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Acquired Resistance to Immune Checkpoint Inhibitors

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Abstract

Immune checkpoint inhibitors (ICI) have rapidly altered the treatment landscape for multiple tumor types, providing unprecedented survival in some patients. Despite the characteristic durability of response to ICI, unfortunately many patients with initial response will later develop acquired resistance. The current understanding of mechanisms of acquired resistance to ICIs is remarkably limited, perhaps restraining effective development of next-generation immunotherapies. Here, we examine the barriers to progress and emerging clinical reports interrogating acquired resistance with the goal to facilitate efforts to overcome acquired resistance to ICIs in the future.

Keywords

Acquired resistance; ICIs; Immunotherapy

Introduction

The advent of immune checkpoint inhibitors (ICIs) has rapidly transformed the treatment paradigm for multiple cancer types. Over the last decade, starting from the initial approval of CTLA-4 inhibitors in metastatic melanoma in 2011, PD-(L)1 inhibitors are now a routine part of treatment for more than 20 different indications. The treatment of patients with lung cancer is just one stark example of this paradigm shift: beginning from the first phase I study of PD-1 blockade where just one of six patients with NSCLC experienced response in a single target lesion (but not overall achieving partial response) to today, where nearly all patients diagnosed with metastatic lung cancer receive PD-(L)1 blockade as a component of treatment (Brahmer et al., 2010; Gandhi et al., 2018; Reck et al., 2016).

This remarkable shift has been driven in large part by unprecedented durability that is characteristic of response to ICIs. Responses *can* last years, if not indefinitely, even without continuous treatment (Antonia et al., 2019; Bernard-Tessier et al., 2018; Fradet et al., 2019;

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Garon et al., 2019; Gettinger et al., 2018a; Hamid et al., 2019; Larkin et al., 2019; Motzer et al., 2015; Schadendorf et al., 2017; Topalian et al., 2019). However, sometimes lost in the understandable enthusiasm related to the clinical impact of ICIs is the reality that the vast majority of patients do not experience benefit from ICIs. The response rate to single agent PD-1 blockade in unselected patients ranges ~40%−70 in some diseases (e.g. melanoma, Merkel cell, Hodgkin's lymphoma, MSI high tumors) (Armand et al., 2018; Chen et al., 2017; Hamid et al., 2019; Larkin et al., 2019; Le et al., 2017; Nghiem et al., 2019) while the response rates in most other approved diseases is limited to 10–25% (Antonia et al., 2019; Balar et al., 2018; Fradet et al., 2019; Mok et al., 2019; Motzer et al., 2015). Furthermore, even among patients who initially respond to ICIs, disease progression can eventually develop. In short, only a minority of patients achieve the long-term, durable response that is the transcendent feature of ICIs, while resistance is the unfortunate experience for most patients.

Resistance to ICIs may be initially classified into two broad categories: 1) primary resistance, generally referring to patients who do not respond at all and instead progress, quickly or eventually, with ICIs, and 2) acquired resistance, which refers to patients who have a period of initial response to therapy followed ultimately by clinical and/or radiologic progression of disease. To confront the challenge of primary resistance, substantial effort has been spent on combination strategies, often with empiric orthogonal therapies, to broaden the responding population. For example, ICIs have been combined with chemotherapy in lung, breast and gastric cancer, as well as multi-target TKIs in RCC and FGFR inhibitors in bladder cancer (Gandhi et al., 2018; Motzer et al., 2019a; Schmid et al., 2018; Tabernero et al., 2019). Further, there has been an extensive search for predictive biomarkers for initial ICI response. PD-L1 expression, tumor mutational burden, tumor infiltrating lymphocytes (TILs) or related gene expression signatures have been explored as potential predictors and various other markers are currently under investigation (Chen et al., 2016; Cristescu et al., 2018; Garon et al., 2015; Hugo et al., 2016; Johnson et al., 2016; Liu et al., 2019; McGranahan et al., 2016; Rizvi et al., 2018; Rizvi et al., 2015; Rodig et al., 2018; Topalian et al., 2012; Tumeh et al., 2014; Van Allen et al., 2015). Conversely, there have been no approved therapeutic breakthroughs yet for circumventing or reversing acquired resistance and there have been remarkably few published investigations on the characteristics or mechanisms of acquired resistance to ICIs.

Unlike primary resistance, the rates of acquired resistance have not been routinely reported and thus are not clearly characterized across tumors types. We have attempted to infer the rates of acquired resistance based upon available duration of response data among different tumor types (Antonia et al., 2019; Armand et al., 2018; Balar et al., 2018; Burtness et al., 2019; Chen et al., 2019a; Cohen et al., 2019; Fehrenbacher et al., 2018; Fradet et al., 2019; Garon et al., 2019; Hamid et al., 2019; Herbst et al., 2016; Janjigian et al., 2018; Larkin et al., 2019; Le et al., 2017; Marabelle et al., 2020; Mok et al., 2019; Motzer et al., 2015; Motzer et al., 2019b; O'Donnell et al., 2019; Overman et al., 2017; Pires da Silva et al., 2020; Powles et al., 2018; Robert et al., 2019; Shah et al., 2019; Tykodi et al., 2019; Wang et al., 2017; Younes et al., 2016; Zandberg et al., 2019). From this data, it appears that rates of acquired resistance vary across multiple disease types (Table 1). For example, an updated report patients with melanoma treated with nivolumab demonstrated 39% of responders had

progressed at five years follow up (Larkin et al., 2019). By contrast, in a recent pooled analysis of patients with NSCLC treated with nivolumab, up to 65% of responders had progressed at 4 years follow-up (Antonia et al., 2019). More generally across tumor types, there appears to be an inverse relationship between the overall response rate to PD-1 blockade and the frequency of acquired resistance among responders (Figure 1). This observation suggests disease-specific features of acquired resistance and contrast with the current typical understanding that, despite variable response rates across tumor types, *if* response occurs it is similarly durable across tumor types. With increasing experience with immunotherapy and longer-term follow-up, it is imperative that future studies report acquired resistance rates among responders to better quantify acquired resistance. Disease-specific or response-rate-related differences in the frequency of acquired resistance may reveal important biological insight into the distinct determinants of initial response, long-term durability, and acquired resistance.

It is instructive to contrast the paucity of data on mechanisms of acquired resistance to immunotherapy with the growing knowledge about resistance to targeted therapy in oncogene driven cancers. For example, in *EGFR*-mutant lung cancer, an archetype for molecularly targeted therapy, knowledge of resistance to first-generation tyrosine kinase inhibitors (TKIs) identified a secondary *EGFR* point mutation which, in turn, led to the development of the third-generation *EGFR*-T790M targeted TKI osimertinib (Mok et al., 2017; Pao et al., 2005). Furthering the cycle, new investigations into the distinct mechanisms of acquired resistance to osimertinib have since yielded even further therapeutic successes (Piotrowska et al., 2018; Ramalingam et al., 2018; Schoenfeld et al., 2020).

Conversely in ICIs, despite the profound number of patients now treated with PD-(L)1 blockade, insight into the underpinnings of and rational therapeutic strategies to treat acquired resistance to ICI remain unrealized. Before reviewing what is known about acquired resistance to ICI, we sought to explore the barriers to progress thus far and propose potential solutions.

Barriers to investigation of acquired resistance mechanisms to immunotherapy

There are challenges to studying mechanisms of acquired resistance to immunotherapy that should be considered when reviewing the currently available data. Primary challenges include (1) *Terminology*: the lack of uniform terminology used to define, classify, and apply acquired resistance, (2) *Tissue*: difficulty acquiring optimal tumor samples for analyses, and (3) *Tools*: the absence of routine, effective tools to comprehensively interrogate and discovery mechanisms of immune resistance in the tumor, host, and/or microenvironment (Table 2). Deeper understanding of these issues is essential to interpreting prior reports and designing studies that can successfully and comprehensively identify mechanisms of acquired immune resistance in the future.

Terminology

First, in order to analyze and interpret the results of multiple studies, a clear and consistent framework for defining *acquired resistance* should be established. For example, in *EGFR*-mutant lung cancer, relatively simple but specific guidelines were developed to define patients with acquired resistance and distinguish this specific disease state from other potentially confounding clinical scenarios. Among other considerations, the *EGFR* criteria stipulated patients should have 1) documented initial objective response (partial or complete response by RECIST or WHO) to therapy OR significant and durable (> 6 months) clinical benefit (stable disease defined by RECIST or WHO) and 2) systemic progression of disease while on continuous treatment with an EGFR-TKI within the last 30 days (Jackman et al., 2010). These criteria may seem so simple as to be self-evident, but have indeed facilitated consistency in evaluating new treatments for resistance and identifying mechanisms. And there are nuanced considerations when applying these criteria in the context of PD-(L)1 resistance.

No such uniform definition has been established for acquired resistance to immunotherapy. The Society for Immunotherapy of Cancer (SITC) has established an anti-PD-(L)1 taskforce and convened an anti-PD-(L)1 resistance workshop from which further discussion is forthcoming. We highlight two (of several) important features to be considered toward the goal of developing such a consensus framework:

1) Depth of initial response—Conceptually, a patient with acquired resistance would have first responded, before later developing resistance. What features constitute “a responder” still require consideration. It is likely uncontroversial to include patients with a RECIST-defined partial or complete response to ICIs, but it is debatable whether to include patients with stable disease of some duration in this definition. As a whole, the stable disease subgroup appears to derive improved survival with ICIs, although to a lesser degree than PR/CR (Antonia et al., 2019; Garon et al., 2019; Gettinger et al., 2018a; Larkin et al., 2019). More fundamentally, the stable disease category is likely a heterogenous mix of some patients with minor responses intermixed with indolent (but non-responsive) and progressive disease. Currently, there are not sufficiently reliable tools to distinguish among patients with stable disease who are responders (despite radiologically stable disease) versus who are non-responders to PD-1 treatment. Therefore, inclusion of stable disease may include this subset of responders whereas it also could also increase heterogeneity and obscure analysis of acquired resistance with patients who are non-responders. With utility far beyond the definition of acquired resistance, tools are needed to better resolve the heterogeneity of stable disease and adjudicate whom within this group should be considered a responder.

Additional nuance of how to interpret depth of response is also needed when considering patients treated with combinations with other active, orthogonal therapy such as chemotherapy. In such combinations, it is presently not feasible to differentiate whether initial response is attributable to either or both therapies, and therefore the nature of the associated resistance is uncertain.

One last consideration includes the importance of differentiating the application of the acquired resistance definition to patient-level response versus the lesion level response. The

depth of response and occurrence of acquired resistance of a patient, using RECIST-defined best overall response or progression for example, may be most relevant for considering clinical trial eligibility and descriptive clinical reports. Meanwhile, the degree of response and acquired resistance at a specific metastatic site may be distinct and important for translational analysis of the underlying biology of acquired resistance at that specific site.

2) Drug exposure—The extent to which continued drug exposure should be required at the time of acquired resistance remains an open question. Unlike molecular therapy where patients will likely progress if therapy is discontinued, prior studies have demonstrated that patients can have ongoing responses long after cessation of ICI therapy. These include patients who have completed 1 – 2 year of therapy, along with patients who have stopped treatment early due to toxicity, even in some cases with minimal drug exposure (Garon et al., 2019; Gettinger et al., 2018a; Larkin et al., 2019; Schadendorf et al., 2017). Conversely, other recent analyses such as KEYNOTE-010 in NSCLC have also shown that 32% of responders had progressive disease after completion of 2 years of treatment (Herbst et al., 2020). And in a study (CheckMate-153) of patients with NSCLC who reached one year of treatment and then were randomized either to discontinue or continue treatment, progression-free survival thereafter was improved in those who continued therapy (Spigel et al., 2017). In general, prior reports on re-challenging patients with ICIs are relatively limited, show varying degrees of success with this strategy, and the patient-specific or tumor-specific features that predict re-response are not known (Garon et al., 2019; Gettinger et al., 2018a; Hamid et al., 2019; Herbst et al., 2020; Pires da Silva et al., 2020; Robert et al., 2019; Wang et al., 2017; Warner et al., 2019). Overall, it is unclear when the emergence of resistance to ICIs is attributable to cessation of therapy, but it appears to be a relative minority of patients and success of re-treatment is far from guaranteed.

Tissue

Importantly, in comparison to molecularly targeted therapy, response to ICI is less common and the occurrence of acquired resistance to ICIs is more idiosyncratic. In EGFR-mutant lung cancer, for example, ~80% patients treated with osimertinib will initially respond and nearly all responders will eventually develop acquired resistance (Soria et al., 2018). In this scenario, it is reasonably efficient to routinely collect pre-treatment tissue with the expectation that the vast majority of patients will both initially respond and later develop resistance, from which additional post-treatment tissue can then be examined. With ICIs, however, a much smaller proportion of patients are initially sensitive to treatment and, among those patients who do respond, the development of acquired resistance is variable. Therefore, in studies interested in acquired resistance specifically, it is inefficient to collect tissue from all patients at baseline knowing a relatively small fraction will respond and even smaller fraction will later develop resistance. Overall, the more unpredictable nature of acquired resistance to ICIs makes devising and executing successful correlative studies difficult.

The oligoprogressive pattern, which may be a common pattern of acquired resistance with ICIs (Gettinger et al., 2018b; Pires da Silva et al., 2020; Wang et al., 2017), also complicates the potentially ideal scenario of comparing site-matched tissue sample (as the acquired

resistance site is unpredictable at baseline). This consideration also contrasts with molecular therapy, where resistance tends to (at least eventually) be systemic and therefore often feasible to interrogate the underlying biology of resistance from biopsy of any tumor site and/or blood (Barron et al., 2018; Ramalingam et al., 2018; Yang et al., 2013). Further, the role of tumor biopsies at the time of progression on immunotherapy may be questioned because of uncertain clinical actionability. In our experience, biopsies can be an essential clinical tool to guide management. Particularly in settings of indeterminate radiologic changes, biopsies can help distinguish inflammation or scar tissue or secondary malignancy or, indeed, acquired resistance. Thus, coordinated efforts to identify patients who have developed acquired resistance to ICIs will be critical. Multicenter and industry-academia cooperation is a potential remedy to small sample sizes and can help overcome the routine challenge of missing pre-treatment and/or post-treatment sampling.

Tools

Finally, the few number published reports on acquired resistance to ICIs may also reflect the few obvious discoveries using common molecular tools for examining the tumor and its microenvironment. By contrast, in targeted therapy analyses, the use of bulk next-generation sequencing of DNA and RNA has been successful to identify primary drivers of acquired resistance. Yet, studies of resistance to ICIs that similarly focused on bulk DNA and RNA sequencing of tumor samples have uncovered a limited number of acquired genomic alterations (discussed in more detail below, largely falling in antigen presentation and interferon-γ signaling pathways) (Anagnostou et al., 2017; Gettinger et al., 2017; Zaretsky et al., 2016). Instead, resistance to ICI may be vastly more nuanced, dynamic, and complex, involving at least three interactions (host, tumor, tumor microenvironment) and two compartments (intratumoral and systemic), necessitating a multifaceted approach and higher resolution investigation of the (epi)genetics, functional state, and geographic interconnections of both tumor and immune cells across broad space. Multiple factors including the evolution of neoantigens and neoantigen presentation, innate and adaptive immune response, epigenomic modifications, and tumoral heterogeneity which have all been associated with immune checkpoint inhibitor efficacy, are not well captured by traditional bulk sequencing from tumor samples and warrant further attention. Further, single cell imaging and/or sequencing approaches may be required to evaluate clonal evolution of tumors and the tumor immune microenvironment in response to ICIs.

With this context in mind, we here present a review of the clinical and pre-clinical studies on acquired resistance to ICIs to date.

Clinical data on acquired resistance to ICIs

By any measure, the number of clinical cases of acquired resistance to ICI reported in the literature is remarkably small. We identified 13 clinical reports comprising 58 total patients with acquired resistance to ICIs across all tumor types (Abdallah et al., 2018; Anagnostou et al., 2017; Ascierto et al., 2017; Gettinger et al., 2017; Hu et al., 2018; Iams et al., 2019; Le et al., 2017; Sade-Feldman et al., 2017; Trujillo et al., 2019; Zaretsky et al., 2016) (Figure 2, Supplemental Table 1). Importantly, these reports use differing inclusion parameters for

acquired resistance and represent a heterogenous population. While some reports require initial partial or complete response to ICI, others include cases of initial stable disease or "mixed responses" (Gettinger et al., 2017; Iams et al., 2019; Kakavand et al., 2017). Detailed analysis of the degree of response and acquired resistance of individual lesions from which tissue samples are derived may also be relevant when considering translational analyses.

Although many cases have uncertain or unknown mechanisms, those with speculated mechanisms of resistance roughly fall into a few categories: 1) defects in antigen presentation, 2) interferon-γ (IFN-γ) signaling 3) neoantigen depletion 4) tumor-mediated immunosuppression/exclusion and 5) additional inhibitory checkpoints (Figure 3).

Defects in antigen presentation machinery

The activation of T-cell mediated immunity is dependent upon the recognition of antigens on major histocompatibility complexes (MHCs) of antigen presenting cells (Blum et al., 2013; Trombetta and Mellman, 2005). Tumor antigen presentation by MHC class I is mediated by the coordinated expression of multiple genes (Blum et al., 2013; Hulpke and Tampe, 2013; Trombetta and Mellman, 2005). One key gene, *beta-2*-microglobulin (*B2M*), is responsible for stabilization of MHC class 1 molecules at the cell surface and facilitation of loading with peptides (Hulpke and Tampe, 2013). Historically, following treatment with adoptive cell therapy or IL-2, loss of function mutations in *B2M* were shown to lead to MHC I loss and represent a molecular route of tumor escape from immune detection (Restifo et al., 1996). More recently, truncating alterations in *B2M* have been one of the few recurring findings in acquired resistance to ICIs. Zaretsky et al., for example, analyzed data from 4 melanoma patients with acquired resistance to immunotherapy and identified 1 patient with a homozygous acquired truncating *B2M* mutation (Zaretsky et al., 2016). Further studies in melanoma and other tumor types, have similarly found acquired defects in antigen presentation. In a study of 5 melanoma patients, Sade-Feldman et al. identified 1 patient with acquired *B2M* loss of heterozygosity (LOH) and 2 frameshift mutations and 1 patient that had 2 frameshift *B2M* alterations at the time of progression (Sade-Feldman et al., 2017). In a study of acquired resistance among 14 patients with lung cancer (10 with paired tumor tissue samples), 1 patient acquired homozygous loss of *B2M* (Gettinger et al., 2017). And in a report of 5 patients with mismatch repair deficient (MMR-d) tumors that received PD-1 blockade and developed progressive disease after initial response, 2 patients had acquired alterations of *B2M* (Le et al., 2017).

Importantly, in 5 of 6 cases of tumors harboring *B2M* genomic alterations described above, the patients' tumors had bi-allelic, or homozygous, alterations of *B2M*. IHC also confirmed that many of these biallelic alterations were linked to expression loss of B2M and MHC class 1. Presumably tumors with a loss-of-function alteration in one allele and loss of heterozygosity (LOH) of the wild-type *B2M* allele have a similar biologic effect due to the lack of presence of functional *B2M* copy, supported by the findings of Sade-Feldman et al (Sade-Feldman et al., 2017). However, the biological and clinical impact of mono-allelic truncating *B2M* alterations with a retained wild-type B2M or missense mutations remains unclear. It is possible that a single copy of the wildtype *B2M* is sufficient to maintain proper

function of the MHC complex and it should not be assumed that all *B2M* alterations will cause ICI resistance.

Despite being one of the more common recurring findings in acquired resistance, biallelic *B2M* alterations appear to be uncommon at baseline and are not a clearly established mechanism of primary resistance to ICIs. For example, in one report examining pre-treatment tissue of patients with NSCLC treated with PD-1 blockade, only 1 of 240 (0.4%) patients had biallelic alterations in *B2M* (Rizvi et al., 2018). Notably, this patient has loss of B2M expression confirmed by immunohistochemistry and nevertheless experienced a partial response to PD-1 blockade. Sade-Feldman found B2M alterations to be enriched in non-responders to anti-CTLA4 therapy compared to responders, but did not find enrichment in a dataset of ant-PD-(L)1 therapy (Sade-Feldman et al., 2017). Further, a recent analysis in MSI-H colorectal cancer has shown that most patients with B2M mutations and protein expression loss were still sensitive to ICIs (Middha et al., 2019), illustrating that mechanisms of acquired resistance may be fundamentally distinct from primary resistance.

Additionally, it is important to distinguish genomic etiologies of MHC loss via *B2M* alterations versus other causes of expression loss of the MHC. In Gettinger et al., significantly reduced levels of MHC class 1 protein and B2M protein were also identified in 4 out of 9 and in 3 out 9 patients, respectively, with no corresponding molecular alterations of *B2M* (Gettinger et al., 2017). In these cases, additional unknown genomic or nongenomic factors may modulate MHC class 1 expression and influence resistance to ICIs.

Defects in IFN-γ signaling

Release of IFN-γ from effector T cells triggers a signaling cascade in tumor cells via the JAK-STAT pathway that mediates both MHC class I and PDL1 expression and can induce tumor cell death in multiple ways (Bach et al., 1997). One of the critical first steps within this pathway is activation of receptor associated kinases JAK1 and JAK2 via IFN-γ binding to the heterodimeric IFNGR1/IFNGR2. Recent clinical reports have revealed cases of inactivating mutations of *JAK1* or *JAK2*, which may contribute to progression on ICIs (Gao et al., 2016; Shin et al., 2017; Sucker et al., 2017; Zaretsky et al., 2016).

In Zaretsky et al., 2 patients with who progressed on ICIs after 1 and 2 years had acquired loss of function mutations in genes encoding *JAK1* (*JAK1* Q503*) or *JAK2* (*JAK2* F547 splice-site mutation) (Zaretsky et al., 2016). The mutations were upstream of the kinase domains and presumably caused truncation or nonsense-mediated decay of the JAK protein. Similar to the scenario with *B2M* alterations described above, no wild-type *JAK1* or *JAK2* allele was retained as the mutations occurred concurrently with the LOH of the wild-type allele at the time of progression. Cell lines established from the patient with the acquired *JAK2* mutation and CRISPR-Cas9 engineered cell lines with analogous *JAK1* or *JAK2* mutations also confirmed a total loss of JAK protein and a loss of tumor cell sensitivity to IFN-γ, with a corresponding lack of MHC class 1 and PDL1 expression (Zaretsky et al., 2016). An additional analysis of cell lines from 6 melanoma patients with alterations of the JAK-STAT pathway (*JAK1* n=4, *JAK2* n=1, *STAT1* n=1) validated that only patients with homozygous deficiency of *JAK* (n = 2) either through LOH or biallelic genomic alterations appeared insensitive to the effects IFN-γ (Sucker et al., 2017). Patients with heterozygous

mutations still demonstrated strong IFN-γ pathway activation with elevated surface expression of HLA class 1 and PD-L1 surface expression (Sucker et al., 2017).

JAK1, *JAK2*, and *IFGNGR* alterations have been reported in the setting of primary resistance to immunotherapy (Gao et al., 2016; Shin et al., 2017). In an analysis of 16 patients with melanoma who received CTLA4 inhibitors, genomic loss of other key IFN-γ genes such as *IRF1* and amplification of critical IFN-γ pathway inhibitors such as *SOCS1* and *PIAS4* were also strongly enriched in tumors of non-responder patients (Gao et al., 2016). However, clinical reports of acquisition of these alterations are lacking and the extent to which additional IFN-γ pathway genomic alterations beyond *JAK1* and *JAK2* contribute to acquired resistance to ICIs is unclear.

Neoantigen depletion

Neoantigen-specific T cells may be key drivers of role response to ICIs (McGranahan et al., 2016; Rizvi et al., 2015; van Rooij et al., 2013). Consequently, loss of the somatic mutations encoding putative tumor-specific neoantigens either through clonal selection, epigenetic repression, or copy number loss may lead to subsequent immune evasion and clinical progression (Rosenthal et al., 2019).

In a clinical report of 4 patients with NSCLC who developed acquired resistance to ICIs, no loss-of-function mutations in HLA genes such as *B2M* or *JAK1* or *JAK2* were identified (Anagnostou et al., 2017). Rather, exome data of pre vs post-treatment tissue identified loss, at the time of acquired resistance, of several mutations computationally predicted to generate neoantigens. In vitro, there was clonal T-cell expansion when stimulated with many of the lost neoantigen peptides, suggesting these predicted neoantigens were immunologically relevant and may have driven the selective pressure to eliminate these clones and permit the outgrowth of acquired resistance. Relatedly, immunoselection via loss of T-cell-recognized antigens after adoptive T-cell transfer has similarly been described (Verdegaal et al., 2016).

Tumor-mediated immunosuppression/exclusion

In preclinical models, loss of the tumor suppressor *PTEN*, which is critical in the regulation of PI3K activity, increases expression of immunosuppressive cytokines and decrease T cell effector IFN-γ leading to inhibition of T-cell mediated infiltration and immunity (Peng et al., 2016). *PTEN* loss has also been observed in cases of acquired resistance. For example, in a recent report of a patient with metastatic uterine leiomyosarcoma who initially had a near complete response to pembrolizumab for greater than 2 years, biallelic *PTEN* loss was acquired at the time of resistance (George et al., 2017). Similarly, in a report of two patients with melanoma who developed acquired resistance to immune therapies, one patient also developed biallelic *PTEN* loss (Trujillo et al., 2019).

Of note, in Trujillo et al, the second patient initially showed a durable partial response to a melanoma-peptide/interleukin-12 vaccine and subsequently developed new treatment-resistant metastases had showed new robust tumor expression of β-catenin (Trujillo et al., 2019). Similar to *PTEN* loss, WNT-β-catenin activity has been linked to the production of immunosuppressive cytokines, alterations in priming of dendritic cells, promotion of regulatory T cells, and lack of significant T cell infiltration in melanoma, supporting B-

catenin's role in ICI resistance (Spranger et al., 2015; Spranger et al., 2017; Yaguchi et al., 2012; Zhao et al., 2018).

Additional inhibitory checkpoints

Some reports have described upregulated expression of other T cell checkpoint at the time of acquired resistance, including TIM3 (Gettinger et al., 2017; Koyama et al., 2016), LAG3 (Gettinger et al., 2017) and V-domain Ig suppressor of T cell activation (VISTA) (Kakavand et al., 2017), although whether these changes are causally associated with resistance is unclear. Although such checkpoints may in some contexts be functionally associated with T cell exhaustion and terminal dysfunction, they may also be associated with T cell activation (Blank et al., 2019). Additional data may be needed to understand the functional T cell state in these examples and, ultimately, the contribution to acquired resistance.

Unclear mechanisms of resistance

We caution that even in many of the reports discussed above, it is challenging to verify or identify a specific mechanism of resistance in many cases. In some reports, no mechanism is proposed and in others, overlapping mechanisms are presented, or the mechanism of resistance is inferred from circumstantial data. Thus, the true landscape of acquired resistance to ICIs remains largely uncertain.

Insights from new tools

The sparse clinical data illustrates that the reliance on common molecular tools may not fully reveal the underlying mechanisms of acquired resistance. Instead, broader and deeper exploration of the relationship between the tumor microenvironment, host immunity, and tumor may be required to understand the functional and geographic nature of immune response and immune checkpoint inhibitor efficacy. There are new emerging techniques may further facilitate this pursuit.

Systematic, genome-wide scale CRISPR-Cas9 mutagenesis screens have identified candidate genes and pathways critical for modulating anti-tumor immunity and, therefore, potentially relevant to understanding acquired resistance (Manguso et al., 2017; Pan et al., 2018; Patel et al., 2017). These studies converge on the importance of the IFN-γ receptor signaling pathway and reveal other potential mediators of ICI sensitivity and resistance. Patel et al. highlighted the role of APLNR, a G-protein-coupled receptor that is mutated in multiple cancer types, in immunotherapy resistance and showed that APLNR regulates T cell response via modulation of JAK1 and IFN-γ signaling (Patel et al., 2017). Manguso et al. demonstrated activating mutations in the tyrosine-protein phosphatase nonreceptor type 2 (Ptpn2), which negatively regulates JAK1 and JAK2, contributes to resistance to ICIs through IFN-γ resistance (Manguso et al., 2017). Finally, Pan et al., which also found Ptpn2 and IFNγ signaling was enriched in ICI resistance, reveals critical components of the SWI/SNF chromatin remodeling complex can suppress IFNγ signaling and thereby mediate T cell antitumor immunity (Pan et al., 2018).

Vredevoogd et al. also performed a genome wide CRISPR-Cas9 screen of IFN-γ receptor-deficient melanoma to identify IFN-γ independent pathways involved in anti-tumor

immunity. In doing so, they uncovered that tumor necrosis factor (TNF) receptor associated factor 2 (TRAF2) inactivation within the TNF signaling pathway contributes to T-cell elimination (Vredevoogd et al., 2019).

Single cell sequencing has also further refine understanding of key regulators of distinct T cell states, which may define the balance between effective anti-tumor immunity or resistance. In particular, better understanding of the characteristic features and mechanisms underlying T cell exhaustion with chronic antigen stimulation may be critical to predicting patterns of response and reinvigorating T cell response after acquired resistance. (Blank et al., 2019).

Sade-Feldman et al. profiled the transcriptomes of individual immune cells (48 samples from 32 patients with melanoma) receiving immunotherapy, enabling the detection of individual T cell populations and associated markers associated with ICI responders and non-responders (Sade-Feldman et al., 2018). In this report, TCF1, a protein within the Wnt/β-catenin signaling pathway, emerged as a top marker of response and linked to a cluster of T cells with expression of genes of memory, activation, and cell survival. TCF1 has previously been described a critical factor for differentiation, self-renewal, and persistence of memory CD8+ T cells and shown to reinvigorate and sustain effective immunity of CD8+ T cells against chronic lymphocytic choriomeningitis mouse virus (LCMV) infection upon anti-PD-1 treatment (Blank et al., 2019; Chen et al., 2019b; Hudson et al., 2019; Im et al., 2016; Utzschneider et al., 2016; Zhou et al., 2010).

In another recent report, Miller et al. compared single-cell expression profiles from TIL CD8+ T cells in murine models of LCMV chronic infections versus tumors to further characterize T cell exhaustion (Miller et al., 2019). They identify similar, overarching traits in both models and highlight two critical subpopulations of T cells in the tumor microenvironment; progenitor exhausted CD8+ T cells and terminally exhausted CD8+ T cells. The report demonstrates that the frequency of these subsets may account for differential responses and resistance to immunotherapy. Further, progenitor T cells, which may proliferate and differentiate into exhausted CD8+ T cells upon PD-1 blockade, may be necessary for sustained anti-PD-1-driven responses. Future clinical studies are required to determine if and when depletion of progenitor T cells may play a role in acquired resistance to ICIs.

Interrogation of peripheral blood samples has also facilitated better understanding of the relationship of systemic and intratumoral T-cell responses to PD-1 blockade. In metastatic melanoma, sequential immune profiling of the peripheral blood has shown increased Ki67 expression among exhausted T cells after PD-1 blockade and evidence that immunologic reinvigoration and response may be detected in the periphery (Huang et al., 2017). Two recent studies have further identified early changes in a subset of peripheral CD8+ memory effector cytotoxic T cells was associated with subsequent response to PD-1 blockade in melanoma (Fairfax et al., 2020; Valpione et al., 2020). Additionally, paired single-cell RNA and T cell receptor sequencing demonstrated that expanded TCRs on-treatment and T cell response to PD-1 blockade may actually be derived from peripheral circulation rather than baseline TILs (Yost et al., 2019). Likewise, serial peripheral sampling and single-cell

analyses may be a valuable, complementary process to paired tumor tissue for investigating and monitoring acquired resistance to immunotherapy and require further exploration in future correlative studies.

Conclusions

Acquired resistance to ICI is a common clinical phenotype about which relatively little mechanistic insight is known. The clinical development of next generation immunotherapies for patients with primary and acquired resistance is robust, but success has been limited. More effective therapeutic strategies may be achieved though deeper understanding of the underlying biology, permitting precision deployment of immunotherapies beyond immune checkpoint inhibitors. Collaborative efforts are needed to overcome the barriers toward a deeper understanding of the underlying biology of acquired resistance to ICIs. This progress will ultimately enable rational future drug and cellular therapy development to prevent, circumvent, or reverse resistance to ICIs.

Supplementary Material

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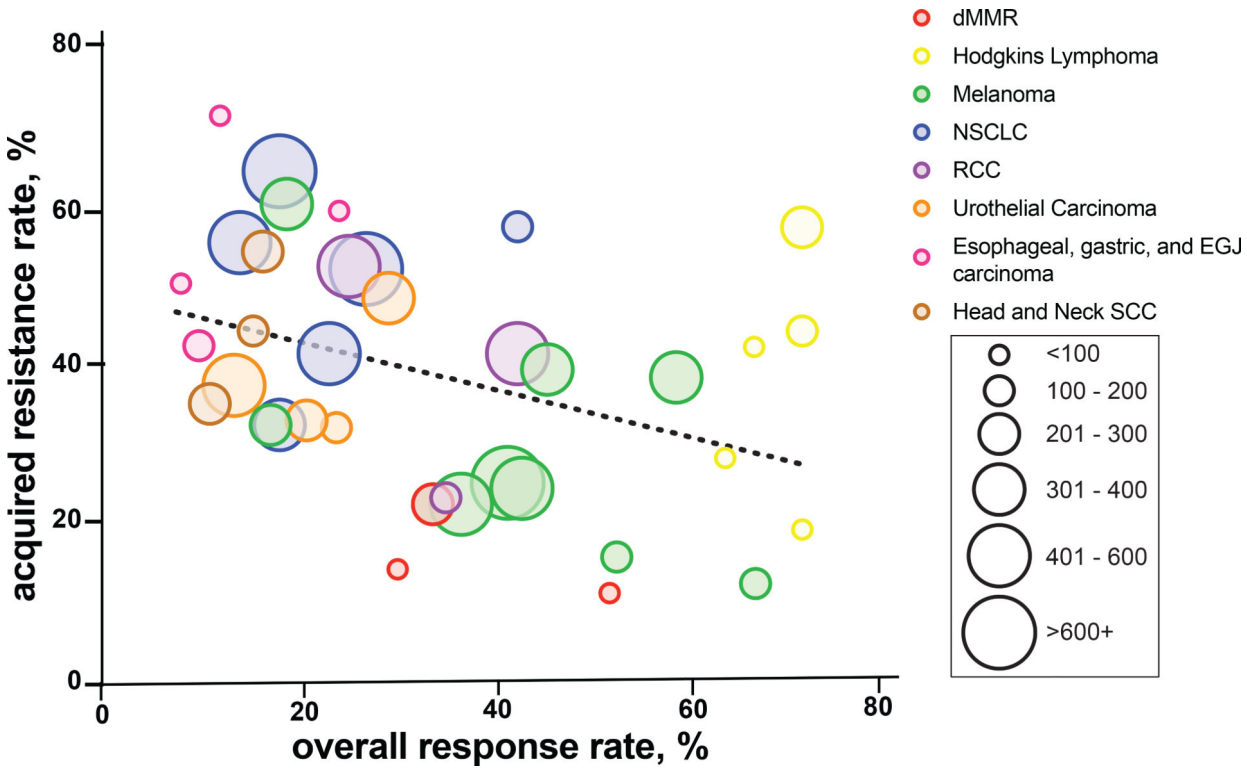

Figure 1. Rate of acquired resistance and overall response by tumor type.

Different tumor types are represented by colored circle and number of patients is represented by size of the circle.

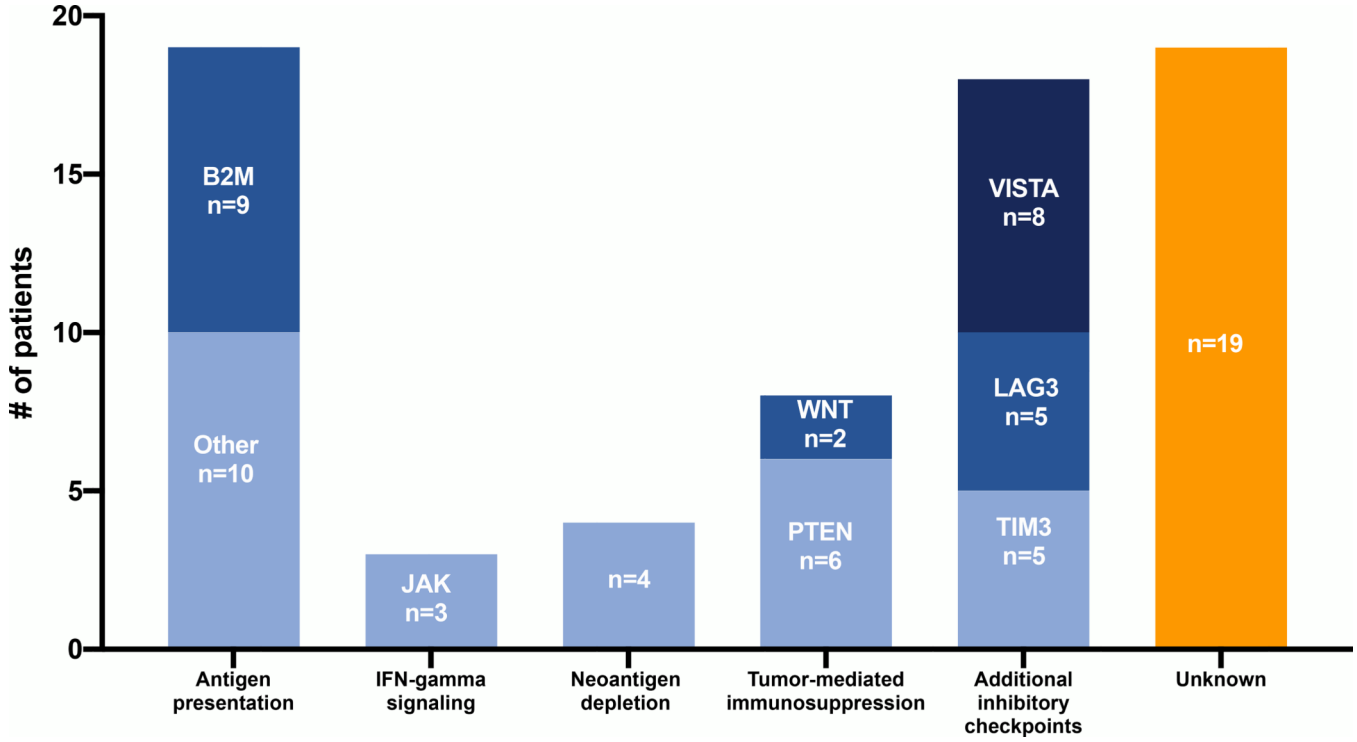

Figure 2. Summary of reported mechanisms of acquired resistance to immune checkpoint inhibitors.

The number of clinical cases by reported mechanism of acquired resistance mechanism are shown. Some cases report multiple acquired resistance mechanisms.

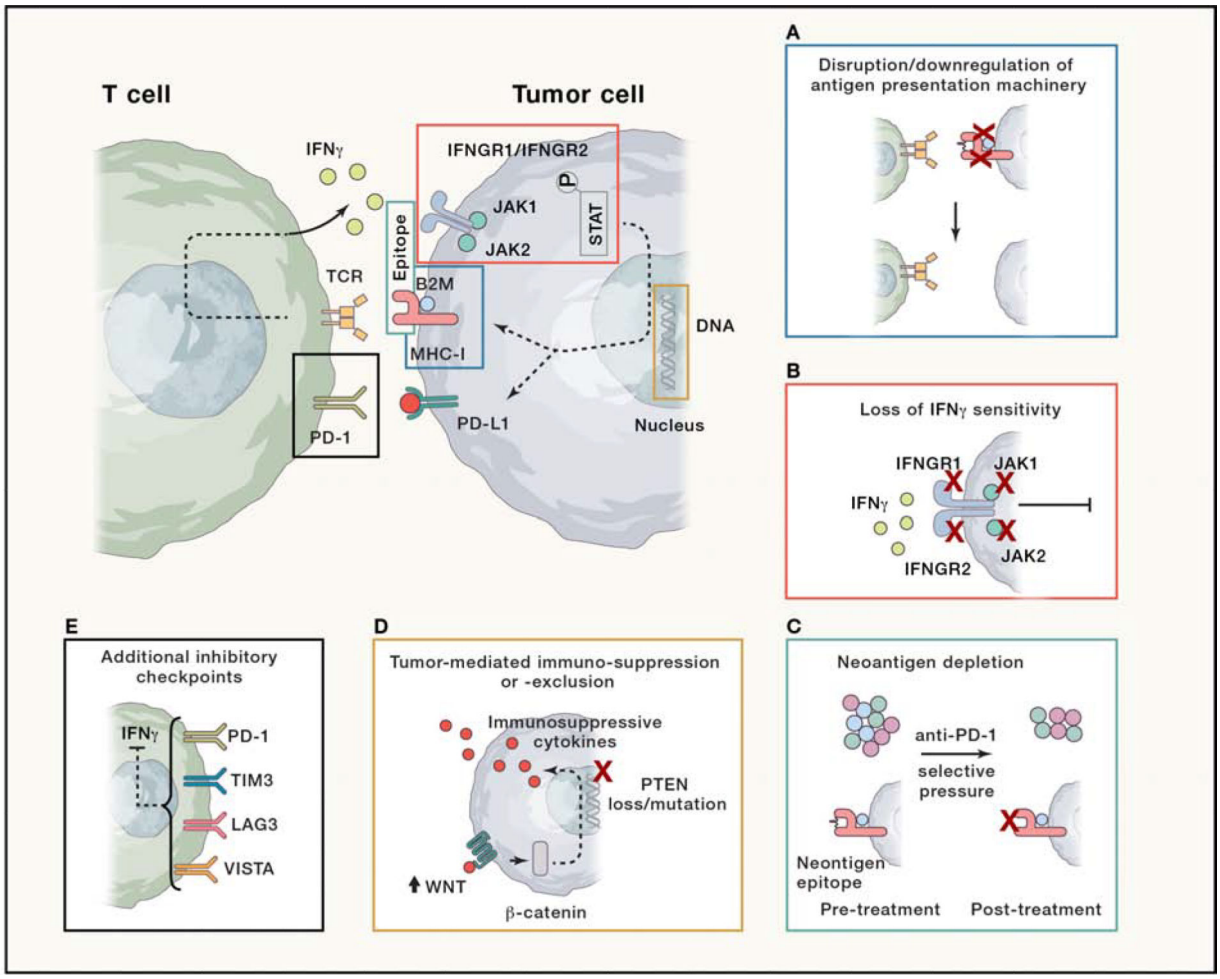

Figure 3. Schema of interaction between T cell and tumor cells, highlighting loci for proposed mechanisms of acquired resistance to immune checkpoint inhibitors.

Central cartoon depicts engagement between T cell (green) and tumor cell (blue). Transmembrane-bound T-cell receptor (TCR) and PD-1 receptor are depicted along T cell membrane, along with elaboration of IFN-γ cytokine granules into the extracellular space. Along the tumor cell membrane are the transmembrane-bound peptide-MHC class I receptor, stabilized by B2M, PD-L1 ligand, and IFNGR1/IFNGR2 heterodimer, bound by JAK1 and JAK2. Downstream of JAK1/JAK2 activates phospho-STAT, which engages with tumor cell DNA to regulate, among other targets, MHC-I and PD-L1 expression. Surrounding inset figures depict individual postulated mechanisms of acquired resistance that modulate or interfere with the normal engagement between a T cell and tumor cell. (A) Disruption/downregulation of antigen presentation machinery: Mutations or loss of MHC-I or *B2M* lead to loss of tumor antigen presentation and lack of TCR engagement. (B) Loss of IFN-γ sensitivity: mutations or loss of *IFNGR1/IFNGR2* or *JAK1/2* lead to insensitivity to IFN-γ in tumor microenvironment and resistance to anti-PD-1 mediated T cell response. (C) Neoantigen depletion: A clonally heterogenous tumor (blue, green, and purple circles) is affected by selective pressure from effective response to anti-PD-1 blockade, leading to loss of clones containing effective neoantigens (with blue circles lost post-treatment). Cartoon below depicts peptide-MHC-B2M complex with neoantigen epitope in pre-treatment setting,

which is then lost post-treatment. (D) Tumor-mediated immunosuppression/exclusion: upregulated WNT signaling leads to stabilization of β-catenin or *PTEN* mutations/loss can ultimately yield immune-suppressive and -exclusionary cytokines that reduce infiltration and function of CD8+ T cells in the tumor microenvironment. (E) Additional inhibitory checkpoints: upregulation of additional immune checkpoints such as TIM3, LAG3, and VISTA can be found at the time of acquired resistance, and may reflect terminal exhaustion and fixed loss of effector function.

Table 1.

Rate of acquired resistance and overall response by tumor type and study

Disease Type	Study Name	Drug(s)	Line of therapy	ORR	Estimated acquired resistance rate	Endpoint used to estimate acquired resistance	Time landmark (months)
dMMR	Keynote 158	Pembrolizumab	2+	34 (80/233)	22	DOR KM curve	24
	Le et al.	Pembrolizumab	2+	53(46/86)	11	Rate	Last follow-up (med 13)
	Checkmate 142	Nivolumab	2+	31(23/74)	14	DOR KM curve	12
Esophageal carcinoma	Keynote 180	Pembrolizumab	3+	10(12/121)	42	Rate	Last follow-up (med 6)
Esophageal, gastric, and EGJ carcinoma	Checkmate 032	Nivolumab 1 + ipilimumab 3	2+	24 (12/49)	59	Rate	Last follow-up (med 24)
	Checkmate 032	Nivolumab 3 + ipilimumab 1	2+	8 (4/52)	50	Rate	Last follow-up (med 22)
	Checkmate 032	Nivolumab	2+	12 (7/59)	71	Rate	Last follow-up (med 28)
Head and Neck SCC	Keynote 040	Pembrolizumab	2+	11 (26/247)	35	Rate	Last follow-up (med 8)
	Keynote 048	Pembrolizumab	1	17 (51/301)	54	DOR KM Curve	24
	HAWK	Durvalumab	2+	16(18/111)	44	Rate	Last follow-up (med 6)
Hodgkin Lymphoma	Keynote 087	Pembrolizumab	2+*	72 (151/210)	57	DOR KM curve	24
	Checkmate 205	Nivolumab	2+*	65 (41/63)	28	DOR KM curve	12
	Checkmate 205	Nivolumab	3+*	68 (54/80)	42	DOR KM curve	12
	Checkmate 205	Nivolumab	3+*	73 (73/100)	44	DOR KM curve	12
	Younes et al.	Nivolumab	3+*	73 (58/80)	19	Rate	Last follow-up (med 9)
Melanoma	Pires da Silva et al	PD-(L)1 inhibitor + ipilimumab	1	66 (93/140)	12	Rate	Last follow-up (med 16)
	Checkmate 067	Nivolumab + ipilimumab	1	58 (183/314)	38	Rate	Last follow-up (med 54.6)
	Keynote 001	Pembrolizumab	1	52 (78/151)	18	Rate	Last follow-up (med 55)
	Checkmate 067	Nivolumab	1	45 (141/316)	39	Rate	Last follow-up (med 36.0)
	Keynote 001	Pembrolizumab	1+	41 (267/655)	27	Rate	Last follow-up (med 55)
	Wang et al.	PD-(L)1 inhibitor	1+	34(166/488)	22	Rate	Last follow-up (med 33)
	Checkmate 067	Ipilimumab	1	19 (60/315)	60	Rate	Last follow-up (med 18.6)

Disease Type	Study Name	Drug(s)	Line of therapy	ORR	Estimated acquired resistance rate	Endpoint used to estimate acquired resistance	Time landmark (months)
	Keynote 006	Pembrolizumab	1+	42 (235/556)	24	DOR KM curve	18
	Keynote 006	Ipilimumab	1+	17 (46/278)	32	DOR KM curve	18
NSCLC	Keynote 001	Pembrolizumab	1	42 (42/101)	57	Rate	Last follow-up (med 61)
	Keynote 042	pembrolizumab	1	27(174/637)	52	DOR KM curve	24
	Keynote 001	Pembrolizumab	2+	23(103/449)	41	Rate	Last follow-up (med 61)
	CheckMate 017, 057, 063, and 003	Nivolumab	2+	18(122/664)	64	DOR KM curve	48
	Keynote 010	Pembrolizumab2	2+	18 (62/344)	32	DOR KM curve	12
	OAK	Atezolizumab	2+	14 (58/425)	55	Rate	Last follow-up (med 28)
RCC	Checkmate 214	Nivolumab + Ipilimumab	1	42 (177/425)	41	Rate	Last follow-up (med 32)
	Checkmate 025	Nivolumab	2+	25 (103/410)	52	Rate	Last follow-up (min 14)
	Keynote-427	Pembrolizumab	1	36 (40/110)	23	DOR KM curve	6
Urothelial Carcinoma	Keynote-052	Pembrolizumab	1	29 (107/370)	48	DOR KM curve	24
	IMvigor210	Atezolizumab	1**	24(28/119)	32	Rate	Last follow-up (med 29)
	Keynote-045	Pembrolizumab	2+	21 (57/270)	32	DOR KM curve	12
	IMvigor211	Atezolizumab	2+	13 (62/467)	37	Rate	Last follow-up (med 17)

Abbreviations: ORR = objective response rate. dMMR = deficient mismatch repair tumors. EGJ = esophagogastric junction. SCC = squamous cell carcinoma. NSCLC = non-small cell lung cancer. RCC = renal cell carcinoma Med = median. Min = minimum. DOR = duration of response. KM = kaplan-meier. DOR KM curve refers to estimates inferred from DOR KM curve. Rate refers ratio of responders who progressed divided by all responders (CR/PR) detailed in report.

* Prior therapies are autologous stem cell transplantation and/or brentuximab vedotin.

** Acquired resistance data only available for first-line cohort.

Table 2.

Barriers to insight in acquired resistance

1. Terminology: • No consensus definition of acquired resistance • Need to differentiate definitions and applications of patient-level and lesion-level acquired resistance
2. Tissue: • Infrequency of response and idiosyncratic acquired resistance stymies effective/efficient biospecimen collection • Need to articulate and demonstrate clinical utility to tissue sampling at acquired resistance
3. Tools: • Analyses using bulk DNA/RNA next-generation sequencing have been helpful in molecularly targeted therapies, but more limited success for immune checkpoint inhibitors • New tools are needed to interrogate the complexity of anti-tumor immunity