) of the growing blastocyst and further can be cultured in an appropriate in vitro microenvironment to support their totipotent state or can be transformed into derived hESCs. The development of protocols to differentiate hESCs into various lineages including hepatocytes, cardiomyocytes, pancreatic beta cells and retinal pigmented cells were the remarkable steps for their potential applications in certain diseases. However, their immunologic properties and the risk of teratoma formation after transplantation have been the major challenges in treatment. On the other hand, clinical trials conducted especially for macular degeneration have promising results on the safety and efficacy of derived hESC applications. In this chapter, the stages of preimplantation development, the alternative sources of hESCs, the immunogenicity of hESCs, and their therapeutic applications in certain diseases will be discussed.

2. Early Preimplantation Embryo Development

The zygote, formed by the fusion of the female gamete (oocyte) and male gamete (sperm), is the first cell of life in the mammalian embryo. After the fertilization and formation of the zygote, the embryo continues to develop, going through a series of special mitotic cell divisions called cleavage. The zygote is a totipotent cell and has the potential to give rise to all types of human cells including extraembryonic lineages. With each embryonic cleavage and further development, although all the cells have the same genetic composition in their nucleus, the pluripotent development potential becomes increasingly restricted and they begin to differentiate following the embryonic cell fate. The dynamic differentiation process of the embryo is controlled by a highly specific and complex cellular control mechanism.

The mammalian embryonic cell cycle is approximately 24 hours. On the 3rd day of the development of the human embryo (8-cell stage), blastomeres begin to compact basolaterally mainly due to E-cadherin activity. Around 5-6th days of the development (blastocyst stage), the first different lineages of the embryo are established. The inner cell mass (ICM) cells give rise to the embryonic tissues whereas the surrounding thin layer of trophectoderm cells develop into the placenta. ICM cells are pluripotent and capable of forming all tissues in the adult organism (Figure 1). The stages of embryonic development are briefly summarized in the following section.

2.1. Zygote

In mammals, with the fusion of the plasma membranes the sperm nucleus and centriole enter ooplasm. Microtubules protruding from the centrioles form sperm aster and the male nucleus moves to the center of the ooplasm through this aster. After the decondensation of the

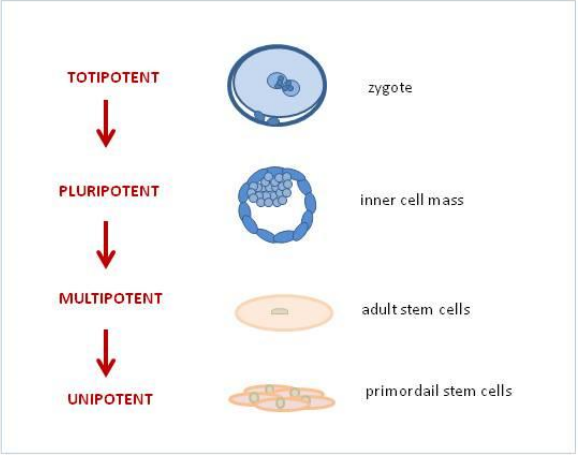


Figure 1: Schematic representation of the loss of potency during embryonic development and cell differentiation

sperm nucleus, male and female pronuclei are formed (1). In assisted reproduction techniques (ART), two centrally aligned pronucleus are observed around 12 hours after intracytoplasmic sperm injection and 16-18 hours after conventional *in vitro* fertilization (Figure 2).

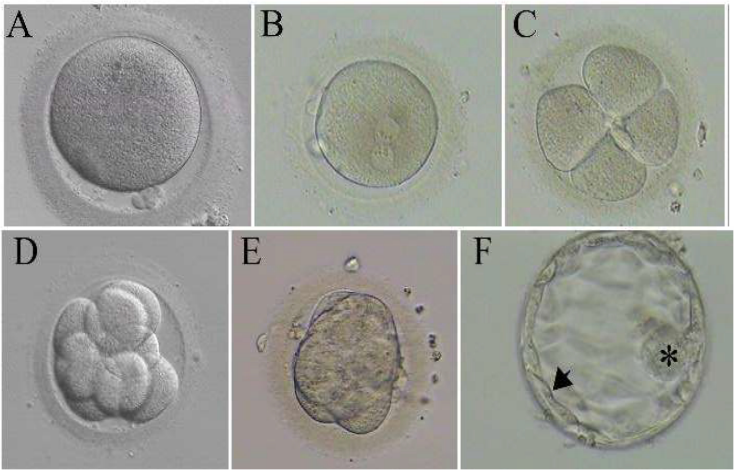


Figure 2: Stages of embryonic development. A. Oocyte, B. Zygote, C. and D. Cleavage stage embryo, E. Compact morula, F. Blastocyst. *: inner cell mass (ICM), arrowhead: trophectoderm cells. Images are from the Image and Photography Archive of Istanbul University, Istanbul Faculty of Medicine, Training Center for Assisted Reproduction Techniques

2.2. Cleavage Stage Embryo

The period from zygote to the formation of a 4-8 cell embryo is defined as the cleavage stage. The observation of the first mitotic division, which occurs around 23-29 hours after fertilization of the *in vitro* developing human embryo, is considered the first reliable marker of embryo viability (2). On the 2nd day and 3rd day after fertilization, the human embryo has

2-4 cells and 6-8 cells of equal size, respectively. In humans, zygotic genome activation takes place at the 8-cell stage however, minor zygotic genome activation is observed earlier (3).

2.3. Compaction

After the 3rd day, the number of cells in the embryo increases with subsequent divisions and forms a compact structure composed of 16-32 cells. During this period, the intercellular junctions (tight junctions, adherens junctions, desmosome, and gap junctions) increase in strength. Some of these junctions bind the cells tightly to each other, forming an impermeable layer, while others selectively transport certain molecules, thus enabling intercellular signaling mechanisms in the later stages of embryo development. At this compaction, the stage embryo is called the morula. In this highly communicating cell mass, blastomeres gain pluripotent properties (Figure 2).

2.4. Blastocyst

Around the 5th day of development, the embryonic cavity begins to form and the embryo is called blastocyst with two clearly distinguishable cell lineages: the trophectoderm and the inner cell mass (ICM). Due to the tight intercellular bonds in the trophectoderm layer, blastocele fluid accumulates in the embryonic cavity. Pluripotent hESCs can be isolated from the ICM of the blastocyst stage embryo (Figure 2).

3. Alternative Sources for Human Embryonic Stem Cells

The development of ARTs enabled the researchers to monitor the developing embryos *in vitro* and gave them the opportunity to research early embryonic development. The use of surplus and donated embryos obtained from couples undergoing ART for the isolation of hESCs is a much-debated topic. Removal of a group of stem cells from the ICM of an embryo at the blastocyst stage may result in the developmental failure of the embryo and even may cause developmental disorders. Moreover, an embryo at this stage is considered as an individual in many countries and therefore hESC studies are considered morally and ethically inappropriate. In general, the following *in vitro*-produced embryo groups are used as stem cell sources in hESC research (4):

a) *Good quality embryos:* Surplus embryos after ART that are donated by the couple for hESC research can be used. This group of embryos probably constitutes the highest quality hESC source since the embryos have apparently normal development capacity.

b) *Poor quality embryos:* Donated surplus embryos after ART embryos with a lower quality that are not suitable for embryo transfer or cryopreservation are another group. In this alternative source, the lower developmental capacity is the main problem and the blastocyst rate is lower in such embryos.

c) *Genetically abnormal embryos:* The genetically abnormal embryos diagnosed by preimplantation genetic test (PGT) can be used to obtain stem cells. However, such embryos

are not genetically normal and are mainly used in studies for certain genetic diseases. They can also be used for drug screening and gene therapy studies.

d) *Parthenogenetic embryos:* Parthenogenetic embryos result from spontaneous or manipulated activation and are followed by subsequent divisions of the oocyte without fertilization by a spermatozoon. Such embryos have only haploid maternal genomes and as a consequence, many of them do not develop further and rarely reach to blastocyst stage.

e) *Embryos obtained by nucleus transfer:* An embryo can be generated by injecting a nucleus isolated from a somatic cell into an enucleated oocyte (somatic cell nuclear transfer, SCNT). The blastocyst obtained by this method serves as a possible cell source for transplantation of the autologous graft.

4. Isolation of Embryonic Stem Cells

Pluripotent ESC lines were first derived from ICM of mouse blastocysts following culture conditions developed for embryonic carcinoma cells such as culturing on feeder cells or with the presence of serum (5). Later, protocols using serum-free media supplemented with certain cytokines such as leukemia inhibitory factor (LIF) preventing ESC differentiation were developed (6). ESCs can differentiate into various cell types and have the potential to form teratocarcinoma when injected into mice. Unlike embryonic carcinoma cells, ESCs are karyotypically normal and result in chimeras that include germline cells. This characteristic can be useful for specific research applications (for detailed review ref. 7). Similar to other animal models, hESCs are mostly derived from ICM of the blastocysts (5) although blastomeres from other developmental stages are also a source for pluripotent hESCs. The first isolation of hESCs from ICM was reported in 1998 much later than the culture of mouse ESCs (8). The reason for this delay was primarily due to insufficient knowledge of species-specific culture characteristics of ESCs and optimal culture media for hESCs.

In the laboratory application to isolate the ICM of the blastocysts, an opening is performed on the zona pellucida layer either mechanically, chemically, or by laser (assisted hatching). Subsequently, ICM can be isolated by different methods that are briefly summarized below.

a) *Mechanical dissection:* It is an efficient way to derive new lines of human embryonic stem cells. The technique is fast and no xeno-component is required. On the other hand, technical expertise is crucial for the proper isolation of ICM cells.

b) *Laser dissection:* This technique is frequently used and appropriate results are achieved in the xeno-free isolation of the ICM.

c) *Immunosurgery procedure:* The disadvantage of the method is the requirement of guinea pig serum.

d) *Microdissection:* It is a relatively easy method to isolate the ICM, but with a lower success rate.

e) *Minimized trophoblast cell proliferation (MTP):* This technique is used to obtain hESCs

from normal, abnormal, and cryopreserved embryos. The reported success rate is around 50% (9).

The first hESC lines were established using immunosurgery, which required the use of animal-derived products, including anti-human serum antibodies and guinea pig complement (10, 11). Later, it was observed that the use of resulting hESCs isolated with animal-derived products may not be suitable for transplantation due to the risk of inducing the host immune system and thus leading to graft rejection. Therefore, it is important that the ICM is isolated without using animal products (12, 13).

After the isolation, ICM cells form three-dimensional (3D) embryoid body structures *in vitro* and these cells can be cultured according to the application or methodology suitable for the research purpose. In recent years, hESC lines are produced following Good Manufacturing Practice (GMP) and several hESC lines have been derived *in vitro* (11, 14). The resulting hESC lines are characterized in terms of having pathogen-free nature, karyotypic stability, and pluripotency (15). Efficient and robust differentiation protocols are currently being developed. In order to use the resulting hESCs in therapeutic applications, it is necessary to obtain pure cell lines and to be able to eliminate undesired lineages and potentially tumorigenic cells (16).

5. Human Embryonic Stem Cells and Derivatives

5.1. Embryoid Bodies

The 3D embryoid body structures of human embryonic stem cells have significant potential to differentiate into many cell lineages and derivatives of all three germ layers. Various cell types such as neural cells, cardiomyocytes, hematopoietic precursors like cells, hepatocytes, and germ cells were developed by inducing embryoid bodies. Using these 3D structures, crucial mechanisms in developmental stages such as cell fate decisions, cell migration or differentiation in gastrulation can be investigated. Additionally, cellular effects after exposure to drugs or other compounds can be analyzed. The lack of proper control mechanisms in differentiation and in tissue localization constitutes a disadvantage for these cells (17).

5.2. Embryonic Stem Cell Derivatives

Following the first isolation of hESCs, many researchers have conducted studies and developed protocols to differentiate hESCs into various adult cell types such as pancreatic β cells, hepatocytes, neural cells, cardiomyocytes or retinal pigmented epithelial cells. These cells are called hESC-derivatives (18). Clinical trials have been conducted for the development of treatment protocols for their use in macular degeneration, spinal cord injury, and type 1 diabetes (18). In the effort to overcome the rejection of hESCs, specific applications such as the formation of patient-specific pluripotent SCs developed by SCNT were also performed in the last decade (19).

6. Immune Properties of hESCs

The expression of MHCs on the cell surface is the major immunologic barrier for the SCs in their therapeutic use. hESCs express MHC class I molecules on the cell surface and do not express MHC class II antigens (20,21). ESCs do not directly activate allogeneic T cells. However, the expression of immunogenic antigen molecules such as MHC class I and Oct4 can activate T cells through antigen-presenting cells. Additionally, the self-renewal features of the ESCs and their capability of spontaneous differentiation is another mechanism that causes the T-cell dependent allogeneic rejection (22). Thus, ESCs have the vulnerability to be immune rejected and eliminated in immunocompetent allogeneic recipients. Furthermore, uncontrolled spontaneous differentiation of the ESCs has the risk of teratoma formation after transplantation. To overcome these challenges studies were focused on the establishment of MHC-matched hESCs (23).

Although the immune suppressant regimen suppresses allogeneic responses, in addition to the side effects of persistent use, the toxic effect of immune suppressants on some of the hESC-derived progenitor cells is another challenge for hESC therapy (24).

There are contradicting reports on the immunogenicity of ESCs and long-term survival of the transplanted cells in different species (25). In some of the studies in mice, immune tolerance and prolonged graft survival was obtained after transplantation of hESC-derivatives (26, 27). However, such success could not be achieved in humans. The therapeutic potential of hESC-derived cells was also investigated using humanized mice (hu-mice). Hu-mice are specifically designed immunodeficient mice models that functional human tissues were transplanted (28). The hu-mice that human primary hematopoietic cells and tissues were engrafted and that do not express MHC class I and II antigens, is particularly used for transplantation of hESC-derivatives (29). In an experiment investigating neovascularization, hESC-derived endothelial cells, human embryonic fibroblasts, and cord blood–derived endothelial progenitor cells were transplanted into hu-mice. In the result of the study, the hESC-derivatives had a lower immunogenicity and this characteristic was related to the low level of MHC class I expression in hESC-derived endothelial cells (30).

7. Therapeutic Use of Embryonic Stem Cells and Derivatives

The possibility of treatment of various degenerative diseases by SC therapy is a promising approach (8). Although hESCs have long-term proliferative potential, unlimited self-renewal capacity in culture, and the ability to differentiate into all cell types in the body, the therapeutical applications were not progressed sufficiently. One of the reasons for this outcome is the involvement of social and ethical concerns about the source of the stem cells being a human embryo. Additionally, ESCs have the vulnerability to be eliminated in immunocompetent allogeneic recipients. One other reason involves the uncertainty about the physiology, maturity, or function of the hESC-derived cells. These cells have divergent

differentiation routes and problems are related to teratoma formation or immune rejection. hESCs have been widely discussed on both public and governmental platforms, and hESC research has been banned in many countries around the world. Although later approved in some countries, hESC research is allowed within strict regulations and there is still not enough information about the safety of their applications in treatment (9, 31-33).

On the other hand, the replacement of damaged or injured tissue with hESC-derived cells is promising potential applications, especially in degenerative diseases. Current research is focused on the development of the optimal protocols to control the differentiation of the derived cells and to produce safe and pure cells with reduced immunogenicity. One of the most researched derivatives is the hESC-derived cells for macular degeneration. Degeneration of retinal pigment epithelium (RPE) causes the loss of photoreceptors in many retinal diseases including macular degeneration, macular dystrophy, and retinitis pigmentosa and leads to progressive vision loss and blindness (34, 35). The mammalian retina has limited regenerative capacity and SC therapy is a promising treatment approach (36). In some rodent models, hESC-derived RPE was able to rescue the photoreceptors and thus loss of vision could be prevented (37). The results of initial experiments in humans had contradicting results regarding graft survival and improvement in vision (38-40). The first human trial using hESC-derived RPE cells was conducted in 13 patients diagnosed with age-related macular degeneration (41). However, complications were reported in the follow-up of these patients due to the use of systemic immunosuppressants (42). On the other hand, promising results were observed as well. After 1-year follow-up, the graft was present in 72% of the patients, and correction in visual acuity was observed in ten patients (42). In another study, the tolerability of transplantation of hESC-derived RPE cells was confirmed in the five years of follow-up (35, 43). Despite these promising results, the long-term safety of hESC-derivatives is still under debate. Studies with larger cohorts and with replicable protocols are still required.

8. Conclusion

Currently, several somatic cell therapy products have been in clinical use for a long time and have proven to be safe. Adult and embryonic stem cells are particularly attractive new sources for cell therapies. However, they still have their own risks in their clinical use. Especially in hESCs, their genomic stability as well as their unlimited proliferation and differentiation capacities makes them more advantageous than adult embryonic stem cells. Nevertheless, there are still technical, regulatory, clinical, ethical, legal risks and challenges related to the use of hESCs in biotechnology, pharmacology, basic scientific studies and regenerative medicine. Clinical treatment with derived cells derived from hESCs is a much newer field than other stem cell types. For this reason, it is vital to support these studies and increase their number in order to ensure that the product obtained can be used safely in a life-threatening manner.

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CHAPTER 15

THE IMMUNOGENICITY OF STEM CELL-BASED THERAPY FOR CARDIOVASCULAR DISEASE

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ABSTRACT

Cell-based therapy approaches for cardiovascular diseases (CVDs) causing heart failure (HF) have become an interesting topic in recent years. In these studies, stem cells often play a role in heart repair in two different ways. The first technique entails the generation of fresh cardiomyocytes to replenish lost heart tissue. This mechanism functions in accordance with the differentiation of stem or progenitor cells. The second application is paracrine stimulation. The best types of stem cells not only perfuse tissue but also discharge paracrine chemicals and extracellular vesicles that control the healing process, affect the host immune system, and aid in cell survival and restoration of heart function. This chapter presents preclinical and clinical research, and the processes and stem cell types that promote heart repair in cardiovascular illnesses.

Keywords: Cardiovascular diseases, cell-based therapy, immunogenicity, stem cells, cardiac repair

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, currently claiming more lives each year than cancer and chronic lower respiratory diseases combined (1). One of the most severe effects of CVD is heart failure (HF), which is induced by both acute and chronic illnesses including myocardial infarctions (MI), infections, or a number of concomitant conditions like obesity, hypertension, diabetes, and chronic kidney disease that lead to cardiomyocyte loss and/or malfunction. In the end, the long-term comorbidities result in molecular alterations in the heart that promote myocardial fibrosis and myocyte stiffness, as well as systemic inflammation, vascular dysfunction, and myocardial oxidative stress (2). The ability of the damaged myocardium to self-heal and create new cardiomyocytes is also quite limited (3). If immunogenicity issues can be resolved, transplantable cell therapy will be a promising treatment option for CVD patients for the regeneration of wounded heart tissue and restoration of post-MI cardiac function. The significance of gene variants in hereditary cardiac illnesses such as cardiomyopathies and primary arrhythmias, however, as well as the molecular mechanisms of action of modifier genes, was evaluated using a variety of induced pluripotent stem cell–cardiomyocyte models (4). This chapter presents studies on cell-based therapies for CVDs as well as applications of induced pluripotent stem cells.

2. Cell-Based Therapy Approaches in Cardiovascular Diseases

Since HF cannot be stopped or reversed in its progression, the majority of patients still have no other options for treatment except a heart transplant. Only a small number of patients can receive a new heart even though heart transplantation is the only remaining option for the treatment of end-stage HF (1, 5). The intended effect is frequently inevitable, even if the majority of pharmaceutical therapies to date have concentrated on changing hemodynamics (lowering afterload, regulating blood pressure, and blood volume) or cardiac myocyte function (1). Cell-based therapies, therefore, appear to be a potential therapeutic choice.

Studies on two approaches for cell-based therapeutics are continuing in light of recent advancements. Utilizing stem cells with healing capabilities is one of them, and using modified immune cells is the second.

According to knowledge gathered in recent years, treating CVDs with novel immunotherapy therapies is also an option. An immune response is used in immunotherapy, a cell-engineered strategy, to treat heart damage and heart fibrosis (6).

3. Cell-Based Treatment Approaches in Cardiovascular Diseases: Stem Cell Types

Current stem cell treatments for cardiovascular disorders have the potential to displace current medications. The use of stem cell therapy for CVDs was suggested in 2001 by preclinical study findings that first revealed enhanced infarction heart tissue repair and ventricular function (7). Twenty years after the initial experiments, researchers are currently assessing the potential for cardiovascular regeneration of several stem cell types (3, 5, 7, 8).

3.1. Embryonic Stem Cells (ESCs) and their Derived Cardiomyocytes (ESC-CMs)

The mouse experiment of Evans et al. (1981) was the first to reveal the existence of embryonic stem cells (ECS), which are specialized pluripotent cells produced from the blastocyst inner cell mass. Human blastocysts can be used to create pluripotent embryonic stem cells (10). The ability of ESCs to self-renew, differentiate into cell types from the three germ layers (endoderm, mesoderm, and ectoderm), and form embryonic bodies has been well investigated and are valuable characteristics for regenerative medicine (10). Although numerous *in vitro* methods have been developed to stimulate cardiomyogenic differentiation in ESCs, it has been difficult to produce significant numbers of mature and naive cardiomyocytes (11, 12). The formation of teratomas (tumors of mixed germ layers) has been linked to the transplantation of undifferentiated ESCs, despite the initial findings in animal studies suggesting that the cardiac environment is sufficient to cause the differentiation of transplanted ESCs into cardiomyocytes to repair damaged host tissue (13, 14).

It has become easier to separate cardiac myocytes from the embryonic body's mixed cell population, however, it is still unclear what kinds of cardiac myocytes are present in differentiated human embryonic bodies. Additionally, the requirement of the destruction of embryos to develop the derivative cells raises many ethical questions, and their use and development are banned in many countries (15). In conclusion, the clinical application of ESCs for the treatment of CVDs is still hampered by the challenges of developing the best *in vitro* techniques, the potential for immune rejection, genetic instability, the risk of teratoma formation, and ethical and legal concerns.

3.2. Induced Pluripotent Stem Cells (iPSCs)

In 2006, Takahashi and Yamanaka used the retroviral transfer of four transcription factors-c-Myc, octamer-binding transcription factor (Oct-3/4), SRY box-containing gene 2 (Sox2), and Kruppel-like factor (Klf4)-to produce pluripotent stem cells from mouse fibroblasts. These cells were named induced pluripotent stem cells (iPSCs), and it was shown that they had several common characteristics with mouse ES cells, including the ability to differentiate, proliferation, morphology, telomerase activity, and the expression of stem cell markers (16). The same team also produced iPSCs from human fibroblast cells a year later (17). Compared to ESCs, patient-specific iPSCs have several advantages, including no ethical concerns and a minimal risk of immunological rejection, which have made them a valuable resource in the treatment of cardiac disease (17-19). Similar to ES cell-derived cardiomyocytes, iPSCs express cardiac genes and can develop into cardiomyocytes with atrial, nodal, and ventricular-like action potential characteristics. Additionally, exactly like ESC-CMs, iPSC-derived cardiomyocytes respond to beta-adrenaline by increasing spontaneous heart rate and decreasing action potential processing (19).

Masumoto et al. created human iPSC-engineered cardiovascular cell sheets (CTSs),

enhanced heart function after transplanting the sheets into the rat MI model, and created a new human iPSC (hiPSC) differentiation protocol for cardiovascular cells. The produced hiPSC-CTSs appear promising for cardiac regeneration treatment, according to this result (20). In a pig MI model, Kawamura et al. demonstrated that their novel culture technique produced significant quantities of very pure hiPS-CM and that hiPS-CM sheet transplantation may enhance heart function following ischemic cardiomyopathy (21). Despite being almost undiscovered, iPSCs have been extensive research because of their potential for heart regeneration. Creating many cardiomyocytes from iPSCs through differentiation and avoiding teratoma formation by eliminating undifferentiated iPSCs are critical challenges, nonetheless. This is an important condition to be considered for the clinical application of human iPSCs (21-23).

3.3. Mesenchymal Stem Cells (MSCs)

To be able to differentiate into diverse mesodermal lineages, such as osteocytes, chondrocytes, adipocytes, hepatocytes, neurons, myocytes, and cardiomyocytes, mesenchymal stem cells (MSCs) should be self-renewing and multipotent (24). The surface markers CD11b, CD14, CD31, CD34, and CD45 are negative, while CD29, CD44, CD90, CD73, CD105, CD106, and CD166 are positive in MSCs grown in a normal culture medium (25). MSCs were initially identified from the bone marrow stroma, but can also be detected in other tissues, including the skin, fat, amniotic fluid, brain, spleen, and cord blood (26).

MSCs have several benefits for clinical use in cardiovascular diseases, including the ability to be derived from multiple tissues, the capacity to respond to the damaged host environment, the release of several factors that promote tissue remodeling, the absence of significant ethical concerns, and low immunogenicity and immunomodulatory properties (27). After transplantation, there is an increase in myocardial-specific marker proteins including troponin T, which is suggestive of the differentiation of MSCs into cardiomyocytes (28). Additionally, MSCs should possess immunosuppressive qualities that enable their administration in an allogeneic setting. A potent strategy to encourage heart healing is the suppression of the inflammatory response (29). MSCs may aid in heart regeneration, according to numerous experimental and clinical investigations. Studies have demonstrated that MSCs primarily assist cardiac repair through paracrine effects, even though they have been discovered to develop into cardiomyocytes and vascular smooth muscle by aiding in angiogenesis (19, 29). Kinnaird et al. have demonstrated that the production of VEGF and FGF by MSCs enhanced arteriogenesis, provided myocardial perfusion, and decreased tissue ischemia (30).

3.4. Cardiac Stem Cells (CSCs) and Cardiac Progenitor Cells (CPCs)

The capacity of the heart to regenerate itself is a subject of intense debate. Recent investigations have refuted the notion that the adult mammalian heart is a postmitotic,

specialized organ that cannot be rejuvenated. The heart can continue to make new cardiomyocytes and microvessels throughout life, albeit at a relatively sluggish rate, according to studies (19, 31). By the age of 25, cardiomyocyte regeneration occurs at a rate of 1% per year, and it steadily decreases with age (32). In a lifetime, less than half of the cardiomyocytes are replaced. This is accomplished by a limited number of self-renewing, multipotent cardiomyocytes known as resident cardiac stem cells (CSCs). The only restriction on CSCs is their myocardial phenotype (31). Considering cardiomyogenic differentiation as a potential of adult stem cells, cardiac progenitor cells (CPCs), which are ”preprogrammed” to become cardiomyocytes, may be a more valuable option for cardiac regeneration (29). Through direct differentiation, studies have demonstrated that CSCs and CPCs can support adult cardiomyogenesis (33-35).

According to their cell surface receptors, heart stem cells can be categorized as islet-1 (isl1), stem cell antigen-1 (Sca-1), MDR-1/ABCG2 (cardiac side population cells), and c-kit receptor (c-kit+). Beltrami et al. demonstrated that CSCs expressing stem cell receptor (c-kit +) can develop into cardiomyocytes, endothelial cells, and smooth muscle cells in the heart, enhancing heart function and shrinking infarct size (36). C-kit+ cells have received a lot of research due to their functional differentiation and integration into the host myocardium.

The microenvironment of the heart supports the maintenance of stem cells in undifferentiated condition and permits their release when required. It was discovered that the cardiosphere, a cell group isolated from atrial or ventricular biopsies, contained c-kit+ cells. Cardiosphere-derived cells (CDCs) have shown promising results in both animal and human studies (36-38). Many experiments were performed for C-kit+ cells due to their functional differentiation and integration into the host myocardium. The microenvironment of the heart supports the maintenance of stem cells in undifferentiated condition and permits their release when required. It was discovered that the cardiosphere, a cell group isolated from atrial or ventricular biopsies, contained c-kit+ CDCs have shown promising results in both animal and human studies (36-38).

3.5. Circulating Angiogenic Cells (CACs)

According to Asahara et al., endothelial phenotype-differentiating hematopoietic progenitor cells isolated from human peripheral blood contributed to the development of new capillaries and endothelial integrity following ischemia. In terms of self-renewal and clonogenicity, so-called endothelial progenitor cells (EPCs) resemble stem cells. On the other hand, EPCs are unipotent cells that can only develop into endothelial cells (19, 39). It is challenging to identify EPCs from endothelial cells and hematopoietic stem cells since they share several cell surface characteristics. EPCs have been discovered to express the hematopoietic stem cell surface marker CD34 (19) and the vascular endothelial growth factor receptor 2 (VEGFR2). EPCs are attractive possibilities for therapeutic usage after tissue

revascularization because of their ascribed vascular regeneration features (19, 40). In addition to all this data, it is currently understood that the therapeutic benefits of EPCs are mediated by extracellular vesicle (EV)-mediated "paracrine" pathways. The use of paracrine factors is an attractive method in regenerative medicine. According to *in vivo* animal studies, the infusion of EPC-derived EVs in a shear-thinning gel (STG) leads to an increase in peri-infarct vascular proliferation, the preservation of ventricular shape, and enhanced post-MI hemodynamic performance. But it is proposed that to use this tactic, significant studies using animal models and ultimately clinical human applications are needed (41).

4. Preclinical Trials of Stem Cell Therapy for Cardiac Regeneration

Cardiovascular diseases remain the leading cause of death globally, despite advancements in treatments. Many people still succumb to the disease within a year of their first heart attack. It is therefore important to develop new treatments for HF and heart attacks. However, before these treatments can be used in humans, it is necessary to test them in animals.

Although stem cell therapy shows great potential, it is crucial to identify the most effective approach that will work in humans. Animal studies are essential for finding the best treatment. Small animals, like rodents, have proved helpful for evaluating new treatments because they can be tested quickly and inexpensively, and their group sizes are large enough to provide acceptable statistical power. However, to determine if the treatments will be functional in humans, it is necessary to test them on other animals like dogs, sheep, and pigs. Such animal studies are expensive but provide a better understanding of how treatments will work in humans (42).

4.1. Preclinical Studies of MSCs

MSCs are commonly used to treat heart problems because they can modulate the immune system. MSCs of the bone marrow and adipose tissue are able to differentiate into bone, fat, and cartilage cells *in vitro* and develop into heart muscle cells. Tests have shown that bone marrow MSCs can support heart function in small and large animals after a heart attack. Studies in various animal models of MI have shown that the therapeutic potential of MSCs contributes to myocardial regeneration by engrafting injected cells into the myocardium (43-45). In an early clinical trial following animal study, they reported significant improvement in left ventricular function compared to the MSC group and control group at six months of follow-up (46). The MESAMI phase 1 study by Guijarro et al. demonstrated the feasibility and safety of MSC transplantation after chronic ischemia (47).

4.1.1. Preclinical Studies of Bone Marrow-Derived MSCs (BM-MSCs)

Bone marrow (BM) is a valuable source of multipotent precursors for stem cell therapy, including MSCs, mononuclear cells, and endothelial progenitor cells. These studies in animals have shown positive results in treating acute myocardial infarction (AMI), including

reduced apoptosis and fibrosis and increased VEGF expression and regional blood flow (48). BM-MSCs have also been shown to be effective in the treatment of idiopathic dilated cardiomyopathy (DCM) in model animals. In these studies, an increase in left ventricular end-systolic pressure (LVESP) and a decrease in left ventricular end-diastolic pressure (LVEDP) was demonstrated (49, 50).

Both autologous and allogeneic types of MSCs derived from bone marrow were effective in treating acute and chronic myocardial infarction in pigs. MSC therapy acts by secreting growth factors, microvesicles, and exosomes, which provide therapeutic benefits (51, 52). It has been discovered that BM-MSC therapy can reduce fibrosis and reverse remodeling in conditions of chronic ischemic cardiomyopathy (44). Allogeneic MSCs were examined in a study employing a pig model of chronic MI and shown to be efficient and secure, enhancing myocardial blood flow, left ventricular ejection fraction (LVEF), and regional contractility when compared to placebo (53).

Ding et al. reported that when they treated BM-MSCs with Jagged1 (a ligand of the Notch pathway), activation of Notch1 signaling promoted differentiation into cardiomyocytes by contributing to multi-lineage differentiation of BM-MSCs (54). In another study, overexpression of miR1-2 in mouse BM-MSCs increases the expression of wnt11, β-catenin, TCF, and JNK in the Wnt/β-catenin signaling pathway was demonstrated. The findings of this study supported the hypothesis that the activation of the Wnt/β-catenin signaling pathway induces cardiomyocyte-like differentiation of BM-MSCs (55). The potential of these stem cells for cell engineering is still under investigation.

4.1.2. Preclinical Studies of Stro-1+ BM-MSCs

Among the BM-MSCs, the Stro-1^{+}/$CD34^{-}$ cells comprise a subpopulation that can differentiate into various cell types, including chondrocytes, adipocytes, chondroblasts, and smooth muscle cells. In animal studies, using MPCs has shown good results. The intracoronary injections of allogeneic MPCs reduced scar size by 40% and increased vascular density by 50% in ovine research with myocardial infarction (56). In a different trial in ovine models, intramyocardial injections of allogeneic MPCs four weeks after a MI was performed, and enhanced LVEF, wall thickness, and vascular density were demonstrated (57). According to a recent study, the administration of two doses of mesenchymal stem cells in rats with dilated cardiomyopathy was found to be more effective than a single dose in restoring left ventricular function (58).

4.1.3. Preclinical Studies of Adipose Stem Cells (AdSCs)

Adipose tissue gives rise to adipose stem cells (AdSCs), which can differentiate into a variety of cell types. AdSCs share similarities with bone marrow stem cells and are easier to isolate. They have been shown to enhance the function of the failing heart. Rasmussen et al. conducted a study that showed AdSCs improved LVEF when injected into rats 1-week

post-MI, but neither cell type reduced infarct size nor promoted angiogenesis (59). Also, adipose-derived cardiomyogenic cells (Ad-CMG), which are isolated from AdSCs, were studied and found to reduce remodeling, increase vasculogenesis, and stabilize EF when injected into the coronary artery of mice three days after the ligation of the left anterior descending artery (LAD) (60). However, not all research has reported a role for AdSCs in cardiac repair. Intracoronary injection of allogeneic AdSCs was determined to increase perfusion but not LVEF in a pig model. The reason for this was attributed to the fact that the efficiency of the cells may have been altered by the delivery method (61).

4.1.4. Preclinical Studies of Umbilical Cord MSCs (UC-MSCs)

Fibroblast-like cells from Wharton's jelly (WJ) in umbilical cords can be extracted without harming the donor and quickly grow in large quantities. Compared to BM-MSCs, UC-MSCs express more genes that involve in cell adhesion, proliferation, immune system activity, and neurotrophic support. In studies with mice and rabbits, hUC-MSCs have been found to improve heart function and reduce inflammation in individuals with heart disease (62). In pigs, hWJ-MSCs have been shown to improve blood flow in the heart, reduce cell death, and improve heart function after a heart attack (63).

4.2. Preclinical Studies of CSCs and CPCs

Cardiovascular progenitor cells, which aid in heart damage repair, are produced by stem cells. However, after significant infarctions, these endogenous stem cells are exhausted and unable to fully repair the damage. In significant studies, CSCs from mice have been shown to produce cardiomyocytes, smooth muscle cells, and endothelial cells after being injected into rats with myocardial infarction (64). In immunodeficient mice with an AMI model, Oskouei et al. compared c-kit+ human CSCs with hMSCs for heart repair. The hMSCs were 30-fold more effective in reducing scar size than the CSCs at improving hemodynamic parameters. In both mouse and pig models of acute MI, overexpressing Pim1 kinase in c-kit+ hCSCs increased their retention and therapeutic efficacy (65). In a study on pigs with chronic ischemia and heart attack, c-kit+ CSCs were given through the coronary artery three months after the heart attack and showed positive results in treatment. In addition to engraftment and differentiation into CMs and vascular structures, the treated group has displayed an improvement in LVEF and regional cardiac function (66).

In a meta-analysis study investigating the therapeutic effect of CSCs, a significant improvement was observed in the LVEF (10.7%) of large and small MI animal models compared with placebo controls. In addition, in animals treated with CSC, a reduction in the healing effect was noted in large animal models compared to small animal models (67). Fanton et al. demonstrated that atrium-derived CSCs stimulate blood vessel formation by paracrine mechanisms as well as improve myocardial function through cardiomyogenic differentiation. The contribution of CSCs to cardiac angiogenesis improved their therapeutic

potential in ischemic heart diseases (68). Until now, although there are no clinical studies of CSC transplantation, preclinical studies suggest that arrhythmia and tumorigenesis have not been observed in *in vivo* transplantation, so the use of CSCs in treatment is considered a safe approach (69, 70).

4.3. Preclinical Studies of Cardiosphere-Derived Cells (CDCs)

Cardiospheres are a group of stem cells from the heart that cluster together. These cells are called cardiosphere-derived cells (CDCs) when expanded *ex vivo*. CDCs have been studied as a therapy for heart attacks. Cardiosphere-derived cells have been shown to improve angiogenesis and heart function while minimizing scar size in animals that had heart attacks (37). In addition, using CDC to improve diastolic dysfunction in senescent rats induced biological rejuvenation of the heart (71). Exosomes produced by the CDC are probably the main cause of the therapeutic effects.

4.4. Preclinical Studies of Bone Marrow Mononuclear Cells (BM-MNCs)

The cell types that constitute BM-MNCs include MSCs, hematopoietic stem cells (HSCs), and EPCs. They are easier to isolate than individual cell types, but clinical trials suggest they are less effective than MSCs. Early studies in rats found that injecting BM-MNCs into the heart muscle promoted vasculogenesis, however, vascularity decreased four weeks later (72). In a different study, direct injection of BM-MNCs into the peri-infarction site after ligation of the LAD showed promising results. BM-MNCs had favorable effects on ventricular function in a canine model (73), but the same effect could not be shown in studies with sheep (74). Preclinical studies demonstrated that BM-MNCs enhance neovascularization and reduce scar size, but large animal models have had variable effects on ventricular function.

4.5. Preclinical Studies of Hematopoietic and Endothelial Progenitor Cells (HSCs and EPCs)

Transferring cells from bone marrow to mice led to the discovery of cells that can regenerate. HSCs and EPCs are $CD34^+/CD133^+$ cells that can differentiate into lymphoid or myeloid cells. They can become endothelial cells and aid in the formation of new blood vessels after transferring from the bone marrow to the circulation as a result of tissue injury.

Studies on mice that had had an AMI revealed enhanced neovascularization, EF, a smaller scar, and the differentiation of HSCs into endothelial cells in the myocardial tissue (75, 76). EPCs can be modified to increase their capacity for repairs. Thal et al. evaluated the capacity of EPCs that had undergone epigenetic reprogramming to differentiate into cardiomyocytes and support cardiac repair following AMI (77). However, it has been reported that modified EPCs (5-azacytidine, valproic acid) function significantly better than unmodified EPCs, which reduce infarct size, decrease left ventricular volume, and increase capillary density (77). Kawamoto et al. showed that ex vivo expanded and intravenously administered EPCs are

involved in myocardial neovascularization foci in ischemic hearts and improve left ventricular function after MI (75). Kupatt et al. reported that transplantation of clonal embryonic EPCs derived from 7.5-day-old mice into pigs (eEPCs) improves myocardial function by reducing ischemia-reperfusion injury (78). In addition, clinical studies have shown that EPCs improve cardiac function in humans (79, 80).

4.6. Preclinical Studies of iPSCs and ESC-CMs

Initial studies in the MI animal model have shown that iPSC-CMs can integrate into host tissue, improve heart function, and attenuate reverse remodeling processes (21, 81-85). But a rat model of MI showed that iPSC-CMs engrafted long-term but failed to produce beneficial effects (86). Although Shiba et al. showed that transplantation of ESC-CMs to the injured heart in the pig MI animal model might protect against arrhythmias, contract simultaneously with host cardiomyocytes, and significantly improve heart function (87), another monkey MI model study reported that ESC-CMs improved remuscularization of the infected heart but still caused potential arrhythmic complications (88). In addition to these complications, studies have remained at the preclinical level due to the risk of immune rejection and the teratoma-forming potential of ESCs. On the other hand, combined stem cell approaches have also been tested in model animals. In a large animal model study using this approach, the combination of hiPSCs and hMSCs was shown to increase capillary density, likely secondary to decreased rates of apoptosis (89). A sheet of hiPSC-CMs generated from dermal fibroblasts enhanced angiogenesis and reduced *left ventricular* (LV) remodeling in an ischemia swine model (21). Studies on ESC- and iPSC-CMs used non-human primates, and after three months, hESC-CMs were found to contribute to development, greatly assist in the re-muscularization of infarcted tissue, and display regular calcium transitions. However, arrhythmia was observed in all monkeys (88). Cells derived from hPSCs have been found to improve cardiac function and reduce apoptosis, but not remuscularization (90).

5. Clinical Trials of Stem Cell Therapy in Cardiovascular Medicine

Stem cell therapy for cardiac repair is a promising approach that has sparked much excitement in the field of cardiovascular medicine. Although it was once believed that the heart could not regenerate, it is now generally accepted that between 1% and 2% of cardiomyocytes are replaced every year, with a rate that decreases with aging (32). Cardiac remodeling is characterized by an ongoing inflammatory response following acute stress and during chronic pathologies: increased oxidative stress, myocyte apoptosis, unbalanced oxygen consumption, energy metabolism, extracellular matrix formation causing scarring, endothelial dysfunction, decreased capillary density, and neovascularization. It is possible to prevent these consequences and encourage heart healing using stem cells and other cell-based therapies. When viewed strictly, stem cells have the capacity for both self-renewal and differentiation,

in contrast to other cell-based therapies that function by transmitting molecules that activate endogenous regenerative pathways.

The transport of small molecules and mitochondria through gap junctions and tunneling nanotubes, respectively are the primary mechanisms of direct communication between stem cells and host cells. Exosomes, growth factors, cytokines, and metalloproteinases are also released by them and aid in cell regeneration. Exosome secretions are also an important way for cardiac cells to communicate (91). Despite the complexity and incomplete understanding of the mechanisms underlying stem cells and other cell-based therapies, current evidence is consistent with the notion that both methods enhance cardiac structure and function.

5.1. Route of Delivery

Intracoronary administration has the advantage of having little inflammation, but it also leads to poor cell retention in the heart (92). Stem cells are directly injected into the myocardium through the endocardium using a procedure called a transendocardial stem cell injection (TESI). Although there is a slight chance of perforation and arrhythmias with this procedure, it is more effective in certain pathologies and retains more cells than other approaches (93). The least intrusive delivery method is intravenous, and it makes use of physiological attraction cues that cause cellular homing to the site of injury (59). However, there are issues with implantation and retention in this application. According to the meta-analysis by Kanelidis et al., TESI has better efficacy than intracoronary administration (93). To determine the best delivery route, more research is required.

5.2. Acute Myocardial Infarction

Over 100 studies have evaluated BM-MNCs, which are excellent for use in the context of AMI. BM-MNC trials were first carried out in an AMI environment. TOPCARE-AMI, one of the earliest clinical trials evaluating the therapeutic efficacy of BM-MNCs, was one of these. The BM-MNCs tested in this study were administered on average five days after an AMI (94). The trial revealed a markedly improved LVEF and a smaller scar. The REPAIR-AMI experiment, which was conducted to evaluate the effectiveness of BM-MNCs, is the largest phase III, double-blinded, placebo-controlled clinical trial to date (94).

Although the initial studies were intriguing, numerous follow-up studies had contradicting results. Because of their ability to produce angiogenic cells, preclinical findings suggested that a subpopulation of $CD34^+$ BM-MNCs would be a better candidate for AMI (95). As a result, the PreSERVE-AMI trial, the biggest stem cell trial for AMI in the US, was carried out. In this trial, there was no improvement in LVEF or myocardial perfusion at rest (96). The WJ-MSC-AMI experiment, which showed that MSCs generated from human umbilical cords enhanced LVEF while lowering end-systolic volume (ESV) and end-diastolic volume (EDV), was conducted (97). The phase I/II, randomized, double-blind, placebo-controlled CAREMI trial also examined the therapeutic efficacy of allogeneic CSCs. Although cardiac stem cells

were shown to not significantly reduce the scar size, left ventricular volumes, LVEF, or local wall motion after one year of follow-up, they could be administered safely with no mortality and low immunity (98).

In the ALLSTAR (Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration) study investigating the efficacy of allogeneic CDCs in improving myocardial function and contractility, it was reported that the administration of CDC in patients with LV dysfunction after MI showed improvement in segmental myocardial function (38).

5.3. Chronic Ischemic Cardiomyopathy (ICM)

Stem cell therapy in AMI has inspired researchers to investigate its use in chronic ICM, seeking to restore normal function. Although mainstream treatment focuses on attenuating left ventricular enlargement, cell therapy aims to be curative rather than palliative. Researchers can utilize and cultivate a variety of cell types to maximize therapeutic effects in chronic conditions like ICM. One of the most studied cell types for use in ICM is BM-MNCs. A study conducted by Perin et al. was the first to investigate the effects of BM-MNCs on patients with ICM (99). The study demonstrated significant increases in LVEF and decreases in ESV in treated patients. Similarly, in the TOPCARE-CHD study, it was determined that BMMNC treatment showed significant gains in LVEF, and even cell therapy did not reduce scar size compared to placebo (100). In the FOCUS-CCTRN study in patients receiving BM-MNC, no significant improvement in LVEF or infarct size was found (101). Cardiospheres are different types of cells that might be used to treat ICM. A phase I randomized trial called CADUCEUS in patients with ICM was founded on promising preclinical research (102). Despite a 42% reduction in scar size in the treatment group, CDC therapy has been shown to fail to increase LVEF, decrease left ventricular volumes, or improve quality of life. The ALLSTAR trial using CDCs had to be suspended because the primary endpoint was not reached (103).

5.4. Non-Ischemic Dilated Cardiomyopathy

Non-ischemic dilated cardiomyopathy (NIDCM) is a serious medical condition characterized by enlargement of the left ventricle of the heart and not caused by clogged blood vessels in the heart. It is the leading cause of death in heart transplant recipients.

To address this issue, medical researchers have conducted several clinical trials to test various cell therapies. The use of bone marrow mononuclear cells (BM-MNCs) has shown positive results in the TOPCARE-DCM (104) trial, improving LVEF and quality of life (QoL) in NIDCM patients. However, the MiHeart trial did not show any significant changes (105).

The use of mesenchymal stem cells (MSCs) is another promising treatment. The immunomodulatory, reverse remodeling, and regenerative abilities of MSCs are well documented. Allogeneic MSCs were discovered to considerably improve functional metrics and LVEF in the POSEIDON-DCM study (106). The study also showed that patients who did not have a pathogenic genetic variation reacted to cell treatment more favorably.

The administration of $CD34^+$ cells has also consistently improved LVEF, 6-minute walk distance (6MWD), BNP levels, and survival in NIDCM patients (107). TESI has been found to produce higher engraftment and therapeutic efficacy compared to other methods of delivery.

6. Immunogenicity in Stem Cell Therapy

Previous preclinical and clinical studies have shown that cell-based therapy for MI and ICM reduces scar size in the heart. These investigations (52, 53, 108, 109) examined the impact of autologous and allogeneic MSCs on restoring cardiac functioning. It has been demonstrated that administering xenogeneic or autologous CSCs along with MSCs to immunosuppressed model animals or humans results in significant improvements in cardiac function (66, 110-112). Although autologous MSCs and CSCs work together to enhance the function of injured cardiac tissue, allogeneic (allo-) MSCs are immunoevasive, and it's debatable whether or not CSCs can do the same. Although this is anticipated to limit the success of allogeneic cell combination therapy (ACCT), a previous study showed that ACCT synergistically promotes cardiac regeneration and left ventricular functional recovery without provoking immunological reactions in a swine model of chronic ischemic cardiomyopathy (113).

Allogeneic MSCs (allo-MSCs) harvested from young healthy donors are more advantageous in potency and availability compared to autologous cells (114), and there is additional evidence from clinical studies that allogeneic cell-based therapy may be superior to autologous cell therapy (107, 115, 116). The use of autologous stem cells in therapy has some disadvantages, such as the individual's own diseases and morbidity risk. On the other hand, although the main concern with allogeneic stem cells is their potential for immune rejection, MSCs may evade the immune response through the release of immunomodulators (117-121), inhibit T-cell proliferation, trigger anti-inflammatory subsets (121), and lack of class II major histocompatibility complex (MHC) (122). Allogeneic CSCs (allo-CSCs) may be used as allografts alone or in combination with MSCs (ACCT) (113), however, the immunoinvasive abilities of these cells and their MHC class I and II expression rates are unclear. Preclinical studies have shown that allo-CSC is tolerated immunologically in rats (37, 123).

The transplantation of pluripotent stem cell-derived cardiomyocytes (PSC-CMs) has some obstacles, including post-transplant tumor formation, arrhythmias, and immune rejection (124). Viral vectors, which are used as the most effective method of transferring Yamanaka's transcription factors (Sox2, Oct3/4, Klf4, and c-Myc) in the formation of induced PSC-CMs (iPSC-CMs), have the possibility of causing mutations after transplantation (125). Sustained and/or non-sustained ventricular tachycardia episodes have been observed after intramyocardial transplantation in primates (126, 127). Long-term use of immunosuppressive drugs used to prevent immune rejection that can be observed after allogeneic transplants can cause life-threatening infections, malignancies, and renal failure (124). Although it is

theoretically predicted that autologous iPSCs will not cause immune rejection, it is claimed that immune rejection may occur during reprogramming due to genetic and epigenetic variations (128, 129). MHC mismatch is the first step in immune rejection, and to avoid it, a previous study used CRISPR-Cas9 (clustered regularly spaced short palindromic repeats/associated proteins 9) to knock out the β2 microglobulin and MHC class II genes in iPSCs. In this study, it was observed that the pluripotency of these cells did not change, and T cell activation did not occur (130). Indeed, it was determined that MHC I-negative cells such as stem cells and cancer cells were rejected by natural killer (NK) cells (131, 132). NK cells infiltrating graft sites have been demonstrated after intramyocardial transplantation of MHC I knockout iPSC-CMs (133).

7. Immune Response After Stem Cell Transplantation

In allogeneic organ or tissue transplantations, immune rejection is observed as a result of immune reactions such as cytokine release, inflammation, and cytotoxic mechanisms caused by immune cells against the graft. In the first stage of this process, the recipient's lymphocytes are activated and proliferate by recognizing the antigens (MHC classes I and II) on the graft. Usually, the severity of the immune rejection response depends on the percentage of MHC mismatches (134). The second stage is an acute process that begins within the first few weeks after transplantation with T cell activation and entails the destruction of the graft, depending on the transplanted tissue or organ (135). In comparison, patients with chronic immune rejection have a reduced acute response through the use of immunosuppressive drugs, however, the rejection occurs after a long period of time or even many years later.

The antigens that are mainly responsible for the genetic background of the different tissues or cells transplanted into an individual are histocompatibility antigens, which are products of tissue compatibility genes. Histocompatibility antigens are encoded at more than 40 loci, and the loci responsible for the most severe allograft rejection reactions are on the major histocompatibility complex (MHC), known as the human leukocyte antigen (HLA) system. Combinations of several minor antigens may elicit strong rejection responses, while other minor antigens alone cause weaker reactions. HLA genes are located on the short arm of chromosome 6 and are co-dominantly expressed. Polymorphic HLA genes have three major class I alleles (HLA-A, -B, and -C) and three major class II alleles (HLA-DR, -DQ, and -DP). HLA mismatch has a significant impact on prolonging graft survival, as a closely HLA-matched graft is less likely to be recognized and rejected (136). The class I molecules of MHC are normally expressed on all nucleated cells in the body, while MHC class II molecules are expressed only on antigen-presenting cells (APCs), such as B cells, macrophages, and dendritic cells.

The immune response consists of both cellular (lymphocyte-mediated) and humoral (antibody-mediated) rejection mechanisms. T cells play a central role in graft rejection.

The immune response to antigens begins only when MHC class I and II molecules form a complex with an antigenic peptide, activating $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells, respectively (135). During T cell activation, hydrolysis of membrane-bound inositol phospholipids to diacylglycerol (DAG) and IP3 leads to cytokine production by stimulating molecular cell pathways including protein kinase C (PKC) activation and activation of nuclear factor kappa B (NFkB). In the immune response, the final common pathway for cytolytic processes is the induction of apoptosis in the target cell (137). After activation of cytotoxic T lymphocytes (CTLs), they dissolve the transplanted tissue by inducing target cell apoptosis by forming cytotoxic granules containing perforin and granzymes (138, 139).

In humans, T-cell-mediated hypersensitivity and cytotoxicity play important roles in graft rejection. Regulatory T cells (Tregs), which help maintain the body's immune tolerance to prevent excessive activation of the immune response, are involved in the prevention of acute and chronic rejection in allogeneic transplantation (140, 141).

It is known that acute activation of the immune response after allogeneic adult stem cell transplantation may be devastating for hosts. However, it has been shown that the acute inflammatory response that occurs in stem cell therapy applied to the damaged area in the heart of patients with MI may provide therapeutic benefits due to the wound healing response (142). In this study, it was shown that the acute immune response after cell transplantation, which leads to the activation of $CCR2^+CX3CR1^+$ macrophages, would change the activity of fibroblasts, decrease myocardial fibrosis in the border region of myocardial ischemia, and also increase the mechanical properties of the infarct region (142).

Cell-based therapies derived from bone marrow or heart tissue and reintroduced to the infarcted heart, called adoptive transfer of autologous stem cell populations, have been generally applied clinically recently (143-145), and as expected, these stem cells mainly transdifferentiate into new cardiomyocytes rather than injured cardiomyocytes. It was suggested that cell-based therapy mainly has modulatory effects on inflammation developed in the tissue (143, 144, 146). The paracrine hypothesis of stem cells has been shown to produce many different types and amounts of cytokines, growth factors, exosomes, and small non-coding RNAs in their surrounding microenvironment (147-149). Factors released from stem cells have been shown to interact directly with neutrophils, M8s, B-cells, and T-cells (147-150). The effectiveness of cell-based therapies in modulating the immune response after myocardial injury varies according to the type of immune cells interacting with stem cells (143).

8. Conclusions

There are many studies with different research designs on stem cell therapies for cardiovascular diseases. Although stem cell therapy approaches are promising, more studies are needed comparing delivery routes and doses of stem cells. In addition, the safety and

efficacy of the cell therapy should be monitored with long-term follow-up studies. With the increasing knowledge in molecular biology, genetics, and tissue engineering, it is predicted that stem cell therapies will be standardized in the near future and will provide more benefits to individuals with cardiovascular disease.

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