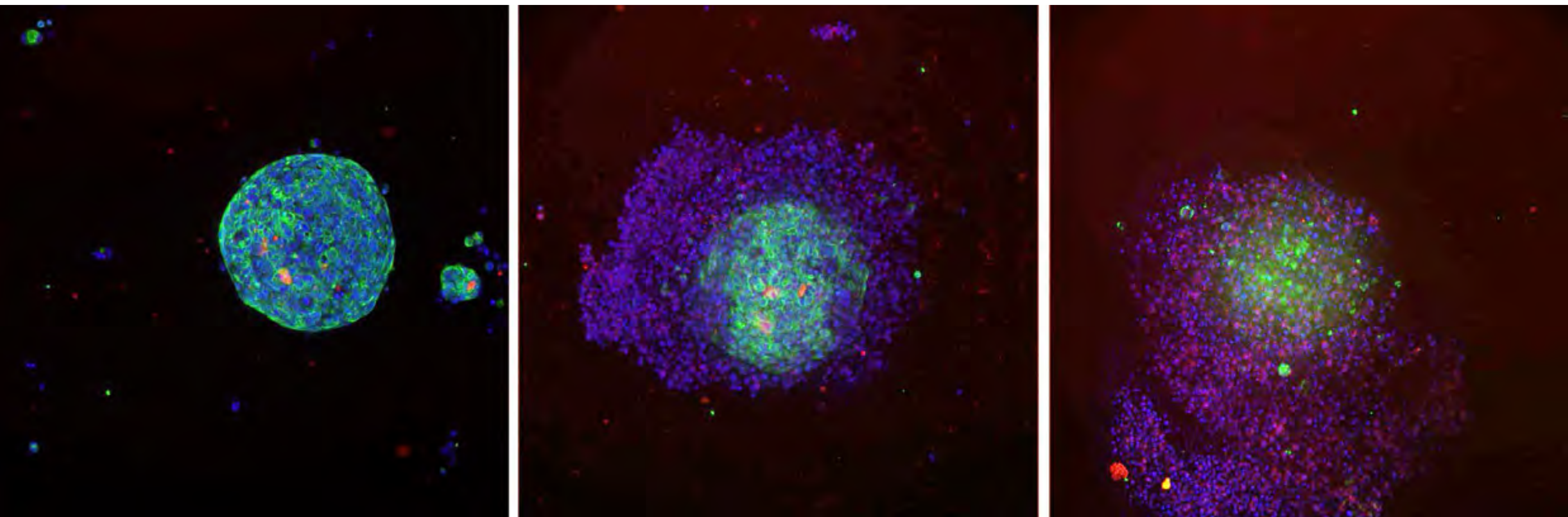

Immuno-oncology: Advances in basic research and translational medicine

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.

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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 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 →

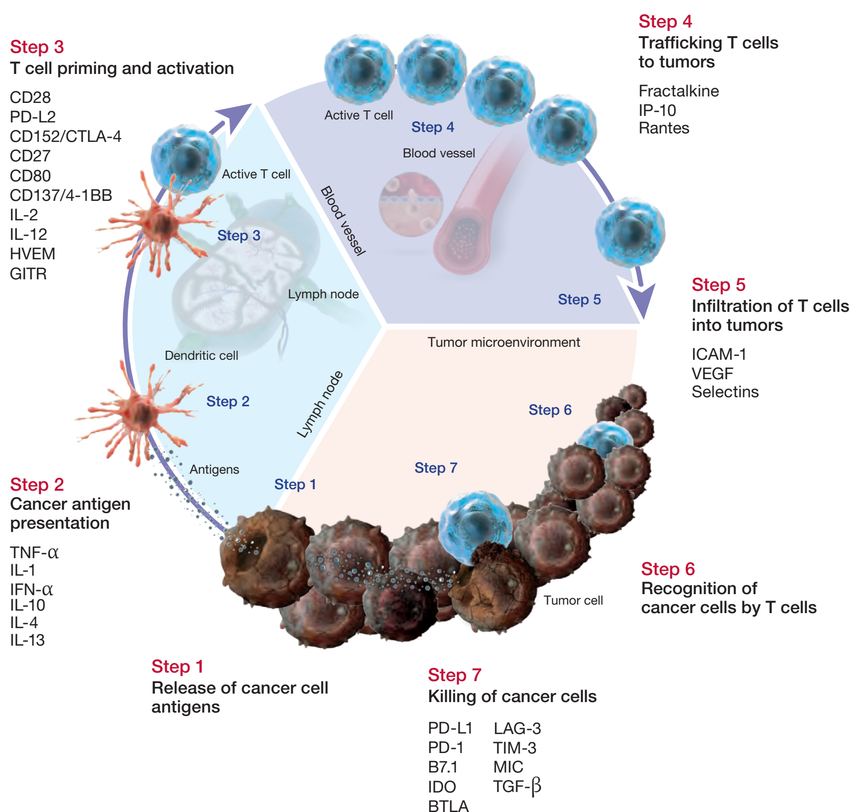

Figure 2. The multistep anti-tumor responses. The cancer immunity cycle involves the coordination of a myriad of checkpoint molecules and other cell-surface receptors, as well as soluble factors such as cytokines and chemokines. The process is initiated by the release of tumor-derived antigens (step 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.

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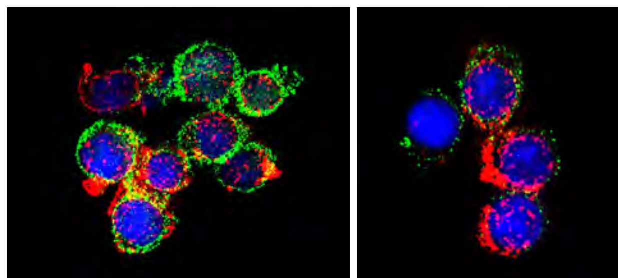

Figure 3. Immunofluorescence analysis of Jurkat cells using anti–CTLA-4 antibody. PMA-treated (left) and untreated (right) Jurkat cells were fixed and permeabilized for detection of endogenous CTLA-4 using Invitrogen™ ABfinity™ anti–CTLA-4 recombinant rabbit monoclonal antibody (clone 11H7L17, Cat. No. 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.

the US Food and Drug Administration (FDA). Table 1 provides details on the first-generation ICIs that are currently approved for indications in cancer. Figure 3 shows an example of Jurkat cells stained with a research antibody that recognizes the human CTLA-4 receptor.

Methods for identifying immune cells in TME, blood, and culture

Immune cell subsets within the TME include, but are not limited to, dendritic cells (DCs), natural killer (NK) cells, B cells, and T cells (including $CD4^+$ T helper and regulatory subsets, $CD8^+$ cytotoxic T cells with the potential to directly eradicate tumor cells, and other T cell subsets). The TME also harbors cells of the myeloid lineages, which may have both tumor-suppressive and tumor-promoting properties. The ability to isolate, characterize, and modulate cells that populate the TME is directly linked to advances in basic biomedical research and translational medicine. To learn more about leukocyte subsets and flow cytometry, access the T cell proliferation and stimulation ecourse at **thermofisher.com/elearningcourses.**

Flow cytometry. 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**.

Immunoassays for soluble proteins. In addition to implementing flow cytometry methods, immunophenotyping may be accomplished

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

Agent	Target	Indication (initial FDA approval year)	Pivotal clinical trial reference
Ipilimumab	CTLA-4	Melanoma (2011) [1]	Hodi FS, O'Day SJ, McDermott DE et al. (2010) *N Engl J Med* 363:711–723.
Pembrolizumab	PD-1	Melanoma, NSCLC, HNSCC, cHL (2014) [2]	Hamid O, Robert C, Daud A et al. (2013) *N Engl J Med* 369:134–144.
Nivolumab	PD-1	Melanoma, NSCLC, RCC, cHL, SCCHN, UC, dMMR solid cancers, CRC, HCC (2014) [3]	Weber JS, D'Angelo SP, Minor D et al. (2015) *Lancet Oncol* 16:375–384.
Atezolizumab	PD-L1	Bladder, NSCLC (2016) [4]	Rosenberg JE, Hoffman-Censits J, Powles T et al. (2016) *Lancet* 387:1909–1920.
Avelumab	PD-L1	MCC, bladder (2017) [5]	Kaufman HL, Russell, J, Hamid O et al. (2016) *Lancet Oncol* 17:1374–1385.
Durvalumab	PD-L1	Bladder (2017) [6]	Antonia SJ, Vilegas A, Daniel D et al. (2017) *N Engl J Med* 377:1919–1929.

Colorectal cancer (CRC); classical Hodgkin lymphoma (cHL); hepatocellular carcinoma (HCC); Merkel cell carcinoma (MCC); mismatch repair deficient (dMMR); non-small cell lung cancer (NSCLC); renal cell carcinoma (RCC); squamous cell carcinoma of the head and neck (SCCHN). 1. Yervoy prescribing information (2015) Bristol-Myers Squibb Company. Accessed 15 January 2018. **accessdata.fda.gov/drugsatfda_docs/label/2015/125377s073lbl.pdf**. 2. Keytruda prescribing information (2014) Merck & Co., Inc. Accessed 15 January 2018. **merck.com/product/usa/pi_circulars/k/keytruda/keytruda_pi.pdf**. 3. Opdivo prescribing information (2018) Bristol-Myers Squibb Company. Accessed 15 January 2018. **packageinserts.bms.com/pi/pi_opdivo.pdf**. 4. Tecentriq prescribing information (2017) Genentech, Inc. Accessed 15 January 2018. **gene.com/download/pdf/tecentriq_prescribing.pdf**. 5. Bavencio prescribing information (2017) EMD Serono, Inc. **emdserono.com/ms.country.us/en/images/Bavencio_PI_tcm115_161084.pdf**. 6. Imfinzi prescribing information (2017) AstraZeneca Pharmaceuticals LP. Accessed 15 January 2018. **azpicentral.com/imfinzi/imfinzi.pdf**.

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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 quantitation 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.

Agent	Target	Development stage
rHIGM12B7	PD-L2	Phase I
Tremelimumab	CTLA-4	Phase I–III
IMP321, BMS-986016	LAG-3	Phase I–II; Phase I–II
TSR-022	TIM-3	Phase I
PBF-509	A2aR	Phase I

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**.

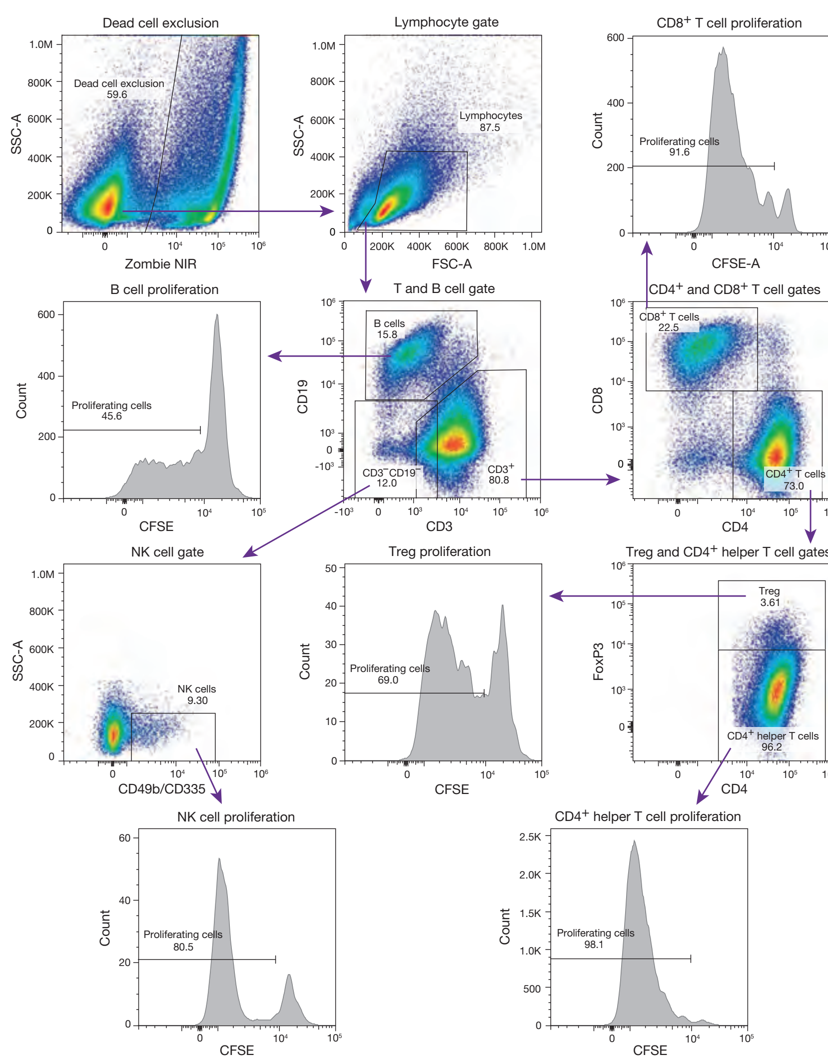

Figure 4. Profile of splenocytes (derived from a Balb/c syngenic CT26 colorectal tumor model) stimulated with anti-CD3 antibody. Tumor-naive and tumor-bearing mice were left untreated or administered anti–mouse CTLA-4 antibody. Subsequently, splenocytes were harvested, preloaded with carboxyfluorescein succinimidyl ester (CFSE), stimulated, stained, 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, MI Bioresearch, Ann Arbor, Michigan, USA.

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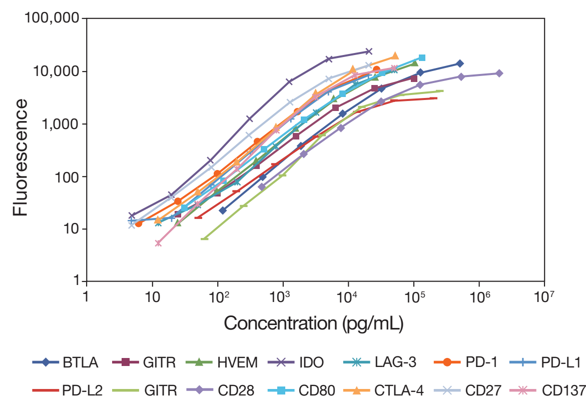

Figure 5. Analysis of 14 soluble protein biomarkers in a single sample using a ProcartaPlex immunoassay. Standard curves are shown for the Invitrogen™ Immuno-oncology Checkpoint 14-Plex Human ProcartaPlex™ Panel 1 (Cat. No. EPX14A-15803-901).

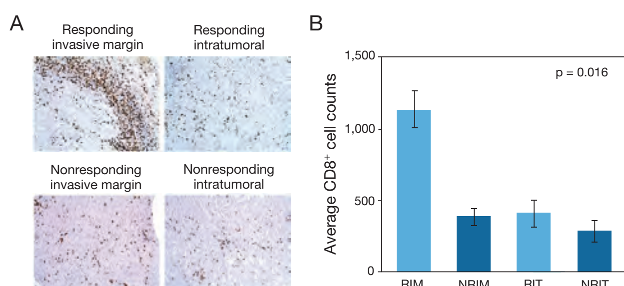

Figure 6. Tumors responding to treatment show increased T cell infiltration prior to therapy. (A) Representative $CD8^+$ immunohistochemical (IHC) staining of the invasive tumor margin and intratumoral region in pretreatment metastatic melanoma tumors (responding N = 4, nonresponding N = 4). Tumor compartments were assessed by a dermatopathologist. **(B)** Average $CD8^+$ cell counts for responding and nonresponding tumor compartments. T cell counts were produced by averaging the counts of 10 randomly selected fields using a 20x objective for each tumor compartment (10 invasive margin; 10 intratumoral). RIM = responding invasive margin; NRIM = nonresponding invasive margin; RIT = responding intratumoral; NRIT = nonresponding intratumoral. Reprinted with permission from Shields B, Mahmoud F, Taylor EM et al. (2017) *Sci Rep* 7:807, and under the Creative Commons Attribution 4.0 International License (**creativecommons.org/licenses/by/4.0/**).

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 immunohistochemical 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 multiparametric 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

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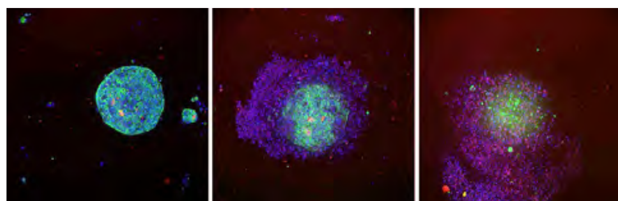

Figure 7. Chimeric antigen receptor (CAR) T cell invasion into cancer spheroids. HCC827 spheroids were formed using spheroid microplates for 48 hr. Then, 24 hr after the addition of EGFR scFv-CD28-CD3ε CAR T cells (ProMab Biotechnologies), spheroids were immunostained for cytokeratin-7 (green) and CD3ε (red), and counterstained with Hoechst™ dye (blue). As the effector-to-target ratio is increased from 10:1 (middle panel) to 40:1 (right panel), invasion of the CAR T cells into the HCC827 tumor spheroid and subsequent tumor cell lysis are visible. Images were obtained on the Thermo Scientific™ CellInsight™ CX7 High-Content Analysis Platform in confocal mode with a 10x objective, and used with permission from Corning Inc.

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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