Immuno-oncology: Advances in basic research and translational medicine

With a focus on immune checkpoint inhibitors and T cell immunotherapy.

Developing methods for the prevention, diagnosis, and treatment of over 100 different types of cancer is the core objective in research laboratories around the world. In 2012, the International Agency for Research on Cancer reported 14.1 million new cancer cases and 8.2 million cancer deaths worldwide, and these numbers are expected to increase as a result of growing and aging global populations [1]. Although there have been improvements in surgery, radiation therapy, and chemotherapy treatments along with a decline in the rate of cancer deaths over the last few decades, metastatic disease is rarely completely controlled with conventional approaches. Moreover, debilitating side effects are frequently associated with radiation and chemotherapy.

Figure 1 (above). Chimeric antigen receptor (CAR) T cell invasion into cancer spheroids. See experimental details in Figure 7 caption.
According to the American Society of Clinical Oncology (ASCO), people living with cancer are benefiting from recent advances in cancer immunotherapy research—a field of study that began more than a century ago. The overarching goal of these novel treatment approaches is to enhance or enable anti-tumor immune responses, to overcome tumor evasion mechanisms, and to promote conditions that favor immune protection (Figure 1). Immunotherapy may offer distinct advantages over standard treatment modalities. For example, tumor-specific immune cells have the ability to migrate to areas of the body that are inaccessible by surgery. Cells of the immune system may also target microscopic disease and disseminated metastases. Furthermore, compared with radiation and chemotherapy, immunotherapy has been shown to act specifically against the tumor, thereby lowering the risk of damage to surrounding healthy tissue and minimizing side effects associated with standard cancer treatments. Nevertheless, severe toxicities may be associated with some particular immunotherapies [2].

**Demonstrated promise of immunotherapies**

Successes were realized in the past two decades with the development of novel cancer therapies known as immune checkpoint inhibitors (ICIs) [3]. ICIs are drugs (typically antibodies) that block either the immunosuppressive proteins on the surface of cancer cells or the T cell proteins that recognize them, thereby allowing T cells to mount an immune response. For example, with respect to certain solid tumors, administration of monoclonal antibodies (mAbs) that block T cell–expressed costimulatory receptors such as PD-1 and CTLA-4 augment the cytolytic activity of CD8+ T cells (killer T cells) within the tumor microenvironment (TME). Multiple studies have shown that these immune modulatory agents (Tables 1 and 2) increase overall survival in preclinical cancer models and are promising for treating human subjects with cancers of the skin, lung, bladder, or other organs. In-depth understanding of T cell biology has also supported advancement of cellular therapies, including T cell adoptive immunotherapies with engineered antigen receptors—approaches that epitomize the concept of personalized medicine.

**Immune modulation of the tumor microenvironment**

The TME is composed of a complex network of tumor cells and cells of the stroma, immune system, vasculature, and extracellular matrices (Figure 2). Although tumor-infiltrating immune cells are present in the TME, the immunosuppressive milieu must be overcome to achieve antigen recognition, T cell priming and activation, and the expansion of tumor antigen–specific cytotoxic T cells. In addition to various cytokines that drive or inhibit immune responses, Figure 2 lists several T cell co-receptors (or their ligands), including ICIs, that are rational drug targets for immunotherapy—some of which are targets of cancer therapy agents approved by

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**Figure 2. The multistep anti-tumor responses.** The cancer immunity cycle involves the coordination of a myriad of checkpoint molecules and other cell-surface receptors, as well as soluble factors such as cytokines and chemokines. The process is initiated by the release of tumor-derived antigens (step 1). The engulfment of these antigens by dendritic cells and their subsequent presentation to T cells (steps 2 and 3) drive the immune response. Once activated, effector T cells acquire the ability to destroy target cells by specifically recognizing tumor peptide–MHC complexes displayed on the tumor cell surface (steps 4–7). Increasing levels of tumor antigens are then released, further driving the progression of the cycle. ICIs have been shown to augment the T cell–mediated tumor rejection.
Figure 3. Immunoﬂuorescence analysis of Jurkat cells using anti-CTLA-4 antibody. PMA-treated (left) and untreated (right) Jurkat cells were fixed and permeabilized for detection of endogenous CTLA-4 using Invitrogen™ ABfinity™ anti-CTLA-4 recombinant rabbit monoclonal antibody (clone 11H7/L17, Cat. No. 702534) in conjunction with Invitrogen™ Superclonal™ goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor™ 488 conjugate (Cat. No. A27034). Cell-surface localization of CTLA-4 protein is represented by the green signal. Nuclei (blue) were counterstained with Invitrogen™ SlowFade™ Gold Antifade Mountant with DAPI (Cat. No. S36938); cytoskeletal F-actin (red) was labeled with rhodamine phalloidin (Cat. No. R415). Compared with the untreated cells in the right panel, cells treated with PMA (5 ng/mL; 48 hr; left panel) show an upregulation of CTLA-4 protein. The images were captured at 60x magnification on a Nikon™ Eclipse™ Ti-E Inverted Microscope.

Figure 4 illustrates how tumor-inﬁltrating lymphocytes (TIL) isolated from mice treated with or without anti–mouse CTLA-4 monoclonal antibody (mAb) may be evaluated ex vivo by multicolor ﬂow cytometry—a powerful technology for phenotyping heterogeneous cell populations [4]. In this study by Draper et al., splenocytes were isolated from mice after administration of a total of 5 doses of anti–CTLA-4 mAb at 10 mg per kilogram of body weight, delivered 3 times weekly. Shown is the gating strategy used to deﬁne B cell, CD4+ helper T cell, Treg, CD8+ T cell, and NK cell populations. Cell proliferation was monitored by detecting the carboxyﬂuorescein succinimidyl ester (CFSE)–labeled leukocyte populations of interest. Use of this representative antibody staining protocol, CSFE labeling method, and gating strategy provides an effective way to evaluate the effects of ICI treatment in mouse tumor models. To learn more about the Invitrogen™ Attune™ NxT Flow Cytometer, ﬂow cytometry reagents, and antibodies, go to thermofisher.com/ﬂowcytometry.

Immunobssays for soluble proteins. In addition to implementing flow cytometry methods, immunophenotyping may be accomplished using Immunoassays for soluble proteins. For more information, visit thermofisher.com/flowcytometry.

Table 1. Approved T cell co-receptor-associated targets of cancer immunotherapy.

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<tr>
<th>Agent</th>
<th>Target</th>
<th>Indication (initial FDA approval year)</th>
<th>Pivotal clinical trial reference</th>
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</table>

using sophisticated immunoassays that measure soluble proteins. The Invitrogen™ Immuno-oncology Checkpoint 14-Plex Human ProcartaPlex™ Panel 1 is a novel immunoassay that utilizes Luminex® xMAP® technology for the multiplex detection of the soluble immune checkpoint protein analytes displayed in Figure 5. An increasing number of studies suggest that these soluble proteins have the potential to function as decoy receptors or as immune adjuvants that may interfere with the efficacy of checkpoint modulator drug candidates. Additionally, evaluating soluble immune checkpoint protein biomarkers in sera or plasma of cancer patients may offer a minimally invasive method for correlating ICI treatment efficacy with analyte concentrations or for enabling identification of individuals that may or may not respond to a given ICI therapy.

This concept was first demonstrated in a study that reported a positive correlation between elevated serum levels of soluble CTLA-4 and clinical outcome benefit in patients treated with ipilimumab [5]. This ProcartaPlex panel is suitable for use with the Luminex 200™, FLEXMAP 3D®, and MAGPIX® systems, and data for multiple analytes may be obtained from small sample volumes (25 µL for serum or plasma samples, 50 µL for cell culture supernatant). Similar to conventional immunoassays, antigen quantification is accomplished using a fluorescently labeled secondary antibody, and signal intensity is proportional to the concentration of protein detected. Importantly, this multiplex assay produces results that overlap with those obtained with singleplex plate-based enzyme-linked immunosorbent assays (ELISA). Visit thermofisher.com/luminex to learn more about multiplex assays using Luminex technology and the ProcartaPlex.

Table 2. Representative examples of investigational ICI agents.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Target</th>
<th>Development stage</th>
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<tbody>
<tr>
<td>rHGM12B7</td>
<td>PD-L2</td>
<td>Phase I</td>
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<tr>
<td>Tremelimumab</td>
<td>CTLA-4</td>
<td>Phase I–III</td>
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<tr>
<td>IMP321, BMS-986016</td>
<td>LAG-3</td>
<td>Phase I–II; Phase I–II</td>
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<tr>
<td>TSR-022</td>
<td>TIM-3</td>
<td>Phase I</td>
</tr>
<tr>
<td>PBF-509</td>
<td>A2aR</td>
<td>Phase I</td>
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</table>

To learn more about ICI antibodies for flow cytometry, IHC, and functional bioassays, see the related article in BioProbes 75 Journal of Cell Biology Applications (May 2017) titled “Harness immune checkpoints to combat tumors” at thermofisher.com/bp75.

Figure 4. Profile of splenocytes (derived from a Balb/c syngenic CT26 colorectal tumor model) stimulated with anti–CD3 antibody. Tumor-naive and tumor-bearing mice were left untreated or administered anti–mouse CTLA-4 antibody. Subsequently, splenocytes were harvested, preloaded with carboxyfluorescein succinimidyl ester (CFSE), stimulated, and analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer. All procedures were performed according to the investigator’s protocols. Data used with permission from David Draper and Alden Wong, Mt Bioresearch, Ann Arbor, Michigan, USA.
immuno-oncology checkpoint panels in particular (Figure 5). Visit thermofisher.com/procartaplex-immunocheckpoints to read the application note “Detection of soluble isoforms of immuno-oncology checkpoint markers”.

Cell imaging analysis. Both cell-imaging microscopes and high-content analysis (HCA) instruments are particularly robust tools that enable researchers to assess the structure, localization, and endogenous expression levels of proteins of interest and other cell characteristics. Invitrogen™ EVOS™ Imaging Systems may be employed for transmitted-light imaging as well as colorimetric and fluorescence imaging for qualitative assessments; quantitative information about protein relocalization or organelle function can be obtained after importing images into data analysis software such as ImageJ. Figure 6 shows an example of data produced using the EVOS FL Auto Imaging System. Compared with observations in nonresponding metastatic melanoma patients treated with anti–CTLA-4 or anti–PD-1 mAbs, this immuno-histochemical analysis indicates that, prior to treatment, statistically significant numbers of CD8+ T cells accumulated in the invasive margin of tumors from patients that responded to ICI therapy [6]. Learn more about EVOS Imaging Systems at thermofisher.com/evos.

High-content imaging and analysis. HCA technology combines high-resolution microscopy and automated image capture with multi-parametric acquisition and data analysis to provide precise quantitative analysis of individual cells in a large and potentially heterogeneous cell population. With the aid of molecular tools such as fluorescent dyes, chemical probes, and targeted antibodies, a wide range of cell events and features can be quantitated, including but not limited to nuclei and DNA counts, nuclear and whole-cell morphology, and cytoskeletal organization, as well as cell motility, migration, and invasion [7]. In addition, these features can be quantitated over time to evaluate temporal and spatial relationships between multiple cellular targets in intact cells. An important advantage of HCA is the ability to perform multiple independent measurements simultaneously, and cell-based HCA assays are increasingly being used to monitor mechanisms critical in immuno-oncology research [8]. To produce the data presented in Figure 7, a Thermo Scientific™ CellInsight™ CX7 High-Content Analysis Platform was used to visualize effector T cell–mediated lysis of HCC827 cancer spheroids. Compared with the negative control conditions shown in the left panel, increasing the effector:target ratio resulted in a greater degree of target cell lysis, as seen in the right panel [9]. To learn more about Thermo Scientific™ high-content imaging and analysis instruments and reagents, go to thermofisher.com/hca.

Alternative ICI T cell targets and additional strategies for cancer immunotherapy

The ICIs listed in Table 1 may become first-line therapies for certain advanced cancers; however, these treatments—either alone or in combination with other immunotherapy or chemotherapy agents—have been successful in a minority of patients. Table 2 provides a nonexhaustive summary of ICI drug candidates for the treatment of various solid and
hematological tumors, administered alone or as combinatorial drug therapies with other ICIs or chemotherapy [10,11].

Beyond developing biotherapeutic antibodies that target immune checkpoint pathways, over the past few decades researchers have harnessed the power of adoptive cell therapies (ACT). For example, in adoptive T cell transfer, a patient’s T cells are extracted, genetically modified to recognize the cancer cells, cultured in vitro, and then reintroduced into the patient. In 1988, autologous T cell adoptive transfer of ex vivo expanded cells was used with relative success to treat patients with metastatic melanoma resistant to conventional therapies, and incremental improvements in efficacy have emerged over time [12].

More recently, investigators have realized gains with the development of chimeric antigen receptor (CAR) T cell therapy for treating certain forms of cancer (Figure 7). In 2017, two CAR T cell therapies were approved by the FDA, opening the door for a new generation of ACTs.

Another emerging field inspiring great interest is immuno-metabolism, which explores intracellular metabolic pathways in immune cells. Among several focus areas, researchers are seeking to understand how drugs may selectively target metabolic pathways that govern T cell function and how alterations in T cell metabolism may potentially boost tumor rejection in vivo [13,14].

**Download the Immuno-oncology Guide**

To learn more about technologies that enable immuno-oncology research, download the Immuno-oncology Flow Cytometry Guide (at thermofisher.com/flow-io), which provides detailed information about workflows for flow cytometry, biomarker profiling, and cell imaging, and reviews several central aspects of cancer research.

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**References**

11. clinictrials.gov