Clinical Trials with Biologic Primary Endpoints in Immuno-oncology: Concepts and Usage

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ABSTRACT

Clinical trials that have a pharmacokinetic or a pharmacodynamic immunologic mechanism of action–based primary outcome could substantially improve the validity and efficiency of early development of immuno-oncology agents. Here, we outline different trial design options in this area, review examples from the literature and their unique immunologic aspects, and highlight how these trials have been underutilized. We illustrate how new technologies and translationally focused approaches can be successfully used to develop different classes of immunotherapeutic agents.

Introduction

Immunotherapy with immune checkpoint inhibitors (ICI) has dramatically altered the treatment landscape for many cancer types such as melanoma and non–small cell lung cancer (NSCLC; refs. 1, 2). However, ICI achieve long-term disease control in only a minority of patients, even in highly responsive tumor subtypes. While many new classes of immunotherapies are in development, the FDA has approved only a few for solid tumors outside of the main ICI with programmed death-1 (PD-1), PD-ligand 1 (PD-L1), and CTL antigen 4 (CTLA-4) antibodies. Consequently, efficient methods to evaluate the large number of immunotherapies and their combinations are needed.

Clinical trials with primary biological rather than clinical outcomes are at the forefront of this effort. Such trials of therapies emerging from the laboratory are typically small (3) but crucial for generating evidence regarding the effects of treatment on specific targets that inform subsequent studies. The statistical design properties of these trials are not highly evolved because they do not fit the conventional clinical trials developmental paradigm. Nonetheless, tissue-based trials with biologic primary endpoints are becoming more widely used, providing critical biologic insights and addressing challenges associated with the development of immunotherapy, even with small sample sizes. We will characterize this loosely defined class as "trials with biologic primary endpoints" and note that they can be found at any stage of therapeutic development.

Types of Trials with a Biologic Primary Endpoint

Sharp definitions are essential in translational immunotherapy to help characterize biological effects and mechanisms of immune response and resistance. In phase II and phase III trials, biomarkers are used often for prediction and classification but infrequently as outcome signals. Early in development, the most helpful outcome may be a specific biological assessment known from animal models. A common goal for such studies is to seek an "irrefutable signal" that summarizes what is known about the vital disease pathway and site of action of the therapy, illustrating how lab models and human trials intersect in the translational space. This yields essential insights into the performance of the immunotherapy beyond preclinical models that might not be an ideal representation of the complex human immune system. In clinical trials, drug activity can be measured through pharmacodynamics, the study of the biochemical and physiologic effects of drugs and their mechanisms of action. Pharmacodynamic biomarkers can be developed to evaluate certain pharmacologic responses that are directly linked to engagement of the primary molecular target by a specific drug. In this review, we discuss how pharmacodynamic endpoints can be used to evaluate the biologic activity of a candidate therapy. For immunotherapy, biologic activity is centered around measuring changes in immune cell population number or function and can also be defined as an "immunologic" endpoint. Several trial designs, including phase 0 and window of opportunity trials, have these characteristics (**Table 1**)—incorporating biological samples taken during treatment to measure changes in a prespecified biologic biomarker.

In 2006, the FDA issued guidance supporting the use of an "exploratory" trial design, commonly referred to as a "phase 0″ clinical trial (4). The guideline recognized that substantial resources are required for preclinical evaluation of candidate drugs, and animal models or *ex vivo* studies may not translate into clinical activity in humans (5). A phase 0 trial is designed to evaluate a new therapy in a small number of patients with limited exposure (often only one to two doses). The drug is typically given at a subtherapeutic dose to differentiate promising drug candidates early in the development process, without exposing patients to excess risk of toxicity. This reduces the number of patients exposed and resources devoted to ineffective therapies. For example, the PARP inhibitor veliparib was initially studied in a phase 0 trial of 13 patients, measuring intratumoral PAR levels, a product of PARP activity (6). The drug demonstrated activity at doses of 25 and 50 mg, with the intended target effect and reduced PAR levels within the tumor microenvironment (TME) on biopsies. Many of the trials we discuss in this review share features with a phase 0 trial, namely a focus on pharmacodynamic or pharmacokinetic outcome and exposure to a limited number of patients. However, immunotherapies are typically not given at a subtherapeutic dose or for a limited duration (outside of neoadjuvant therapy) which differs from the classical phase 0 trial design.

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Table 1. Examples of clinical trial designs with a biologic endpoint.

Design	Goal	Features	Potential disadvantages
Translational	Introduce novel agents for cancer therapy in humans	– Build evidence for efficacy trials – Uses reproducible endpoints in a defined patient population	– Undefined time to effect – Clear definition of PD is required – Best evaluated in a non-rapidly progressing population
Phase 0	Focus on PK and PD endpoints to determine whether the investigational agent achieves therapeutic levels and hits its intended target supporting the proposed mechanism of action.	Appropriate for cytotoxic and potentially targeted therapies that may have predictable and dose dependent PK and PD effects	– Small sample size – Classically, uses a subtherapeutic dose
Surgical window of opportunity	Evaluate whether drug reaches tumor and/or whether the immune therapeutic induced the desired response in the tumor microenvironment Some overlap with phase 0 and neoadjuvant designs	– Design and endpoints provide insight into the requirements for local drug effect – Allows study of mechanisms of action	Potential challenges with biospecimen acquisition Dosing and frequency strategies may be tailored to surgical intervention, compared with typical schedules
Neoadjuvant	Employs immunotherapy strategies on treatment-naïve tumor with sample being available for correlative analysis post-immunotherapy	– Evaluates antitumor response in a tumor landscape not altered by adjuvant radiotherapy and chemotherapy	– Difficult to achieve large sample size – Pretreatment biopsy is pivotal to guide choice of therapies – Potentially greater toxicity in a patient population awaiting surgery
Early developmental trials with biopsies	Translational but in phase I and II trials that mandate biopsies before and after therapy to assess the impact of treatment.	Can be incorporated to most trials with clinical primary endpoints	Not strictly biomarker driven On-treatment biopsy may not be feasible in some patients

Abbreviations: PK, pharmacokinetics; PD, pharmacodynamics.

A surgical window of opportunity trial typically accrues a small number of treatment-naïve patients given an investigational therapy for a limited period prior to standard-of-care surgical resection. The tumor specimen is then examined for the presence of therapeutic effects. Phase 0 and window of opportunity studies are distinct entities. While they both assess biologic target modulation, phase 0 is often a pre-phase I step in drug development. In contrast, window of opportunity trials typically test established agents (such as from the metastatic setting) to assess activity in earlier disease. Nevertheless, window of opportunity trials can contribute to the mechanistic understanding of drug activity by comparing pre-treatment (diagnostic biopsy) and on-treatment tumor specimens (surgical resection).

Unique Aspects of Early-Phase Immunotherapy Trial Designs

Immunotherapeutic agents require unique early developmental designs due to less predictable toxicity profiles. Unlike traditional chemotherapy or targeted therapies, toxicity may not increase uniformly with dose and dose-limiting toxicities (DLT) may not be seen in dose-escalation trials (7). In addition, the onset of immune-related adverse events (irAE) can occur outside of the initial treatment cycles or 4 to 6 weeks DLT window. In an analysis of 576 patients treated with nivolumab, the median onset of irAE ranged from 5 weeks for cutaneous to 15.1 weeks for renal toxicity (8). A recent multicenter retrospective review ($n = 999$) reported 5.3% irAEs with onset >1 year after commencing anti-PD-1 (9). These late irAEs were more likely to be high grade as compared with earlier onset irAEs. Therefore, standard dose escalation might not be an appropriate design for phase I immunotherapy studies.

Moreover, preclinical modeling of immunotherapies has limitations due to complexities in the human immune system and substantial differences between mouse and human tumor–immune interactions (5). Many preclinical studies use transplanted syngeneic tumor model systems due to reproducibility and ease of use. However, these models lack immunosuppressive microenvironments and are genetically homogenous compared with human tumors, limiting ability for clonal evolution and immune evasion. Thus, these models likely generate overly favorable results with a novel immunotherapeutic (10).

Authochthonous genetically engineered tumor model systems do represent a more natural growth cycle and develop in a native organ microenvironment but substantial differences between mouse andhuman immune systems remain (11). Xenograft models which are commonly used for drug development outside of immune oncology require NSG mice lacking functional immune systems. Transfer of autologous human stem cell or peripheral blood mononuclear cells (PBMC) into the xenograft model can be utilized but is limited by feasibility and the development of GVHD in the mouse (5). Thus, while murine systems remain a valuable tool for preclinical modeling, to date the model systems have been less reliable for evaluating immunotherapies.

Because of the limitations of preclinical modeling in immunotherapy, even if a therapy is tolerated from a safety perspective in phase I studies, it may not yield sufficient biologic activity to justify its advancement developmentally (7). However, relying on clinical response rates alone may not capture biologic activity of immune-oncology monotherapies. If clinical response is not seen but a significant biologic effect is measured, combination therapies may be most appropriate as opposed to terminating development. Early-phase trial designs though, typically do not explore the combination dose sufficiently to determine optimal dose or schedule. Reducing doses of established standard of care agents with proven efficacy, when evaluated

in combination is generally not accepted. This is problematic when true synergy might occur at a lower dose (and correspondingly higher for some other component). Novel early-phase trial designs such as determining a "maximal biologic dose" evaluate changes in pharmacodynamic endpoints in addition to safety with dose escalation. Alternatively, given the general favorable safety profile of ICI and other immune therapies, a multiple dosing response seeking design has been proposed to initially enroll patients at several dose levels to detect clinical or biological activity, while maintaining a safety stopping boundary.

Furthermore, if late toxicities are expected and play a critical role in defining the MTD, alternative designs, such as time-to-event continual reassessment method (TITE-CRM), could be considered, in which the occurrence of a DLT event is managed as a time to event endpoint. In a comparison with the 3+3 design or a standard CRM design, the implementation time of a TITE-CRM design could be shorter when toxicity observation times are long, treat more patients at or above the MTD, identify the MTD more accurately (12).

Examples from the Literature of Trials with Biologic Endpoints in Different Immunotherapy Classes

To evaluate how the oncology field has addressed these challenges, we conducted a literature search identifying examples of immunotherapy trials designed with biologic primary endpoints (**Fig. 1**). The identified trial examples represent broad classes of immunotherapies including tumor vaccines, antibodies or small molecules, cell therapy, recombinant cytokines, oncolytic viruses and Toll-like receptor agonists (**Table 2**).

Vaccines

Vaccine trials are currently the most represented class of agents utilizing primary pharmacodynamic endpoints in clinical trials. Because the target antigen is known, methods of identifying an immunologic response in the peripheral blood have been well established. Assays to assess immune response include the enzyme-linked immunosorbent spot (ELISpot) assay, flow cytometry to detect an antigen-TCR (T-cell receptor) multimer, delayed hypersensitivity (DTH) skin tests, or ELISA detection of antibodies. These are performed both pre- and post-therapy to determine the effect of the vaccine compared with baseline activity.

Cancer vaccine trials have previously used biologic endpoints to evaluate the efficacy of vaccine adjuvants. Boudewijns and colleagues evaluated a dendritic cell (DC) vaccine with or without cisplatin in patients with melanoma-expressing gp100 (NCT02285413; ref. 13). The preclinical rationale suggested that cisplatin may serve favorable immunomodulatory function including depleting myeloid-derived suppressive cells (MDSC) and regulatory T cells (Treg) and therefore augment the vaccine-induced immune response. However, the clinical

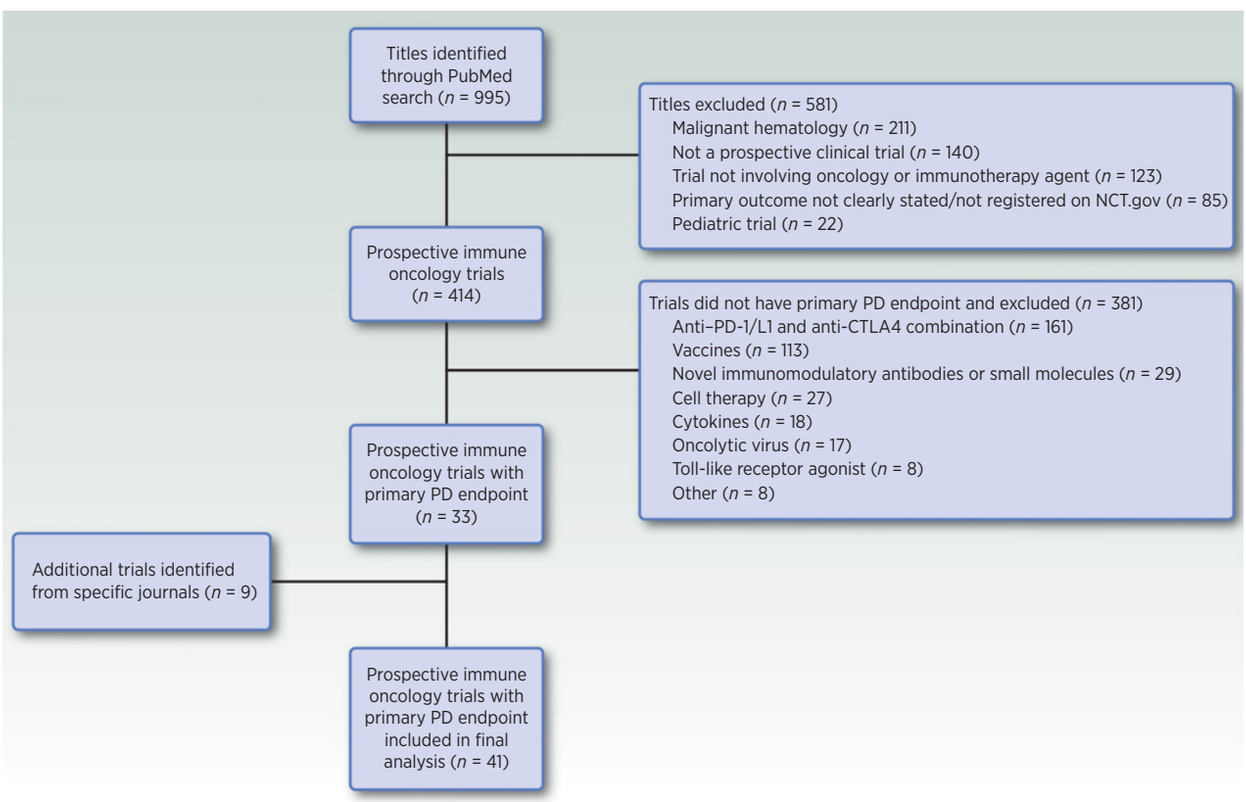

Figure 1.
Diagram of the literature search strategy to identify the use of primary biologic endpoints in phase I and II clinical trials involving immune therapies for solid tumors. PD, pharmacodynamics.

Table 2. Characteristics of selected immune oncology trials with a pharmacodynamic primary endpoint.

Study ID	Patient population	Study agent	PD or nonclinical outcome
Cancer vaccines			
Bhardwaj et al., *Nature Cancer* 2020 (14) (NCT02129075)	Fully resected stage IIb through IV melanoma	Fusion antibody vaccine targeting CD205, linked to NY-ESO-1. Given with TLR3 agonist in combination with or without FLT3 ligand	To determine whether immune response to NY-ESO-1 elicited by vaccination (measured by IFNγ ELISpot assay) was significantly increased by administration of CDX-301 (FLT3 ligand)
Boudewijns et al., *Cancer Immunology, Immunotherapy* 2020 (13) (NCT02285413)	Stage III or IV melanoma expressing gp100	Autologous DC vaccination (gp100 and tyrosinase) with or without cisplatin	Immunologic response rate as measured by DTH skin test to intradermally injected DC
Immunomodulatory antibodies and small molecules			
Dominguez et al., *Clinical Cancer Research* 2017 (19) (NCT02076451)	Advanced solid tumors or lymphoma refractory to standard treatment	TRAIL-R2 agonistic antibody	Measuring the presence of various populations of MDSC in PBMC before and after treatment
Zappasodi et al., *Nature Medicine* 2019 (20) (NCT01239134)	Solid tumors that had relapsed or progressed following standard therapy	TRX518, an agonist anti-GITR antibody	Define a maximum single dose at which there are tolerable side effects and/or maximum PK/PD parameter changes and effect of TRX518 on lymphoid cell subsets
Checkpoint blockade			
Ferrarotto et al., *Clinical Cancer Research* 2020 (16) (NCT03144778)	Stage II–IVA or locoregionally recurrent oropharyngeal cancer amenable to resection	Neoadjuvant durvalumab or durvalumab plus tremelimumab	To assess the differences between $CD8^+$ TIL evaluated by IHC staining in the post-treatment surgical specimens as compared with baseline
Schalper et al., *Nature Medicine* 2019 (18) (NCT02550249)	Newly diagnosed or recurrent glioblastoma undergoing surgical resection	Neoadjuvant nivolumab	Changes in percentage and level of expression of PD-L1 by tumor cells and lymphocytes, assessed at baseline and following neoadjuvant nivolumab
Cell therapy			
Stadtmauer et al., *Science* 2020 (27) (NCT03399448)	Subjects with a confirmed diagnosis of relapsed refractory multiple myeloma, melanoma, synovial sarcoma, or myxoid/round cell liposarcoma (MRCL)	Autologous NY-ESO-1 TCR therapy with TRAC, TRBC, and PDCD1 CRISPR knockout	Evaluate percentage of manufacturing products that do not meet release criteria for vector transduction efficiency, gene disruption T-cell product purity, viability, sterility or due to tumor contamination
Weathers et al., *Clinical Cancer Research* 2020 (28) (NCT02661282)	Recurrent glioblastoma, CMV seropositive	Autologous *ex vivo* expanded CMV-specific T cells	Immunologic effects in tumor tissue measured by levels of IFN, IL2, TNFα, perforin, and granzyme B

Abbreviations: DC, dendritic cell; IFN, interferon; MDSC, myeloid derived suppressor cells; PBMC, peripheral blood mononuclear cells; PD, pharmacodynamic; PD-L1, programmed death-ligand 1; PK, pharmacokinetic; TIL, tumor infiltrating lymphocytes; TLR, Toll-like receptor.

trial did not find that cisplatin improved immunologic responses as measured by induction of gp100 multimer specific T cells following a stimulatory skin test (13). In contrast, Bhardwaj and colleagues examined a FLT3 ligand to promote differentiation and expansion of DCs prior to administration of an NY-ESO-1–based vaccine and a TLR3 agonist in patients with resected melanoma (NCT0212907; ref. 14). They demonstrated an increase in NY-ESO reactive T cells (via ELISpot), and an increase in the number of peripheral blood DCs, B cells, natural killer (NK) cells, CD8, and CD4 T cells with the FLT3 ligand as compared with control (14). Thus, FLT3 ligand is considered a promising therapy to augment vaccine response.

Novel approaches such as neoantigen vaccines have also evaluated antigen-specific T-cell responses as a focus of early-phase trials. Ott and colleagues studied a personalized vaccine comprising 20 peptides based on neoantigens predicted to bind to HLA molecules in patients with resected melanoma (NCT01970358; ref. 15). The primary outcome was feasibility demonstrating sufficient neoantigen targets to manufacture a vaccine for 8 of 10 patients. They also demonstrated immunogenicity by showing ELISpot T-cell activity to 58 (60%) and 15 (16%) of CD4 and CD8 neoantigen-specific peptide targets, respectively. The immune responses were further characterized by flow cytometry using antigen-specific tetramer staining. On the basis of this biologic activity, the investigators are now studying neoantigen vaccines in combination with ICI (NCT03929029).

Immunomodulatory Antibodies

ICIs with anti–PD-1 or anti-CTLA4

Given the efficacy and generally favorable safety profile established by ICI in the metastatic setting, clinical trials are now evaluating ICI in the neoadjuvant setting, representing a "window of opportunity" design. Here pre-treatment biopsy samples can be compared with standard-of-care surgical resection specimens to evaluate many parameters including changes in the presence or quantity of immune cell

population and gene expression changes suggestive of immunologic activity.

Ferrarotto and colleagues randomized patients with surgically resectable oropharyngeal cancer to durvalumab or durvalumab plus tremelimumab (NCT03144778). This trial was powered to investigate whether combination therapy would increase the ratio of posttreatment to pretreatment CD8 tumor-infiltrating lymphocytes (TIL) density. They found that the combination arm did not increase CD8 TIL density or pathologic response compared with durvalumab monotherapy (16). This study demonstrates how an adequately powered biologic endpoint can provide an early signal of an ineffective therapy in a particular disease setting. These results are also consistent with previous studies that the tremelimumab did not provide clinical benefit when added to durvalumab in recurrent/metastatic squamous cell carcinoma of the head and neck (HNSCC; ref. 17).

In another trial, Schalper and colleagues enrolled 30 patients undergoing resection for glioblastoma (GBM; NCT02550249) and demonstrated that neoadjuvant nivolumab led to enhanced expression of chemokine transcripts, higher immune cell infiltration and augmented TCR clonal diversity in post-treatment resected tumor tissue compared with pre-treatment tumor tissue (18). Thus, while ICI monotherapy may have limited clinical activity in GBM, these results confirm that there is immunologic activity beyond the blood–brain barrier. Consequently, ICI may be considered in combination regimens to augment immune responses in future early-phase trials in GBM.

Additional immunomodulatory antibodies and small molecules

Novel immunotherapy agents target additional immunosuppressive cell populations or proteins. Immune correlative analysis may focus on measuring changes in cell population of interest including Tregs and MDSCs.

Dominguez and colleagues evaluated a TRAIL-R2 agonistic antibody (NCT02076451), demonstrating that the TRAIL-R2 antibody lowered circulating MDSC levels and intratumoral MDSCs in 50% of patients who had on-treatment biopsies. However, peripheral MDSC levels rebounded to pre-treatment levels by day 42 despite repeated dosing (19). Thus, the TRAIL-R2 antibody could be evaluated further as an initial component of a combination regimen; however, the limited duration of activity would question its use as a maintenance therapy.

Zappasodi and colleagues evaluated an agonistic anti-GITR antibody (TRX518) with a dose-escalation design to assess safety (NCT01239134). However, the primary endpoint for dose escalation included measurement at the dose for which there was a maximal change in peripheral immune subsets. The trial found the agonistic antibody reduced Tregs but did not increase CD8 effector T cells or yield clinical responses (20). Given the Treg depleting effect, the authors highlighted that TRX518 might provide synergistic therapy with anti-PD-1 despite its limited activity as a monotherapy. This combination is now being studied in an ongoing trial (NCT02628574). Other examples include trials that show that clinically available small molecules tadalafil (NCT00894413), or ATRA (NCT02403778) produced changes in peripheral MDSC quantity and function (21, 22).

Several examples in the literature highlight unanticipated target effects of immunotherapeutic agents, limiting their efficacy in humans. An antibody targeting killer immunoglobulin-like receptors (KIR) on NK cells was studied in multiple trials within smoldering myeloma and head and neck cancer. In murine models, the KIRD2 antibody IPH2101 was shown to augment NK-mediated killing of HLA-C–expressing tumor cells by blocking KIR-mediated inhibitory pathways in NK cells (23). However, after poor response rates in early-phase trials, pharmacodynamic studies later showed that in humans, the antibody actually led to $KIRD2^{+}$ NK-cell contraction and hyporesponsiveness through a previously unrecognized Fc-mediated interaction with monocytes and neutrophils (24). This further highlights the differences in preclinical models and the human immune system and confirms the need to understand mechanism of action before exposing large numbers of patients.

The examples in this section illustrate how many immunotherapy agents do not act directly on CD8 T cells, but function through an intermediate step. In this case, pharmacodynamic outcomes may require determining both whether the target of interest is achieved, and whether this target has biologically significant immunologic activity. An example is the clinical development of the indoleamine 2,3-dioxygenase (IDO) inhibitor epacadostat. IDO is an enzyme active in the TME that leads to the catabolism of tryptophan, generating an immunosuppressive effect by depleting T cells of an essential amino acid. A phase I trial of epacodostat did include a robust correlative pharmacodynamic outcome by measuring plasma levels of kynurenin, a downstream metabolite of tryptophan catabolism. The trial demonstrated a dose-dependent reduction in plasma kynurenine levels, with near maximal changes at doses >100 mg twice daily, suggesting that epacodostat efficiently inhibits IDO (25). However, this trial did not find significant changes in plasma proteins related to immune function and there was not an analysis of the direct impact of IDO inhibition on CD8 T-cell prevalence or function in peripheral blood or the TME. Despite significant optimism for epacadostat, the pivotal phase III did not show a benefit of adding epocadostat to pembrolizuab in metastatic melanoma (26). It may be speculated that an emphasis on analyzing the end effect on CD8 T-cell function could have led to an earlier detection that epocadostat does not lead to broad clinical activity, at least in unselected patient populations.

Cell-Based Therapy

Cell therapies with TILs, CAR-T, or TCR therapies are being investigated as personalized therapies with potential for durable treatment responses. Because of the technical challenges in producing these therapies and the heterogeneity in cell products, early-phase trials have focused on feasibility and analyzing the immunologic function of the cell therapy product itself. Given the expense of producing cellular therapies, small trials focused on characterizing biologic activity are desired prior to moving to larger efficacy trials.

Stadtmauer and colleagues studied an autologous NY-ESO-1 TCR transgenic T-cell product with CRISPR knockout of PD1 and the endogenous TCR (NCT03399448). As a co-primary outcome, this approach was feasible, with up to 30% of the NY-ESO-1–transduced T cells having at least two CRISPR gene edits. Surprisingly, in 1 patient, the NY-ESO-1 TCR-transduced T cells that had PD-1 knockout did not appear to generate persistent memory T cells. However, the NY-ESO-1 TCR-transduced T-cell product did appear to persist longer than historical controls of TCR adoptive cell therapies, suggesting that the endogenous TCR knockout may provide a fitness advantage that the PD-1 knockout did not (27).

A phase I trial in GBM evaluated autologous CMV-specific T cells that were expanded *ex vivo* from patient PBMCs in the presence of a CMV peptide (NCT02661282). In 1 patient with a post-treatment resection, CMV-specific T cells were located in the tumor vasculature with only a minor portion in the TME. The T cells failed to produce effector cytokines following stimulation and had upregulated PD-1. The authors concluded that prior temozolomide treatment of the

patients in the trial led to impaired immune activity in the starting PBMC population, limiting CMV T-cell culture expansion and quality. This contrasts with previous work that had shown feasibility and cytotoxic function of CMV-specific T cells expanded from healthy donors (28).

Challenges and Directions for Future Research

The studies reviewed above illustrate how insights from clinical trials with biologic primary endpoints can be carried forward to guide further drug development (**Fig. 2**). Biologic signals can inform dosing schedules, maximization of clinical activity, rational combinations, or alternatively to reach a "no-go" decision to terminate development. A major challenge in the utilization of pharmacodynamic endpoints for immune therapy is determining how to measure and defining what is a meaningful change in an immune cell population of interest. This has led to an underutilization of primary pharmacodynamic endpoints in early-phase immunotherapy trials. As immune-based correlative studies measuring biologic effect are increasingly incorporated into trials, a better understanding of relevant endpoints designed with statistical power are needed. A first step in initiating trials in this setting is to establish the definition of a pharmacodynamic "response" for the specific agent based on the mechanism of immunomodulation, and how this response will be measured. Multiple novel technologies (discussed below) are now available to measure these parameters. The second step is to define what constitutes a promising observed pharmacodynamic response rate for a specific dose level and what fraction of patients must demonstrate a pharmacodynamic response for the dose level to be declared biologically active. As trials reviewed above have shown, this can be incorporated into dose-escalation safety designs to achieve a maximally effective biologic dose.

To date, there has been a significant emphasis on characterizing pretreatment immune populations, with less evaluation of on-treatment change of immune parameters as predictors of clinical response. Indeed, in addition to PD-L1 and tumor mutation burden, the baseline presence of several immune cell populations in the TME [CD8 T cells (29), DCs (30), NK cells (30)] or T cell–inflamed gene signatures (31) have been correlated with response to ICI. Studies evaluating meaningful on-treatment changes in immune parameters (rather than only at the baseline timepoint) are needed to define pharmacodynamic endpoints measuring biologic activity. A few retrospective studies have evaluated this, demonstrating that patients with treatment response to anti-PD-1 have increased density of CD8 T cells and gene signatures suggestive of immune activation in on-treatment compared with pretreatment biopsies (32, 33). Characterization of a meaningful magnitude of change in additional immune cell subsets (DCs, NK cells, Tregs, MDSCs) also needs to be defined in future studies. Importantly, it is remains unclear if ultimately a CD8 T-cell response needs to be generated to achieve antitumor immune activity. However as studies

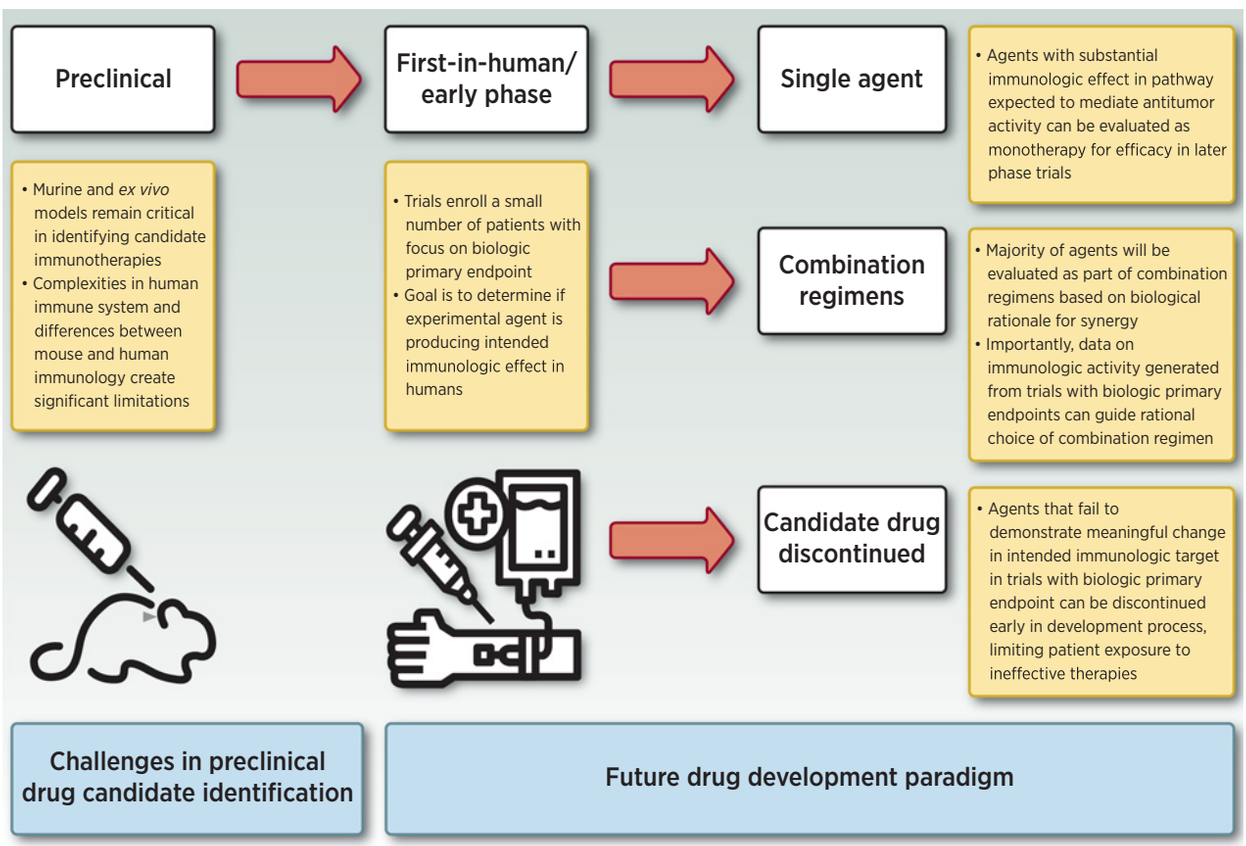

Figure 2.
Drug development pathway incorporating clinical trials with a biologic primary endpoint.

have demonstrated (20), it may be that therapies that only modulate one component (MDSCs, Tregs) without enhancing effector T-cell activity may need to be used as combination therapy rather than advancing as monotherapies.

Another significant challenge is patient selection. Ideally, baseline immune parameters matching the target of interest may select patients for specific clinical trials. A recent phase II trial evaluated the combination of the histone deacetylase inhibitor, entinostat with pembrolizumab in ICI refractory NSCLC (NCT02437136; ref. 34). Prior preclinical and clinical evidence suggested antitumor activity for entinostat was mediated through epigenetic effects in the myeloid compartment. While the trial did not meet the primary specified response rate endpoint, treatment benefit was enriched in patients with high baseline circulating classical monocytes ($CD14^+/CD16^-$/HLA-DR hi). A phase II/III trial has been designed to evaluate pembrolizumab and entinostat stratified by baseline classical monocyte count (high vs. low).

While immune biomarker patient selection holds significant promise, there remain challenges (**Fig. 3**). Expression of targets (biomarkers) can often be induced, may be inconsistent over time and space and be impacted by the investigational immunotherapy itself. An example would be a regimen of an immunotherapy influencing the lymphoid compartment (such as PD-L1), combined with immunotherapy influencing the immune-suppressed TME (such as MDSCs). The target in the lymphoid compartment may not be expressed in an "immune desert" tumor, but might be induced with expansion of tumor-reactive T cells if the immunosuppressive mechanism is reversed. Thus, an in depth understanding of the dynamic nature of immune cell subtypes after treatment with novel immune therapy agents is required to advance predictive biomarkers.

The Impact of New Technologies

The major challenge to incorporating biologic endpoints has been the difficulty of measuring dose-dependent immunologic effects, their magnitude and duration in a validated manner. Novel technologies offer opportunity to better assess the on-treatment changes in immune populations and may help prioritize candidate immunotherapies.

On-treatment biopsies

Interrogation of the TME at single-cell resolution provides a powerful platform for construction of immune phenotype outcomes, which is now being transitioned from the laboratory to clinical trials (35–38). New methods have been developed to characterize composition of cell populations for single-cell RNA sequencing (RNA-seq) platforms (39–42). Immunotherapy trials can benefit by characterizing cell populations based on transcriptomic profiles using unsupervised methods or based on *a priori* defined gene matrix signatures (43–46). Once cell populations are identified, immune phenotypes can be constructed on the basis of cell composition

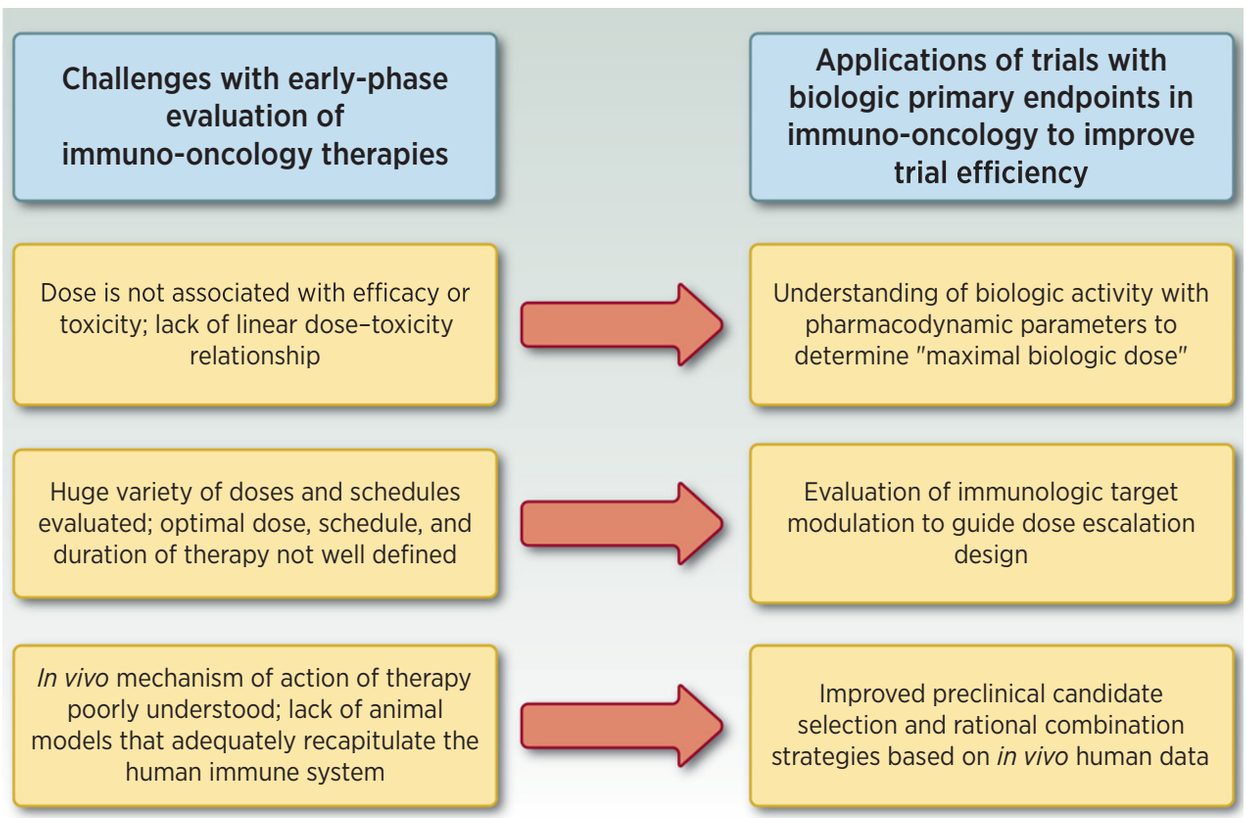

Figure 3.
Applications of clinical trials with a biologic primary endpoint to address challenges with early-phase trials.

proportions, or alternatively based on gene expression profiles within specific cell populations of interest. The ability to study T- and B-cell repertoires (through TCR or B-cell receptor sequencing algorithms) and cell-surface markers (through CITE-seq, a method of using DNA-barcoded antibodies to detect protein expression) at the single-cell resolution offers the potential to enhance the utility of this approach. A recent window of opportunity trial in HER2$^-$ breast cancer (NCT03197389) utilized single-cell RNA-seq, TCR sequencing (TCR-seq), and CITE-seq to evaluate immune cell populations before and after neoadjuvant pembrolizumab (47). This approach demonstrated on-treatment clonal expansion of T cells within specific T-cell subsets. Additional immune cell subsets including DCs and macrophages were quantified and correlated with T-cell expansion. Multiple retrospective studies have similarly used multiparameter flow cytometry or mass cytometry to characterize immune cell populations at the single-cell level (48–50). However, cytometry-based approaches (51) often require resected tumor as they may not feasible with limited cell numbers from needle biopsies, limiting the use for serial measurement in clinical trials.

While single-cell approaches require fresh dissociation of tumors, IHC offers analysis of spatial distribution and density of immune cells in the intact TME. Quantifying TILs by measuring the area of CD8 T-cell infiltration on an formalin-fixed, paraffin-embedded (FFPE) slide has been validated as a prognostic biomarker in breast cancer (51). IHC evaluation of TILs has also been previously used as a pharmacodynamic endpoint in neoadjuvant immunotherapy trials (16). Furthermore, multiplexing with chromogenic IHC or immunofluorescence allows for detection of multiple specific immune subtypes of interest. In the trial evaluating an agonist GITR antibody described above, immunofluorescence using markers for FOXP3 and CD4 demonstrated a decrease in Treg density following treatment (20). In addition, pathology slides are increasingly digitalized and analyzed by computational platforms to improve reproducibility. A recent trial evaluating neoadjuvant atezolizumab in NSCLC (NCT02927301) demonstrated that artificial intelligence quantification of standard hematoxylin and eosin stains could detect increased immune cell density in post-treatment biopsies (52). Finally, imaging technologies including multiplexed ion beam imaging by time of flight (53) and expansion microscopy (54) allow for detection of substantially more proteins on FFPE slides. Digital spatial profiling with DNA barcode tags can even allow transcriptional profiling in addition to protein quantification (55). However, the technical requirements and expense of these techniques have to date limited their application in clinical trials.

Peripheral blood

Longitudinal sampling of tumor tissue poses practical challenges, but may be more feasible with blood-based assays. In the peripheral blood, analysis of PBMCs with flow cytometry can measure changes in immune cell populations of interest such as MDSCs (19) or Tregs (20). Novel approaches to gene expression analysis and TCR-seq can also analyze changes in peripheral T cells. A recent analysis of 69 patients with melanoma who had serial blood samples taken during treatment with pembrolizumab evaluated peripheral T-cell gene expression and TCR clonality. At day 21, responding patients had overexpression of TCR-encoding genes and large TCR clones detected in comparison with nonresponding patients (56). This represents one potential method which is antigen agnostic (in comparison with ELISpot assays which require knowledge of the tumor-specific antigens). Immune monitoring can also be achieved with other methods, such as using whole blood with complex stimuli such as TLR ligands or microbes and measuring associated response (39, 57).

Imaging studies

Imaging studies offer noninvasive measurement of systemic immune response and may better characterize heterogeneity across multiple tumor sites. Clinically validated imaging modalities such as CT or FDG-PET are limited for immunotherapeutic agents, because they may not accurately represent immune-mediated tumor response. Increased immune cell infiltration may make lesions appear larger or intensify FDG signal, known as pseudoprogression. Novel approaches seek to use labeling of specific immune molecules to overcome this and more specifically measure T-cell activity.

The use of an immuno-PET approach has been studied in a phase I trial with IAB22M2C, an anti-CD8 minibody radiolabeled with ^{89}Zr (NCT03107663; ref. 58). The minibody is biologically inert as it does not interact with Fc receptors, does not deplete or impact CD8 T-cell proliferation and does not cause cytokine release. The trial established the safety of the immuno-PET with metastatic lesion uptake seen in 2 of 6 patients including one deltoid muscle lesion, found to have intratumoral CD8 T-cell infiltration when excised for clinical purposes. A phase II trial (NCT03802123) is underway evaluating the ^{89}Zr-Df-IAB22M2C PET tracer in patients with solid tumors receiving standard-of-care ICI. PET uptake will be compared with CD8 T-cell infiltration by IHC in on-treatment biopsies. Additional radiolabeled tracers have been developed to measure T-cell activation including metabolic targets such as AraG (NCT04186988), or effector molecules such as granzyme B (NCT04169321). Finally, although a predictive biomarker rather than demonstrating on-treatment activity, PD-1/L1 PET using radiotracers conjugated to nivolumab, pembrolizumab, durvalumab, or atezolizumab have been developed. One study demonstrated that PET activity was a better predictor of atezolizumab response than validated IHC-based PD-L1 assays (59, 60).

For cellular therapies, labeling T cells for reporter gene imaging offers potential to assess the biodistribution, honing to tumor site, and persistence of adoptively transferred cells. In one example, an IL13 CAR-T for GBM was also transfected with herpes simplex virus type 1 thymidine kinase (HSV1-TK). [^{18}F]FHBG, a radiolabeled probe (analog of peniciclovir) was phosphorylated by HSV1-TK and trapped within the CAR-T cell, allowing detection by PET imaging. *Ex-vivo* studies suggest that this construct does not adversely affect CAR-T cell function, and in a phase I trial (NCT00730613) demonstrated an increase in PET signal after CAR-T administration in GBM lesions (60).

By studying immune populations across repeat biopsies, serial plasma collection, or on-treatment immune-PET imaging, immunotherapy trials with biologic endpoints can be designed on the basis of longitudinal quantitative endpoints (**Fig. 3**). This, of course, assumes changes from baseline quantify something that is biologically, and clinically relevant and that the minimum effect size is quantifiable and feasible. Despite the enthusiasm for novel technologies, research in this area remains retrospective or based small prospective cohorts. As costs and technical barriers become less challenging in the future, these analyses may be more feasible and integrated into larger prospective trials. As new technologies are incorporated into trial design, we stress the importance of defining meaningful biologic endpoints based on available data, and incorporating these endpoints into the primary objectives of the trial design.

Conclusions

A better understanding of immunotherapy trials that measure specific biologic endpoints can help the development of more effective agents. Merely repeating conventional trial designs is insufficient for defining the role for the large number of immunotherapies in development. Trial designs with biologic primary endpoints can determine the changes in an immune parameter in parallel with dose escalation to determine the "maximal biologic dose," or alternatively that no immunologic effect occurs.

Future clinical trials of immunotherapeutic agents should incorporate aspects of translational trial design, including valid and transferable data generated on the basis of immune assays, with significant harmonization and standardization of techniques (61). In parallel, for each immune-oncology agent under development, a good understanding of the immunologic complexities and what is being targeted is essential.

Concurrent early-phase clinical trials and preclinical studies may help the selection of biological endpoints that can be compared in near real-time, thus helping to establish assay cut-offs in patients and integrate the bench to bedside and back to bench translational paradigm.

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