The Memorial Thesis of the Best Poster Award (Recommended article)**Recommender: Division of Cellular Signaling, Institute for Advanced Medical Research,
Keio University School of Medicine, Prof. Yutaka KAWAKAMI****Prospects for personalized combination immunotherapy for solid tumors
based on adoptive cell therapies and immune checkpoint blockade therapies**Daiki KATO^{*1,2}, Tomonori YAGUCHI^{*1}, Takashi IWATA^{*1}, Kenji MORII^{*1},
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summary

Immune checkpoint blockade (ICB) and adoptive cell therapies (ACT) with antigen-receptor gene-engineered T cells have been shown to be successful for a limited number of patients with solid tumors. Responders to ICB therapy typically have T cell-inflamed tumors. Thus, it is important to develop strategies that convert non-T cell-inflamed tumors to T cell-inflamed tumors. Although chimeric antigen receptor transduced T (CAR-T) cell therapy targeting hematological malignancies demonstrated durable clinical responses, the success of gene-engineered T cell therapies in solid tumors is hampered by a lack of unique antigens, antigen loss in cancer cells, and the immune-suppressive tumor microenvironment (TME) of solid tumors. However, gene-engineered T cells possess strong killing activity and cytokine production capacity, which can induce antigen spreading and modulate the TME of non-T cell-inflamed tumors seen in non-responders to ICB therapy. Immune responses against cancer are highly heterogeneous, not only between tumor types, but also within a patient or between different patients with the same type of cancer, indicating that personalized immunotherapy should be employed, based on the immune status of the individual patient. Here, we offer our perspective for personalized combination immunotherapy for solid tumors based on ACT and ICB therapies.

Key words——tumor immunology; tumor microenvironment; immune checkpoint blockade therapy; adoptive cell therapy; chimeric antigen receptor transduced T (CAR-T) cell therapy

Immune checkpoint blockade therapies

Cytotoxic T cells can specifically recognize tumor antigen (tumor Ag) and eliminate cancers^{1–4}. As demonstrated by recent evidence from study of immune checkpoint blockade (ICB) and adoptive T cell therapies, an entirely immunologic approach can treat a wide range of human cancers. ICB therapies, which use monoclonal antibodies (mAbs) that target inhibitory immune checkpoint molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4)⁵ and programmed cell death protein 1 (PD-1)/programmed cell death-ligand protein 1 (PD-L1)⁶, have demonstrated durable clinical responses in various cancers, including melanoma^{7, 8}, lung cancer^{8, 9}, renal cell carcinoma^{8, 10}, bladder cancer¹¹, ovarian cancer¹², triple-negative breast cancer¹³, gastric cancer¹⁴, Hodgkin's lymphoma¹⁵, and gastrointestinal and endometrial cancers associated with defects in DNA mismatch repair¹⁶. In melanoma, lung cancer, and renal

cell carcinoma, clinical response rates as high as 20–30% have been reported^{8, 9}. The observed anti-tumor responses were associated with the activation and expansion of tumor Ag-specific T cells^{17, 18}, and responding patients showed high pre-treatment tumor infiltration of T cells compared to non-responding patients¹⁹. This evidence suggests that conversion of non-T cell-inflamed tumors to T cell-inflamed tumors would improve response to ICB therapies.

Adoptive cell therapies

T cells are considered the final effectors of immune-mediated cancer regression, hence direct administration of tumor Ag-specific T cells has been developed into adoptive T cell therapies (ACT)²⁰ (**Fig. 1**). To prepare T cells for ACT, tumor-infiltrating lymphocytes (TILs) are harvested from surgically removed tumor metastases and expanded *ex vivo*. Administration of TILs demonstrated durable clinical responses in patients

with melanoma and cervical cancer^{21, 22}). TIL therapy for patients with advanced melanoma showed a 52–72% objective response rate based on the Response Evaluation Criteria in Solid Tumors (RECIST), and 19 of the 93 patients (20%) achieved complete tumor regression beyond 3 years²²). To broaden the efficacy of ACT, the antigens recognized by TILs have been studied extensively. A recent report has demonstrated that mutated antigens in a non-melanoma epithelial cancer (i.e. gastrointestinal cancer) can give rise to immune responses, despite the low number of nonsynonymous somatic mutations in these cancers³). While TILs can be grown *in vitro* from virtually all tumors, TILs that demonstrate a clinical response have only been produced from melanomas²⁰).

Gene-engineered cell therapies

Progress in genetic engineering technologies has enabled the efficient generation of antigen-receptor gene-engineered T cells, including T cell receptor transduced T (TCR-T) cells and chimeric antigen receptor transduced T (CAR-T) cells (Fig. 1). Use of gene-engineered T cells obviates the requirement for surgery because these T cells can be isolated from blood samples, and viral or non-viral transduction of receptors confers tumor Ag specificity²³). As the same antigen receptor can be used for a large population of cancer patients and gene-engineered T cells can potentially be produced on a large scale, gene-engineered T cells may be useful as an off-the-shelf formulation. Recent experience with sipuleucel-T (trade name Provenge), a gene-engineered cell product for prostate cancer, demonstrated the feasibility of processing patient immune cells in a central manufacturing facility and returning them to the patient in a manner that has gained US Food and Drug Administration (FDA) regulatory approval²⁴).

TCR-T cell therapies

TCR-T cells are made by transduction of a cloned T cell receptor (TCR) that recognizes tumor Ag presented within the groove of a specific major histocompatibility complex (MHC). The engineered TCR triggers T cell activation via the signal transduction machinery employed by endogenous TCR²⁵). Several reports have been published regarding clinical trials of TCR-T cell therapy. In an early clinical trial, 17 patients with advanced melano-

ma displaying human leukocyte antigen (HLA)-A*0201 were treated with TCR-T cells specific for a HLA-A2-restricted epitope derived from melanocyte-differentiating antigen (MART-1). The study resulted in long-term persistence of the infused cells and accompanying tumor regression in 2 of 17 patients, without toxicities attributed to the transferred cells²⁶). A subsequent study with an affinity-enhanced MART-1-specific TCR reported tumor regression in 6 of 20 patients (30%). Some patients showed moderate but non-lethal toxicity in tissues where melanocytes are present, such as the skin, eyes, and ears²⁷). A recent study using modified high-avidity TCR targeting a HLA-A2-restricted epitope derived from a cancer germline antigen (NY-ESO-1) demonstrated a good response in 11 of 20 patients (55%) with advanced melanoma (4 CRs, 3 of these CRs were ongoing after > 36 months) and 11 of 18 (61%) patients with synovial cell sarcoma, a non-melanoma solid tumor (1CR). No toxicities attributed to the transferred cells were recorded^{28, 29}). However, in a study using affinity-enhanced TCR against HLA-A*01-restricted MAGE-A3, 2 patients with melanoma and myeloma developed cardiogenic shock and died within a few days of T-cell infusion. This adverse effect was caused by recognition of an unrelated peptide derived from cardiomyocytes by the affinity-enhanced TCR^{30, 31}).

CAR-T cell therapies

TILs are difficult to generate in non-melanoma cancers, and TCR-T cells recognize only specific MHC alleles, limiting their use to a restricted subset of patients and making them vulnerable to MHC downregulation and immune escape³²). To address these limitations, a new concept of gene-engineered T cells has been developed. A versatile class of receptors known as chimeric antigen receptors (CARs) are generated by combining the antigen-binding domain of a single-chain variable fragment (scFv) from a mAb that recognizes tumor Ag with intracellular signaling motifs that are capable of T cell activation³³). Because their ability to recognize antigens is enabled by antibody binding, CARs can mimic endogenous TCR-mediated activation without the drawbacks of classical MHC restriction. Moreover, although endogenous TCRs are restricted by selection in the thymus, antibody-redirected CARs can accommodate

Fig. 1. Summary of concepts of adoptive T cell therapies (ACT)

Three types of tumor Ag-specific T cells are used for ACT. For tumor-infiltrating lymphocyte (TIL) therapy, TILs are harvested from surgically removed tumor tissue and expanded *ex vivo*. Because TILs recognize a variety of tumor antigens, TIL therapy is a polyclonal antigen-targeting therapy. For T cell receptor transduced T (TCR-T) cell therapy, tumor Ag-specific TCR sequences are obtained from tumor Ag-specific T cells. Monoclonal antigen-targeting TCR-T cells are generated by transfection of these tumor Ag-specific TCR genes in patient T cells derived from PBMCs. For chimeric antigen receptor transduced T (CAR-T) cell therapy, CARs are generated by connecting intracellular signaling domains and a single-chain variable fragment (scFv) from a mAb that recognizes tumor Ag. The CAR genes can also be transduced into T cells from patient-derived PBMCs.

Fig. 2. Prospects for combination adoptive immunotherapies

The immune status of tumors can be categorized into four types, based on mutation-derived immunogenicity, mainly passenger mutations, and mutation-derived immunosuppression, mainly driver mutations. The tumor shown in panel A possesses a large amount of immunogenic mutations; hence, tumor Ag-specific T cells have infiltrated strongly and immune checkpoint blockade (ICB) therapies would be effective. In contrast, the tumor shown in panels C and D possesses a highly immunosuppressive tumor microenvironment (TME), hence modulation of the TME would be needed. Because the tumor in panel B shows very weak immunogenicity, treatment for enhancement of immune recognition (e.g., immunogenic cell death) would be needed. Because patient immune status is highly heterogeneous, personalized combination immunotherapy should be considered.

infinite antigenic diversity and operate at affinities in the nanomolar range^{34, 35}. As an additional advantage, the signaling domain of CAR constructs can be modulated to exhibit various T cell functions, including expansion, survival, cytokine secretion, tissue-selectivity, and safety^{36–40}.

Clinical trials of CAR-T cell therapies

Many clinical trials of CD19-specific CAR-T cell therapies targeting B cell malignancies demonstrated objective regression of cancer in patients with acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and various other types of B cell lymphoma. The first successful application of CD19-specific CAR-T cell therapy was observed in patients with follicular lymphoma⁴¹, and the treatment target was subsequently extended to other chemotherapy-refractory B cell cancers^{42, 43}. For example, CAR-T cell therapy of pediatric and adult patients with ALL demonstrated a complete remission rate of approximately 90%, with sustained remission for up to 2 years⁴⁴; 57 % of patients with CLL showed an objective response, including complete remission (29%)⁴⁵. Because normal B cells express CD19 antigen, B cell aplasia and deficiencies in circulating immunoglobulins (Igs) are frequently observed in patients treated with CD19-specific CAR-T cell therapies. These toxicities can be managed with infusion of Igs. Many patients also exhibit cytokine release syndrome after T cell infusion. In most cases, these side effects can be managed with aggressive supportive care or immunosuppression including steroids and cytokine-specific antibodies⁴⁶. Because CD19-specific CAR-T cell therapy has shown a favorable and lasting clinical outcome compared with conventional radio- or chemotherapy, CD19-specific CAR-T cell therapy may enter mainstream care for many B cell malignancies in the next a few years and the FDA has recognized it as a “breakthrough therapy”. Although CAR-T cell therapies are effective in treating hematological malignancy, use of CAR-T cell therapies for solid tumors has encountered many difficulties, including limitation of tumor-specific antigens expressed on the surface of solid tumors and the need for T cell infiltration into tumor tissue where the environment is heavily immunosuppressive. A trial with GD2-specific CAR-T cells resulted in tumor regression

in 3 of 11 patients with neuroblastoma, including 2 sustained CRs, without severe adverse effects⁴⁷. In addition, a study of CD171-specific CAR-T cells demonstrated a partial response in 1 of 6 patients with neuroblastoma⁴⁸. Because shared tumor Ags are occasionally expressed in normal tissues and recognized by CAR-T cells, substantial unexpected on-target off-tumor toxicities have occurred. For example, a trial of CAR-T cells targeting carbonic anhydrase IX (CAIX), overexpressed in renal cell carcinoma, caused severe liver dysfunction due to cholangitis, without cancer regression. The expression of CAIX in epithelial cells of the bile duct results in on-target toxicities³⁴. In addition, a lethal adverse effect was reported in a study of ERBB2-specific CAR-T cells expressing scFv from the humanized mAb trastuzumab, resulting in fatal respiratory failure, with inflammatory cytokine release in the lung within 15 minutes after intravenous infusion of 10^{10} cells⁴⁹. It was speculated that many of the administered T cells localized in the lungs and were activated by recognition of ERBB2 on lung epithelial cells, leading to extreme cytokine release. In contrast, a recent trial employing infusion of 10^8 cells of ERBB2-specific CAR-T cells using a different anti-ERBB2 Ab clone (FRP5, a mouse anti-human ERBB2 mAb) demonstrated no significant toxicities, although no significant tumor reduction was observed in this trial⁵⁰. These results from clinical trials might indicate the potential of ERBB2 as a therapeutic antigen and the importance of careful selection of the Ab clones used for production of CAR-T cells.

Clinical trials of ACT with gene-engineered T cells, such as NY-ESO-1 TCR-T cells and GD2-specific CAR-T cells, have shown that gene-engineered T cells have the potential to show anti-tumor effects without damaging normal tissue in patients with solid tumors. However, on-target off-tumor toxicity of gene-engineered T cell therapies causes serious adverse effects, which have been reported for TCRs with mouse-derived gp100, mouse-derived CEA, affinity-enhanced MART-1, affinity-enhanced MAGE-A3, and CARs directed against ERBB2 and CAIX^{27, 31, 49, 51}. The most important factor is the selection of the target antigen; the most desirable antigen must be expressed only on tumor cells and not on normal tissue. However, even if the target antigen is expressed in normal cells, it can be a possible therapeutic target. For

example, no severe adverse effects have been reported for mesothelin-specific CAR-T cell therapies, even though a substantial amount of mesothelin is expressed in normal mesothelial cells; some objective responses to these therapies have been documented. There might be an unknown mechanism for the specific recognition of the antigen expressed on cancer cells by CAR-T cells⁵². This case might lead to relaxation of the restriction of target antigens for gene-engineered T cell therapies.

Immunosuppressive TME and ACT therapies

The immunosuppressive status of the tumor micro-environment (TME) is a major problem in cancer immunotherapies, as it reduces the anti-tumor effects of immunotherapies. Two types of immunosuppression mechanisms operate in the TME^{53, 54}. One is tumor-intrinsic immunosuppression, which is induced by genetic alterations related to cancer. The other is local adaptive immunosuppression, which is triggered by tumor-specific T cells infiltrated in tumors. The former operates via multiple mechanisms that employ various immunosuppressive molecules triggered by genetic alterations, including activation of various oncogene pathways such as the Wnt- β -catenin signaling pathway^{55, 56}, the MAPK signaling pathway^{57, 58}, the JAK-STAT3 signaling pathway^{57, 59}, and the NF- κ B signaling pathway⁶⁰. The activation of tumor-intrinsic oncogene pathways induces tumor cells to produce suppressive molecules such as cytokines and chemokines, mediating exclusion of T cells from the TME. Small molecule inhibitors, such as β -catenin and BRAF inhibitors, can modulate the activated oncogene pathways^{60–62}. Based on current knowledge, it is conceivable that combination therapies of ACT with inhibition of the activated oncogene pathways using small molecule inhibitors could work synergistically to exhibit anti-tumor effects through mechanisms that restore T-cell infiltration. In a recent study, BRAF inhibitors improved the anti-tumor activity of ACT in mouse models⁶².

The latter mechanism, local adaptive immunosuppression, is triggered by tumor-specific cytotoxic lymphocytes (CTLs), whereby cytokines, such as IFN- γ produced by anti-tumor T cells, induce immune-checkpoint molecules such as PD-L1, immunosuppressive indoleamine (IDO), which catabolizes tryptophan, or induce chemokines. We and others reported the potential

effectiveness of combination therapies of ACT with ICB therapies targeting molecules such as PD-1, TIGIT, LAG3, and Tim3 that are expressed on tumor Ag-specific T cells^{63–65}. In ACT therapies, adaptive immunosuppression can theoretically occur, and it is also conceivable that combination therapies of ACT with ICB therapies could be effective through restoration of T-cell function.

Modulation of the TME by gene-engineered T cell therapies

Gene-engineered T cell therapies, which typically target monoclonal antigens, have a disadvantage compared to TIL therapies, which use polyclonal TILs. This disadvantage was exemplified by a CD19-specific CAR-T cell therapy, which led to the loss of CD19 expression in leukemia cells⁴⁴. Although it might be not easy to cure bulky tumors using only gene-engineered T cell therapies, their strong killing and cytokine production abilities could be employed for tumor Ag releasing and modulation of the immunosuppressive TME. The evidence for contribution of antigen spreading to favorable outcomes in CAR-T cell therapy was suggested in an immunocompetent mouse model. This study showed that the cured mice which were treated with ACT of murine EGFRvIII-specific CAR-T cell therapy did not develop tumors upon rechallenge with EGFRvIII negative matched tumor⁶⁶. This study suggests that the strong anti-tumor abilities of gene-engineered T cell therapies could potentially trigger anti-tumor immune reaction and convert the non-T cell-inflamed TME seen in ICB non-responders to the T cell-inflamed TME.

CD4⁺ CAR-T cell therapies

Because CD8⁺ T cells have the ability to directly target, engage, and destroy tumor cells, current basic and clinical research on CAR-T cells focuses mainly on improving CD8⁺ CAR-T cell-mediated cellular immunotherapy. Because most tumors do not express MHC class II molecules, CD4⁺ T cells cannot recognize tumor cells. However, CD4⁺ T cells transduced with CAR can potentially recognize target Ag and produce various cytokines. Modulation of the immunosuppressive TME has been reported to attract anti-tumor T cells to the tumor site by production of Th cytokines from transferred CD4⁺ T cells. Moreover, there have been many evidence that

tumor infiltration and interaction of CD4⁺ T cells with immune cells at the tumor site are associated with a good prognosis⁶⁷⁾.

Th1 and Th2 cells are the best-characterized subtypes of helper T cells. Th1 cells are characterized by production of cytokines such as IFN- γ , TNF- α , monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 α (MIP1 α), and are thought to be primarily responsible for immune responses against intracellular pathogens either by enhancing the CD8⁺ T cell response or by directly activating macrophages to phagocytose intracellular pathogens. Th2 cells produce interleukin IL-4, -5, and -10, and mainly respond to extracellular pathogens by orchestrating humoral immunity and promoting allergic inflammatory responses. Although IFN- γ -secreting Th1 cells may be more potent, both of these subtypes have been reported to demonstrate anti-tumor function^{68, 69)}. The major anti-tumor effects of Th1 cells may result from the secretion of large amounts of IFN- γ , as well as chemokines that enhance priming and expansion of CD8⁺ T cells. Th1 cells also help to recruit natural killer (NK) cells and type I macrophages to tumor sites, and act in concert for tumor eradication^{70–72)}. Clinical data have also demonstrated the anti-tumor effects of Th1-phenotype CD4⁺ T cell adoptive transfer. Tran et al. showed the anti-tumor effect of IFN- γ -producing CD4⁺ TIL therapy that is specific to tumor-mutational Ag in a patient with cholangiocarcinoma⁷³⁾. Hunder et al. reported a durable response in a patient with advanced melanoma treated with *in vitro* generated autologous IFN- γ -producing CD4⁺ T cell clones that recognize the melanoma-associated antigen NY-ESO-1⁷⁴⁾. This patient experienced a complete response and has remained disease-free for 2 years, with persistence of the transferred cells. In addition, induction of endogenous CD8⁺ T cells against various melanoma antigens other than NY-ESO-1, such as MAGE-3 and MART-1, was detected. Although this result was only published as a case report, it suggests that Th1 cells have the potential to induce antigen spreading and tumor Ag-specific CD8⁺ T cells.

Other Th subsets have also been demonstrated to show anti-tumor effects. Th17 are characterized primarily by production of the cytokines IL-17A and IL-17F⁷⁵⁾. IL-17A in turn induces the expression of multiple chemokines, such as CCL2, CCL7, CXCL1, and CCL20, that promote

inflammatory responses. Although the influence of Th17 cells and IL-17A has been reported for multiple patients with cancer, their contribution to tumor progression and immune protection remains unclear⁷⁶⁾. Antigen-specific Th17 cells differentiated *in vitro* showed antitumor activity against murine B16 melanoma stronger than that of Th1 cells^{77, 78)}. These Th17 cells promoted production of CCL20, which led to recruitment of dendritic cells and effector T cells into the tumor site, as well as the induction of tumor Ag-specific T cells.

A pioneering study revealed the ability of a new subset of Th9 cells for control of tumor growth⁷⁹⁾. In a B16 melanoma model, Purwar et al. showed that the antitumor effects of adoptive transfer of antigen-specific Th9 cells were greater than those of transferred Th1 or Th17 cells. Another report demonstrated the anti-tumor mechanism of Th9 cells, which induced strong endogenous tumor Ag-specific CD8⁺ T cell responses by promoting Ccl20/Ccr6-dependent recruitment of DCs to the tumor site, combined with CD8⁺ T cell priming⁸⁰⁾. In addition, IL21 produced by Th9 and Th17 cells is a well-known stimulator of IFN- γ production, and it enhances the cytolytic activity of NK cells and CD8⁺ T cells. IL-21 enhances generation of human tumor Ag-specific CD8⁺ T cells and promotes the effector functions of generated Ag-specific CTLs⁸¹⁾.

Together, these data provide a strong impetus to investigate the anti-tumor efficacy of adoptive transfer of CD4⁺ Th cells. Adoptive transferred CD4⁺ Th cells possess the potential for TME modulation, enhancement of effector T cell function, and induction of Ag-specific endogenous effector T cells against patient-specific abundant neo-Ags.

Conclusion

Recent progress in cancer immunotherapy has provided direction for the future improvement of cancer immunotherapy. Particularly, ICB therapies and T cell-based ACT showed robust clinical responses; however, these therapies have only been effective in a limited number of patients. Clinical responses to ICB were correlated with the non-synonymous mutation burden, which can potentially generate immunogenic neo-Ags^{17, 18)}, and pre-existing effector T cell infiltration¹⁹⁾. Notably, immune infiltrates are highly heterogeneous, not only between

tumor types, but also within a patient or between different patients with the same cancer type, indicating that personalized immunotherapy should be employed based on the immune status of the individual patient. Thus, we propose classification of tumors into four groups, on the basis of their immunogenicity and immune suppression in the TME⁸²⁾ (**Fig. 2**). Patients with sufficient pre-existing anti-tumor T cells and local adoptive immune suppression, including PD-1-related suppression (shown in Panel A), will show a better response to ICB therapies, such as anti-PD-1 Ab therapies. In patients with few pre-existing anti-tumor T cells, due to low tumor immunogenicity (shown in Panel B), an immune reaction needs to be induced. Because these patients need immunogenic tumor Ag release, treatment with immunogenic cell death, cancer vaccine therapies, or ACT with gene-engineered T cells⁵²⁾ would convert the TME from B to A (arrow 1). In patients with few pre-existing anti-tumor T cells and strong tumor-intrinsic immune suppression (shown in Panel C), the immunosuppressive TME needs to be modulated. Because various mechanisms of immune suppression may be operating in the TME of these patients, oncogene pathway-targeted therapies, TME cytokine modulation by ACT of CD4⁺ CAR-T cells⁷⁴⁾, or other immune suppression blockades could be used to convert the TME from C to B (arrow 2). In patients with moderate pre-existing anti-tumor T cells, because of tumor immunogenicity and strong tumor-intrinsic immune suppression (shown in Panel D), the immunosuppressive TME needs to be modulated. In these patients, oncogene pathway-targeted therapies, other immune suppression blockades, or TIL therapies could be used to convert TME from D to A (arrow 3). Overall, personalized combination immunotherapy should be considered for improvement of cancer immunotherapy.

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