# Characterizing functional immuno-oncology

## Using flow cytometry and high-content imaging to evaluate innate immune function.

The field of immuno-oncology has expanded dramatically in the last few decades, producing novel immunotherapies designed to enhance or enable antitumor immune responses, overcome tumor evasion mechanisms, and promote conditions that favor immune protection. Immunotherapy may offer distinct advantages over conventional treatment modalities [1]. For example, tumor-specific immune cells have the ability to migrate to areas of the body that are inaccessible by surgery, to target microscopic disease and disseminated metastases, and to potentially act specifically against the tumor, thereby lowering the risk of damage to surrounding healthy tissue; nevertheless, severe toxicities may be associated with some particular immunotherapies.

In 2013, Chen and Mellman defined the series of immune system functions that specifically target and kill cancer cells as the cancer-immunity cycle [2], which involves the coordination of a myriad of checkpoint molecules and other cell-surface receptors, as well as soluble factors such as cytokines and chemokines. This complex series of anticancer immune responses is typically initiated by the release of tumor-derived antigens. The engulfment of these antigens by dendritic cells and their subsequent presentation to T cells drive the immune response. Once activated, effector T cells acquire the ability to destroy target cells by specifically recognizing tumor antigens displayed on the tumor cell surface (Figure 1). Trafficking of T cells to tumors, followed by infiltration of the T cells into tumors, leads to cancer cell death. Increasing levels of tumor antigens are then released, further driving the progression of the cycle.

Understanding the mechanisms of T cell–based immunity has led to many revolutionary cancer therapies, including (1) checkpoint blockade immunotherapies, which 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, and (2) chimeric antigen receptor (CAR) T cell therapy, a form of adoptive cell therapy in which T cells are extracted from a patient, genetically engineered to express a CAR specific for a tumor antigen, cultured *ex vivo*, and then reintroduced back into the patient to attack tumor cells.

These therapeutic modalities, while clinically successful for many patients, are limited in their focus to T cells of the adaptive immune system. Recently, translational research has increasingly focused on the role of the innate immune system in antitumor immunity, as summarized by Demaria and colleagues in the cancer–innate immunity cycle [3]. These efforts have resulted in novel therapeutics such as CAR natural killer (NK) cells [4] and engineered antibodies designed to better recruit the cancer cell killers of the innate immune system: complement proteins, NK cells, and macrophages [5]. These approaches will hopefully benefit a larger percentage of patients and provide more scalable manufacturing, while reducing potential drug toxicity. Here we describe methods and assays to characterize the targeted killing of cancer cells by three different innate immune mechanisms: complement-dependent cell killing, NK cell–mediated cytotoxicity, and phagocytosis by macrophages (Figure 1).

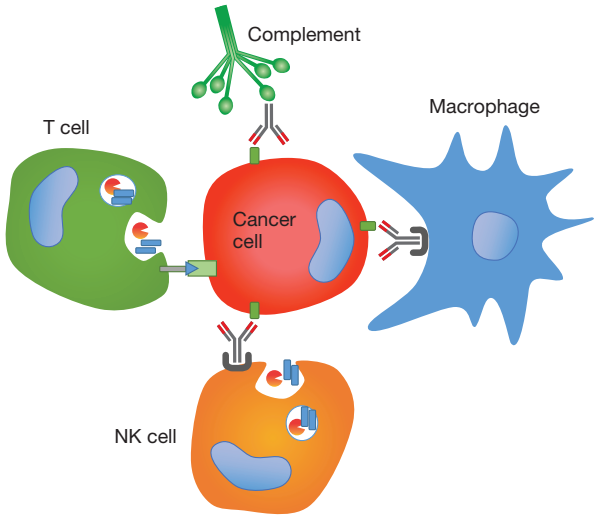


**Figure 1. Mechanisms of cancer immunotherapy that employ both the adaptive and innate immune system.** T cells form the backbone of the adaptive immune system, whereas the innate immune system is mediated by complement-dependent cytotoxicity (CDC), natural killer (NK) cell recruitment, and phagocytosis by macrophages.

### Antibody-mediated complement-dependent cytotoxicity

When an antibody drug such as rituximab (an anti-CD20 monoclonal antibody) binds to the CD20 membrane protein on cancer cells in the presence of human serum, complement proteins in the serum initiate

a complement cascade, ultimately leading to CD20+ cell death while leaving CD20– cells unaffected. Although the mechanism of rituximab-mediated complement activation is not well understood, the structure of the rituximab–CD20 complex has recently been published and suggests a model for complement recruitment that involves rituximab crosslinking CD20 molecules into circular super-assemblies specifically designed to engage complement [6].

We used flow cytometry to evaluate complement-dependent cytotoxicity (CDC) induced by therapeutic antibodies. The specificity of complement-dependent killing for cells expressing CD20 can be demonstrated by first labeling CD20+ Ramos B and CD20– Jurkat cell lines with spectrally distinct Invitrogen™ CellTrace™ cell proliferation stains—we used CellTrace Violet and CellTrace Far Red dyes. The labeled cells were then combined in equal quantities, followed by the addition of fresh human serum, rituximab, and the cell viability dye Invitrogen™ SYTOX™ Green Dead Cell Stain, which is also spectrally distinct from the proliferation dyes used. After a 1-hour incubation at 37°C, cells were analyzed with the Invitrogen™ Attune™ NxT Flow Cytometer with Autosampler. This combination of three bright, spectrally distinct fluorescent dyes analyzed on the efficient (up to 10x faster than traditional cytometers), and flexible (up to 4 lasers and 16 detection channels) Attune NxT Flow Cytometer enables clear discrimination of targeted killing in a mixed population of cells. Figure 2 shows that CD20+ cells (labeled with CellTrace Violet dye) undergo significant cell death whereas CD20– cells (labeled with CellTrace Far Red dye) are unaffected.

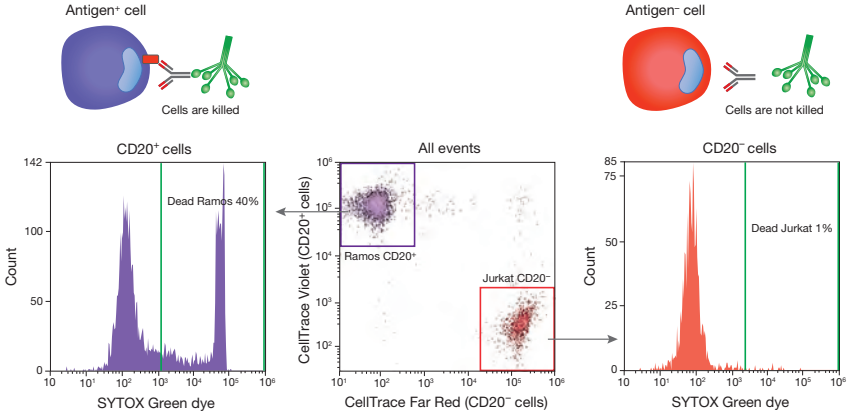


**Figure 2. Specific complement-dependent cytotoxicity assay.** Antibody binding to antigen-positive cells triggers the classical complement pathway, leading to cell death. CD20+ Ramos B cells were labeled with Invitrogen™ CellTrace™ Violet dye from the Invitrogen™ CellTrace™ Violet Cell Proliferation Kit (Cat. No. C34557). CD20– Jurkat cells were labeled with Invitrogen™ CellTrace™ Far Red dye from the Invitrogen™ CellTrace™ Far Red Cell Proliferation Kit (Cat. No. 34564). The two cell types were mixed 1:1 and incubated with 10 nM rituximab and 10% fresh human serum for 1 hr. Viability was measured with the Invitrogen™ SYTOX™ Green Dead Cell Stain (Cat. No. S34860). Cell analysis on the Invitrogen™ Attune™ NxT Flow Cytometer with Autosampler (Cat. No. A29004, Cat. No. 4473928) shows that cell death occurs only in antigen-positive (CD20+) cells.

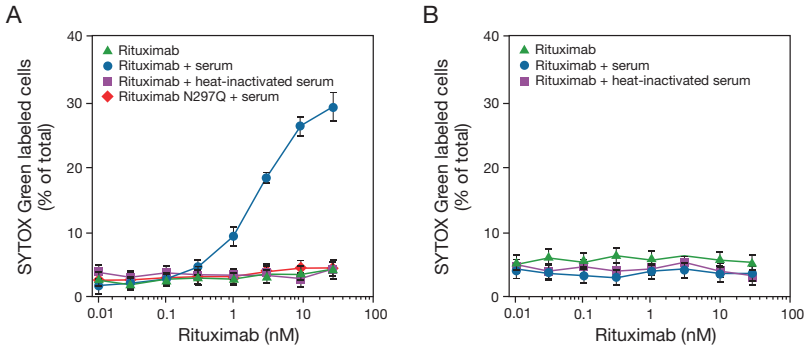


**Figure 3. Flow cytometry screening for complement-dependent cytotoxicity.** Using the cytotoxicity assay described in Figure 2 and either **(A)** CD20+ Ramos B cells or **(B)** CD20– Jurkat cells, we found that cell death only occurs with functional rituximab (not with mutated rituximab N297Q), active serum, and antigen-positive (CD20+) cells.

When characterizing the functional behavior of a therapeutic antibody, it is essential to test a range of antibody concentrations and conditions. This kind of screening experiment requires a robust flow cytometer and careful selection of reagents to provide clear results. Figure 3 shows the results of a typical antibody screening experiment using rituximab and CD20– and CD20+ cells, with analysis on the Attune NxT Flow Cytometer. We tested antibody concentrations across an 8-point dose-response curve, demonstrating the specificity of complement-dependent →

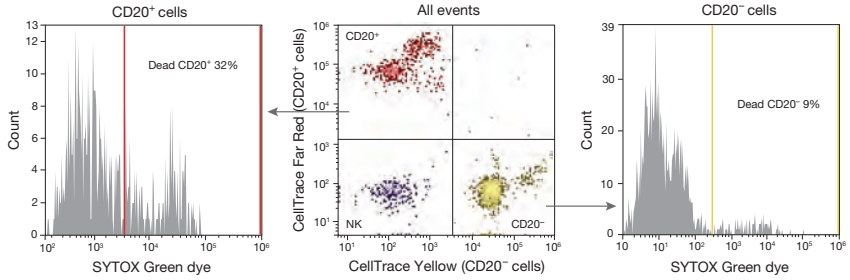


**Figure 4. Specific antibody-dependent natural killer (NK) cell killing assay.** NK cells were isolated from human peripheral blood mononuclear cells (PBMCs) using the Invitrogen™ Dynabeads™ Untouched™ NK Cells Kit (Cat. No. 11349D). NK cells were labeled with Invitrogen™ CellTrace™ Violet dye from the Invitrogen™ CellTrace™ Violet Cell Proliferation Kit (Cat. No. C34557); CD20+ Ramos B cells were labeled with Invitrogen™ CellTrace™ Far Red dye from the Invitrogen™ CellTrace™ Far Red Cell Proliferation Kit (Cat. No. 34564); and CD20– Jurkat cells were labeled with Invitrogen™ CellTrace™ Yellow dye from the Invitrogen™ CellTrace™ Yellow Cell Proliferation Kit (Cat. No. C34567). Cells were mixed 1:1:1 with 10 nM rituximab for 1 hr at 37°C, labeled with Invitrogen™ SYTOX™ Green Dead Cell Stain (Cat. No. S34860), and analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer (Cat. No. A29004). The three cell types appear as distinct populations on a dot plot. NK cell antibody-dependent cell cytotoxicity (ADCC) is observed in CD20+ cells, while minimal death is observed in CD20– cells.

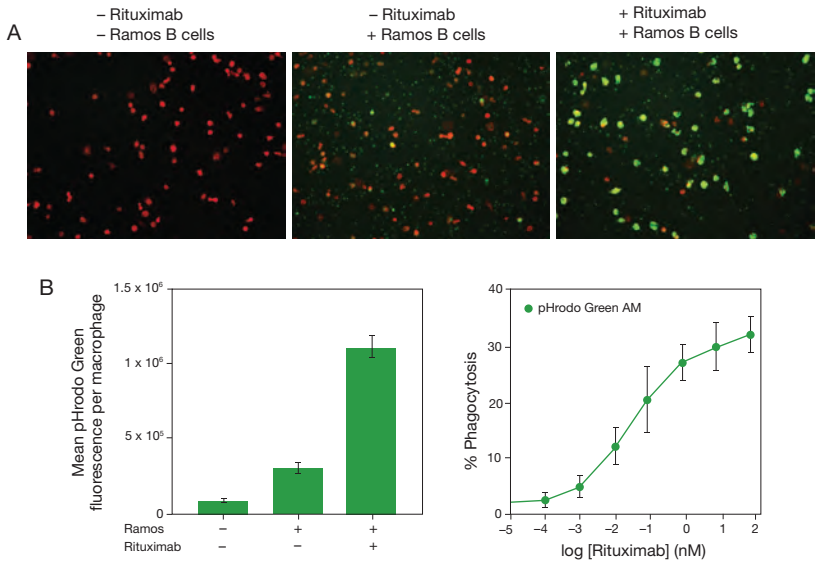


**Figure 5. Quantitative imaging of antibody-dependent phagocytosis.** Monocytes were isolated from human peripheral blood mononuclear cells (PBMCs) using Invitrogen™ Dynabeads™ Untouched™ Human Monocytes Kit (Cat. No. 11350D), then differentiated into macrophages for 7 days in Gibco™ RPMI 1640 Medium (Cat. No. 11875168) with 10% Gibco™ Fetal Bovine Serum (Cat. No. 26140087), 8% human serum, 1% Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061), 20 mM HEPES, 1% Gibco™ Penicillin-Streptomycin (10,000 U/mL, Cat. No. 15140122), and 50 ng/mL Gibco™ M-CSF Recombinant Protein (Cat. No. PHC9501). Human monocyte–derived macrophages were cultured in a 96-well microplate before labeling with Invitrogen™ CellTrace™ Far Red dye (Cat. No. C34564). CD20+ Ramos B cells were labeled with Invitrogen™ pHrodo™ Green AM Intracellular pH Indicator (Cat. No. P35373) and added to the labeled macrophages with or without 10 nM rituximab and incubated for 4 hr at 37°C. Cells were **(A)** imaged and **(B)** analyzed with the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform (Cat. No. CX7A1110LZR). Image-based analysis of pHrodo Green fluorescence in single cells provides a direct quantitative indication of antibody-mediated phagocytic events.

cell killing using one positive control (cells treated with rituximab and fresh human serum) and three negative controls (cells treated with rituximab only, rituximab and heat-inactivated serum, or mutated (nonfunctional) rituximab N297Q and serum). In CD20+ Ramos B cells, complement-dependent cytotoxicity only occurs with functional rituximab and active serum (Figure 3A). In contrast, CD20– Jurkat cells are completely unaffected by rituximab-mediated, complement-dependent cell killing (Figure 3B).

## Antibody-mediated NK cell cytotoxicity

NK cells exhibit potent tumor-specific cytotoxicity based on a combination of activating and inhibitory receptors on their cell membrane surface. They can also kill target cells based on antibody-dependent cell cytotoxicity (ADCC), in which IgG antibodies (known as opsonizing antibodies) bind to tumor-associated antigens on cancer cells and to Fc receptors on NK cells, resulting in NK cell activation and cancer cell death.

The specificity and potency of ADCC can be demonstrated with an *in vitro* flow cytometry experiment. CD20+ Ramos B cells labeled with CellTrace Far Red dye and CD20– Jurkat cells labeled with CellTrace Yellow dye were combined with rituximab and labeled NK cells (previously isolated from peripheral blood and labeled with CellTrace Violet dye). After a 1-hour incubation, cells were labeled with SYTOX Green stain and analyzed using the Attune NxT Flow Cytometer (Figure 4). CD20+ cancer cells can be easily identified by the CellTrace Far Red tracking dye, and subsequently analyzed for SYTOX Green dye labeling to distinguish live and dead cell populations. The experimental controls demonstrate that

this cytotoxicity is entirely dependent on the presence of opsonizing antibody, as cell killing does not occur without sufficient rituximab (data not shown).

## Antibody-dependent phagocytosis by macrophages

Antibody-opsonized cancer cells can also be recognized by Fc gamma receptors (FcγRs) on macrophages, leading to phagocytosis and killing of the cancer cells. This antibody-dependent cell phagocytosis (ADCP) is a major mechanism of action of therapeutic antibodies, which have been specifically developed to take advantage of the role macrophages play in the innate immune system. Accurate measurement of ADCP requires the use of fluorescent reagents that directly indicate the uptake of a cancer cell by a macrophage. Here we use the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform to visualize and quantitatively analyze activation of the phagocytic pathway with the pH-sensitive Invitrogen™ pHrodo™ Green AM Intracellular pH Indicator. The pHrodo pH indicator, which is weakly fluorescent at neutral pH but increasingly fluorescent as the pH drops, can be used to quantify cytosolic pH in the range of 9–4, with a pKa of ~6.5 and excitation/emission of 509/533 nm.

CD20+ Ramos B cells were first labeled using the cell-permeant pHrodo Green AM ester and then added to macrophages labeled with CellTrace Far Red dye in the presence of varying concentrations of rituximab (Figure 5). With an adequate concentration of rituximab, the macrophages are observed to phagocytose the B lymphoma cells, which exhibit significantly more green fluorescence due to the acidification of the pH-sensitive pHrodo dye in the phagosome.

## Instruments and reagents for immuno-oncology research

Thermo Fisher Scientific is developing new reagents and adapting existing reagents and instrumentation for the study of the immune system and its role within the cancer environment. To learn more about tools and technologies that enable immuno-oncology research, go to **thermofisher.com/immunooncology**, where you can download the Immuno-Oncology Product Resource Guide. Or, go to **thermofisher.com/flow-io** to download the Immuno-Oncology Flow Cytometry Guide, which reviews several central aspects of cancer research and provides detailed information about workflows for flow cytometry, biomarker profiling, and high-content cell imaging. ■

### References


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| Product | Quantity | Cat. No. |
|---|---|---|
| **Instruments** | | |
| Attune™ NxT Flow Cytometer, blue/red/yellow/violet6 | 1 each | A29004 |
| Attune™ NxT Flow Cytometer, blue/red/yellow/violet | 1 each | A24858 |
| Attune™ NxT Flow Cytometer Autosampler | 1 each | 4473928 |
| CellInsight™ CX7 LZR High Content Analysis Platform | 1 each | CX7A1110LZR |
| CellInsight™ CX7 LZR High Content Analysis Platform and Store Standard Edition (SE) Software | 1 each | CX7B1112LZR |
| CellInsight™ CX7 LZR High Content Analysis Platform with Store Standard Edition (SE) Software and Orbitor™ RS Plate Mover | 1 each | CX7C1115LZR |
| **Culture and labeling reagents** | | |
| CellTrace™ Far Red Cell Proliferation Kit, for flow cytometry | 20 assays<br>180 assays | C34572<br>C34564 |
| CellTrace™ Violet Cell Proliferation Kit, for flow cytometry | 20 assays<br>180 assays | C34571<br>C34557 |
| CellTrace™ Yellow Cell Proliferation Kit, for flow cytometry | 20 assays<br>180 assays | C34573<br>C34567 |
| Dynabeads™ Untouched™ Human Monocytes Kit | 1 kit | 11350D |
| Dynabeads™ Untouched™ Human NK Cells Kit | 1 kit | 11349D |
| Fetal Bovine Serum | 100 mL<br>500 mL<br>10 x 50 mL | 26140087<br>26140079<br>A3160502 |
| GlutaMAX™ Supplement | 100 mL<br>20 x 100 mL | 35050061<br>35050079 |
| M-CSF Recombinant Human Protein | 10 µg<br>100 µg | PHC9504<br>PHC9501 |
| Penicillin-Streptomycin (10,000 U/mL) | 20 mL<br>100 mL<br>20 x 100 mL | 15140148<br>15140122<br>15140163 |
| pHrodo™ Green AM Intracellular pH Indicator | 50 µL | P35373 |
| RPMI 1640 Medium | 1 L<br>5 L<br>6 x 1 L | 11875085<br>11875168<br>11875135 |
| SYTOX™ Green Dead Cell Stain, for flow cytometry | 1 mL | S34860 |