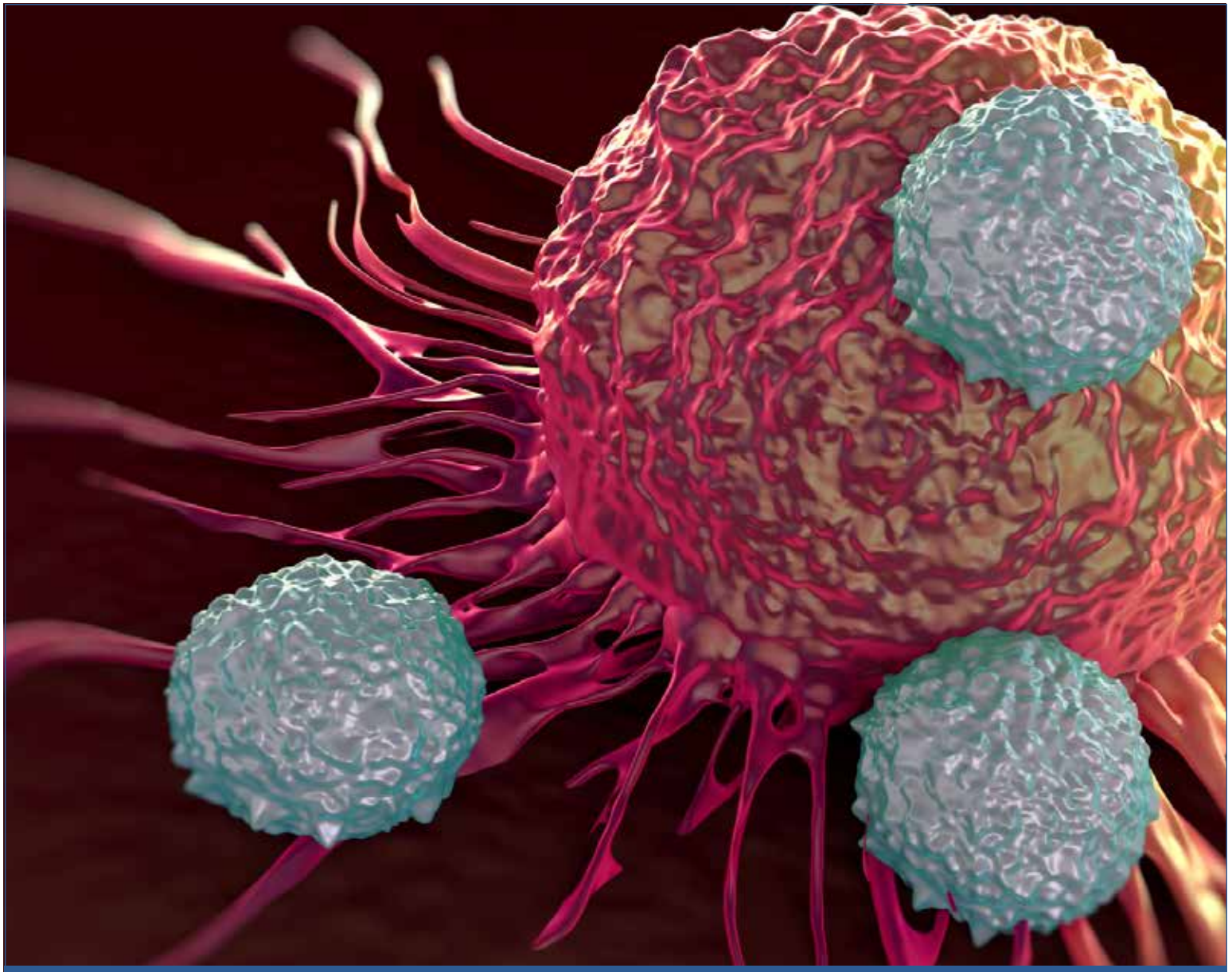

IMMUNO-ONCOLOGY RESEARCH AND FLOW CYTOMETRY:

Transforming Cancer Treatment in the 21st Century

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Cancer treatment is undergoing a revolution. Decades of basic and clinical research have revealed how the immune system can be harnessed to destroy tumors specifically or prevent metastasis. Along the way, flow cytometry has been a powerful tool for determining the mechanism of action of certain therapeutic treatments, monitoring responses in clinical trials, and tracking the effectiveness of treatment in patients.

Immuno-oncology (IO) research is a burgeoning field with incredible potential to transform cancer treatment. Researchers are applying cutting edge discoveries in basic immunology to developing novel cancer immunotherapies. These areas include T cell, B cell, and NK cell-mediated anti-tumor responses, cytokines, co-stimulation, chemotaxis and immune cell migration, and immune tolerance. Basic immunology findings provide critical insights into how certain immune cells and molecules can be used to specifically target tumor cells or instruct the immune system. All of these aspects of the immune system can be characterized and measured using different flow cytometry techniques.

This white paper will highlight different IO-based therapies that are transforming cancer treatment, and how flow cytometry is an ideal technique for bolstering research and driving preclinical and clinical decisions in this growing biomedical research sphere.

The Immune System's Anti-Tumor Toolkit

The immune system is constantly surveilling the body for abnormal or foreign cells. Immunologists have observed that tumor cells can

appear as foreign or abnormal by displaying tumor antigens on the cell surface. T cells recognize these cells as foreign and can orchestrate a multi-pronged anti-tumor response that destroys the tumor cell and generates a memory response that can squelch identical tumor cells that may re-emerge. This anti-tumor response is not always effective at destroying tumors or keeping cancer progression in check. The breakdown in these responses can be caused by tumors eluding immune surveillance or hiding in immune-privileged sites like the brain.

Immune System Molecules As Cancer Treatment

Since the advent of oncology treatments in the 19th century, protocols have been based on surgery, radiation, or chemotherapy. These interventions were effective for certain types of cancers but had many potential side effects. Immuno-oncology (IO) uses immune system cells or molecules to specifically target an immune response against a cancer. The potential of IO began to emerge in the 1980s as molecular biology was used more widely for immunology research, and more immune molecules and immune cells were being defined at a molecular level. A groundbreaking clinical trial out of NIH in 1985 demonstrated that administration of the interleukin 2 (IL-2) was sufficient to cause regression of metastatic melanoma and metastatic renal cancer[1]. Basic research discoveries in the 1980s began to reveal the molecular mechanisms behind T cell signaling, and T cell activation and differentiation, which provided essential insights for the development

[1] *Rosenberg, SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Vetto JT, et al. 1985. Ob servations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N. Engl. J. Med. 1985. 313: 1485–1492.*

of IO therapies that harness T cells. Natural killer (NK) cells were also characterized in 1986 as a novel immune cell type that may be critical for detecting abnormal or malignant cells[2]. Humanized monoclonal antibodies were developed in the 1980s, and this technological breakthrough was critical to the development of many antibody-based IO therapies[3]. These advancements in understanding the immune system and producing monoclonal antibodies drove the innovation of flow cytometry as an analytical tool, and flow cytometry became a standard approach used regularly by immunologists and clinical researchers across the globe.

The field of IO has matured and within the last decade, several new groundbreaking IO treatments have been approved for clinical use. These treatments can be classified as active or passive therapies based on their mechanism of action and are effective at treating a wide spectrum of cancers, including those previously impervious to other treatments.

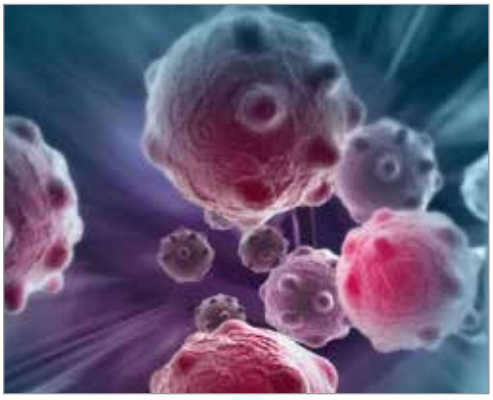
Passive IO Therapies

Passive therapies include monoclonal antibodies that bind specifically to tumor cells. Antibody binding can trigger different anti-tumor responses, such as halting the tumor cell proliferation or targeting the tumor cell for destruction by the immune system through antibody-dependent cellular cytotoxicity (ADCC). Monoclonal antibodies were among the first IO therapies approved for clinical use in the 1990s, and are some of the

[2] *Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986. 391: 675-678.*

[3] *Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature. 1986. 321: 522-525.*

most effective treatment options today. Rituximab was one of the first approved anti-tumor antibody therapies approved by the FDA in 1997 for the treatment of non-Hodgkin's lymphoma[4]. It is a monoclonal antibody that recognizes CD20, a marker expressed on B cells and is used to treat B cell lymphomas and leukemias. Trastuzumab (also known as Herceptin®) was also among the first monoclonal antibodies approved to treat cancer. It targets breast cancer cells expressing the HER2/neu receptor and disrupts tumor cell proliferation[5].



Cell-based passive therapies are also being explored, including the use of tumor-infiltrating lymphocytes, which are isolated from the tumor of a patient, expanded in vitro, then re-infused back into patients. This experimental immunotherapy is still in development and has been tested as a treatment option for metastatic melanoma[6]. Flow cytometry and Fluorescent Activated Cell Sorting (FACS) are essential to this research area because tumor-associated lymphocytes must be identified and sorted from other tumor cells in the sample matrix, so the correct tumor-specific cells can be grown in vitro and re-infused for treatment.

Active IO Therapies

Active therapies activate the immune system through different mechanisms to target and destroy tumor cells. This area of IO research

[4] *Scott SD. Rituximab: a new therapeutic monoclonal antibody for non-Hodgkin's lymphoma. Cancer Pract. 1998. 6 (3): 195–7.*

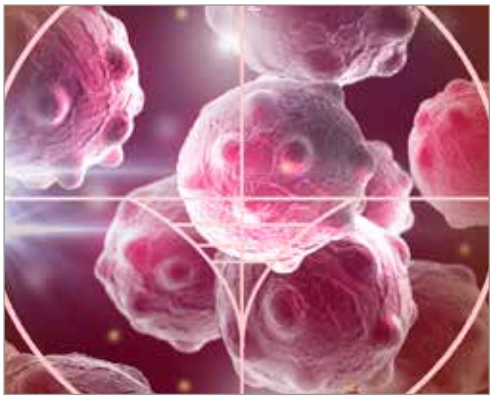
[5] *Le XF, Pruefer F, Bast R. HER2-targeting antibodies modulate the cyclin-dependent kinase inhibitor p27Kip1 via multiple signaling path ways. 2005. Cell Cycle 4 (1): 87–95.*

[6] *Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, Wunderlich J, Restifo NP, Thomasian A,Downey SG, Smith FO, Klapper J, Morton K, Laurencot C, White DE, Rosenberg SA. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. 2008. J. Clin. Oncol. 26(32):5233-9.*

is currently very active because many specific immune mechanisms can be used individually or in combination therapies. Flow cytometry techniques are used throughout research and development of these different IO therapies and are critical to clinical monitoring.

Cytokines

Cytokines are proteins produced by different cells, especially cells of the immune system, and have diverse roles, including stimulating immune cell expansion or inhibiting cancer cell proliferation. IL-2 was one of the first cytokines approved to treat cancer, as described above, for use in metastatic melanoma and metastatic renal cancer. The interferon (IFN) family of cytokines has potent anti-proliferative properties and can activate the anti-tumor activity of multiple immune system cells. IFN-alpha was also another early IO therapy approved in the 1980s for treatment of hairy cell leukemia[7] and is now used as part of treatment protocols for a variety of cancers. IFN-alpha activates multiple responses in dendritic cells and cytotoxic T cells that lead to durable anti-tumor responses[8] and is now being explored as a combination therapy with other cancer treatments.



Flow cytometry assays can evaluate the effectiveness of different cytokine therapies by monitoring changes in the phenotypes of immune cells, including measurement of biomarkers for activation, proliferation, and migration. Multi-parametric flow cytometry panels provide a snapshot of the immune system in a single assay including

[7] *Quesada JR, Reuben J, Manning JT et al. Alpha interferon for induction of remission in hairy-cell leukemia. N Engl J Med 1984. 310:15-18.*

[8] *Belardelli F, Ferrantini M, Proietti E, Kirkwood JM. Interferon-alpha in tumor immunity and immunotherapy.Cytokine Growth Factor Rev. 2002. 13(2):119-34.*

dendritic cells, NK cells, and different T cell subsets. The flexibility and customizable nature of flow cytometry assays makes them well suited for monitoring changes in the phenotypes of diverse immune cells, including measuring biomarkers for activation, proliferation, and migration.

Cell-Based Cancer Immunotherapy (Cancer Vaccines)

One goal of IO research is to find ways to tailor cancer treatments to an individual's specific tumor. Immunologists have worked for decades to determine how tumor antigens are seen by the immune system and how the immune response can be targeted specifically to destroy tumors. Dendritic cells (DCs) are a type of antigen-presenting cell (APC) recognized by T cells, and when DCs present tumor antigens to cytotoxic T cells, this interaction triggers a potent and specific anti-tumor response. The basic understanding of this mechanism was used to develop the first cell-based cancer immunotherapy, which is also considered a cancer vaccine. Sipuleucel-T (Provenge®) was developed for the treatment of prostate cancer[9] and uses a patient's own APCs, which are removed by leukapheresis, treated with a fusion protein comprised of a prostate antigen and a molecule to promote APC maturation (GM-CSF) and then re-infused back into the patient. This APC-based vaccine can specifically activate cytotoxic T cells in the patient and induce anti-tumor responses.

This type of therapy requires the use of flow cytometry, particularly intracellular cytokine staining in order to monitor and validate the

[9] *Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF. IMPACT Study Investigators. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med. 2010. 363: 411-422.*

phenotype of the cells that are modified in vitro. Flow cytometry is also critical to measuring the effectiveness of treatment in patients and monitoring the durability of the response, such as the development of memory T cells. The field of immuno-oncology research has worked together to standardize and validate flow cytometry methods, such as those for T cell assays, so they can be used in preclinical development and clinical trials[10].

T Cell Response Modifiers

Within the last two years, several T cell response modifiers, also known as checkpoint inhibitors, have been approved for cancer treatment and have significantly changed the landscape of IO treatment options. T cell modifiers are monoclonal antibodies that bind to inhibitory signaling molecules on the surface of the cytotoxic T cell and alter how these cells respond. T cell signaling and activation is driven primarily by a T cell receptor binding to a peptide antigen presented on a major histocompatibility complex (MHC) on another cell, such as an APC. This engagement of the T cell receptor triggers signaling events that will drive activation of cytotoxic T cells that have potent anti-tumor activity

Costimulatory molecules, including CD28 on T cells and B7 on APCs are engaged during TCR-peptide/MHC signaling and are part of activating T cell responses. Alternatively, APCs also express inhibitory signals that block T cell signaling and activation, which in turn blocks anti-tumor responses.

CTLA-4 is one such inhibitory molecule on T cells, and a human monoclonal antibody that targets CTLA-4 (ipilimumab) has been shown to improve melanoma survival[11] and was approved by the FDA for

[10] *Britten CM, Janetzki S, Butterfield LH, Ferrari G, Gouttefangeas C, Huber C, et al. T Cell Assays and MIATA: The Essential Minimum for Maximum Impact. Immunity. 2012.37(1):1-2.*

[11] *Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melan ma. N Engl J Med. 2010. 363: 711-723.*

melanoma treatment in 2011. Pembrolizumab is a monoclonal antibody that targets PD-1 on T cells and blocks inhibitory signaling triggered by PD-1/PD-L1 interactions. Pembrolizumab has been associated with anti-tumor responses in patients[12] and was approved by the FDA in 2014.

These checkpoint blockade therapies are now being studied for use against other cancers or in combination with existing cancer therapies. Flow cytometry is essential to studies of checkpoint inhibitors in order to assess the pharmacodynamics of these molecules and characterize changes in T cell phenotypes during the course of treatment.

Flow Cytometry And The IO Pipeline

Many novel IO therapies are currently in the research pipeline and build on existing IO therapies or take different approaches. Current research includes adoptive cell transfer of genetically engineered T cells that express a chimeric antigen receptor (CAR) that specifically recognizes a tumor antigen. These CAR T cells have been associated with anti-tumor activity in patients with certain types of leukemia[13] and lymphoma[14] and may become a standard treatment in the future.

These engineered T cells, as well as new types of cancer vaccines, monoclonal antibodies and cytokines are under development for

[12] *Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I, Topalian SL. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol. 2010. 28: 3167-3175.*

[13] *Brentjens RJ1, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblat T, Maslak P, Frattini M, Sadelain M. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leuke mia. Sci Transl Med. 2013. 5 (177): 177ra38.*

[14] *Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M, Yang JC, Phan GQ, Hughes MS, Sherry RM, Raffeld M, Feldman S, Lu L, Li YF, Ngo LT, Goy A, Feldman T, Spaner DE, Wang ML, Chen CC, Kranick SM, Nath A, Nathan DA, Morton KE, Toomey MA, Rosenberg SA. Chemotherapy-Refractory Diffuse Large B-Cell Lymphoma and Indolent B-Cell Malignancies Can Be Effectively Treated With Autologous T Cells Expressing an Anti-CD19 Chimeric Antigen Receptor. J Clin Oncol. 2015. 33(6):540-9*

many types of cancers. Flow cytometry assays are critical to these studies at all phases of development. These assays can be used for determining specific mechanisms of action (such as ADCC, ADCP, CDC, trogocytosis or apoptosis), monitoring cell migration, and measuring pharmacodynamics and immunotoxicology. Clinical flow cytometry applications include sorting of cells targeted for in vitro modification and expansion, and evaluating changes in specific peripheral immune cells during the course of treatment.

Immuno-oncology is changing the nature of cancer treatment and new therapies will be coming to market in the coming years. Flow cytometry is a powerful and versatile tool for IO research and development and will be essential to the advancement of future therapies.

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