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Review Article

Clinical Development of Immune Checkpoint Inhibitors

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Recent progress in cancer immunotherapy has been remarkable. Most striking are the clinical development and approval of immunomodulators, also known as immune checkpoint inhibitors. These monoclonal antibodies (mAb) are directed to immune checkpoint molecules, which are expressed on immune cells and mediate signals to attenuate excessive immune reactions. Although mAbs targeting tumor associated antigens, such as anti-CD20 mAb and anti-Her2 mAb, directly recognize tumor cells and induce cell death, immune checkpoint inhibitors restore and augment the antitumor immune activities of cytotoxic T cells by blocking immune checkpoint molecules on T cells or their ligands on antigen presenting and tumor cells. Based on preclinical data, many clinical trials have demonstrated the acceptable safety profiles and efficacies of immune checkpoint inhibitors in a variety of cancers. The first in class approved immune checkpoint inhibitor is ipilimumab, an anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) mAb. Two pivotal phase III randomized controlled trials demonstrated a survival benefit in patients with metastatic melanoma. In 2011, the US Food and Drug Administration (FDA) approved ipilimumab for metastatic melanoma. Several clinical trials have since investigated new agents, alone and in combination, for various cancers. In this review, we discuss the current development status of and future challenges in utilizing immune checkpoint inhibitors.

1. Introduction

In this decade, remarkable progress has been made in the clinical application of cancer immunotherapies. Most notable is the emergence of immune checkpoint inhibitors. Large-scale clinical trials have shown their feasibility and efficacy for patients with advanced malignancies. The therapeutic targets, or "immune checkpoints," are also known as coinhibitory molecules or costimulatory molecules expressed on T cells.

As the name implies, costimulatory/inhibitory molecules mediate positive/negative signals that modify MHC-TCR (major histocompatibility complex-T-cell receptor) signaling pathways. These signals each regulate T-cell survival, proliferation, differentiation, or responsiveness to cognate antigens. The net effect depends on the balance among signals [1]. T-cell activation requires costimulatory signals. If they contact antigens without costimulatory ligands on antigen presenting cells (APCs), T cells remain inactivated in a state of anergy.

Coinhibitory molecules induce T-cell dysfunction (so called "T-cell exhaustion") or apoptosis. Employing this inhibitory pathway, the immune system can attenuate excessive immune reactions and ensure self-tolerance, which is important for maintaining immune homeostasis. These functions involve programmed cell death protein-1 (PD-1), programmed cell death-1 ligand-1/2 (PD-L1/2), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin mucin-3 (TIM-3), and B and T lymphocyte attenuator (BTLA). Tumor cells harness

these suppressive effects as one of their "immunoediting" mechanisms [2]. As shown in recent clinical trials, immune checkpoint blockade with monoclonal antibody promotes endogenous antitumor activities of immune cells and achieves clinically significant benefits for cancer patients [3, 4].

In this review, we focus on the current development status of and future challenges in utilizing immune checkpoint inhibitors, especially CTLA-4, PD-1, and PD-L1.

2. Anti-CTLA-4 Antibody

CTLA-4 (also known as CD152) is a member of the CD28 family of receptors [21, 22]. CTLA-4 is inducibly expressed on the surfaces of activated conventional $CD4^+$ and $CD8^+$ T cells. CTLA-4 binds to ligands B7.1 (CD80) and B7.2 (CD86) on APCs, where it competes with costimulatory receptor CD28 to bind with shared ligands. As CTLA-4 binds with higher affinity than CD28, it reduces CD28-dependent costimulation. CTLA-4 also mediates direct inhibitory effects on the MHC-TCR pathway [23]. CTLA-4 recruits 2 phosphatases, SHP-2 and PP2A, to its intracellular YVKM domain. SHP-2 dephosphorylates the CD3ζ chain, attenuating the TCR signal. PP2A inhibits downstream Akt phosphorylation, further impairing TCR signaling. Furthermore, CTLA-4 is constitutively and highly expressed on $CD4^+CD25^+FOXP3^+$ regulatory T cells (T regs) and plays a role in their suppressive functions [24–26]. CTLA-4 knockout mice have a lethal autoimmune-like syndrome. Prominent infiltration of $CD4^+$ T cells is detected in multiple organs. Thus, CTLA-4 is considered to be indispensable for maintaining immune homeostasis.

In the tumor microenvironment, CTLA-4 suppresses antitumor immune activities. In animal models, it has been shown that CTLA-4 blockade leads to reactivation of the antitumor immune response and tumor shrinkage [27–29]. The mechanism of action has not yet been fully elucidated. Observations made to date suggest that anti-CTLA-4 antibodies function not only by blocking inhibitory signals from reaching effector T cells but also by depleting regulatory T cells in the tumor microenvironment [30, 31]. For use in humans, based on preclinical studies, two anti-CTLA-4 antibodies have been developed: ipilimumab (Bristol-Myers Squibb) and tremelimumab (Pfizer).

2.1. Ipilimumab. Ipilimumab is a fully humanized IgG1 monoclonal antibody that inhibits CTLA-4 [32, 33].

Early clinical trials evaluated ipilimumab in patients with a variety of malignancies, including melanoma, prostate cancer, renal cell carcinoma, and non-Hodgkin lymphoma [34–45]. Some of these studies combined ipilimumab with a peptide vaccine, chemotherapy, or IL-2. Based on preclinical data, ipilimumab was administered at a dose range of 0.1–20 mg/kg, employing single or multiple dosing schedules (every 3-4 weeks).

A phase I study evaluated a single 3 mg/kg dose of ipilimumab for patients with metastatic hormone-refractory prostate cancer. Two (14%) of 14 patients showed ≧50% decline in prostate specific antigen. One (7%) patient developed grade 3 rash/pruritus requiring systemic corticosteroid administration [36]. Another phase I trial combined ipilimumab (administered at 3 mg/kg every 3 weeks) with a glycoprotein (gp) 100 peptide vaccine for patients with metastatic melanoma. Three (21%) of 14 patients responded to this treatment, including 2 showing complete responses (CRs). Grade 3 to 4 immune-related adverse events (irAEs) occurred in 6 (43%) patients. These irAEs included dermatitis, enterocolitis, hepatitis, and hypophysitis [34]. On the whole, irAEs were mild and manageable with therapy discontinuation and/or appropriate treatments, including corticosteroids.

A phase II trial compared 3 doses (0.3, 3, or 10 mg/kg) administered every 3 weeks for a total of 4 doses. Eligible patients were permitted to receive reinduction therapy (at a dose of 10 mg/kg) or maintenance therapy (administered at the previously assigned dose level every 12 weeks). The overall response rate (ORR) in the 10 mg/kg arm was superior to those in the other arms (11.1% versus 4.2% versus 0.0%), but irAEs were also higher in the 10 mg/kg arm [43]. The optimal dosing and scheduling are as yet unknown. A phase III randomized trial (NCT01515189) is currently comparing 2 doses (3 mg/kg versus 10 mg/kg). No consensus has yet been reached on the relative significance of reinduction versus maintenance therapy [46, 47]. A prospective study comparing reinduction therapy versus the physician's choice of chemotherapy (NCT00495066) is currently underway.

Based on pivotal phase III randomized controlled trials (RCTs) showing survival benefit, ipilimumab was approved by the US Food and Drug Administration (FDA) for metastatic melanoma [5, 6]. In the landmark phase III trial for patients with previously treated metastatic melanoma, ipilimumab (administered at 3 mg/kg every 3 weeks for a total of 4 doses) with or without the gp 100 peptide vaccination was compared with the gp 100 peptide vaccine alone. Eligible patients were permitted to receive reinduction therapy. The median OSs in the ipilimumab-containing arms were significantly superior to that in the gp 100 alone arm (10.1 months in ipilimumab/gp 100, 10.0 months in ipilimumab alone, and 6.4 months in gp 100 alone, hazard ratio (HR) 0.68; $P < 0.001$). Grade 3 to 4 irAEs were seen in 10–15% of patients in the ipilimumab-containing arms, while 3% in the gp 100 alone arm experienced irAEs. There were 14 treatment-related deaths (2.1%), including 7 patients with irAEs [5]. Long-term follow-up analysis confirmed an approximately 20% survival rate for patients in the ipilimumab-containing arms. Safety profiles in long-term survivors were comparable among the 3 groups, and new onset irAEs after the last dose of ipilimumab were infrequent (8%; all grades) [48]. The other phase III trial compared ipilimumab (at 10 mg/kg every 3 weeks for 4 doses)/dacarbazine with dacarbazine/placebo, followed by maintenance therapy with ipilimumab or placebo administered every 12 weeks for eligible patients. Overall survival (OS) was significantly longer in the ipilimumab/dacarbazine arm (11.2 versus 9.1 months), and the higher survival rates were durable (47.3% versus 36.3% at 1 year, 28.5% versus 17.9% at 2 years, 20.8% versus 12.2% at 3 years, HR for death 0.72; $P < 0.001$). Grade 3 to 4 AEs were seen in more patients in the ipilimumab/dacarbazine arm (56.3% versus 27.5%;

$P < 0.001$). No drug-related deaths occurred among those in the ipilimumab/dacarbazine arm [6].

The analysis of the collected data from 12 previous clinical trials, which include 1861 ipilimumab-treated patients with advanced melanoma, demonstrated a median OS of 11.4 months and 3-year OS rate of 22%. The OS curve started to show plateau around year 3, which was independent of the dose of ipilimumab (3 or 10 mg/kg), therapy line (treatment-naïve or not), or use of maintenance therapy [49].

2.2. Tremelimumab. Tremelimumab is a human IgG2 monoclonal antibody that blocks CTLA-4 [50].

Early clinical trials on tremelimumab monotherapy showed response rates of 2–17%, and these responses were durable (>150 days) [51–57]. Based on preclinical and clinical data, the standard regimen is 15 mg/kg every 90 days. Most adverse events were mild and manageable. These adverse events included skin rash, diarrhea, and endocrine abnormalities.

A phase III study compared tremelimumab (15 mg/kg every 3 months) with chemotherapy (physician's choice) in patients with untreated advanced melanoma [7]. This study demonstrated no benefits in either ORR (10.7% versus 9.8%) or OS (12.6 mo versus 10.7 mo), but a superior response duration was seen (35.8 versus 13.7 months). This observation might be explained by patient selection bias (exclusion of patients with lactate dehydrogenase (LDH) >2x upper limit of normal), drug crossover (to ipilimumab) in the control arm, and even a potentially suboptimal dosing regimen. Tremelimumab is still being investigated for other tumors, both alone and as combination therapy (Table 1).

3. Anti-PD-1 Antibodies

Programmed cell death protein-1 (PD-1; also known as CD279), like CTLA-4, is a coinhibitory CD28-family molecule [22]. While CTLA-4 works in the early phase of naïve-T-cell activation, PD-1 functions mainly in the late phase, in which PD-1 induces exhaustion or anergy in effector T cells. Thus, PD-1 is considered to play an important role in chronic inflammation such as that associated with viral infection or tumor exposure [58]. PD-1 is expressed on activated T cells, T regs [59], activated B cells, NK cells, and monocytes. It binds to the B7-family ligands PD-L1 (programmed death ligand-1, B7-H1) and PD-L2 (programmed death ligand-2, B7-DC) on APCs. PD-1 has cytoplasmic domain motifs known as ITIM (immunoreceptor tyrosine-based inhibitory motif) and ITSM (immunoreceptor tyrosine-based switch motif) [23]. When these motifs are phosphorylated, they recruit two inhibitory phosphatases, SHP-1 and SHP-2 (SHP: SH2-containing-phosphatase). These phosphatases dephosphorylate the CD3ζ chain, decreasing TCR signaling. Although the inhibitory mechanisms of CTLA-4 and PD-1 have some similarity in terms of inhibiting Akt activation, CTLA-4 can also interfere with Akt independently via PP2A [23]. PD-1 knockout mice show a milder lupus-like syndrome than CTLA-4 knockout mice [60].

Tumor cells utilize the PD-1-PD-L1/2 pathway to evade immune-cell attack [61]. Blockade of this pathway was shown to restore and augment antitumor immune activities [62].

3.1. Nivolumab (BMS-936558/ONO-4538). Nivolumab is a fully humanized IgG4 monoclonal antibody that blocks PD-1 [62].

Phase I studies tested nivolumab in such cancers as melanoma, non-small cell carcinoma of the lung (NSCLC), ovarian cancer, and renal cell carcinoma. These studies showed response rates of approximately 20–30%, durable tumor regression (>1 year), and an acceptable safety profile, with Grade 3 to 4 irAEs developing in about 20% of patients [8, 9, 63–65]. In long-term follow-up of the phase I trial for advanced melanoma, median OS was 16.8 months and survival rates were 62% at 1 year and 43% at 2 years. The patients requiring discontinuation of treatment maintained their tumor responses for at least 16 months (16–56 months). Long-term safety profiles were acceptable and similar to those described in a previous report [8]. The preliminary results of a phase I study evaluating nivolumab (at 3 mg/kg q2w) for untreated advanced NSCLC were recently reported. The ORR was 30% with 2 complete remissions (CRs), as measured by RECIST. ORR and progression-free survival (PFS) correlated with PD-L1 positivity (67% versus 0% for ORR, 45.6 mo versus 36.1 mo for median PFS). AEs were generally manageable and grade 3 to 4 AEs occurred in 3 patients, including rash, increased transaminase, and hyperglycemia [66].

Recently the interim analysis report of a phase III study (NCT01721746), comparing nivolumab monotherapy (at 3 mg/kg q2w) with investigator's choice chemotherapy in ipilimumab-refractory advanced melanoma, was shown. The ORRs were 32% in the nivolumab arm and 11% in the control arm, with the median duration of response in the nivolumab arm not reached. Grade 3 to 4 drug-related AEs were less frequent in the nivolumab arm (9% versus 31%) [10]. Another phase III study (NCT01721772) compared nivolumab monotherapy (at 3 mg/kg q2w) with dacarbazine in 418 patients with previously untreated stage III or IV melanoma. This study was stopped ahead of schedule and unblinded after independent data monitoring committee found significant survival superiority in nivolumab over dacarbazine. The results from the double-blind part of the study before the stoppage showed that the OS rate at 1 year was significantly higher in the nivolumab arm (72.9% versus 42.1%, HR for death 0.42; $P < 0.001$), and the median PFS was also significantly longer in the nivolumab arm (5.1 versus 2.2 months, HR for death or progression 0.43; $P < 0.001$). Grade 3 to 4 drug-related AEs occurred in more patients in the dacarbazine arm (11.7% versus 17.6%). No drug-related deaths occurred in both arms [11]. A phase II study (NCT01927419) of nivolumab in combination with ipilimumab compared with ipilimumab alone for advanced melanoma is currently ongoing (recruitment has been completed).

In 2013, nivolumab received Fast Track designation for the treatment of NSCLC, melanoma, and renal cell carcinoma (RCC) from the FDA. In April 2014, a rolling submission to the FDA for nivolumab in third-line pretreated NSCLC was started. In May 2014, nivolumab received a Breakthrough

Table 1

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
CTLA-4	Ipilimumab	III	Completed (NCT00094653)	Melanoma	676	Endpoint: safety/efficacy Ipi + gp100 versus Ipi versus gp100	Ipi + gp100: ORR 5.7%; SD 14.4%	Ipi + gp100 versus gp100: 10.1 versus 6.4 mos	Ipi + gp100: drug-related 17.4%; irAEs 10.2%; diarrhea 4.5%; fatigue 5.0%; dyspnea 3.7%; anemia 2.9%; endocrine abnl. 11%; AST↑ 0.5%; ALT↑ 0.3%	[5]
		III	Completed (NCT00324155)	Melanoma	502	Endpoint: efficacy Ipi + DTIC versus PBO + DITC	Ipi + DTIC: ORR 15.2%; SD 18.0%	Ipi + DTIC versus PBO + DTIC.: 11.2 versus 9.1 mos	Ipi + DTIC: immune-related 41.7%; pruritus 2.0%; rash 1.2%; diarrhea 4.0%; colitis 6.1%; AST↑ 17.4%; ALT↑ 20.7%	[6]
	Tremelimumab	III	Completed (NCT00257205)	Melanoma	655	Endpoint: efficacy treme. versus chemo.	ORR 10.7%	Treme. versus chemo.: 12.6 versus 10.7 mos (NS)	52%; diarrhea/colitis 18%; fatigue 6%; rash 2%; pruritus 1%; dyspnea 3%; hypothalamus and pituitary disorders 1%; hepatitis 1%	[7]

Table 1: Continued.

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
PD-1	Nivolumab (BMS-936558/ONO-4538)	I	Ongoing (not recruiting) (NCT00730639)	Melanoma	107	Endpoint: safety/efficacy 5 dosing regimens	ORR 30.8%; median duration of response 104 wks; SD (≧24 wks) 6.5%	OS 16.8 mos; PFS 3.7 mos	22.4%; fatigue 1.9%; diarrhea 1.9%; abdominal pain 1.9%; lymphopenia 2.8%	[8]
		I	Ongoing (not recruiting) (NCT01176461)	Melanoma	90	Endpoint: safety/efficacy 3 dosing regimens	ORR 25%; SD (≧24 wks) 21%	PFS (at 24 wks) 46%	5.6%; rash 2.2%; interstitial pneumonitis 2.2%	[9]
		III	Ongoing (not recruiting) (NCT 01721772)	Melanoma	370	Endpoint: efficacy Nivo. versus ICC	ORR 32% versus 11%	NA	9% versus 31%	[10]
		III	Completed (NCT01721772)	Melanoma	418	Endpoint: efficacy Nivo. versus dacarbazine	ORR 40.0% versus 13.9%	OS (at 1 yr) 72.9% versus 42.1%, median PFS 5.1 versus 2.2 mo	11.7% versus 17.6%; fatigue 0.5%; diarrhea 1.0%; rash 0.5%; vomiting 0.5%	[11]
	Pidilizumab (CT-011)	II	Completed (NCT01435369)	Melanoma	103	Endpoint: safety/efficacy 2 dosing regimens	ORR 5.9%	OS (at 1 yr): 64.5%	NA	[12]
		I	Ongoing (not recruiting) (NCT01295827)	Melanoma	135	Endpoint: safety/efficacy 3 dosing regimens	ORR 38% by RECIST and 37% by irRC	Median PFS >7 mos	13%; hypothyroidism 1%; diarrhea 1%; fatigue 1%; AST↑ 1%; renal failure 1%; rash 2%; pruritus 1%	[13]
	Pembrolizumab (MK-3475)	I	Ongoing (not recruiting) (NCT01295827)	Untreated NSCLC	57	Endpoint: safety/efficacy 3 dosing regimens	ORR 26% by RECIST and 47% by irRC	Median OS NR; OS at 1 yr 80%; median PFS 45.6%; PFS at 24 wks 70%	CK↑ 2%; pericardial effusion 2%; pneumonitis 2%; acute kidney injury 2%	[14]
		I	Ongoing (not recruiting) (NCT01848834)	Head and neck cancer	60	Endpoint: safety/efficacy single arm	ORR 19.6% in total, 20.0% in HPV+, and 19.4% in HPV−;	NA	Gr3–5 16.7%; Rash 3.3%	[15]
		I	Ongoing (not recruiting) (NCT01848834)	Gastric cancer	39	Endpoint: safety/efficacy single arm	ORR 30.2% by RECIST	NA	7.7%; hypoxia 2.6%; peripheral neuropathy 2.6%; pneumonia 2.6%	[16]

Table 1: Continued.

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
	BMS-936559	I	Ongoing (not recruiting) (NCT00729664)	Melanoma	52	Endpoint: safety 4 dose levels	ORR 17%; SD (≧24 wks) 27%	PFS (at 24 wks) 42%	9%; fatigue 1%; infusion reaction 1%; lymphopenia 1%	[17]
				NSCLC	49		ORR 10%; SD (≧24 wks) 12%	PFS (at 24 wks) 31%		
				Ovarian cancer	17		ORR 6%; SD (≧24 wks) 18%	PFS (at 24 wks) 22%		
				Renal cell carcinoma	17		ORR 12%; SD (≧24 wks) 41%	PFS (at 24 wks) 53%		
PD-L1	MPDL3280A	I	Recruiting (NCT01375842)	Urothelial bladder cancer	68	Endpoint: safety/efficacy/ biomarker single arm	ORR: PD-L1 + 43% (at 6 wks) and 52% (at 12 wks); PD-L1 − 11% (at 6 wks);	NA	4%; no irAE	[18]
	MEDI4736	I	Recruiting (NCT01693562)	Advanced solid tumors	26 (as of Jan 2014)	Endpoint: safety/efficacy single arm	PR 15.4%; disease control rate (≧12 wks) 46%	NA	Any Gr 34%; Gr3/4 0%; no DLT; no MTD	[19]
	MSB0019718C	I	Recruiting (NCT01772004)	Refractory malignancies	27 (as of Jan 2014)	Endpoint: safety single arm	NA	NA	Treatment discontinuation 52.2% (8.7% for AEs); drug-related AEs 11.1%; DLT 3.7% (CPK↑, myositis, and myocarditis)	[20]

Abbreviations: NSCLC, non-small cell lung cancer; Ipi, Ipilimumab; gp100, glycoprotein 100 peptide vaccine; DITC, dacarbazine; PBO, placebo; ORR, objective response rate; PR, partial response; SD, stable disease; mo, month; wk, week; RECIST, response evaluation criteria in solid tumors; irRC, immune-related response criteria; HPV, human papillomavirus; NA, not available; NS, not significant; NR, not reached; OS, overall survival; PFS, progression-free survival; AE, adverse event; irAE, immune-related adverse event; Gr, Grade; abnl., abnormality; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase; DLT, dose limiting toxicity; MTD, maximum tolerance dose; ICC, investigator's choise chemotherapy.

Therapy designation for non-Hodgkin lymphoma from the FDA. In Japan, in July 2014, nivolumab received manufacturing and marketing approval for unresectable melanoma from the domestic regulator, the Ministry of Health Labor and Welfare, which made nivolumab the first in anti-PD-1 antibody to receive regulatory approval in the world.

3.2. Pidilizumab (CT-011). Pidilizumab (CT-011) is a humanized IgG-1κ monoclonal antibody that blocks PD-1. In animal models, an antitumor effect was achieved with BAT monoclonal antibody (a murine mAb developed against a membrane preparation of a Burkitt lymphoma cell line), from which pidilizumab is derived [67, 68].

In humans, the safety and tolerability of the single dose regimen were shown in a phase I study of patients with advanced hematologic malignancies [69]. No treatment-related toxicities occurred and the maximum tolerated dose was not identified in this trial (0.2–6 mg/kg).

Pidilizumab has been tested in phase II trials, as monotherapy for patients with diffuse large B-cell lymphoma after autologous hematopoietic stem-cell transplantation [70] and as combined therapy with rituximab for relapsed follicular lymphoma [71]. Both trials showed promising efficacies even in high-risk patients.

The results of a phase II trial in patients with pretreated advanced melanoma were recently reported. ORR was 5.9%, measured by immune-related response criteria (irRC), and the OS rate at 1 year was 64.5%. The patients who had been pretreated with ipilimumab (51% of patients) tended to experience a higher rate of immune-related stable disease (irSD) and longer PFS (2.8 mo versus 1.9 mo) [12].

3.3. Pembrolizumab (MK-3475, Formally Known as Lambrolizumab). Pembrolizumab (MK-3475) is a humanized monoclonal IgG-4κ antibody that blocks PD-1.

A phase I dose-escalation study evaluated three dose levels, 1 mg/kg, 3 mg/kg, and 10 mg/kg, administered every 2 weeks, in patients with multiple solid tumors [72]. All dose levels were found to be safe, and the maximum tolerated dose was not identified. Clinical responses were observed at all dose levels. Another phase I study tested 3 regimens (2 mg/kg every 3 weeks and 10 mg/kg every 2 or 3 weeks) in patients with advanced melanoma [13]. AEs were generally mild and grade 3 to 4 AEs were seen in 13% of patients. The ORRs ranged from 38% to 52%, in the biweekly 10 mg/kg cohort (measured by RECIST), showing no significant differences. These responses were durable, with the median PFS exceeding 7 months for all three regimens.

An ongoing phase II trial is now comparing 2 dose levels of pembrolizumab with investigator-choice chemotherapy in patients with previously treated advanced melanoma (NCT01704287). Another ongoing phase II trial is also evaluating 2 dose schedules of pembrolizumab (10 mg/kg q2w or q3w) compared with ipilimumab (3 mg/kg q3w) for advanced melanoma (NCT01866319).

In April 2013, pembrolizumab received the Breakthrough Therapy designation for advanced melanoma from the FDA. After being reviewed under the FDA's Accelerated Approval program, in September 2014, pembrolizumab received approval for treatment of patients with advanced melanoma by the FDA.

Besides melanoma, several early trials have showed the tolerability and antitumor effects of pembrolizumab in other tumors. The preliminary results of another phase I study evaluating pembrolizumab in untreated PD-L1-positive NSCLC were recently reported. The overall objective response rate was 25% (33% in the 2 mg/kg q3w, 20% in the 10 mg/kg q3w, and 31% in the 10 mg/kg q2w group), as measured by RECIST. AEs were generally mild and grade 3 to 4 AEs occurred in 3 patients, including pneumonitis requiring treatment discontinuation [14]. Another preliminary result was reported for the phase I trial of pembrolizumab as monotherapy, administered at 2 mg/kg every 2 weeks, to 60 patients with recurrent/metastatic head and neck cancers. Grade 3 to 4 drug-related AEs were reported in 16.7% of patients. The best ORR was 20% in all patients (assessed by RECIST 1.1). Efficacies were comparable between human papilloma virus- (HPV-) positive and HPV-negative patients (20.0% versus 19.4%) [15]. Another phase I study (NCT01848834) assessed pembrolizumab in the patients with previously treated advanced gastric cancer that expressed PD-L1. The enrolled 39 patients were treated with pembrolizumab at 10 mg/kg q2w. Median follow-up period was 6 months. Treatment-related AEs occurred in 24 patients (61.5%), and those of grade 3 to 5 occurred in 3 patients (pneumonitis, peripheral neuropathy, and hypoxia). ORR was 30.8% and disease control rate was 43.6%. Responses were mostly ongoing and the median response duration was not reached [16].

4. Anti-PD-L1 Antibodies

PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-DC or CD273) are inhibitory B7-family molecules that bind the PD-1 receptor. PD-L1 is inducibly expressed on a variety of hematopoietic and nonhematopoietic cells, including most human tumor cells and cells within the tumor microenvironment [61]. PD-L1 expression has been shown to correlate inversely with the clinical outcomes of some malignancies. PD-L2 is expressed on hematopoietic cells. PD-L1 knockout mice show infiltration of lymphocytes into nonlymphoid organs and exacerbation of preexisting autoimmune diseases [73, 74].

As mentioned above, the PD-1-PD-L1 axis is one of the main mechanisms by which cancer cells evade immune-cell attack [61]. Blockade of this pathway was shown to reinforce antitumor immune activities [62]. Because PD-L1 also interacts with CD80 [75, 76], anti-PD-L1 antibody might have optimal clinical potency against PD-1.

4.1. BMS-936559. BMS-936559 is a fully humanized IgG4 monoclonal anti-PD-L1 antibody. It inhibits the binding of PD-L1 to PD-1 and CD80. A phase I dose-escalation study evaluated BMS-936559 in 207 patients with selected cancers, including melanoma, NSCLC, ovarian cancer, and renal cell carcinoma. The study drug was administered at 4 dose levels (0.3–10 mg/kg) every 14 days, 3 times in each 6-week course for up to 16 cycles, when either CR or disease progression was confirmed. The ORRs were 6–17% and efficacy was durable

(>1 year in 8 of 16 patients who responded). Grade 3 to 4 irAEs, seen in 9% of the patients, were treatment-related in 5% [17].

4.2. MPDL3280A. MPDL3280A is a humanized IgG-1κ monoclonal anti-PD-L1 antibody. It is genetically engineered to modify the Fc domain, thereby impairing the antibody-dependent cellular cytotoxicity of PD-L1 expressing cells [77, 78].

A phase I trial of MPDL3280A as monotherapy for advanced melanoma achieved a response rate of 26% and PFS of 35% at 24 weeks. Grade 3 to 4 AEs were seen in 33% of patients [79]. The results of another phase I trial were recently reported. MPDL3280A was tested in patients with pretreated metastatic urothelial bladder cancer. ORR in PD-L1-positive patients was superior to that in PD-L1-negative patients (43% versus 11% at 6 weeks). ORR at 12 weeks was 52% in PD-L1-positive patients. Grade 3 to 4 AEs were seen in 4% of patients, with no irAEs [18]. The FDA has granted the Breakthrough Therapy designation to MPDL3280A.

4.3. MEDI4736. MEDI4736 is a humanized IgG-1κ monoclonal antibody that blocks PD-L1. MEDI4736 demonstrated tumor regression and improved survival in a mouse model.

A "first-time-in-human" phase I study evaluating the safety, tolerability, and pharmacokinetics of this agent in patients with advanced solid tumors is currently underway (NCT01693562). The interim report was recently presented. As of January 2014, 26 patients were receiving dose-escalation treatments and had been given a median of 5 (1–25) q2w and 4.5 (1–7) q3w doses of MEDI4736 across 6 cohorts (0.1–10 mg/kg q2w; 15 mg/kg q3w). No dose limiting toxicities (DLTs) or maximum tolerated dose was identified. Treatment-related AEs occurred in 34% of patients, but all were grade 1 to 2 and did not lead totreatment discontinuation. Four of the 26 patients showed partial responses (PRs). The rate (PR + stable disease ≧ 12 weeks) was 46%. Clinical responses were durable, with 11 patients remaining in the study (2+ to 14.9+ months) [19]. Another phase I trial is now testing the combination of MEDI4736 plus tremelimumab (NCT01975831).

4.4. MSB0010718C. MSB0010718 is a fully humanized IgG1 monoclonal antibody directed to PD-L1. A phase I trial is currently testing MSB0010718 to assess its safety, tolerability, and pharmacokinetics in patients with refractory malignancies (NCT01772004). As of January 2014, 27 patients had been enrolled and were participating in a dose-escalation study (3 + 3 design; 1, 3, 10, and 20 mg/kg, q2w). Twenty-three patients had been followed for at least 4 weeks. Discontinuation of the treatment had been necessary in 12 patients (52.2%): 9 (39.1%) due to progression of disease, 2 (8.7%) for AEs, and 1 (4.3%) because the patient died. Grade 3 to 4 drug-related toxicities included laboratory abnormalities in 3 patients. One DLT was observed in 1 patient at dose level 4 (20 mg/kg): an irAE with creatine kinase elevation, myositis, and myocarditis [20].

5. Combination Therapy

Recent clinical trials have actively investigated the potential for synergistic effects by combining immune checkpoint inhibitors with other agents. The partner agents/therapies include other checkpoint agents, cytotoxic agents, anticancer vaccines, cytokines, and radiotherapy.

A phase I study evaluated combined therapy with ipilimumab plus nivolumab in patients with advanced melanoma [80]. The patients received ipilimumab once every 3 weeks for 4 doses and nivolumab once every 3 weeks for 8 doses concurrently. Then, eligible patients were permitted to receive both once every 12 weeks up to 8 doses. Grade 3 to 4 treatment-related AEs were seen in 53% of the concurrent-cohort patients but were mild and manageable. The maximum tolerated dose was 3 mg/kg of ipilimumab and 1 mg/kg of nivolumab, a dosing regimen at which 53% of patients showed responses. Recent follow-up surveys confirmed OS to be 94% at 1 year and 88% at 2 years in this cohort. An expansion cohort, with the patients receiving 3 mg/kg of ipilimumab and 1 mg/kg of nivolumab every 3 weeks for 4 doses and 1 mg/kg of nivolumab every 2 weeks until disease progression, is currently being evaluated in a phase II/III study [81]. A phase III trial (NCT01844505) evaluating this combination is currently ongoing (recruitment has been completed).

6. Biomarkers for Predicting Clinical Benefits and Adverse Reactions

Although immune checkpoint inhibitors have shown promising safety and efficacy, to date only a small proportion of patients have achieved long-term survival, with severe irAEs occurring on occasion. Biomarkers predicting clinical benefit may enable physicians to select individualized treatments for their patents and thereby maximize clinical benefits. Thus, there is an urgent need to identify "baseline (pretreatment)" biomarkers predicting responses or toxicities. Several biomarkers for examining T-cell proliferation or activation and other forms of antigen-specific immunity have been assessed in the context of immune checkpoint inhibitors.

Immunohistochemical PD-L1 expression in a tumor specimen is among the potential markers for PD-1-PD-L1-directed therapies. In a phase I study of nivolumab, though the data obtained are preliminary, an objective response was seen only in the patients who showed immunohistochemical PD-L1 expression in pretreatment tumor specimens [63]. These observations may support the strategy of selecting PD-L1-positive patients for therapy. However, PD-L1 expression on tumor cells is inducible and is susceptible to influences of the tumor microenvironment. Furthermore, technical advances in PD-L1 immunostaining are still needed. Also, the value of PD-L1 IHC staining as a predictive biomarker for combination therapy with nivolumab plus ipilimumab has yet to be validated [80]. As yet, the applicability and significance of PD-L1 expression as a baseline biomarker must be interpreted with caution and further prospective evaluations are needed, including the results of ongoing randomized

clinical trials that are prospectively evaluating PD-L1 IHC as a companion diagnostic platform (NCT01721746).

Another potential biomarker is pretreatment levels of monocytic myeloid-derived suppressor cells (m-MDSCs) [82, 83]. A recent retrospective study suggested higher pretreatment quantities of $Lin^{-}CD14^{+}HLA\text{-}DR^{low/-}$ m-MDSC to be associated with inferior OS in patients with metastatic melanoma treated with ipilimumab [83].

Recent genetic analysis using whole-exome sequencing showed the significance of somatic mutational load as predictive biomarker of clinical benefit in melanoma patients treated with CTLA-4 blockade. The neopeptide signature associated with clinical response was identified and predicted mutant peptides were verified to activate patient T cell *in vitro* [84].

Other potential predictive/prognostic biomarkers include the gene expression profiles obtained employing tumor biopsies [85, 86], CRP level [87], absolute lymphocyte and eosinophil counts [88], and LDH levels [89]. These possibilities await further research.

7. Conclusion

Immune checkpoint inhibitors have opened a new era of cancer immunotherapy. Since the FDA approval was obtained for the anti-CTLA-4 monoclonal antibody ipilimumab, several large-scale clinical trials have evaluated new agents both alone and in combinations with other conventional or new therapies. Future challenges include exploring new target molecules and immune cells, optimizing dosing regimens and combination therapies, validating the safety and efficacy of these novel treatment strategies in many other malignancies, establishing an immunomonitoring system to be applied during therapy, and identifying biomarkers predicting clinical responses and toxicities. Active, ongoing investigations are anticipated to provide further clinical benefits for patients with cancers that are currently refractory to treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Cancer Immunology Research

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Research Article

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Computational Algorithm-Driven Evaluation of Monocytic Myeloid-Derived Suppressor Cell Frequency for Prediction of Clinical Outcomes

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Abstract

Evaluation of myeloid-derived suppressor cells (MDSC), a cell type implicated in T-cell suppression, may inform immune status. However, a uniform methodology is necessary for prospective testing as a biomarker. We report the use of a computational algorithm-driven analysis of whole blood and cryopreserved samples for monocytic MDSC (m-MDSC) quantity that removes variables related to blood processing and user definitions. Applying these methods to samples from patients with melanoma identifies differing frequency distribution of m-MDSC relative to that in healthy donors. Patients with a pretreatment m-MDSC frequency outside a preliminary definition of healthy donor range (<14.9%) were significantly more likely to achieve prolonged overall survival following treatment with ipilimumab, an antibody that promotes T-cell activation and proliferation. m-MDSC frequencies were inversely correlated with peripheral $CD8^+$ T-cell expansion following ipilimumab. Algorithm-driven analysis may enable not only development of a novel pretreatment biomarker for ipilimumab therapy, but also prospective validation of peripheral blood m-MDSCs as a biomarker in multiple disease settings. *Cancer Immunol Res; 2(8); 812–21. ©2014 AACR.*

Introduction

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of granulocyte- and monocyte-like cells that inhibit T-cell function (1, 2). Clinically significant MDSC accumulation has been observed in many challenges to the immune system in humans including chronic infection, transplant, and multiple malignancies (3–10). Diversity in phenotype and methods used for analysis creates challenges in prospectively and reproducibly defining the clinical import of this cellular subset. Monocytic MDSCs (m-MDSC) are frequently characterized as $CD14^+$/HLA-$DR^{low/-}$ cells in humans; however, HLA-DR expression is typically a broad distribution, making identification of a specific subset of cells susceptible to inter-user variability. Nevertheless, increased $CD14^+$/HLA-$DR^{low/-}$ cells in the peripheral blood have been designated as m-MDSCs in individual datasets based on this cell population's ability to suppress lymphocyte function and are prognostic in patients with hematologic cancers (chronic lymphocytic leukemia and multiple myeloma), solid tumors (hepatocellular carcinoma, non–small cell lung cancer, melanoma, and others), chronic infection (HIV), cirrhosis, and allotransplantation (5, 8, 11–17).

In melanoma, m-MDSCs correlate with melanoma disease activity and are independently prognostic of overall survival (OS) in patients with stage IV disease (6, 18–20). Levels of m-MDSC inversely correlate with the presence of NY-ESO-1–specific T cells and seem to be increased in ipilimumab nonresponders (20, 21). This finding suggests a link between m-MDSC and antigen-specific immunity *in vivo* and provides additional rationale for routinely evaluating m-MDSCs as a biomarker in the context of immunotherapy clinical trials. However, a uniform methodology that corrects for artifacts introduced by cell processing, cryopreservation, and analysis needs to be developed to enable routine measurement of m-MDSCs for prospective testing as a biomarker (22).

Immunomodulatory therapy, which has emerged as a promising treatment approach for metastatic melanoma and other cancers, is an area in which biomarker development may enable selection of therapy for individuals more likely to achieve prolonged OS. Ipilimumab, an antibody that blocks the function of the immune inhibitory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4), was the first immunomodulatory antibody to gain regulatory approval as a cancer therapeutic based on two phase III studies demonstrating

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significant increases in OS in patients with metastatic melanoma (23, 24). However, only 20% to 30% of patients achieve long-term survival following therapy (25). This finding not only supports the need to define biomarkers in this context, but also to identify mechanisms of resistance that could lead to additional therapeutic targets for improved outcomes.

A number of biomarkers examining T-cell proliferation or activation and antigen-specific immunity have been assessed in the context of ipilimumab therapy. Gene expression profiling on tumor biopsies collected from 45 patients with melanoma before and after ipilimumab treatment showed that an immunologically active tumor microenvironment favors clinical response to ipilimumab (26, 27). In peripheral blood, sustained ICOS elevation in $CD4^+$T cells, higher percentage of $EOMES^+$ $CD8^+$ T cells or $Ki67^+EOMES^+CD8^+$ T cells, and an NY-ESO-1–specific $CD8^+$ T-cell response in patients with NY-ESO-1–seropositive metastatic melanoma have all shown an association with clinical benefit and survival following ipilimumab therapy (28, 29).

Absolute lymphocyte count (ALC), the most clinically accessible biomarker, available through a routine complete blood count, has been shown to correlate with OS in several single-institution, noncontrolled studies (30). More recently, an analysis of almost 2,000 ipilimumab-treated patients in multiple studies, including randomized, controlled, and phase III studies, has demonstrated that an ALC increase is a specific pharmacodynamic biomarker of ipilimumab. In the absence of concomitant chemotherapy, the degree of this pharmacodynamic increase in lymphocyte count at the commercially available ipilimumab dose (3 mg/kg) is associated with OS (Postow et al.; submitted for publication), suggesting that ALC is worthy of further investigation in the context of risk-adapted clinical trial design.

We report the development of methods to enable uniform analysis of m-MDSCs that overcome issues related to blood processing and inter-user variability. This is achieved by deriving a measure of m-MDSCs using coefficient of variation (CV) to assess HLA-DR spread on $CD14^+CD11b^+$ cells and through the evaluation of stabilizers of HLA-DR levels in whole blood. We validate these methods by demonstrating that $CD14^+HLA\text{-}DR^{low/-}$ m-MDSC quantity derived from CV values is both inversely correlated with pharmacodynamics markers of ipilimumab function and also associated with OS among patients undergoing treatment with ipilimumab.

Materials and Methods

Patients

We identified 83 patients who were treated on a clinical study with ipilimumab between February 2008 and March 2012 and had cryopreserved peripheral blood samples in our tissue banks. Peripheral blood from healthy donor volunteers was obtained at the time of the current study and from samples in our institutional tissue bank. MDSC analyses were performed between December 2011 and March 2012. We excluded 4 and 11 samples in the 10-mg/kg and 3-mg/kg ipilimumab groups, respectively, because of an overnight delay between phlebotomy and processing time, which validation studies confirmed affects levels of HLA-DR (Fig. 1E). Patients and healthy donors provided informed consent for the clinical studies and the collection of blood and tumor tissue on a correlative biospecimen protocol. Patients were treated with ipilimumab on Bristol-Myers Squibb studies CA184045 (NCT00495066) or CA184-087 (NCT00920907), with four doses of ipilimumab at 10 mg/kg or 3 mg/kg i.v. every 3 weeks during induction therapy, followed by maintenance ipilimumab at the same dose every 12 weeks, starting at week 24. Clinical benefit was determined by investigators at week 24 imaging based on interpretation of radiographic stable disease or better by modified World Health Organization (mWHO) or RECIST criteria. All studies were approved by the Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY) Institutional Review Board.

MDSC staining

Blood was collected and cryopreserved using BD Vacutainer CPT tubes (BD pharmingen) from patients with melanoma and healthy donors for the retrospective analysis. Samples were collected from patients and healthy donors in Cyto-Chex (Streck), Vacutainer CPT, or standard heparin vacutainer tubes for comparative analysis. Peripheral blood mononuclear cells (PBMC; 5×10^5) from patients with melanoma or healthy donors were washed with 2 mL FACS buffer (PBS containing 2% bovine serum albumin and 0.05 mmol/L EDTA). The following antibodies were then added for 30 minutes at 4°C: Lineage (CD3/CD16/CD19/CD20/CD56) cocktail FITC (BD Pharmingen), CD14-PerCP Cy5.5, CD11b-APC Cy7, CD33-PE-Cy7 (BD Pharmingen), and HLA-DR-ECD (Beckman Coulter). Isotype controls included the appropriate fluorochrome-conjugated mouse IgG1, IgG 1k, IgG2a, or IgG2b k (BD Pharmingen; Beckman Coulter; R&D Systems). Whole blood samples were lysed for 10 minutes in BD Phosflow Lyse/Fix after staining (BD Pharmingen). Stained cells were detected using a LSR Fortessa with FACS Diva software (BD Biosciences). Analysis was carried out using FlowJo (TreeStar). m-MDSCs were quantified as described. Briefly, scale values for HLA-DR within a singlet, live, lineage-negative (Lin^-; CD3, CD16, CD19, CD20, and CD56) cell population that expressed $CD14^+CD11b^+$ were exported from FlowJo and analyzed using code written in R software to derive the CV, a ratio of standard deviation (SD; σ) and geometric mean fluorescence intensity (GMFI). A %m-MDSC frequency defined as the $\%HLA\text{-}DR^{low/-}$ among $CD14^+$ $CD11b^+$ cells was derived using a nomogram based on the 99th percentile $CV_{HLA\text{-}DR}$ among $CD14^+CD11b^+$ cells from healthy donors. Absolute number of m-MDSC (/μL) in peripheral blood was estimated using the formula: (%m-MDSC) × (number of monocytes (/μL) from a complete blood count on the same day.

T-cell suppression assay

A T-cell suppression assay was performed as described previously (31). Briefly, $CD14^+$ PBMCs magnetically separated using MACS beads (Miltenyi Biotec) were cultured with 2×10^5 CFSE-labeled autologous $CD14^-$ PBMCs in 96-well flat-bottom α-CD3–specific Ab-coated plates (OKT3, 100 μL at 0.5 μg/mL

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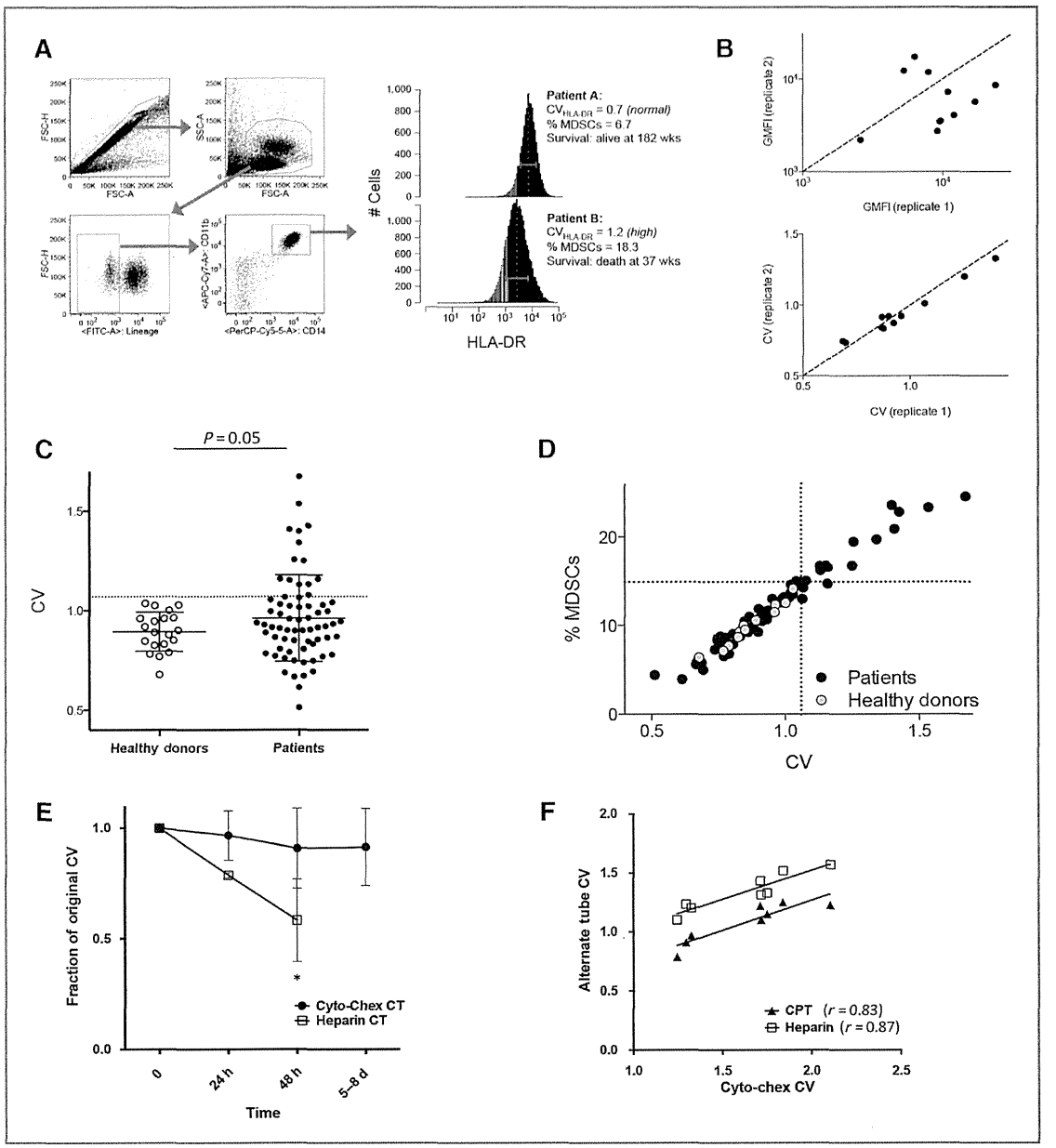

Figure 1. Analysis of MDSC frequency. PBMCs from patients with advanced melanoma and from healthy donors were stained with surface antibody and analyzed by multicolor flow cytometry. We defined monocytic myeloid cells based on the presence of CD14, CD11b in a CD3, CD16, CD19, CD20, and CD56 in a Lin^- population. Within this monocytic cell population, m-MDSCs were isolated on the basis of their low levels of HLA-DR expression. A, gating strategy to isolate myeloid-derived cells as $CD14^+CD11b^+Lin^-$ cells. On the basis of the 99th percentile of healthy donor values, a cutoff for low expression of HLA-DR was set to isolate the population of m-MDSC (shaded in gray). B, m-MDSC composition by HLA-DR GMFI is subject to *fluctuations in staining acquisition and sample handling.* CV_{HLA-DR} *represents a self-normalizing measurement and is stable among replicate* measurements. C, comparison of CV for HLA expression within the myeloid compartment reveals a larger spread for patients pretreatment, compared with healthy donors and large differences in CV between patients (healthy donors vs. patients; $P < 0.05$). D, normogram plotting relationship between CV values and m-MDSC frequency. E, evaluation of whole blood collected in standard heparin or Cyto-Chex tubes ($n = 9$) for m-MDSC frequency and stored at room temperature for the specified interval between analysis and acquisition. Data are expressed as a percentage of total m-MDSCs present at baseline. *, $P = 0.002$. F, correlation between m-MDSC analysis of samples ($n = 8$) cryopreserved using BD Vacutainer CPT tubes, standard heparin tubes, and collected in Cyto-Chex tubes.

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for 2 hours at 37°C) in RPMI-1640 medium supplemented with 10% FBS and IL2 (10 IU/mL; Roche). After 5 days, cells were harvested, stained with CD3-PECy7, CD4-ECD, and CD8-APC Cy7 (BD Pharmingen), and CFSE signal of gated $CD8^+$ T cells ($CD3^+$ $CD4^-$) was measured by flow cytometry. The stimulation index (SI) was calculated by dividing the proliferation measured in the absence of m-MDSC by proliferation measured in the presence of m-MDSC, as previously described (31).

Statistical analysis

Patient characteristics were described using median and range for continuous variables and frequency and percentages for categorical variables. The primary endpoint for this retrospective analysis was OS, which is defined as the time from pretreatment %m-MDSC assessment to death or last follow-up. Landmark analysis from week 6 was also performed. Patients alive at last follow-up are censored. Maximally selected log-rank statistics was used to find a cutoff value for %m-MDSC. The Kaplan–Meier method and log-rank test were used to compare differences in survival for categorical variables. Univariate and multivariate Cox proportional hazards regression was used to assess the association between clinical variables and OS. A Student t test with Welch correction was used for comparisons of %m-MDSC frequency in the patient and healthy donor groups. Pearson correlation was used to evaluate for relationships between %m-MDSC and lymphocyte subsets.

Results

Measuring HLA-DR spread using a computational algorithm removes user bias and inter-replicate variability in m-MDSC assessment

Published reports of m-MDSC frequency have evaluated this cellular subset by gating on Lin^- $CD14^+CD11b^+$ $HLA\text{-}DR^{low/-}$ cells in the peripheral circulation. We similarly developed a flow cytometric strategy to define m-MDSC based on high abundance of CD14, CD11b, and low or absent HLA-DR expression in a CD3, CD16, CD19, CD20, CD56, Lin^- population (Supplementary Fig. S1). HLA-DR expression on myeloid cells displayed a wide continuous distribution rather than distinct populations. Log-rank tests based on different gating cutoffs resulted in a broad range of m-MDSC cutoff values and highly variable survival curves. Thus, selection of an accurate gate for a low or negative HLA-DR fraction is challenging and prone to user bias and experimental unreliability. However, we observed distinct spreads for the HLA-DR distribution between individual patients, suggesting that evaluating this parameter on $CD11b^+CD14^+$ cells could serve as a measure of m-MDSC. Thus, we gated on $CD11b^+CD14^+$ cells and measured HLA-DR GMFI, SD, and the CV, a ratio between GMFI and SD (Fig. 1A). Evaluating CV corrects for shifting GMFI due to staining protocol and nearly eliminates inter-replicate variability (Fig. 1B), enabling measurement of HLA-DR distribution on myeloid cells objectively and independently of staining fluctuations (32). Measurements across a cohort of healthy donors ($n = 20$) and patients with melanoma ($n = 68$) revealed a higher value of $CV_{HLA\text{-}DR}$ among patients' myeloid cells (Fig. 1C). Furthermore, we found a cohort of patient samples with $CV_{HLA\text{-}DR}$ levels above the range for healthy donors (defined by the 99th percentile in CV values among healthy donors). For these patients, the higher CV value indicates a higher HLA-DR spread, representative of abnormal elevations in the number of m-MDSC ($HLA\text{-}DR^{low}$ cells). To explicitly quantify the number of m-MDSCs, we use the upper limit of CVs for healthy donors (again, the 99th percentile, = X) as a "cutoff" and generate a nomogram to calculate an *ad hoc* quantitative measure of MDSC frequency (%m-MDSC). By translating the mean-normalized variance in the data to a concrete percentage of the population, we relate $CV_{HLA\text{-}DR}$ to a classical immunophenotyping measurement (Fig. 1D).

Given the potential for changes in HLA-DR expression that may occur during blood processing or transport to significantly alter m-MDSC evaluation, we evaluated our methods in whole blood stored at room temperature as well as cryopreserved Ficoll purified PBMCs. We noted that $CV_{HLA\text{-}DR}$ was significantly reduced as the interval between phlebotomy time and analysis increased: A 48-hour delay until processing demonstrated a nearly 50% reduction from baseline. Levels of $CV_{HLA\text{-}DR}$ were, however, consistent over time in Cyto-Chex blood collection tubes even if whole blood was stored at room temperature before processing for up to 8 days after phlebotomy (Fig. 1E). Actual $CV_{HLA\text{-}DR}$ values were different but clearly correlated between Cyto-Chex BCT, vacutainer CPT cell preparation tubes ($r = 0.83$), and standard heparin tubes ($r = 0.87$; Fig. 1F).

m-MDSCs occur with relatively higher frequency among patients with metastatic melanoma than in healthy donor controls

Using our $CV_{HLA\text{-}DR}$/%m-MDSC conversion nomogram, we determined the relative frequency of m-MDSCs for 68 patients with melanoma treated with ipilimumab at either 10 mg/kg ($n = 28$) or 3 mg/kg ($n = 40$) for whom pretreatment and week 6 PBMC samples were processed the same day as phlebotomy and stored in our tissue repository. We again used healthy donors as controls. The baseline characteristics of the patients and healthy donors are described in Table 1. The overall median time from initial m-MDSC measurement to last recorded follow-up was 13.6 months (range, 0.66–63.9).

We found that the relative frequency of peripheral blood m-MDSCs was increased among patients with metastatic melanoma ($P = 0.05$) when compared with a group of healthy individuals (Fig. 2A). Pretreatment m-MDSC frequency did not differ significantly in our cohort between patients who were treated with different doses of ipilimumab (Fig. 2B).

Pretreatment m-MDSC quantity correlates with OS in patients treated with ipilimumab

To evaluate the hypothesis that lower frequency of m-MDSCs was associated with OS, we parsed our patients according to their %m-MDSC at baseline and after two doses of ipilimumab (week 6). On the basis of log-rank statistics within our ipilimumab-treated cohort, we defined 14.9% as the

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Table 1. Patient and healthy donor characteristics

Characteristics	Ipilimumab 10 mg/kg	Ipilimumab 3 mg/kg	Healthy donors[a]
Number of patients	28	40	20
Median age (range)	62 (34–83)	60 (34–80)	38 (26–58)
Sex (%)			
Male	17 (61)	29 (73)	10 (50)
Female	11 (39)	11 (27)	7 (35)
Stage of disease (%)			
III (unresectable)	0	1	—
M1a	3	0	—
M1b	4	5	—
M1c	21	34	—
Median number of prior therapies (range)	1 (0–3)	1 (0–5)	—
Median LDH (range)	209 (113–968)	211 (117–816)	—
≥Upper limit of normal (% of available LDH)	13 (46)	28 (70)	—
<Upper limit of normal (% of available LDH)	15 (54)	12 (30)	—
MDSC frequency			
%HLA-DR$^{low/-}$ in Lin$^-$CD14$^+$CD11b$^+$ (range)[b]	11.4 (3.9–24.5)	11.2 (5.8–20.9)	10.3 (6.4–14.3)
≥14.9 (%)	7 (25)	7 (18)	0 (0)
<14.9 (%)	21 (75)	33 (82)	20 (100)
Median baseline ALC (range)	1,250 (500–5,100)	1,100 (600–8,100)	—
≥1,000/μL (%)	19 (68)	25 (63)	—
<1,000/μL (%)	9 (32)	15 (37)	—

[a]Data for anonymously donated blood bank samples are unavailable.
[b]Baseline values.

cutoff between "high" and "low" %m-MDSC. The distribution of m-MDSC frequencies among analyzed patients is summarized in Table 1.

Having less than 14.9% m-MDSC pretreatment was associated with improved OS among 68 patients treated with ipilimumab (Fig. 2C and Table 2) with a HR of 0.35 [95% confidence interval (CI), 0.18–0.70; $P = 0.003$]. When analyzed by individual dose groups, the difference was seen in patients treated at 10 mg/kg, but not at 3 mg/kg (Supplementary Table S1). We performed univariate (Table 2) and multivariate analyses (Table 3) to evaluate the impact of ALC, lactate dehydrogenase (LDH), and monocyte counts on survival in our patient cohort. %m-MDSC < 14.9% was correlated with superior OS on both univariate and multivariate analyses. Monocyte quantity was not predictive, suggesting that %m-MDSC represents a relative activation state within the monocyte compartment and is not a direct reflection of monocyte numbers.

At treatment week 6, the frequency of m-MDSCs correlates with OS in patients treated with ipilimumab

We also evaluated associations between %m-MDSC at week 6 and OS similarly to what has been evaluated previously for ALC (Table 2 and Supplementary Table S1; refs. 30, 33). %m-MDSC below 14.9% at week 6 was associated with superior OS (Fig. 2D) in patients receiving ipilimumab treatment with a HR of 0.38 (95% CI, 0.19–0.75; $P = 0.005$). As expected, ALC greater than or equal to 1,000 at week 6 was associated with improved OS in our cohort and normal LDH (<250) at week 6 correlated with improved OS in patients treated with ipilimumab. To address potential confounding by ALC and LDH, a multivariate analysis was performed and week 6 %m-MDSC remained significantly associated with OS, even when accounting for both LDH and week 6 ALC (Table 3).

%m-MDSC is inversely correlated with CD8$^+$ T-cell rise on therapy and suppresses T-cell proliferation *in vitro*

Ipilimumab has a specific pharmacodynamic effect on ALC, but data on the specific subset of cells affected are limited. Our group previously reported on a cohort of 35 patients treated with ipilimumab at 10 mg/kg in which the relationships between increases in CD8$^+$ T cells, CD4$^+$ T cells, and CD4$^+$CD25$^+$ regulatory T cells, and clinical outcome were analyzed. In this analysis, the majority of patients had increases in all three lymphocyte subsets, but only the mean increase in CD8$^+$ T cells was significantly associated with clinical benefit (34).

Because m-MDSCs are defined by the ability to suppress CD8$^+$ T-cell proliferation, we examined whether m-MDSC frequency affects T cells *in vivo* or *in vitro*. We first sought to explore whether relationships between ALC and m-MDSC were observed to be consistent with m-MDSC suppressive function *in vivo*. On the basis of the known biologic functions of m-MDSCs, we reasoned that a greater frequency of m-MDSCs would limit the T-cell proliferative response to ipilimumab. However, we did not find correlations between