Editorial

Scientific Rationale for Combination Immunotherapy of Hepatocellular Carcinoma with Anti-PD-1/PD-L1 and Anti-CTLA-4 Antibodies

Masatoshi Kudo

Department of Gastroenterology and Hepatology, Kindai University Faculty of Medicine, Osaka-Sayama, Japan

Prof. M. Kudo

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Introduction

The outcomes of immune checkpoint inhibitor therapies against advanced cancer have greatly exceeded initial expectations since the early clinical studies reported in 2010 [1] and 2012 [2]. It is now evident that immune checkpoint inhibitors are highly effective against multiple solid tumors in addition to malignant melanoma, which was the first malignancy to be studied. Although researchers were initially skeptical about the practical use of conventional cancer immunotherapies that solely enhance immune responses, scientists in both industry and academia are vigorously pursuing the development of anticancer immunotherapies using immune checkpoint inhibitors. In fact, the journal *Science* designated cancer immunotherapy as the “Breakthrough of the Year” in 2013, and the journal *Nature* named immune checkpoint blockade in cancer a paradigm shift in cancer treatment. Since then, various industry-academia collaborations have been initiated, and the remarkable strides in this field have been hailed as “the dawn of the new cancer therapy era” [3] and the “renaissance of cancer immunotherapy” [4].

The first immune checkpoint molecule to be described, programmed cell death protein 1 (PD-1), was identified in 1992 by Dr. Tasuku Honjo and colleagues at Kyoto University [5].

Masatoshi Kudo
Department of Gastroenterology and Hepatology
Kindai University Faculty of Medicine
377-2 Ohnohigashi, Osaka-Sayama 589-8511 (Japan)
E-Mail m-kudo@med.kindai.ac.jp

Later it was shown to be involved in immune suppression, and a study with gene-deficient mice revealed that PD-1 serves as a “brake” on the immune response [6]. In 2000, a collaborative research study between Dr. Honjo’s group, the Genetic Institute, and a group at Harvard University identified the ligands of PD-1, programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) [7, 8]. In 2002, Iwai et al. [6] found that blocking interaction between PD-1 and its ligands enhanced immune activation, resulting in markedly stronger antitumor responses. Based on these findings, a fully human anti-PD-1 antibody was developed in 2005 by Ono Pharmaceutical Co. and Medarex (later acquired by Bristol-Myers Squibb). Nivolumab was approved as an investigational new drug by the FDA in 2006, and clinical trials were initiated in the United States [9]. In 2009, Bristol-Myers Squibb and Ono Pharmaceutical Co. conducted a joint clinical study; in 2014, after several additional clinical trials, nivolumab was approved in Japan for the treatment of malignant melanoma [10]. This was the first time that a PD-1 inhibitor received regulatory approval anywhere in the world. In Japan, nivolumab is currently approved for treatment of non-small-cell lung cancer, renal cell carcinoma, non-Hodgkin’s lymphoma, head and neck cancer, gastric cancer, malignant pleural mesothelioma, and malignant melanoma. Merck’s anti-PD-1 antibody pembrolizumab is also approved for treating malignant melanoma, non-small-cell lung cancer, Hodgkin’s lymphoma, urothelial carcinoma, and unresectable microsatellite instability-high or mismatch repair-deficient metastatic solid cancers.

A series of clinical trials assessing these two anti-PD-1 antibodies for use in the treatment of other types of cancers, including breast cancer, hepatocellular carcinoma (HCC), and bladder cancer, are currently underway, and interim results were reported at meetings of the American Society of Clinical Oncology (ASCO) and the European Society for Medical Oncology. Based on these ongoing investigations, anti-PD-1 and anti-PD-L1 antibodies are now being approved for treatment of many types of cancer.

Meanwhile, in 1995 Krummel and Allison [11] (University of Texas, USA) reported that a different molecule, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), also negatively regulates immune cells, and that administration of an inhibitory antibody against CTLA-4 resulted in tumor regression in mouse models [12]. CTLA-4 is also an immune checkpoint molecule, and in March 2011 the USA approved the anti-CTLA-4 antibody ipilimumab (developed by Bristol-Myers Squibb) for treatment of malignant melanoma; subsequently, it was approved in Europe in July 2011 [13] and in Japan in 2015. Drs. Tasuku Honjo and James Allison won the 2018 Nobel Prize in Physiology or Medicine for their discovery of the inhibitory immune checkpoints PD-1 and CTLA-4 and for their immense contributions to cancer therapy.

Several clinical studies assessing anti-PD-1, anti-PD-L1, and/or anti-CTLA-4 antibodies as monotherapies or combination therapies for treatment of HCC are currently underway, and this field is rapidly advancing. This paper outlines the rationale for clinical studies assessing combination immunotherapy using anti-PD-1/PD-L1 and anti-CTLA-4 antibodies.

The Cancer Immunity Cycle and Immune Escape

Immune responses to tumors can be classified into two phases: the priming phase, which occurs in the lymph node, and the effector phase, which occurs in the tumor tissue. Released cancer antigens are bound by major histocompatibility complex (MHC) molecules expressed on antigen-presenting cells (APCs). These cells migrate to lymph nodes where the antigens are presented to T cell receptors (TCRs) expressed by immature T cells. Immature T cells are not activated solely by antigen stimulation (the first signal), but require an additional costimulatory signal (the second signal) in order to become fully activated; this second signal is

Fig. 1. Activation of cytotoxic T cells. Prepared by the author based on the findings of Yarchoan et al. [15].

delivered by engagement of B7 family molecules (CD80/B7-1 and CD86/B7-2) on APCs with CD28 on T cells (the priming phase). In the priming phase, IL-2 released by CD4-positive cells that are activated by stimulation via MHC class II molecules induces differentiation of CD8-positive T cells into cytotoxic T lymphocytes (CTLs) and promotes their proliferation (Fig. 1). Activated T cells travel via the bloodstream to the tumor, where they infiltrate the tumor tissue. Within the tumor, activated T cells attack tumor cells by releasing perforin and granzyme upon recognition of cancer antigens presented by MHC molecules on tumor cells by TCRs expressed by activated T cells (the effector phase). The cancer antigens are also recognized by APCs, which migrate to lymph nodes to further activate CD8-positive T cells. This is the cancer immunity cycle (Fig. 2) [14]. Additionally, among the three classes of cancer antigens, neoantigens that are unique to cancer cells are the most antigenic (Table 1) [15].

T cell attack seems to be effective initially, but weakens over time. When activated CD8-positive cells release humoral factors such as perforin and granzyme, they also release interferon- γ (IFN- γ), which binds to IFN- γ receptors on cancer cells. This triggers expression of PD-L1 on the cancer cell surface via the signal transducer and activator of transcription 3 (STAT3) pathway, which is one mechanism that contributes to cancer cell escape from immune response (immune escape in cancer, Fig. 1).

There are two main mechanisms of cancer immune escape: one occurs in the lymph nodes and the other at the cancer site.

Fig. 2. The cancer immunity cycle. Adapted from Chen and Mellman [14].

Table 1. Classification of cancer antigens (outline of the immune system)

Antigen type	Description	Examples of antigen type
Tumor-specific antigens (neoantigens)	Completely absent from normal host cells Arise in cancer cells from oncogenic viral proteins or nonsynonymous somatic mutations	HPV oncoproteins E6 and E7 (HPV-associated cancers of the cervix, anus, and oropharynx) Individual KRAS mutations (pancreatic, colon, lung, and various other cancers)
Tumor-associated antigens	Low levels of expression on normal host cells Disproportionately expressed on tumor cells Often result from genetic amplification or posttranslational modifications Can be selectively expressed by the cell lineage from which the cancer evolved	Her2 (breast cancer etc.) Mesothelin (pancreatic cancer and mesothelioma) CD19 on B cell malignancies
Cancer/testis antigens	Absent on normal adult cells, except in reproductive tissues (e.g., testes, fetal ovaries, and trophoblasts) Selectively expressed by various tumor types	MAGE (various cancers) NY-ESO-1 antigen (various cancers)

Adapted from Yarchoan et al. [15].

Fig. 3. Inhibitory mechanism of CD8+ T cell activation at the lymph node (priming phase).

Mechanisms of Immune Escape in Cancer

Immune Escape in the Lymph Nodes (Priming Phase)

Two mechanisms are involved in suppressing T cell activation during the priming phase, resulting in decreased activation and proliferation of cancer-specific naïve T cells. One such mechanism is blockade of the B7/CD28-mediated costimulatory signal by upregulation of CTLA-4 on naïve CD8-positive cells (Fig. 3a). CTLA-4 has 10-fold higher affinity to B7 family costimulatory receptors (CD80/B7-1 and CD86/B7-2) than CD28; thus, it outcompetes CD28 for binding to these receptors, thereby inhibiting transmission of the second costimulatory signal. Under normal conditions, CTLA-4 terminates physiologically unnecessary T cell activity, thereby regulating excessive T cell immune responses. However, in cancer immunology, CTLA-4 inhibits activation and proliferation of activated T cells that recognize cancer antigens (Fig. 3a).

Another key player is regulatory T cells (Tregs; Fig. 3b, 4). CTLA-4 is constitutively expressed on Tregs, which suppress the activity of both dendritic cells (DCs) and CD8-positive T cells. Tregs downregulate expression of costimulatory molecules on DCs directly in a CTLA-4-dependent manner. When naïve CD8-positive T cells are activated by these DCs, there is no transmission of the B7-1/B7-2-CD28 costimulatory signal, resulting in induction of anergy rather than activation (Fig. 3b, 4). Tregs also express the high-affinity form of the IL-2 receptor (CD25) and thus compete with naïve CD8-positive T cells for IL-2, which is essential for acti-

Fig. 4. Regulatory T cell function at the priming phase. Modified from Laidlaw et al. [16] and Tanaka and Sakaguchi [17].

vation of naïve T cells (Fig. 4) [16–21]. At the same time, Tregs decrease production of IL-12 and IL-15 by DCs, thereby inhibiting CD25 expression on naïve CD8-positive T cells and consequently preventing their activation (Fig. 4) [16–21].

Anti-CTLA-4 therapies aim to release the “brake” from T cell activation in the lymph node. As mentioned earlier, the first example of anti-CTLA-4 therapy was demonstrated by Krummel and Allison [11], who showed that tumors in mice could be eradicated by administering antibodies that block CTLA-4. Anti-CTLA-4 antibodies have two roles (Table 2): they block CTLA-4 to restore costimulatory signaling triggered by B7/CD28 binding on T cells (Fig. 3c), and they eliminate Tregs via antibody-dependent cellular cytotoxicity, thereby enhancing T cell activation (Fig. 3d).

At present, clinical studies are investigating the efficacy of the anti-CTLA-4 antibodies ipilimumab and tremelimumab against numerous solid tumors.

Immune Escape at the Tumor Site (Effector Phase)

PD-1, an immunosuppressive accessory signal receptor expressed on activated T cells, B cells, and cells of the myeloid lineage, binds to PD-L1 and PD-L2 to suppress T cell activation in an antigen-specific manner. PD-L1 is expressed widely in blood vessels, cardiac muscle, lung, and placenta as well as on DCs, whereas PD-L2 is expressed only on DCs.

PD-1 is not highly expressed in the peripheral blood of normal mice or healthy humans. After an immune response has been triggered by infection or inflammation, it becomes expressed selectively on T cells in the late stage of activation. Expression is particularly strong on effector T cells in peripheral tissues.

Table 2. Inactivation mechanism of CD8+ cells at the lymph node (priming phase) and activation by anti-CTLA-4 and PD-1/PD-L1 antibody

Activation mechanism of CD8+ T cells	Inactivation mechanism of CD8+ T cells	Activation of CTLs by combination of PD-1/PD-L1 and CTLA-4
(1) TCR of naïve CD8+ T cells recognizes cancer antigen presented on MHC class I on APCs	(1) CTLA-4 expressed on Tregs inhibits maturation of DCs and decreases antigen presentation	(1) Anti CTLA-4 antibody decreases Treg function and number, leading to activation
(2) Costimulatory signal transduction by binding CD80 on CD8+ T cells and B7-1/B7-2 (CD80/86) on APCs	(2) Tregs inhibit CTLs by consuming IL-2 and by cAMP transport	
(3) Differentiation to and upregulation of CTLs by receiving IL-2 by IL-2 receptor expressed on CD8+ T cells, which is produced by CD4+ T cells	(3) CTLA-4 on activated CTLs upregulates the binding between CTLA-4 and B7-1/B7-2, resulting in downregulation of the CTLs	(2) Anti-CTLA-4 antibody upregulates CTL activation
	(4) PD-L1 expressed on APCs binds to PD-1, leading to inactivation of CTLs	(3) Anti-PD-1/PD-L1 activates CTLs

APCs, antigen-presenting cells; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DCs, dendritic cells; MHC, major histocompatibility complex; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; TCR, T cell receptor; Treg, regulatory T cell.

In contrast to PD-1, PD-L1 is expressed constitutively in normal peripheral tissues and by almost all immune cells (including T and B cells) after activation. PD-L1 is expressed by most cancer cells through the mechanism described below.

Meanwhile, expression of PD-L2 is limited to professional APCs; thus, it is thought to be involved only in T cell activation in the lymph node. For this reason, it is generally thought that PD-1 antibodies and PD-L1 antibodies work via a similar mechanism, whereas the utility of anti-PD-L2 antibodies in cancer immunotherapy is limited.

When TCRs on activated T cells recognize cancer antigens presented on MHC molecules, T cells release perforin and granzyme to attack tumor cells directly; they also release cytokines (e.g., IFN- γ), which act on cancer cells. In cancer cells, activation of IFN- γ receptors results in induction of STAT3 signaling, eventually leading to increased cell surface expression of PD-L1. Upon binding of PD-L1 to PD-1 on CTLs, a negative signal is transmitted, which weakens T cell-mediated attacks on cancer cells (immune escape; Fig. 5).

This brake can be released by administration of anti-PD-1/PD-L1 antibodies, which restores immune attack against tumor cells (Fig. 5). In other words, this approach restores preexisting anticancer immunity [22–33]; thus, it is completely different from conventional chemotherapies and molecular targeted therapies. Anti-PD-L1 antibodies are thought to have a similar effect [2]. PD-L1 is a biomarker for the efficacy of anti-PD-1 therapy [34]; however, it must be noted that anti-PD-1 therapy is effective against some tumors that lack PD-L1 expression.

The role of Tregs in suppressing T cell activation during the priming phase was described earlier. Tregs are also involved in immune escape at the tumor tissue during the effector

Fig. 5. Regulatory T cells are associated with cancer immune escape both at the priming and effector phases.

phase (Fig. 6). Peripheral Tregs are naïve T cells, while most Tregs at cancer sites are effector T cells that produce immunosuppressive cytokines (e.g., transforming growth factor- β [TGF- β], IL-10, or IL-35) and cytotoxic molecules (e.g., granzyme and perforin), which attack or inhibit activated T cells (Fig. 6) [18–21]. Anti-CTLA-4 antibodies are effective at suppressing these actions of Tregs (Table 3).

The Importance of Tregs and the Rationale Underlying Combined Use of Anti-PD-1/PD-L1 and Anti-CTLA-4 Antibodies

Because of the constitutive and high-level surface expression of CTLA-4 by Tregs, anti-CTLA-4 antibodies restore activation of CTLs in the lymph nodes and activate CTLs at cancer sites by modulating the immunosuppressive microenvironment. For this reason, combined use of anti-PD-1/PD-L1 and anti-CTLA-4 antibodies is both rational and reasonable.

Combination Therapies Using Anti-PD-1/PD-L1 and Anti-CTLA-4 Antibodies

The efficacy of such combination therapies for treatment of malignant melanoma has already been demonstrated [35], and these combinations are currently being tested for use in the treatment of HCC [36–38]. The rationale for combination therapies using anti-PD-1/

Fig. 6. Restoration of tumor immunity by blocking the CTLA-4 and PD-1/PD-L1 pathways. ADCC, antibody-dependent cellular cytotoxicity.

Table 3. Immune escape and immune activation at the cancer tissue level

Immune response	Immune escape	Immune activation by a combination of PD-1, PD-L1, and CTLA-4 antibodies
(1) TCRs on CTLs recognize the cancer antigen presented on MHC class I of cancer cells, leading to attack on cancer cells by releasing perforin and granzyme	(1) IFN- γ produced by attack on cancer cell by activated CTLs, leading to PD-L1 expression through STAT3 signaling, which results in inhibition of activated CTLs	(1) PD-1/PD-L1 antibody binds to PD-L1 expressed on tumor cells or macrophages, leading to activation of CTLs
	(2) Tregs in the tumor microenvironment inhibit and destroy CTLs by releasing perforin, granzyme, or inhibitory cytokines (TGF- β , IL-10, IL-35, etc.)	(2) CTLA-4 antibodies eliminate Tregs by ADCC activity

ADCC, antibody-dependent cellular cytotoxicity; CTLA-4, cytotoxic T lymphocyte-associated protein 4; CTLs, cytotoxic T lymphocytes; IFN- γ , interferon- γ ; MHC, major histocompatibility complex; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; STAT3, signal transducer and activator of transcription 3; TCRs, T cell receptors; TGF- β , transforming growth factor- β ; Tregs, regulatory T cells.

Fig. 7. Direct action of vascular endothelial growth factor on immune cells and tumor microenvironment. Modified from Fukumura et al. [41].

PD-L1 and anti-CTLA-4 antibodies is that blockade of the PD-1/PD-L1 pathway does not induce antitumor immunity if antigen-specific CD8-positive T cells are not present in cancer tissues; however, blockade of the B7-CTLA-4 pathway leads to increased activation of CD8-positive cells in the lymph nodes as well as increased infiltration of activated CD8-positive T cells into the tumor. Based on this rationale, several studies assessing the combination of anti-CTLA-4 and anti-PD-1/PD-L1 antibodies for treatment of HCC are currently under-way [38].

The results of the CheckMate 040 study, which tested different dose levels and dosing intervals of nivolumab and the anti-CTLA-4 antibody ipilimumab, were reported at ASCO 2019 [37]. In addition, a phase I/II study investigating combination of the anti-PD-1 antibody (durvalumab) with an anti-CTLA-4 antibody (tremelimumab) showed a favorable objective response rate of 25.0% in 40 patients with HCC [39]. The phase III study (HIMALAYA) is currently underway.

Problems with Modulating the Immunosuppressive Microenvironment

As described earlier, the combination of nivolumab and ipilimumab was more effective against melanoma than monotherapy [40]. However, under hypoxic conditions, which are a characteristic feature of solid cancers such as liver cancer, vascular endothelial growth factor

Fig. 8. Immunosuppressive tumor microenvironment produced by vascular endothelial growth factor. Adapted from Voron et al. [43].

(VEGF) influences immunosuppressive Tregs as well as tumor-associated macrophages and myeloid-derived suppressor cells, which release immunosuppressive cytokines such as IL-10 and TGF- β (Fig. 7, 8) [41–43]. It may be difficult to modulate such complex immunosuppressive microenvironments solely using anti-PD-1/PD-L1 antibodies and anti-CTLA-4 antibodies.

Various studies testing combinations of an immune checkpoint inhibitor and an agent targeting VEGF are currently underway. A nonclinical study showed that combination therapy with pembrolizumab and lenvatinib modulated an immunosuppressive tumor microenvironment rich in tumor-associated macrophages and Tregs. Combination therapy reduced secretion of TGF- β and IL-10, increased secretion of IL-12, and inhibited expression of PD-1 and Tim3, thereby inducing an antitumor immune response [44]. In fact, both response rate and disease control rate were highest with the pembrolizumab-lenvatinib combination among several combination trials (Table 4) [37, 39, 45–50].

Therefore, combination of a PD-1/PD-L1 antibody, an anti-CTLA-4 antibody, and a well-tolerated anti-VEGF antibody such as bevacizumab or ramucirumab is both rational and ideal, and clinical studies of such triple regimens are anticipated.

Conclusions

Sharma and Allison [13, 51] suggested that long-term survival can be expected in patients who achieve a clinical response to immune checkpoint inhibitors, and that the particularly high efficacy of combination therapies may offer an opportunity to achieve real

Table 4. Updated results of combination immunotherapy in HCC assessed by RECIST v1.1

	Anti-PD-1 monotherapy		Anti-PD-1/PD-L1 plus TKI/anti-VEGF			Anti-PD-1/PD-L1 plus anti-CTLA-4		
	nivolumab [45] (n = 214)	pembrolizumab [46] (n = 278)	pembrolizumab + lenvatinib [47] (n = 30)	atezolizumab + bevacizumab [48] (n = 104)	camrelizumab + apatinib [49] (n = 18)	avelumab + axitinib [50] (n = 22)	durvalumab + tremelimumab [39] (n = 40)	nivolumab + ipilimumab [37] (arm A) (n = 50)
ORR (95% CI)	20% (15–26)	18.3% (14.0–23.4)	53.3% (34.3–71.7)	36%	38.90%	13.6% (2.9–34.9)	25%	32%
DCR (95% CI)	64% (58–71)	62.20%	90.0% (73.5–97.9)	71%	83.30%	68.2% (45.1–86.1)	57.5% (>16 weeks)	54%
PFS, months (95% CI)	4.0 (2.9–5.4)	3.0 (2.8–4.1)	9.7 (7.7–NE)	7.3 (5.4–9.9)	7.2 (2.6–NE)	5.5 (1.9–7.4)	NA	NA
OS, months (95% CI)	NR ¹	13.9 (11.6–16.0)	14.6 (9.9–NE)	NR	NR	12.7 (8.0–NE)	NA	22.8 (9.4–NE)
DOR, months	9.9 (8.3–NE)	13.8 (1.5–23.6)	8.3 (3.8–11.0)	NR, ≥12 (30%)	NE	5.5 (3.7–7.3)	NA	17.5 (4.6–30.5+)

CI, confidence interval; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DCR, disease control rate; DOR, duration of response; HCC, hepatocellular carcinoma; NA, not available; NE, not evaluable; NR, not reached; ORR, overall response rate (RECIST 1.1); OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor. ¹ Nine months: 74%.

CI, confidence interval; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DCR, disease control rate; DOR, duration of response; HCC, hepatocellular carcinoma; NA, not available; NE, not evaluable; NR, not reached; ORR, overall response rate (RECIST 1.1); OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor. ¹ Nine months; 74%.

cures. Immune checkpoint inhibitors are expected to improve survival of those with HCC. Their combination with other therapies that modulate the immune status of the tumor microenvironment, such as anti-VEGF therapies, may lead to a paradigm shift in liver cancer treatment.

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