How Combination Therapy Supporting Immunotherapy for Tumor Disease

Abstract: Chemoimmunotherapy was a "confused phenomenon" in an early clinical study. Cytotoxic chemotherapy was immunosuppressive while, in the clinic, chemoimmunotherapy demonstrated supporting immunotherapy. To increase ACT (adoptive cell transfer) immunotherapy and decrease the effects of chemotherapy for Tumor-infiltrating lymphocytes (TILs) to treat solid tumors, we began to study chemoimmunotherapy by isolating and culturing primary tumor cells and immune cells from the removed solid tumor tissues before 1994. After about 30-40 years of efforts, results of combination treatment have demonstrated that chemotherapy combined with immunotherapy (called chemoimmunotherapy) is better than monotherapy; now there are four fields to be developed for the combination, including (1) Lymphodepletion (LD) supporting ACT immunotherapy for Advanced Cancer, (2) Chemoimmunotherapy (CI) supporting treatment for Advanced Cancer, (3) Immune Checkpoint Inhibition (ICI) combined with immunotherapy, and (4) Precision Medicine (PM) supporting immunotherapy for Advanced Cancer. Precision therapy can cover all three, as described above, as well as LD, chemoimmunotherapy, and ICI combination. Techniques for precision therapy include tumor tissue biobanks, single cell technique, clinical genomics, and artificial intelligence to support the combination treatment. Overall, the latest generation of combination therapies is more specific and sensitive in treating neoplastic diseases than older versions with fewer side effects. Based on 30–40 years of R&D to improve immunotherapy for patients with advanced cancer and based on increasing research, it is time to define combinations treatment and evaluate the efficacy of combination treatments for oncological diseases

Introduction

In 1986, Dr. Rosenberg discovered that isolated and cultured Tumor-infiltrating lymphocytes (TILs) from tumor tissue could be applied to the adoptive cell transfer (ACT) to treat advanced melanoma [1]. In addition, they also used a combined CTX before TIL infusion therapy for the treatment of tumor patients so that CTX could eliminate lymphocytes *in vivo* to support ACT immunotherapy [2]. Lymphodepletion (LD) is routinely used in ACT immunotherapy, such as CAR-T and TIL therapy [3-4]. After about four decades of efforts, some research on combination therapy has demonstrated that chemotherapy coordinating with immunotherapy (called chemoimmunotherapy) is better than monotherapy; however, understanding regimens of combination therapy also includes chemotherapeutic drugs, optimal drug dose, administration time, and sequence of chemoimmunotherapy is still not clear [5-6].

We have discovered the "confused phenomenon" in our early study. To address the phenomenon, we began to study both tumor cells and immune cells from the removed same solid tumor tissues, which were published in 1994 [7]. We aimed to increase TIL ACT efficacy and decrease chemotherapy toxicity to ACT immunotherapy at that time. Our strategies included that both TIL and primary tumor cells culture from the solid tumor tissues so that the cultured TILs from solid tumors are activated and then infused into patients for ACT immunotherapy. At the same time, the primary tumor cells from the tumor tissues are utilized as *in vivo* and *in vitro* chemosensitivity tests for specific chemotherapy drugs to decrease chemotherapy toxicity to ACT immunotherapy. As our early report for combination therapy for patients with solid tumors by TIL re-infusion and chemotherapy using an *in vitro* chemosensitivity test, we discovered the combination was better than the monotherapy. Following thirty years of research for combination

treatment, we have developed precision medicine, including tumor tissue biobanks, single cell technique, clinical genomics, and artificial intelligence to support the combination treatment. Overall, the latest generation of combination therapies is more specific and sensitive in treating neoplastic diseases than older versions with fewer side effects [8-12].

Based on 30–40 years of R&D to improve immunotherapy for patients with advanced cancer and based on increasing research, it is time to define combinations treatment and evaluate the efficacy of combination treatments for oncological diseases. According to the current publications, the manual will introduce four sections for combination therapy: (1) Lymphodepletion (LD) supporting ACT immunotherapy for Advanced Cancer, (2) Coordinated Therapy (Chemoimmunotherapy) supporting treatment for Advanced Cancer, (3) Immune Checkpoint Inhibition (ICI) combined with immunotherapy, and (4) Precision Medicine supporting Immunotherapy for Advanced Cancer.

Lymphodepletion supporting ACT immunotherapy to treat advanced cancer.

Over the past 40 years, adoptive cell transfer (ACT) for the treatment of malignancies has been one of the most dynamic and fruitful advances in cancer therapy [13-14]. To support ACT efficacy, in 1986, Dr. Rosenberg first used CTX as Lymphodepletion (LD) to support the TIL reinfusion for overcoming immune tolerance in patients with metastatic melanoma [15]. Other solid tumor diseases have also routinely used LD to support TIL reinfusion in clinics [16-17], as shown in Fig-1. Moreover, CAR-T (chimeric antigen receptors) has become an essential antitumor therapy component [18]. However, a significant number of these patients still relapse after CAR-T therapy or become resistant to CAR-T therapy [19-20], so they need to improve the efficacy of CAR-T therapy by LD to play an essential role in overcoming the resistance to CAR-T therapy [21-22].

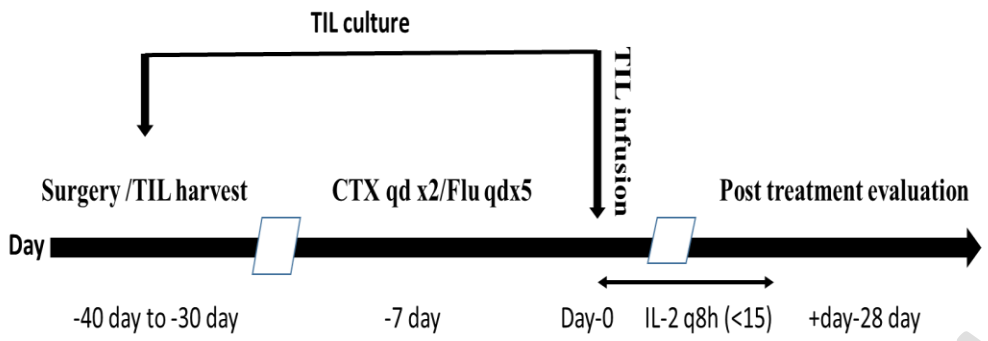

Fig-1. Clinical strategies for lymphodepletion to support ACT

The main goals of LD are to (1) reduce endogenous lymphocytes to inhibit the engraftment of CAR-T infusions and support their long-term activity; (2) reduce tumor cells to avoid rapid exhaustion of CAR-Ts; and (3) prepare and reprogram the microenvironment and soluble factors to ensure optimal engraftment, homing, and long-term survival of CAR-T cells [23-25]. As in Table 1, there are several LDs, such as fludarabine (Flu) and cyclophosphamide (CTX, Cy), and their combination is selectively used for individual situations. LD regimens and doses vary depending on the target disease (such as ALL, NHL, MM, or solid tumors) and the source of T cells (autologous vs. allogeneic) [26-28]. Because a patient receives LD with individual differences, personalized regimens still need to be considered for LD selection with their doses for the new TIL or CAR-T therapy approaches.

Table-1. Lymphodepletion

Types	Components	Common lymphodepletion	Common usage
Fludarabine	purine analogue	Car-T	CLL and indolent NHL
Cyclophosphamide	an alkylating agent	TIL or Car-T	Melanoma, lymphoma, and indolent NHL
Flu and Cy	both action	Car-T	CALL

Bendamustine	an alkylating agent	Car-T	CLL and other B- and T-cell lymphomas
Alemtuzumab	Targeting	Car-T	lymphoproliferative diseases
Oxaliplatin/cy	Targeting	Car-T	CAR-T to solid tumors
Clofarabine	anti-leukemic agent	Car-T	clinical remission

Chemoimmunotherapy combinations supporting immunotherapy.

Chemotherapy has been a critical part of cancer treatment for more than 70 years [29]. The cytotoxic drugs kill tumor cells and inhibit their proliferation through DNA damage, inhibiting DNA replication and arresting mitosis. However, cytotoxic chemotherapy is widely considered to be immunosuppressive because it can cause dose-dependent myelosuppression, suggesting antagonism with immunotherapy [30]. Before 1994, to increase ACT immunotherapy and decrease side effects from chemotherapy for TIL to treat solid tumors, we began to study chemoimmunotherapy with techniques for primary tumor cells and immune cell separation and culture from the removed solid tumor tissues [31-33]. That time, to increase TIL efficacy and decrease toxic chemotherapy, we study chemoimmunotherapy through which the cultured TILs from solid tumors are activated and then infused into patients for Adoptive Immunotherapy. At the same time, the primary tumor cells from the tumor tissues are utilized as *in vivo* and *in vitro* chemosensitivity tests for chemoimmunotherapy [34-35], as shown in Fig-2.

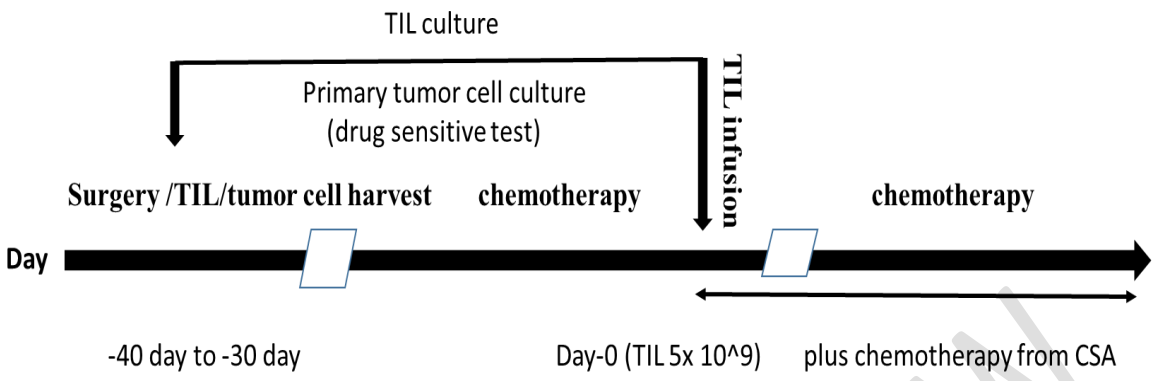

Fig-2. Early clinical chemoimmunotherapy strategies increasing TIL efficacy

After more than thirty years effort, now chemoimmunotherapy combination regimens may have shown advantages over monotherapy: (1) they can maximize cancer elimination within the range of tolerable toxicity; (2) target a wider range of tumor cells with different genetic and epigenetic abnormalities in a heterogeneous tumor population; (3) slow the development of drug resistance; (4) can shrink the primary tumor mass, reduce the number of ACT cells providing an opportunity for their combination with immunotherapy; (5) some chemotherapeutic agents may directly stimulate antitumor immunity with low infiltration of effector T cells within the tumor [36-38]. Now, we all know that the rationale in a chemoimmunotherapy combination can eliminate disseminated and metastatic cancer cells, although chemotherapy can cause myelosuppression.

Immune checkpoint inhibiting immune suppression supporting immunotherapy.

Now, a suppression of tumor growth is emerging through target checkpoints as a new generation of antitumor therapy. There are two kinds of molecularly targeted checkpoint inhibitors. The first one is specifically killing tumors within the tumor microenvironment (TME), inhibiting the occurrence and development of tumors [39], and the second one can target and inhibit immune-pathway molecules called the tumor immune microenvironment (TIME) [40] so that they can

restore immune cell activity and improve the body's antitumor immune function. Currently, molecular target checkpoints increasingly studied within TIME are named Immune checkpoint inhibitors (ICIs) for PD-1, PD-L1, CTLA-4, TIM-3, LAG-3, and Siglec-15 by their corresponding molecular target inhibitors.

Table-2. ICI combination

Types	Common ICI type	supporting item
ICI-ICI	nivolumab	ipilimumab
ICI-ACT	nivolumab	Car-T/TIL
ICI-chemotherapy	nivolumab	chemotherapy
ICI antiangiogenic	nivolumab	Antiangiogenic therapy
ICI-vaccine	nivolumab	HPV16 specific peptide vaccine
ICI-radiation	nivolumab	ionizing radiation
ICI-TME	nivolumab	targeting TGFβ
ICI-cytokine	nivolumab	IL2/IFN-gamma

ICIs were initially approved for the treatment of melanoma. Due to single-agent ICIs' failure, combination regimens involving ICIs have been investigated, such as ICI-ACT, ICI-chemotherapy, and ICI antiangiogenic doublets, as shown in Table 2. Nivolumab and ipilimumab have shown initial success in melanoma and renal cell carcinoma (RCC). ICI-ICI combination therapy is a viable approach to overcome treatment resistance. Novel combination strategies to overcome ICI resistance rapidly evolve, with many clinical trials underway. Bispecific antibodies (bsAbs) allow the targeting of specific resistance mechanisms in a single molecule, and dual checkpoint inhibition of PD-L1 and LAG-3 is an example that has shown promising preclinical results. Other bsAbs combine ICIs with non-ICI immunotherapies, such as PD-L1 antibodies and transforming growth factor-β (TGF-β) traps, a key player in the development of an immunosuppressive tumor microenvironment (TME). Other forms of ICI immunotherapy are also encouraging, including

immunostimulatory cytokines (e.g., recombinant interleukin-2, interferon-α), cancer vaccines, and adoptive cell therapy. Combining vaccine therapies with ICIs may enhance antitumor effects in preclinical models. Combining CAR-T with ICIs may overcome some of the resistance mechanisms encountered. In addition, a combination of ICIs with ionizing radiation and ICIs with targeted therapies for cellular processes, including DNA damage repair, phosphatidylinositol 3-kinase, and histone deacetylase pathways. A logical approach to combination study design is needed to maximize patient benefit [41].

A new generation of precision medicine supporting immunotherapy

According to those discussed above, distinct LD, chemotherapy, and ICI are different for individual patients. Of course, all combinations should differ from one person to another. Following thirty years of research, a new generation of precision medicine techniques is emerging [42-44].

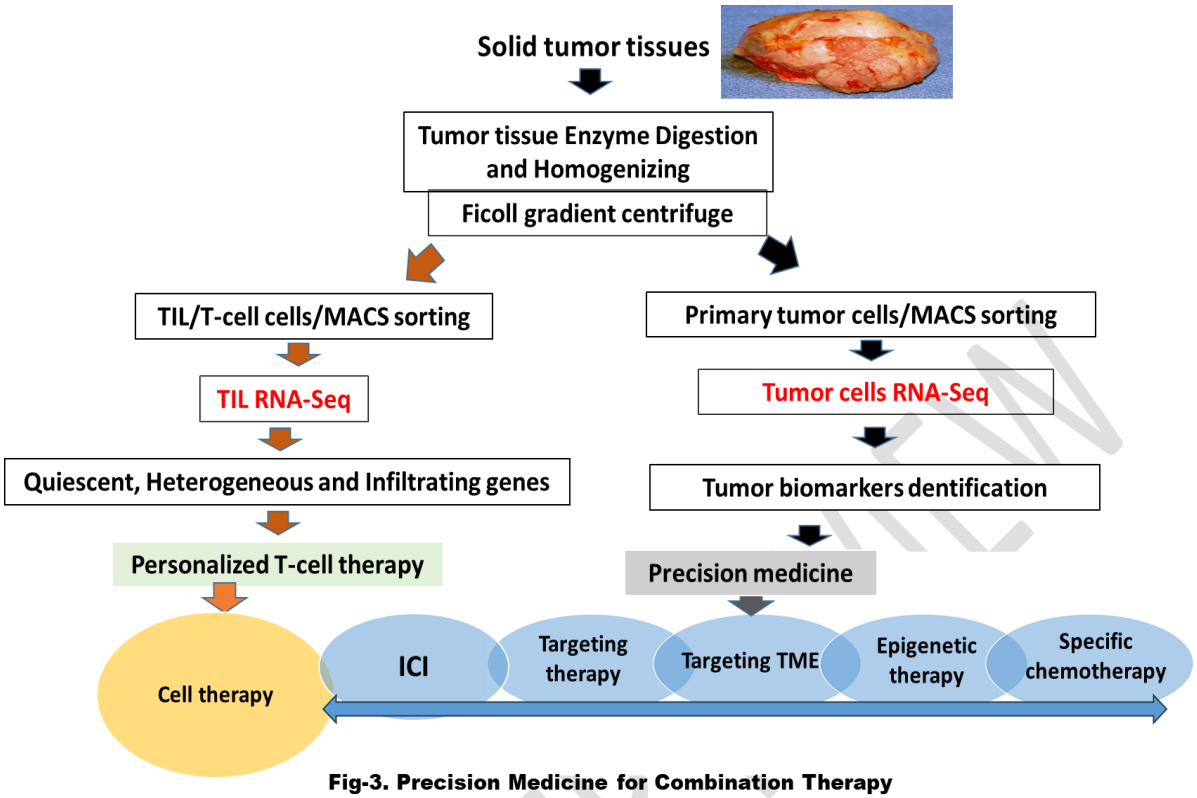

Fig-3. Precision Medicine for Combination Therapy

To address the issues, successful precision medicine for tumor disease has involved immune cells/primary cell culture, tumor tissue biobanks, single cell technique, clinical genomics, and artificial intelligence in our laboratory [45-50]. Moreover, precision medicine is discovering driver genes, tumor proliferation, and tumor metastasis genes according to the updated strategy [51]. Once we find distinct biomarkers in DNA level (SNP and epigenetics), RNA level microRNA, picoRNA, non-coding RNA), and protein level, we can develop precision medicine as Fig-3 to support immunotherapy.

A. Specific personalized combination immunotherapy can be discovered.

In the early period, we developed TIL culture for clinical applications by cultured TIL infusion *in vivo* for adoptive cell therapy (ACT) from solid tumors. After twenty-five efforts, we successfully developed our laboratory's single-cell techniques, clinical genomics, and artificial intelligence. After, we used single-cell genomics analysis to discover a set of upregulated quiescent

genes such as Tob, LKLF, TGF-β, ERF, and REST/NRSF from the T-cells [52-55]. Moreover, after thirty years of effort, we know that TIL is a group of heterogeneous immune cells, so we can perform *ex vivo* heterogeneous TIL determining immune characteristics to kill autologous tumor cells and then treat the tumor patients based on immune characteristics for the tumor diseases [56]. Now we can measure quiescent status in the heterogeneous immune cells such as CD3+ T-cell (CD8+ T-cell and CD4+ T-cell), CD19+ B-cell (tumor-infiltrating B-cells, TIL-B), CD16+/CD56+ NK cell (Natural killer Cell), CD16+/CD56+/CD3+ NKT cell (Natural Killer T-cell), and other immune-cells (macrophage and neutrophil). Finally, the specific immune cells that have been specifically contacted to correspond with the tumor antigen of tumor cells will be cultured for precision immunotherapy. As we all know above, if we discover the higher expression of checkpoint-inhibiting molecules such as PD-1, which blocks immune cells, we can also further stimulate immune cells with PD-1 inhibitors to combine with the specific immune cells for personalized immunotherapy [57-58].

B. Molecular therapeutic targeting can be discovered.

Molecular targeted therapies are advanced therapeutic techniques that interfere with specific molecules to block cancer growth, progression, and metastasis [59-60]. Now, molecular targeted therapies approved by the Food and Drug Administration (FDA) have demonstrated remarkable clinical success in the treatment of a myriad of cancer types, including breast, leukemia, colorectal, lung, and ovarian cancers. We can also further develop immune cells with molecular targeted therapies to combine with specific immune cells to create personalized immunotherapy.

C. TME targeting gene can be discovered

Tumor microenvironment (TME) and tumor cells in tumor tissue take many strategies to evade the host immune response by creating many immune-suppressive factors [61]. Thus, we can use the strategy from TME to be personalized therapy. TME consists of tissues, cells, and signaling molecules in tumor tissue, affecting the immune response to tumor cells. Furthermore, TME elements of tissues, cells, and molecule factors include those during the early period of tumor tissues and those in an aggressive period in tumor tissues. Identifying and regulating TME cells and regulating molecules such as extracellular matrix (ECM) and pathways such as adenosine (ADO) and indole-2,3-dioxygenase (IDO) may guide a new generation of precision medicine so that we can use TME targeting components to combine with the specific immune cells to personalized immunotherapy.

D. Epigenetics targeting can be discovered

Epigenetic therapy is based on methylation assay and PTM histone assay [62-65]. Methylated cytosines recruit protein complexes that promote functionally inactive heterochromatin under a global decrease in DNA methylation, such as 5-azacitidine (AZA) and decitabine (DEC), by inhibiting DNMT1. Epigenetic therapy based on PTM histone assay is HDAC inhabitation, which removes the acetyl groups from the lysine residues to apply for an option for tumor treatment so that we can use epigenetic therapy to combine with the specific immune cells for personalized immunotherapy.

E. Specific chemotherapy can be discovered.

Customized chemotherapy has now been applied to the field of cancer [66-70]. According to genomic data, we can also use personalized chemotherapy to develop immunotherapy with specific immune cells and specific chemotherapy for personalized immunotherapy.

Conclusion

The "foe" or "friend" of chemotherapy combined with immunotherapy is a long-term puzzling issue. Cytotoxic chemotherapy is considered to be immunosuppressive while it is recommended

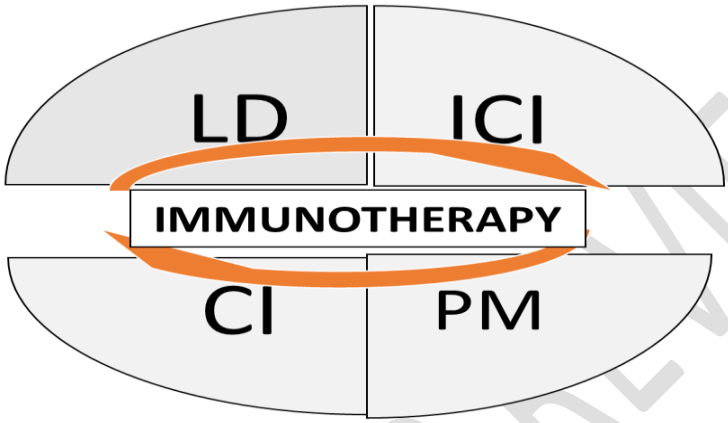

Fig-4. Combination Therapy

to support immunotherapy in clinical application, to increase ACT immunotherapy, and to reduce the side effects of chemotherapy, we have experienced several decades to study the mechanism using isolating and culturing primary tumor cells and immune cells from patient specimens such as resected solid tumor tissues. After about 30-40 years of effort, the combination mechanism has proved that chemotherapy combined with immunotherapy is superior to monotherapy. Based on our current publications, including other clinical laboratory work, the manual introduced four areas of combination therapy: lymphodepletion (LD), chemoimmunotherapy (CI), ICI combined with immunotherapy, and precision medicine (PM) supporting Immunotherapy as Fig-4. Precision medicine based on patients' information includes patient specimen biobanks, single-cell technologies, clinical genomics, and artificial intelligence to support combination therapy. Based on 30-40 years of R&D to improve patient immunotherapy, now is an excellent time to define combination therapy and evaluate its efficacy in oncological diseases.

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Figure Legend

Fig 1. ***Strategy for LD to increase TIL efficacy.***

A lymphodepletion regimens adding TIL treatment to increase TIL efficacy.

Fig 2. ***Clinical strategies for chemoimmunotherapy to increase TIL efficacy.***

A clinical procedure combining TILs with sensitive chemotherapeutic agents, which were screened *chemo-sensitivity assay (CSA) from TIL cytotoxicity experiment of* patient's autogenous tumor cell to increase treatment response.

Fig 3. ***Strategies for precision medicine for personalized T-cell therapy to increase T-cell efficacy.***

A) Improving TIL immune-response in experiment such as discover quiescent genes for TIL and neo-antigen from tumor cells; and rebuilding immune-response for TIL or set up Car-T or TCR-T cells; B) ICI improving T-cell to attack tumor cells; C) Targeting block to increase T-cell activity; D) TME block to increase T-cell activity; D) epigenetics treatment improving T-cell to attack tumor cells; and E) chemotherapy to increase T-cell attack tumor cells.

Fig 4 ***Strategies for combination therapy to support immunotherapy.***