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Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy

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SUMMARY

Cancer immunotherapy can induce long lasting responses in patients with metastatic cancers of a wide range of histologies. Broadening the clinical applicability of these treatments requires an improved understanding of the mechanisms limiting cancer immunotherapy. The interactions between the immune system and cancer cells are continuous, dynamic, and evolving from the initial establishment of a cancer cell to the development of metastatic disease, which is dependent on immune evasion. As the molecular mechanisms of resistance to immunotherapy are elucidated, actionable strategies to prevent or treat them may be derived to improve clinical outcomes for patients.

Introduction

Metastatic cancers remain an incurable disease for the great majority of patients, as the intrinsic genomic instability common to all cancers facilitates the escape from cytotoxic or targeted therapies. The recent breakthroughs in the understanding of tumor immune biology and the development of newer generation of cancer immunotherapies have opened a brand new chapter in the war against cancer. This change in landscape is based on the discovery of cancer immune checkpoints and the success of checkpoint inhibitors, as well as the advances in technology to generate genetically modified immune cells (Miller and Sadelain, 2015). The focus of treatment has shifted from the tumor itself to the host's immune system, to mobilize immune cells to recognize and eventually eliminate the cancer cells. A hallmark of immunotherapy is the durability of responses, most likely due to the memory of the adaptive immune system, which translates into long-term survival for a subset of patients.

Early efforts to harness the immune system in cancer control, pioneered by Dr. William B. Coley in the 1890s (Coley, 1910), were overlooked due to the lack of consistency in response and were soon overwhelmed by the development of more effective treatments such as radiotherapy and chemotherapy. However, investigations continued to unravel and elucidate the interactions between the immune system and cancer cells. The concept of cancer immunosurveillance, which was proposed by Paul Ehrlich (Ehrlich, 1956) and enriched by Burnet and Thomas (Burnet, 1971) in the 1950s, stated that the emergence of malignant cells is a frequent event but is suppressed by the host's natural immunity, that cancer develops when this immunity is weakened, and that lymphocytes are responsible for this process. Finally, the cancer immune-editing concept was eluci-

dated by Schreiber and colleagues in 2002 (Dunn et al., 2002), recognizing a dual role of the host's immunity, both as an extrinsic tumor suppressor and a facilitator of tumor growth and progression, acting across three sequential phases—elimination, equilibrium and escape—through constant interactions between tumor cells, immune cells, and the tumor microenvironment. Importantly, host immune responses and tumor genomics are tightly related, as illustrated by the notion that neoantigens arising from genomic mutations may shape immune responses (Schumacher and Schreiber, 2015); however, these responses may prove ineffective against a heterogeneous and evolving tumor microenvironment.

The process of T cell activation involves antigen presentation by the major histocompatibility complex (MHC) molecules on the antigen-presenting cells (APCs) to the corresponding T cell receptor (TCR) on naive T cells. The interaction of costimulatory molecules CD28 and B7 is required for full activation, which is tightly regulated by inhibitory checkpoints to avoid collateral damage and autoimmunity. The CTLA-4 receptor on activated effector T cells and regulatory T cells (Tregs) was discovered in the 1980s (Brunet et al., 1987). Seminal work by James Allison and colleagues showed that CTLA-4 competes with CD28 for B7 ligands and inhibits proliferation and IL-2 secretion by T cells (Krummel and Allison, 1995) and that CTLA-4 blocking antibodies could treat tumors in immune competent animal models (Leach et al., 1996). Subsequent clinical testing resulted in the approval of ipilimumab for treatment of advanced melanoma in 2011, the first in class CTLA-4 checkpoint inhibitor approved by the US Food and Drug Administration (FDA) (Hodi et al., 2010; Robert et al., 2011). Pooled data from clinical trials of ipilimumab confirmed durable clinical responses, depicted by a plateau in the survival curve beginning around year 3, that lasted

Table 1. Terminology for Different Resistance Mechanisms to Immunotherapy

Term	Description
primary resistance	A clinical scenario where a cancer does not respond to an immunotherapy strategy. The mechanistic basis of lack of response to immunotherapy may include adaptive immune resistance.
adaptive immune resistance	A mechanism of resistance where a cancer is recognized by the immune system but it protects itself by adapting to the immune attack. Given the evolving nature of the immune/cancer cell interaction, this could clinically manifest as primary resistance, mixed responses or acquired resistance.
acquired resistance	A clinical scenario in which a cancer initially responded to immunotherapy but after a period of time it relapsed and progressed.

10 years or more in a subset of approximately 21% of patients (Schadendorf et al., 2015). In 2015, ipilimumab was also approved by the FDA as adjuvant therapy for locally advanced melanoma. Due to enhanced immune responses, possibly during early stages of T cell activation, significant immune-related toxicities have been observed, but most can be managed by systemic steroid therapy.

Another checkpoint receptor expressed by activated T cells, programed death 1 (PD-1), was cloned in 1992 (Ishida et al., 1992), and subsequently its ligand PD-L1 was characterized (Dong et al., 1999; Freeman et al., 2000). PD-L1 expression can be constitutive or induced in many tumors to evade immune attack. Since PD-L1 expression can be induced by IFNγ, which is expressed during an active anti-tumor immune response, it has been referred to as a mechanism of adaptive immune resistance (Table 1). Antibodies blocking the PD-1 and PD-L1 inhibitory axis can unleash activated tumor-reactive T cells and have been shown in clinical trials to induce durable anti-tumor responses in increasing numbers of tumor histologies, including the tumor types that are not traditionally considered immunotherapy sensitive (Okazaki et al., 2013; Zou et al., 2016). This led to the approval of two anti-PD1 antibodies (pembrolizumab and nivolumab) and one anti-PD-L1 antibody (atezolimumab) for the treatment of advanced melanoma, non-small-cell lung cancer, renal cell carcinoma, head and neck squamous carcinoma, Hodgkin's lymphoma, and bladder cancer. Currently there are over ten anti-PD-1 and anti-PD-L1 antibodies in various stages of clinical testing in many different tumor types. Interestingly, there have been thousands of patients receiving PD-1 blockade therapy thus far, with similar immune related toxicities as observed for anti-CTLA-4 but with generally lower frequency, possibly because the PD-1 and PD-L1 checkpoint may act later in the T cell response, resulting in a more restricted T cell reactivity toward tumor cells, with the majority of patients tolerating treatment well (Larkin et al., 2015b). Due to the non-overlapping mechanism of action of anti-CTLA4 and anti-PD1 antibodies (Das et al., 2015; Gubin et al., 2014), clinical testing of the combination of these two classes of checkpoint inhibitors showed improved clinical response (up to 60%) in melanoma at the expense of significantly increased frequency of toxicities (Larkin et al., 2015a). The combination of CTLA4 and PD-1 and PD-L1 checkpoint blockade has been approved as front line therapy for advanced melanoma patients and is being tested in other tumor types with different dose levels and intervals of anti-CTLA4 to reduce toxicity.

Cell-based immunotherapy was pioneered by many investigators, including Alex Fefer, Phil Greenberg, Zelig Eshhar, Steven Rosenberg, and colleagues in the 1980s, inspired by the correlation of the number of tumor infiltrating lymphocytes (TILs) and survival in some cancers. This process required TILs to be isolated from the patient's surgical specimen, expanded in vitro, and re-infused back to the lymphocyte-depleted patient. In these studies, sufficient TILs could not be isolated or expanded from tumors of approximately 50%–60% of patients, which limited the number of patients who could be treated. For patients who could be treated with the expanded TILs, the reported response rate was 50% for melanoma, including 20% complete responses, and 95% of these complete responders had more than 5 years of survival (Rosenberg et al., 2011). This approach, however, requires large surgical samples, experienced academic centers, and tumors enriched with anti-tumor T cells, which is a rare event for most tumor types. The recent advance of gene transfer technologies and T cell engineering has enabled more versatile approaches, including adoptive cell transfer (ACT) of the patient's peripheral T cells that are genetically modified to target cancer specific antigens, via physiological TCRs or chimeric antigen receptors (CARs) (Sadelain, 2016; Yang and Rosenberg, 2016). TCRs are usually cloned from TILs that are reactive to specific cancer antigens with no or very limited expression in normal adult tissue but are widely expressed by cancer cells. Such TCRs recognize tumor antigen presented in the context of the MHC. Clinical success has been documented (Yee et al., 2015). The TCR approach allows intracellular antigen targets but is MHC restricted and can be subject to treatment failure for tumors that have downregulated their MHC surface expression. CAR technology was first developed by Eshhar et al., 1993, who genetically engineered T cells with chimeric genes, linking single chain antibodies (scFv) targeting tumor cell surface antigens to intracellular signaling adaptors for TCR: in the first generation, to the T cell specific activating ζ chain of the CD3 complex. Subsequent modification with co-stimulatory molecules CD28 (second generation) and 4-1BB (third generation) has enabled the expansion of T cells while retaining function upon repeated antigen exposure. CAR T cells do not require MHC restriction and can be engineered to enhance T cell function. Recent clinical success with CD19 targeting CAR to treat CD19+ B cell malignancy has shown great success, with a remarkable 90% complete remission in a cohort of 30 patients with relapsed or refractory pediatric acute lymphoblastic leukemia (ALL), and two thirds of these patients remained in remission after 6 months (Maude et al., 2014). The biggest challenge facing the field of ACT is the identification of target tumor antigens that are not expressed by normal tissues, both to maximize specificity and efficacy and to minimize toxicity (Fesnak
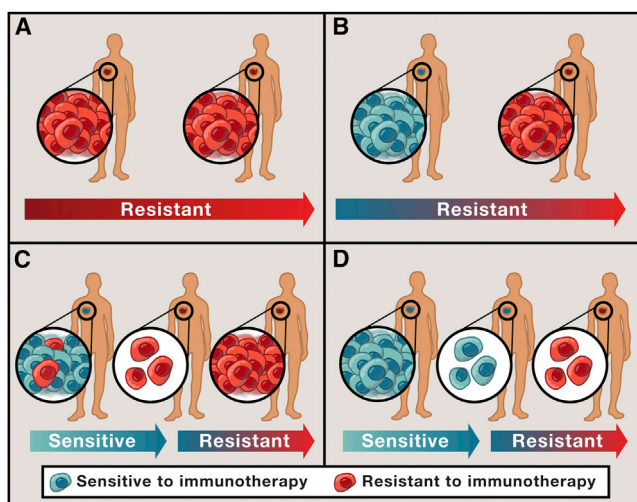
Figure 1. Clinical Scenarios of Primary, Adaptive, and Acquired Resistance to Immunotherapy
(A) Patient's tumor is resistant to immunotherapy with no active immune response.
(B) Patient's tumor is resistant to immunotherapy; active anti-tumor immune response, but turned off by checkpoints or other adaptive resistance mechanisms.
(C) Patient has an initial response to immunotherapy but later progressed; heterogeneous population and selection of resistant clones that were present before treatment started.
(D) Patient has an initial response to immunotherapy but later progressed; true acquired resistance during the immunotherapy.

Table 2. Mechanisms of Primary and Adaptive Resistance to Immunotherapy

	Mechanism	Examples
tumor cell intrinsic	absence of antigenic proteins	low mutational burden lack of viral antigens lack of cancer-testis antigens overlapping surface proteins
	absence of antigen presentation	deletion in TAP deletion in B2M silenced HLA
	genetic T cell exclusion	MAPK oncogenic signaling stabilized b-catenin mesenchymal transcriptome oncogenic PD-L1 expression
	insensibility to T cells	mutations in interferon gamma pathway signaling
tumor cell extrinsic	absence of T cells	lack of T cells with tumor antigen-specific TCRs
	inhibitory immune checkpoints	VISTA, LAG-3, TIM-3
	immunosuppressive cells	TAMs, Tregs

et al., 2016). A commonly seen toxicity in ACT therapy is cytokine release syndrome, which can be life-threatening and requires prompt management with steroids and IL-6 receptor antibody (tocilizumab).

Despite the unprecedented durable response rates observed with cancer immunotherapies, the majority of patients do not benefit from the treatment (primary resistance), and some responders relapse after a period of response (acquired resistance). Several common cancer types have shown very low frequency of response (breast, prostate, and colon cancers), and heterogeneous responses have been seen even between distinct tumors within the same patient (Figure 1). For the purposes of this review, we have categorized primary, adaptive, and acquired resistance as described in Table 1, in keeping with the most typical conceptualization for practicing clinicians. However, in considering resistance mechanisms to immune-based therapies, it is important to remember that the immune response is dynamic and constantly evolving in each patient, either as a result of the patient's own environmental and genetic factors or as a result of treatment interventions, including surgery, chemotherapy, radiation therapy, and immunotherapy. Anti-tumor immune responses that are ongoing throughout the course of a patient's disease may be affected by many of these factors, and the establishment of resistance mechanisms relevant to immunotherapeutic failure may pre-date immunotherapy challenge. Without recourse to detailed immune and tumor characterization, these resistance mechanisms can be divided, clinically, into those that prevent a patient from ever responding

to an immunotherapy or those that facilitate relapse after an initial response. Thus, although resistance to immunotherapies may manifest at different times, in many cases, similar or overlapping mechanisms enable tumor cells to evade anti-tumor immune responses. We discuss known resistance mechanisms and provide rationale for combination therapies to overcome resistance.

Primary and Adaptive Resistance to Immunotherapy
Patients who have primary resistance to checkpoint inhibitors do not respond to the initial therapy. Ongoing studies indicate that both tumor-cell-intrinsic and tumor-cell-extrinsic factors contribute to the resistance mechanisms (Table 2). The most straightforward reason why a tumor would not respond to immune checkpoint therapy or ACT is lack of recognition by T cells because of absence of tumor antigens (Gubin et al., 2014). Alternatively, cancer cells may have tumor antigens but develop mechanisms to avoid presenting them on the surface restricted by MHC, due to alterations in the antigen-presenting machinery (such as proteasome subunits or transporters associated with antigen processing), beta-2-microglobulin (B2M), or MHC itself (Marincola et al., 2000; Sucker et al., 2014). B2M is required for HLA class I folding and transport to the cell surface, and its genetic deficiency leads to lack of CD8 T cell recognition (Figures 2 and 3).

Tumor-Cell-Intrinsic Factors for Primary and Adaptive Resistance
Tumor-cell-intrinsic factors that contribute to immunotherapy resistance include expression or repression of certain genes and pathways in tumor cells that prevent immune cell infiltration or function within the tumor microenvironment. These mechanisms may exist at the time of initial presentation, highlighting primary resistance mechanisms, or these mechanisms may
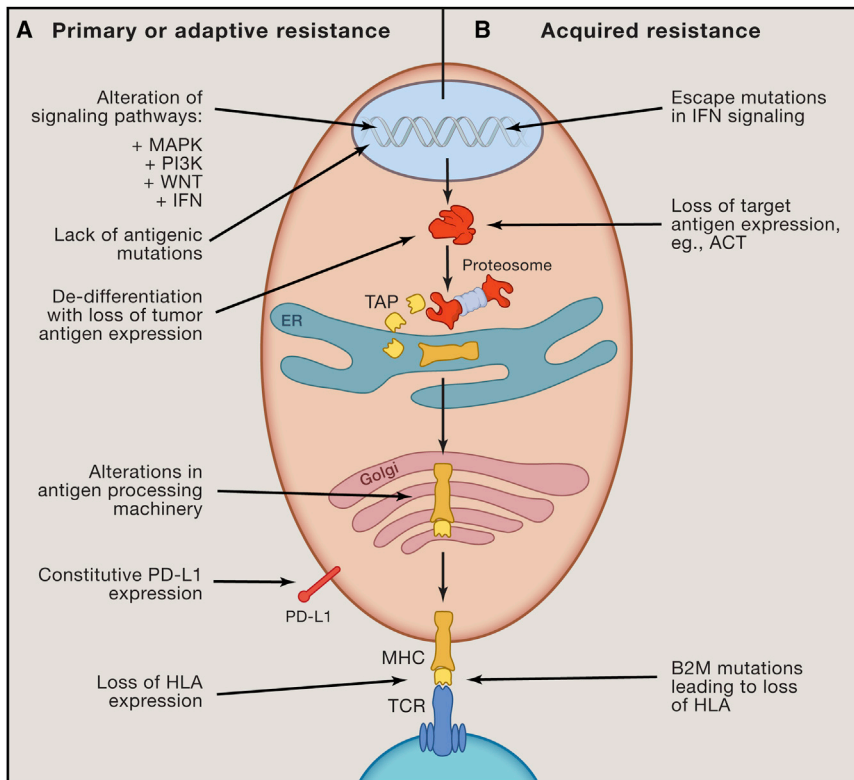
Figure 2. Known Intrinsic Mechanisms of Resistance to Immunotherapy
(A) Intrinsic factors that lead to primary or adaptive resistance including lack of antigenic mutations, loss of tumor antigen expression, loss of HLA expression, alterations in antigen processing machinery, alterations of several signaling pathways (MAPK, PI3K, WNT, IFN), and constitutive PD-L1 expression.
(B) Intrinsic factors that are associated with acquired resistance of cancer, including loss of target antigen, HLA, and altered interferon signaling, as well as loss of T cell functionality.

that attracts CD103+ DCs. In addition, murine tumors lacking β-catenin responded effectively to immune checkpoint therapy whereas β-catenin-positive tumors did not. Non-T-cell-inflamed human melanoma tumors, which lacked T cells and CD103+ DCs in the tumor microenvironment, had significantly higher expression of tumor intrinsic β-catenin signaling genes.

Cancer cells that constitutively express immunosuppressive cell surface ligands like PD-L1 may actively inhibit anti-tumor T cell responses. A genetic amplification of a locus in chromosome 9 that contains the genes for the two ligands of PD-1 (PD-L1 and PD-L2) and the interferon gamma receptor signaling molecule Janus kinase 2 (JAK2) is termed the PDJ amplicon (Ansell et al., 2015; Green et al., 2010; Rooney et al., 2015). PDJ is amplified in the malignant Reed-Sternberg cells in Hodgkin's disease, and anti-PD-1 therapy results in objective responses in over 80% of patients with chemotherapy-refractory Hodgkin's disease (Ansell et al., 2015). Other mechanisms that have been described as leading to constitutive PD-L1 expression by cancer cells include PTEN deletions or PI3K and/or AKT mutations (Lastwika et al., 2016; Parsa et al., 2007), EGFR mutations (Akbay et al., 2013), MYC overexpression (Casey et al., 2016), CDK5 disruption (Dorand et al., 2016), and an increase in PD-L1 transcripts stabilized by truncation of the 3′ UTR of this gene (Kataoka et al., 2016). It is currently unclear whether constitutive PD-L1 expression resulting from these oncogenic signaling processes results in decreased or increased likelihood of responding to anti-PD-1 and PD-L1 therapy, but it may indeed result in lack of response to other cancer immunotherapy strategies by actively inhibiting anti-tumor T cells.

The interferon-gamma pathway is emerging as a key player in primary, adaptive, and acquired resistance to checkpoint blockade therapy (Gao et al., 2016; Pardoll, 2012; Ribas, 2015; Shin et al., 2016; Zaretsky et al., 2016). It has both favorable and detrimental effects on anti-tumor immune responses. Interferon-gamma produced by tumor-specific T cells that have recognized their cognate antigen on cancer cells or APCs induces an effective anti-tumor immune response through (1) enhanced tumor antigen presentation that occurs as a result of increased expression of proteins, such as MHC molecules, involved in antigen presentation, (2) recruitment of other immune
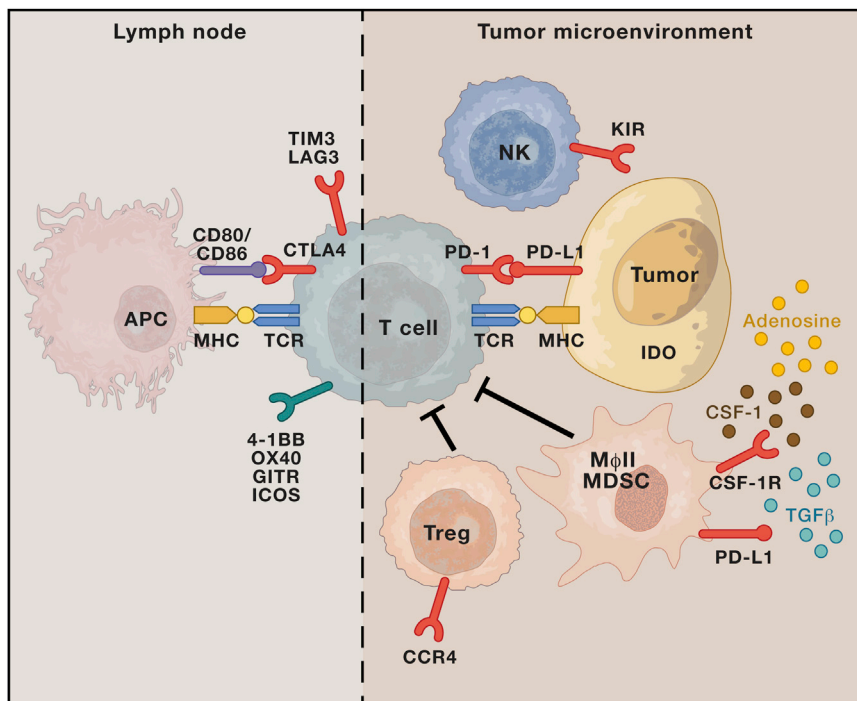
evolve later, highlighting adaptive resistance mechanisms. Multiple tumor-intrinsic mechanisms have recently been identified and include (1) signaling through the mitogen-activated protein kinase (MAPK) pathway and/or loss of PTEN expression, which enhances PI3K signaling, (2) expression of the WNT/β-catenin signaling pathway, (3) loss of interferon-gamma (IFNγ) signaling pathways, and (4) lack of T cell responses as result of loss of tumor antigen expression.

Oncogenic signaling through the MAPK pathway results in the production of VEGF and IL-8, among many other secreted proteins, which have known inhibitory effects on T cell recruitment and function (Liu et al., 2013). Similarly, loss of PTEN, which enhances PI3K signaling and is a common phenomenon across several cancers, including 30% of melanomas, was found to be associated with resistance to immune checkpoint therapy (Peng et al., 2016). PTEN loss in tumors of the Cancer Genome Atlas (TCGA) melanoma dataset correlated with significantly decreased gene expression of IFNγ, granzyme B, and CD8+ T cell infiltration; importantly, the frequency of PTEN deletions and mutations was higher in non-T-cell-inflamed tumors as compared to T-cell-inflamed tumors. In a murine model, PTEN-knockout tumors were less susceptible to adoptive cell therapy than PTEN-expressing tumors.

The potential of oncogenic signaling pathways to induce T cell exclusion from cancers has also been described through the stabilization of β -catenin resulting in constitutive WNT signaling (Spranger et al., 2015). In a murine model, tumors with elevated β-catenin lacked a subset of dendritic cells (DCs) known as CD103+ DCs, due to decreased expression of CCL4, a chemokine

Figure 3. Known Extrinsic Mechanisms of Resistance to Immunotherapy
This includes CTLA-4, PD1, and other immune checkpoints, T cell exhaustion and phenotype change, immune suppressive cell populations (Tregs, MDSC, type II macrophages), and cytokine and metabolite release in the tumor microenvironment (CSF-1, tryptophan metabolites, TGF-β, adenosine). Abbreviations are as follows: APC, antigen-presenting cells; MHC, major histocompatibility complex; TCR, T cell receptor; Treg, regulatory T cell; MDSC, myeloid-derived suppressor cell; Mϕ II, type II macrophage.

cells, and (3) direct anti-proliferative and pro-apoptotic effects on tumor cells (Platanias, 2005). But continuous interferon-gamma exposure can lead to immunoediting of cancer cells, resulting in immune escape (Benci et al., 2016; Shankaran et al., 2001). One mechanism by which cancer cells could escape the effects of interferon gamma is by downregulating or mutating molecules involved in the interferon gamma signaling pathway, which goes through the interferon gamma receptor chains JAK1 and/or JAK2 and the signal transducer and activators of transcription (STATs) (Darnell et al., 1994). In cell line and animal models, mutations or epigenetic silencing of molecules in the interferon receptor signaling pathway results in loss of the anti-tumor effects of interferon gamma (Dunn et al., 2005; Kaplan et al., 1998). Analysis of tumors in patients who did not respond to therapy with the anti-CTLA-4 antibody ipilimumab revealed an enriched frequency of mutations in the interferon gamma pathway genes interferon gamma receptor 1 and 2 (IFNGR1 and IFNGR2), JAK2, and interferon regulatory factor 1 (IRF1) (Gao et al., 2016). Any of these mutations would prevent signaling in response to interferon gamma and give an advantage to the tumor cells escaping from T cells, thereby resulting in primary resistance to anti-CTLA-4 therapy. Mutations in this pathway would additionally result in lack of PD-L1 expression upon interferon gamma exposure, thereby resulting in cancer cells that would be genetically negative for inducible PD-L1 expression. In such a scenario, blocking PD-L1 or PD-1 with therapeutic antibodies would not be useful, and these would be patients who are primary resistant to anti-PD-1 therapy (Shin and Ribas, 2015; Shin et al., 2016).

An additional cancer-cell-intrinsic mechanism of primary resistance to immunotherapy is expression of a certain set of genes that were found to be enriched in tumors from patients who did not respond to anti-PD-1 therapy, termed innate anti-PD-1 resistance signature, or IPRES (Hugo et al., 2016). These genes that lead to lack of response are related to mesenchymal transformation, stemness, and wound healing and are preferentially expressed by cancers that seldom respond to PD-1 blockade therapy, such as pancreatic cancer.

Epigenetic modification of the DNA in cancer cells may lead to changes in gene expression of immune-related genes, which can impact antigen processing, presentation, and immune evasion (Karpf and Jones, 2002; Kim and Bae, 2011). Therefore, demethylating agents may enable re-expression of immune related genes, with potential for therapeutic impact, especially in the setting of combination treatment with immunotherapy. (Héninger et al., 2015). Histone deacetylase inhibitors led to increased expression of MHC and tumor-associated antigens, which synergized with ACT therapy to improve anti-tumor responses in a murine melanoma model (Vo et al., 2009). Similarly, in a lymphoma model, hypomethylating agents were found to increase CD80 expression on tumor cells, with aconcomitant increase in tumor-infiltrating CD8+ T cells (Wang et al., 2013). These pre-clinical data indicate the potential to reverse the epigenetic changes in cancer cells, which may enable enhanced immune recognition and response to immunotherapy.

Tumor-Cell-Extrinsic Factors for Primary and Adaptive Resistance
Tumor-cell-extrinsic mechanisms that lead to primary and/or adaptive resistance involve components other than tumor cells within the tumor microenvironment, including Tregs, myeloid derived suppressor cells (MDSCs), M2 macrophages, and other inhibitory immune checkpoints, which may all contribute to inhibition of anti-tumor immune responses.

Tregs, which can be identified by expression of the FoxP3 transcription factor, have a central role in maintaining self-tolerance (Rudensky, 2011). The existence of suppressor T cells that could downregulate immune responses of antigen-specific T cells was first identified nearly four decades ago in thymectomized, lethally irradiated, bone-marrow-reconstituted mice (Gershon and Kondo, 1970). Tregs are known to suppress effector T cell (Teff) responses by secretion of certain

inhibitory cytokines, such as IL-10, IL-35, and TGF-β, or by direct cell contact (Oida et al., 2003; Sakaguchi et al., 2008; Sundstedt et al., 2003). Published data indicate that many human tumors are infiltrated by Tregs (Chaudhary and Elkord, 2016; Ormandy et al., 2005; Woo et al., 2002). A vast number of murine studies have shown that the depletion of Treg cells from the tumor microenvironment can enhance or restore anti-tumor immunity (Linehan and Goedegebuure, 2005; Viehl et al., 2006). In murine models, response to anti-CTLA-4 therapy was shown to be associated with an increase in the ratio of Teffs to Tregs (Quezada et al., 2006). This shift in the ratio of Teffs to Tregs was found to be a result of both an increase in Teffs and depletion of Tregs in a murine tumor model (Simpson et al., 2013). These data suggest that tumors for which immunotherapy is unable to increase Teffs and/or deplete Tregs to increase the ratio of Teffs to Tregs are likely to be resistant to treatment, either initially or during the relapsed disease setting. However, it is possible that tumor-infiltrating Tregs may co-exist with other immune cells, indicating a potentially immune-responsive tumor. A retrospective study of patients treated with anti-CTLA-4 reported that a high baseline expression of FoxP3^{+} Tregs in the tumor was associated with better clinical outcomes (Hamid et al., 2011). Additional studies are ongoing to determine the impact of tumor-infiltrating Tregs on clinical outcomes for patients who receive treatment with immunotherapy agents.

Myeloid-derived suppressor cells (MDSCs) have emerged as major regulators of immune responses in various pathological conditions, including cancer. MDSCs were initially defined in murine models and were characterized by the expression of CD11b (CR3A or integrin αM) and Gr-1 markers (Bronte et al., 1998; Talmadge and Gabrilovich, 2013). Human MDSCs express markers such as CD11b^{+} and CD33^{+} but are mostly negative for HLA-DR and lineage-specific antigens (Lin), including CD3, CD19, and CD57. Monocytic MDSCs are HLA-DR^{−}, CD11b^{+}, CD33[+,] and CD14^{+} and granulocytic MDSCs are HLA-DR^{−}, CD11b^{+}, CD33^{+}, CD15^{+}; however, mature monocytes express HLA-DR (Wesolowski et al., 2013). MDSCs have been implicated in promoting angiogenesis, tumor cell invasion, and metastases (Yang et al., 2004; Yang et al., 2008). Furthermore, clinical findings have shown that the presence of MDSCs correlates with reduced survival in human cancers, including breast cancer and colorectal cancer (Solito et al., 2011). Reports suggest that the presence of MDSCs in the tumor microenvironment correlates with decreased efficacy of immunotherapies, including immune checkpoint therapy (Meyer et al., 2014), adoptive T cell therapy (Kodumudi et al., 2012), and DC vaccination (Laborde et al., 2014). Therefore, eradicating or reprogramming MDSCs could enhance clinical responses to immunotherapy. Indeed, in melanoma, breast cancer, and head and neck murine tumor models, selective inactivation of macrophage PI3Kγ synergized with immune checkpoint inhibitors to promote tumor regression and increase survival (De Henau et al., 2016; Kaneda et al., 2016). In one study, the investigators demonstrated that mice lacking PI3Kγ or tumor-bearing mice treated with PI3Kγ inhibitors (TG100-115 or IPI-549) had reduced tumor growth, which was associated with enhanced expression of pro-inflammatory cytokines and inhibition of immune-suppressive factors in the tu-

mors (Kaneda et al., 2016). Moreover, genes and proteins associated with immune activation were upregulated in macrophages that were treated with PI3Kγ inhibitors or those from mice lacking PI3Kγ. These data established PI3Kγ as a molecular switch that regulates macrophage function. The investigators also demonstrated that a PI3Kγ inhibitor (TG100-115) plus anti-PD-1 led to improved tumor rejection and survival of tumor-bearing mice (Kaneda et al., 2016). In a second study, tumor-bearing mice treated with triple-combination therapy, a PI3Kγ inhibitor (IPI-549) plus anti-CTLA-4 and anti-PD-1, had improved tumor regression and long-term survival as compared to dual therapy with anti-CTLA-4 plus anti-PD-1 (De Henau et al., 2016). These pre-clinical studies highlight inhibitors of PI3Kγ as a therapeutic potential for combination strategies with immune checkpoint therapy in cancer patients.

Tumor-associated macrophages (TAMs) are another subset of cells that seem to affect responses to immunotherapy. TAMs include both M1 macrophages, which are involved in promoting anti-tumor immunity, and the M2 macrophages, which possess pro-tumorigenic properties (Chanmee et al., 2014). M1 and M2 macrophages can be distinguished based on the differential expression of transcription factors and surface molecules and the disparities in their cytokine profile and metabolism (Biswas and Mantovani, 2010; Hu et al., 2016). Clinical studies have shown an association between higher frequencies of TAMs and poor prognosis in human cancers (Hu et al., 2016). In a chemically induced mouse model of lung adenocarcinoma, depletion of TAMs reduced tumor growth as a result of downregulation of M2 and/or TAM recruitment, possibly due to the inactivation of CCL2 and/or CCR2 signaling (Fritz et al., 2014). Likewise, depletion of M2 macrophages in various murine tumor models, including cutaneous T cell lymphoma (Wu et al., 2014), colon cancer, lung cancer, breast cancer (Luo et al., 2006), and melanoma (Ries et al., 2014; Ruffell et al., 2014; Tham et al., 2015), have shown similar results. Several reports have discussed the role of macrophages in mediating therapeutic resistance in cancer (De Palma and Lewis, 2013; Ruffell et al., 2014; Ruffell and Coussens, 2015). Reports suggest that macrophages can directly suppress T cell responses through programmed death-ligand 1 (PD-L1) in hepatocellular carcinoma (Kuang et al., 2009) and B7-H4 in ovarian carcinoma (Kryczek et al., 2006). To overcome the potential resistance mechanism of macrophages, investigators tested blockade of CSF-1R, a receptor for macrophage-colony stimulating growth factor, in a murine model of pancreatic cancer and demonstrated decreased frequencies of TAMs, with subsequent increase in interferon production and restrained tumor progression. Importantly, neither PD-1 nor CTLA-4 blockade could significantly reduce tumor growth in the murine model, results that were similar to findings from single agent studies in patients with pancreatic cancer (Le et al., 2013; Zhu et al., 2014). However, CSF1R blockade in combination with either an antibody against PD-1 or CTLA-4, in addition to gemcitabine, led to improved tumor regression (Zhu et al., 2014). These data suggest that CSF-1R blockade induced reduction of TAMs, which enabled response to immune checkpoint therapy. Similarly, in a melanoma model, CSF-1R inhibitor was shown to synergize with ACT therapy (Mok et al., 2014). Several early phase clinical trials

are underway to test the combination of CSF-1R inhibition with checkpoint inhibitors (Table 3).

The immune response is dynamic and signals that enhance anti-tumor immune responses also tend to turn on inhibitory genes and pathways in order to tightly regulate the immune response. For example, initial T cell activation, via TCR signaling and CD28 co-stimulation, eventually leads to increased expression of the inhibitory CTLA-4 immune checkpoint (Leach et al., 1996). Similarly, effector T cell responses such as increased IFNγ production leads to increased expression of the PD-L1 protein on multiple cell types, including tumor cells, T cells and macrophages, which can engage the PD-1 receptor on T cells to suppress anti-tumor immunity (Chen, 2004; Dong et al., 2002). Apart from this, IFNγ may additionally promote the expression of immunosuppressive molecules such as indolaimine-2, 3-deoxygenase (IDO), a tryptophan-metabolizing enzyme that can contribute to peripheral tolerance and can have a direct negative effect on effector T cell function (Gajewski et al., 2013). Similarly, carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1), seems to be another inhibitory molecule that is induced by IFNγ (Takahashi et al., 1993), (Gray-Owen and Blumberg, 2006). Therapeutic antibodies blocking CEACAM1 (Ortenberg et al., 2012) and TIM-3 have resulted in enhanced anti-tumor immune responses (Pardoll, 2012; Sakuishi et al., 2010). A recent study in an immunocompetent mouse model of lung adenocarcinoma demonstrated that recurrent tumors after anti-PD-1 treatment were due to increased expression of TIM-3 on T cells. Notably, anti-PD-1 plus anti-TIM-3 led to improved responses in the tumor bearing mice. Similarly, two lung cancer patients who developed recurrent disease after anti-PD-1 treatment were found to have increased TIM-3 expression on T cells (Koyama et al., 2016).

Immune suppressive cytokines are often released by tumor or macrophages for local suppression of anti-tumor immune responses. Transforming growth factor β (TGF-β) is a cytokine that plays important roles in angiogenesis and immunosuppression by stimulating Tregs (Lebrun, 2012). Increased levels of TGF-β are associated with poor prognosis in multiple tumor types (Lin and Zhao, 2015; Massagué, 2008). Preclinical models have shown synergy combining TGF-β receptor kinase inhibitor with anti-CTLA-4, which led to anti-tumor responses in a melanoma model (BRAF^{V600E}PTEN$^{-/-}$) (Hanks et al., 2014). Another pre-clinical study consisting of radiation therapy combined with TGF-β inhibition also demonstrated anti-tumor responses (Vanpouille-Box et al., 2015). Adenosine was shown to inhibit T cell proliferation and cytotoxic function via the A2A receptor on T cells (Zhang et al., 2004) as well as to promote metastasis via the A2B receptor on tumor cells (Mittal et al., 2016). In addition, CD73 is the enzyme that dephosphorylates adenosine monophosphate (AMP) to form adenosine, thus also suppressing immune function and promoting tumor cell metastasis (Stagg et al., 2010), and also stimulates angiogenesis (Allard et al., 2014). High expression of CD73 is associated with poor prognosis in different cancer types (Leclerc et al., 2016; Loi et al., 2013; Turcotte et al., 2015). CD73 is also a potential biomarker for anti-PD-1 therapy, with high expression limiting anti-PD-1 efficacy, which can be rescued by concomitant A2A blockade (Beavis et al., 2015).

Specific chemokines and chemokine receptors are important for trafficking of MDSCs and Tregs to the tumor. For example, tumors secrete ligands CCL5, CCL7, and CXCL8, bind to their receptors CCR1 or CXCR2 expressed on subtypes of MDSCs (Highfill et al., 2014), and attract MDSCs in the tumor microenvironment. Inhibitors of these chemokine receptors could abrogate immune evasion and improve anti-tumor T cell responses. CCR4 is highly expressed by Tregs in the blood and tumors (Sugiyama et al., 2013), and anti-CCR4 inhibits Treg recruitment as well as promotes antibody-dependent cell-mediated cytotoxicity (ADCC), further reducing the Treg population (Chang et al., 2012). CXCR4 is a receptor for the chemokine CXCL12, which has been shown to promote an immunosuppressive tumor microenvironment through several mechanisms, including Treg localization (Gil et al., 2014).

Acquired Resistance to Immunotherapy

A hallmark of cancer immunotherapy has been the induction of long lasting tumor responses. However, with higher activity and broader use of immunotherapies, the denominator of patients with a tumor response has increased and the chances of finding patients who responded for a period of time and then progressed, termed acquired resistance, increases. It is becoming clear that approximately one fourth to one third of patients with metastatic melanoma who have objective responses to checkpoint blockade therapy with anti-CTLA-4 or anti-PD-1 will relapse over time, even despite receiving continued therapy (Schachter et al., 2016). The potential mechanisms of relapse include loss of T cell function, lack of T cell recognition by downregulation of tumor antigen presentation, and development of escape mutation variants in the cancer (Figures 2 and 3). There is evidence that each of these mechanisms can lead to acquired resistance to checkpoint inhibitor therapy or ACT.

If the anti-tumor T cells change their functional phenotype and stop exerting their cytotoxic activity, then a patient who responded to immunotherapy may develop a tumor relapse even if everything else continues to be the same. Acquired resistance to TCR-engineered ACT is rather frequent, with high initial anti-tumor response followed by a high frequency of tumor relapses within months. This has been evident with the ACT of T cells expressing TCRs to melanosomal antigens (MART-1, gp100) and to cancer testis antigens (NY ESO-1) (Chodon et al., 2014; Morgan et al., 2006; Robbins et al., 2011). By studying how the TCR transgenic T cells change their functionality after ACT to humans, it has been reported that the initial highly cytolytic profile when administered shifts over time to a Th2-type cytokine release and lack of cytotoxic functions in late time points when recovered from patients at the time of tumor relapse (Ma et al., 2013; Ma et al., 2011).

It was already well documented by the 1990s that some patients who initially respond to cancer immunotherapies with IL-2 or TIL ACT might develop acquired resistance through loss of the shared component of all HLA class I molecules, B2M, which leads to absence of surface expression of HLA class I (D'Urso et al., 1991; Restifo et al., 1996). B2M is required for HLA class I folding and transport to the cell surface, and its genetic deficiency would lead to lack of CD8 T cell recognition. This mechanism of acquired resistance has also been

Table 3. Examples of Combination Therapies Being Developed to Overcome Resistance to Cancer Immunotherapy

Broad Approach	Specific Approach	Examples in Clinical Testing
combination checkpoint blockade	anti-PD-1/L1 plus anti-CTLA4	Durvalumab + tremelimumab Nivolumab + ipilimumab Pembrolizumab + ipilimumab
	anti-PD-1 plus anti-PD-L1	MEDI0680 + durvalumab PDR001 + FAZ053
	anti-PD-1/L1 plus anti-TIM 3	Nivolumab + TSR022 PDR001 + MBG453
	anti-PD-1/L1 plus anti-LAG 3	Nivolumab + BMS 986016 PDR001 + LAG525 Pembrolizumab + IMP321 REGN2810 + REGN3767
checkpoint blockade plus immune-stimulatory agents	anti-PD-1/L1 plus anti-41BB/CD137	Avelumab + utomilumab Nivolumab + urelumab Pembrolizumab + utomilumab
	anti-CTLA4 plus anti-OX40 anti-PD-1/L1 plus anti-OX40 anti-CTLA4 plus Anti-PD-1/L1 plus anti-OX40 anti-41BB/CD137 plus anti-OX40	Atezolimumab + MOXR0916 ± bevacizumab Avelumab + PF-04518600 Durvalumab + MEDI0562 Pembrolizumab + GSK3174998 Tremelimumab + durvalumab + MEDI6469 Tremelimumab + MEDI0562 Utomilumab + PF-04518600
	anti-CTLA4 plus anti-CD40 anti-PD-1/L1 plus anti-CD40	Atezolimumab + RO7009789 Tremelimumab + CP870893
	anti-PD-1/L1 plus anti-GITR	Nivolumab + BMS986156 PDR001 + GWN323
	anti-PD-1/L1 plus anti-ICOS	Nivolumab + JTX-2011
checkpoint blockade plus metabolic modulators	anti-CTLA-4 plus IDO inhibitors anti-PD-1/L1 plus IDO inhibitors	Atezolizumab + GDC0919 Ipilimumab + epacadostat Ipilimumab + indoximid Nivolumab + BMS986205 Pembrolizumab+ epacadostat
	anti-PD-1/L1 plus A2AR inhibitors or anti-CD73	Atezolizumab + CPI-444 Durvalumab + MEDI9447 PDR001+ PBF509
checkpoint blockade plus other immune modulators	anti-PD-1/L1 plus TGFβ inhibitors	Nivolumab + LY2157299 PDR001 + NIS793
	anti-PD-1/L1 plus CXCR4 inhibitors	Nivolumab + ulocuplumab Durvalumab + LY2510924
	anti-PD-1/L1 plus CCR4 inhibitors	Nivolumab + mogamulizumab
	anti-PD-1/L1 plus anti-CD27	Nivolumab + varlilumab Atezolizumab + varlilumab
	anti-PD-1/L1 plus CD122-biased cytokine	Nivolumab + NKTR-214
	anti-PD-1/L1 plus yeast-derived soluble β-glucan	Pembrolizumab + Imprime PGG
	anti-PD-1/L1 plus anti- TRAIL-DR5	Nivolumab + DS-8273a
	anti-PD-1/L1 plus glutaminase inhibitor	Nivolumab + CB839
	anti-PD-1/L1 plus IAP inhibitor	PDR001 + LCL161
checkpoint blockade plus macrophage inhibitors	anti-CTLA4 plus CSF1R inhibitors anti-PD-1/L1 plus CSF1R inhibitors	Durvalumab + Pexidartinib (PLX3397) Durvalumab + LY3022855 Nivolumab + FPA008 Pembrolizumab + Pexidartinib PDR001 + BLZ945 Tremelimumab + LY3022855

Table 3. *Continued*

Broad Approach	Specific Approach	Examples in Clinical Testing
checkpoint blockade plus injectable therapies	anti-CTLA-4 plus oncolytic viruses anti-PD-1/L1 plus oncolytic viruses	Ipilimumab + Talimogene Laherparepvec Nivolumab + Talimogene Laherparepvec Pembrolizumab + DNX2401 Pembrolizumab + Talimogene Laherparepvec
	anti-CTLA4 plus TLR agonists anti-PD-1/L1 plus TLR agonists	Ipilimumab + MGN1703 Pembrolizumab + CMP001 Pembrolizumab + SD101 Tremelimumab + PF-3512676
checkpoint blockade plus cancer vaccines	anti-CTLA4 plus DC vaccine anti-PD-1/L1 plus DC vaccine anti-PD-1/L1 plus peptide vaccine anti-PD-1/L1 plus neoantigen vaccine	Durvalumab + ADXS11-001 Durvalumab + TPIV200/huFR-1 Ipilimumab + GVAX Nivolumab + GVAX + CRS207 Nivolumab + CIMAvax Nivolumab+ CV301 Nivolumab + NEO-PV-01 Nivolumab + Viagenpumatucel-L (HS-110) Pembrolizumab + ADXS31-142 Durvalumab ± tremelimumab + IMCgp100
checkpoint blockade plus adoptive cell transfer (ACT)	anti-CTLA4 plus ACT anti-PD-1/L1 plus ACT anti-PD-1/L1 plus anti-CD137 plus ACT	Atezolimuamb + KTE-C19 Ipilimumab + NYESO TCR ACT Nivolumab + NYESO TCR ACT Nivolumab + urelumab + TIL ACT Pembrolizumab + TIL ACT Ipilimumab + modified CD8 T cell ACT Pembrolizumab + modified CD8 T cell ACT
checkpoint blockade plus targeted therapies	anti-CTLA4 plus BRAF+MEK inhibitors anti-CTLA4 plus VEGF inhibitors anti-PD-1/L1 plus BRAF+MEK inhibitors anti-PD-1/L1 plus EGFR inhibitors anti-PD-1/L1 plus VEGF inhibitors anti-PD-1/L1 plus PI3K delta inhibitor	Atezolizumab + bevacizumab versus sunitinib Atezolizumab + trametinib Atezolizumab + vemurafenib ± cobimetinib Durvalumab + ensartinib (ALK inhibitor) Durvalumab + gefitinib Durvalumab + trametinib ± dabrafenib Ipilimumab + bevacizumab Ipilimumab + dabrafenib ± trametinib Ipilimumab +vemurafenib Nivolumab + sunitinib or pazopanib Nivolumab + trametinib ± dabrafenib PDR001 + sorafenib Pembrolizumab + dabrafenib + trametinib Pembrolizumab + lenalidomide Pembrolizumab + nintedarnib Pidilizumab + lenalidomide Tremelimumab + sunitinib Nivolumab + SYM004
	anti-PD-1/L1 plus PARP inhibitors	Atezolizumab + Veliparib Durvalumab + olaparib BGB-A317 + BGB-290
	anti-PD-1/L1 plus mTOR inhibitor	PDR001 + everolimus
	anti-PD-1/L1 plus pan RAF inhibitor	PDR001 + LXH254
	anti-PD-1/L1 plus glutaminase inhibitor	Nivolumab + CB839
checkpoint blockade plus radiation therapy (RT)	anti-CTLA4 plus RT anti-PD-1/L1 plus RT anti-CTLA4 plus Anti-PD-1/L1 plus RT	Atezolizumab + stereotactic radiation therapy Pembrolizumab + cisplatin/radiotherapy Pembrolizumab + sterotactic body radiotherapy Pembrolizumab + hypofractionated radiotherapy

Table 3.   *Continued*

Broad Approach	Specific Approach	Examples in Clinical Testing
checkpoint blockade plus chemotherapy	anti-CTLA4 plus chemotherapy anti-PD-1/L1 plus chemotherapy anti-CTLA4 plus Anti-PD-1/L1 plus chemotherapy	Atezolizumab + carboplatin/paclitaxel Atezolizumab + carboplatin/gemcitabine Durvalumab + paclitaxel Ipilimumab +carboplatin/paclitaxel Ipilimumab +dacarbazine Nivolumab + platinum doublets Pembrolizumab + carbo/paclitaxel or carbo/pemetrexed
checkpoint blockade plus epigenetic modifications	anti-PD-1/L1 plus histone deacetylase inhibitors anti-PD-1/L1 plus hypomethylating agents	Azacitidine + entinostat followed by nivolumab Atezolizumab + azacitidine Nivolumab + RRX001 Pembrolizumab + CC486 Pembrolizumab + CC486 + romidepsin Pembrolizumab + romidepsin Pembrolizumab + vorinostat + tamoxifen PDR001 + panobinostat
checkpoint blockade plus NK activation	anti-CTLA4 plus anti-KIR anti-PD-1/L1 plus anti-KIR	Ipilimumab + lirilumab Nivolumab + lirilumab

documented in a case of late acquired resistance to anti-PD-1 therapy, where the resistant cells had a new and homozygous truncating mutation in B2M, leading to lack of surface expression of HLA class I (Zaretsky et al., 2016). In two other cases of tumor relapse, there were copy-number-neutral loss-of-function mutations in *JAK1* or *JAK2*, concurrent with loss of heterozygosity due to deletion of the wild-type allele, which were absent in the baseline biopsies. These mutations allowed the cancer cells to escape from the anti-proliferative effects of interferon gamma (Zaretsky et al., 2016). Additional evidence of loss of antigen-presenting machinery leading to acquired resistance to cancer immunotherapy is provided by a case of a patient with metastatic colorectal carcinoma who responded to TIL ACT. The therapeutic TIL recognized mutated *KRAS G12D* presented by HLA-C*08:02, resulting in an objective tumor response for 9 months, followed by an isolated relapse in a lesion that had lost HLA-C*08:02 in chromosome 6 (Tran et al., 2016). Therefore, acquired resistance to anti-PD-1 therapy and ACT could be mediated through genetic mechanisms that altered antigen-presenting machinery and interferon gamma signaling.

Because anti-tumor T cells are specific for cancer cells that express their cognate antigen, it is possible that cancers may develop acquired resistance through decreased expression or mutations in these tumor antigens. Data suggest that anti-tumor T cells turned on by checkpoint blockade therapy primarily recognize mutational neoantigens (Schumacher and Schreiber, 2015; van Rooij et al., 2013). Therefore, genetic deletions, mutations, or epigenetic changes that would lead to loss of expression of these mutational neoantigens presented by MHC molecules might result in acquired resistance to checkpoint blockade therapy. However, thus far there has not been evidence of such mechanisms in the clinic. CAR T cells are also antigen-specific, but they rely on the whole protein expression on the cancer cell surface. In some cases of patients with ALL who responded initially to CD19 CAR T cell ACT, it has been documented that the epitope in the CD19 protein sequence that is recognized by the CAR can be selectively deleted at pro-

gression (Ruella et al., 2016) and that preexisting alternatively spliced CD19 isoforms might predispose to acquired resistance (Sotillo et al., 2015). Therefore, there is evidence from the clinic that loss of the target of the anti-tumor T cells can result in progression to cancer immunotherapy.

This yin and yang of the immune response, which results in immune editing and eventually immune escape, is clearly a factor as we administer immunotherapeutic agents and attempt to drive anti-tumor immune responses, which may encounter a multitude of inhibitory pathways, either during initial treatment or at the time of relapsed disease. Additional inhibitory immune checkpoints that are often expressed in the tumor microenvironment include LAG-3, TIGIT, VISTA, and many more that are being identified in ongoing studies (Topalian et al., 2015). Several clinical trials are currently underway to test antibodies against these inhibitory pathways, both as monotherapy and combination therapy strategies (Anderson et al., 2016; Sharma and Allison, 2015). To date, the combination of anti-CTLA-4 (ipilimumab) plus anti-PD-1 (nivolumab) has demonstrated improved clinical outcomes as compared to monotherapy, and this combination was recently FDA-approved for patients with metastatic melanoma (Larkin et al., 2015a). We will need data from ongoing and future clinical trials to determine whether combination therapies targeting other inhibitory pathways, either as doublets or triplets in concurrent or sequential treatment strategies, will effectively overcome the resistance mechanisms that act to regulate immune responses and provide additional clinical benefit.

Monitoring Resistance Mechanisms

There are significant efforts underway to identify reliable predictive biomarkers of response and resistance to checkpoint inhibitors in baseline tumor biopsies in patients on immune checkpoint blockade. To date, the best predictive biomarkers identified include total tumor mutational load (Roszik et al., 2016; Snyder et al., 2014), as well as markers of an effective immune infiltrate within a tumor signifying a "hot" tumor
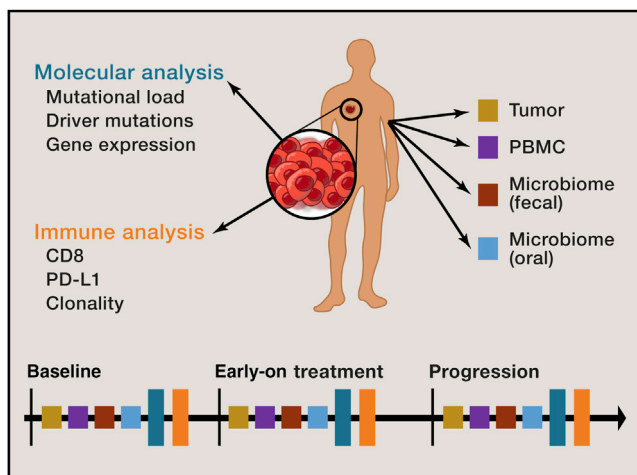
Figure 4. Schema for Analysis of Baseline and Longitudinal Tumor, Blood, and Other Samples
(A) Baseline assessment of the tumor microenvironment typically involves molecular analysis for mutational load, driver mutations, and gene expression, with immune profiling including analysis of CD8+ T cells, PD-L1 expression, and T cell clonality.
(B) Longitudinal evaluation of fresh serial human specimens (tumor, blood, serum, and microbiome) during treatment (at pre-treatment, early-on-treatment, and progression time points) allows for deep analysis to unveil potential mechanisms of therapeutic resistance.

microenvironment, typified by an increased number of CD8+ cytotoxic T lymphocytes in proximity to PD-L1-positive cells (Taube et al., 2014; Tumeh et al., 2014). Mutational load is highly relevant, given that tumors with a higher mutational load exhibit higher levels of neoantigens capable of inducing anti-tumor immune responses, translating into a higher likelihood of response to immune checkpoint blockade across several cancer types (Rizvi et al., 2015; Snyder et al., 2014; Van Allen et al., 2015). In addition to genomic markers and immune regulatory gene expression profiles (Hugo et al., 2016), immune markers in pre-treatment biopsies, including the density and distribution of CD8+ T lymphocytes, PD-L1 expression, and T cell clonality (Taube et al., 2014; Tumeh et al., 2014), have also been associated with differential responses to immune checkpoint blockade, although significant limitations exist when each of these biomarkers is assessed in isolation. Integrative approaches incorporating analysis of several of these features have also been developed, such as the cancer immunogram, which incorporates analysis of seven distinct features within the tumor microenvironment: tumor sensitivity to immune effectors, tumor foreignness, general immune status, immune cell infiltration, absence of checkpoint molecule expression, absence of soluble inhibitors such as interleukin-1 and interleukin-6, and absence of inhibitory tumor metabolism (Blank et al., 2016). These efforts are critical and will ultimately contribute to more personalized treatment strategies for cancer immunotherapy.

An emerging strategy in elucidating mechanisms of response and resistance to immune checkpoint blockade involves the assessment of longitudinal tumor samples throughout the course of treatment. This approach is powerful because it transcends conventional analysis of static time points and seeks to identify superior predictive biomarkers by assessing dynamic responses to cancer treatment. Such an approach has been employed to better understand response and resistance to immune checkpoint blockade (Chen et al., 2016; Hugo et al., 2016; Madore et al., 2015; Tumeh et al., 2014) and has yielded important information that would not have been elucidated through analysis of static unpaired biopsies. A key example is in a recent report describing immune markers in longitudinal tumor samples of patients on immune checkpoint blockade, demonstrating that although pre-treatment markers were largely non-predictive, immune markers in early-on-treatment samples were highly predictive of treatment response (Chen et al., 2016). In addition to this, resistance mechanisms were identified via pairwise comparison of gene expression profiles in pre- to on-treatment tumor samples of responders versus non-responders, including defects in interferon signaling as well as antigen processing and presentation (Chen et al., 2016). This approach is currently under-utilized but is gaining traction in light of advantages over assessment of static baseline biomarkers (Figure 4), as well as an increasing need to better understand responses to a growing number of immunotherapeutic approaches. However nuances exist with regard to immune monitoring in the tumor microenvironment (Wargo et al., 2016), and an appreciation of the importance of concurrent monitoring in the peripheral blood is growing, though the ideal assays to perform are still being elucidated.

Overcoming Resistance to Immunotherapy
On the basis of insights gained (Hugo et al., 2016; Snyder et al., 2014; Van Allen et al., 2015), efforts are currently underway to derive actionable strategies to combat therapeutic resistance to immunotherapy. This includes fundamental efforts to transform immunologically "cold" tumors into "hot" tumors through the use of several approaches (Corrales et al., 2015; Holmgaard et al., 2013; Tang et al., 2016) and also involves tactics to either enhance endogenous T cell function (Gubin et al., 2014; Hodi et al., 2010; Miller et al., 2002; Redmond et al., 2007; Ribas et al., 2015; Weber et al., 2015) or to adoptively transfer antigen-specific T lymphocytes via ex vivo expansion of tumor-infiltrating lymphocytes (Rosenberg et al., 2011) or via administration of antigen-specific engineered T cells (via transduction with CARs or TCRs) (Beatty et al., 2014; Kalos et al., 2011).

Though some of these approaches involve treatment with drugs as monotherapy (including monoclonal antibodies), the majority of contemporary approaches focus on combination strategies in an effort to overcome resistance associated with treatment with single-pronged efforts (Table 3) (Hicklin et al., 1998; Moon et al., 2014; Ninomiya et al., 2015). A prime example of enhanced efficacy with combination therapy is the use of combined therapy with blocking antibodies against two key immune checkpoints, CTLA-4 and PD-1, which results in significantly higher response rates to therapy and improved survival in patients with metastatic melanoma (Larkin et al., 2015a; Postow et al., 2015; Wolchok et al., 2013). The rationale for this combination approach is several fold, as blocking several checkpoints on anergized tumor-specific T cells has been shown to be more efficacious (Berrien-Elliott et al., 2013; Curran et al., 2010; Redmond et al., 2014; Spranger et al., 2014) and CTLA-4 blockade may itself facilitate the conversion of a tumor

microenvironment from "cold" to "hot" (Simpson et al., 2013). Indeed, each of these checkpoint inhibitors has been shown to have both overlapping and unique effects on tumor-specific T cells (Gubin et al., 2014), substantiating the use of these in combination. Numerous other strategies combining immune modulation of the tumor microenvironment with immune checkpoint inhibitor therapy are currently being tested in clinical trials (Puzanov et al., 2016) (NCT02263508, NCT02626000, NCT02565992, NCT02043665, NCT02501473). Vaccine strategies against identified neoantigen epitopes are also being combined with immunotherapeutic approaches, though mature data are not available regarding efficacy.

Another combination strategy with strong clinical and pre-clinical rationale involves the use of molecularly targeted therapy in conjunction with immunotherapy. The most extensively studied cancer type treated with this strategy is melanoma, though the concept is now being widely extended across solid and liquid tumors. The rationale for combining these treatments is that treatment with molecularly targeted therapy can have a substantial effect on anti-tumor immunity and potential synergy when used with immunotherapy (Homet Moreno et al., 2015; Hu-Lieskovan et al., 2015; Koya et al., 2012). Perhaps most illustrative of this is oncogenic BRAF in melanoma. Though treatment with BRAF-targeted therapy alone provides limited durable disease control (Chapman et al., 2011; Hauschild et al., 2012), it is associated with favorable effects in the tumor microenvironment, including increased antigen (Boni et al., 2010) and HLA expression (Bradley et al., 2015), increased T cell infiltrate, reduced immunosuppressive cytokines (Frederick et al., 2013; Wilmott et al., 2012), and improved T cell function (Comin-Anduix et al., 2010). Thus, treatment with molecularly targeted therapy may indeed help convert a "cold" microenvironment to a "hot" one, with resultant increased expression of PD-L1 via the phenomenon of adaptive resistance (Taube et al., 2012), further supporting a multi-modality treatment approach. Emerging strategies to enhance responses to immunotherapy are being developed based on novel insights into T cell and overall immune function. Examples of this include insights into metabolic reprogramming of T cells to enhance therapeutic responses (Buck et al., 2016; Chang and Pearce, 2016) and via modulation of the gut microbiome to augment responses to cancer immunotherapy (Sivan et al., 2015; Vétizou et al., 2015).

Complexities exist when attempting to validate these combination strategies given that the extent of possible combinations far outnumbers the human and technical resources available. There is an urgent need to test these combinations in appropriate preclinical models and expedite clinical translation through novel approaches to clinical trial design. In addition, we need to have a deep understanding of the kinetics of the immune response to each of these agents in isolation as well as in combination in order to narrow the search space of biologically promising and optimal combination strategies. Immune responses to targeted agents may be short-lived (Cooper et al., 2014), thus proper timing and sequence of therapy must be strongly considered.

Conclusions

Great advances have occurred in the field of cancer immunotherapy as a result of elegant research work conducted to eluci-date the mechanisms that regulate anti-tumor T cell responses, including eventual translation of these concepts to the clinic. This has allowed the rational design and clinical development of treatment strategies that might result in tumor regression and long-term survival for patients with metastatic cancer. However, the benefit, to date, has been limited to a minority of patients with certain cancer types. In addition, as a result of more successful immunotherapy treatments, we now have a significant subset of patients who initially respond but eventually relapse. Bringing clinical benefit to the majority of patients requires a complete understanding of the mechanisms that would lead to an effective anti-tumor response and the different tumor-cell-intrinsic and -extrinsic factors that would result in primary, adaptive, and acquired resistance to immunotherapy. Elucidation of these mechanisms will reveal important clues as to the next steps that need to be taken to potentially overcome resistance to immunotherapy.

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