A flow cytometric assay to measure the modulation of phagocytosis

Immuno-oncology research applications for the BD Accuri™ C6 Plus flow cytometer

Features

- Screen a variety of cancer cell lines for relative expression of target antigens
- Assess levels of phagocytosis mediated by macrophages

Phagocytosis by macrophages is one of the innate immune system's first line of defense mechanisms against pathogens and transformed cells. To ensure self-tolerance, this process must be highly regulated. Inhibitory *don't eat me* signals based on recognizing "markers of self" dominate over positive *eat me* signals, thus preventing phagocytosis of normal cells.

CD47, expressed by all normal cells, is a critical regulator of phagocytosis. By binding to the ligand signal regulatory protein α (SIRP-α) on macrophages, it induces activation of immuno-receptor tyrosine-based inhibition motifs (ITIM) and recruits Src homology phosphatases (SHP-1 and SHP-2), which prevent phagocytosis by blocking myosin-IIA accumulation at the phagocytic synapse. This inhibitory mechanism is exploited by tumor cells that express high levels of CD47.

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The observation that blocking CD47:SIRP-α interaction enables increased phagocytosis of tumor cells led to several clinical trials based on blocking CD47 through therapeutic monoclonal antibodies. However, high variability in the response to CD47 blockade has been observed across different cancer cell lines, suggesting the existence of alternative inhibitory mechanisms. For example, a second inhibitory mechanism based on the interaction between MHC class I and leukocyte immunoglobulin-like receptor subfamily B2 (LILRB2) has recently been discovered, leading to the possibility of combined therapy blocking both inhibitory axes for tumors expressing high levels of CD47 and MHC class I.[1]

Study design: Our goal in this study was to assess the effects of blocking CD47 antibodies on phagocytosis of cancer cells by monocyte-derived macrophages. Based on a simple, rapid screen of a variety of different cancer cell lines, we selected three cell lines that differed in CD47 expression. We then derived macrophages from monocytes and performed a potency assay to measure their phagocytic activity. Macrophages were co-cultured with CFSE-labeled target cells and phagocytosis was measured as a function of the macrophages' CFSE uptake. Upon CD47 blocking, we expected increased phagocytosis for cell lines that express high levels of CD47, and little to no effect on the cell lines that express much lower levels of CD47.

To determine the relative levels of CD47 expression, 16 cancer cell lines were stained with mouse anti-human CD47 FITC and analyzed for signal-to-noise ratio (Figure 1A). Combining easy-to-use fixed voltage gain settings with a seven-decade dynamic range, the BD Accuri™ C6 Plus personal flow cytometer allows reliable characterization of these cells that varied broadly in size and CD47 antigen density.

Figure 1A

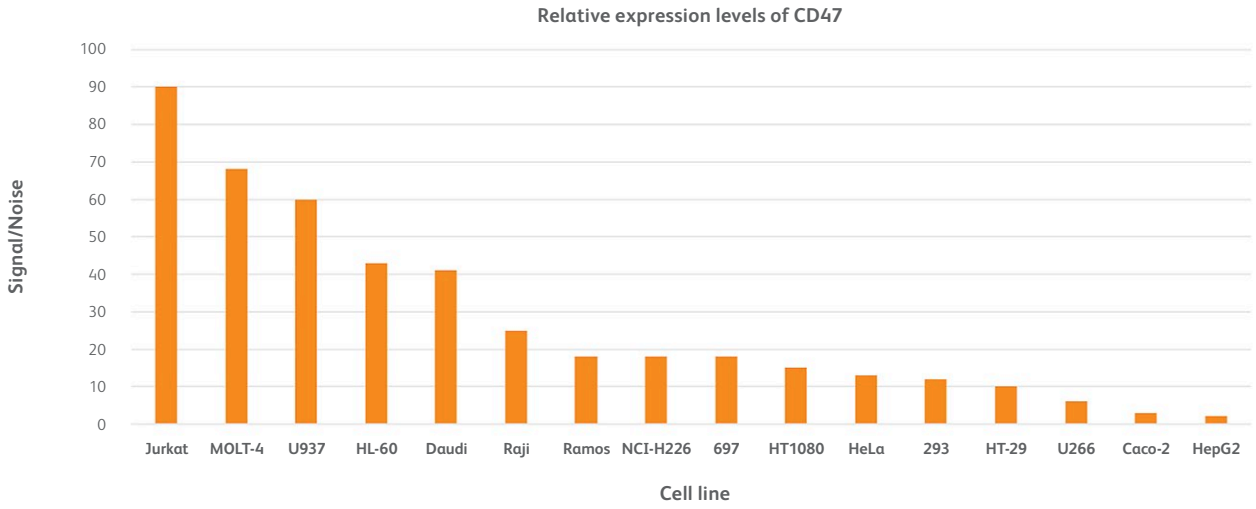

Figure 1B

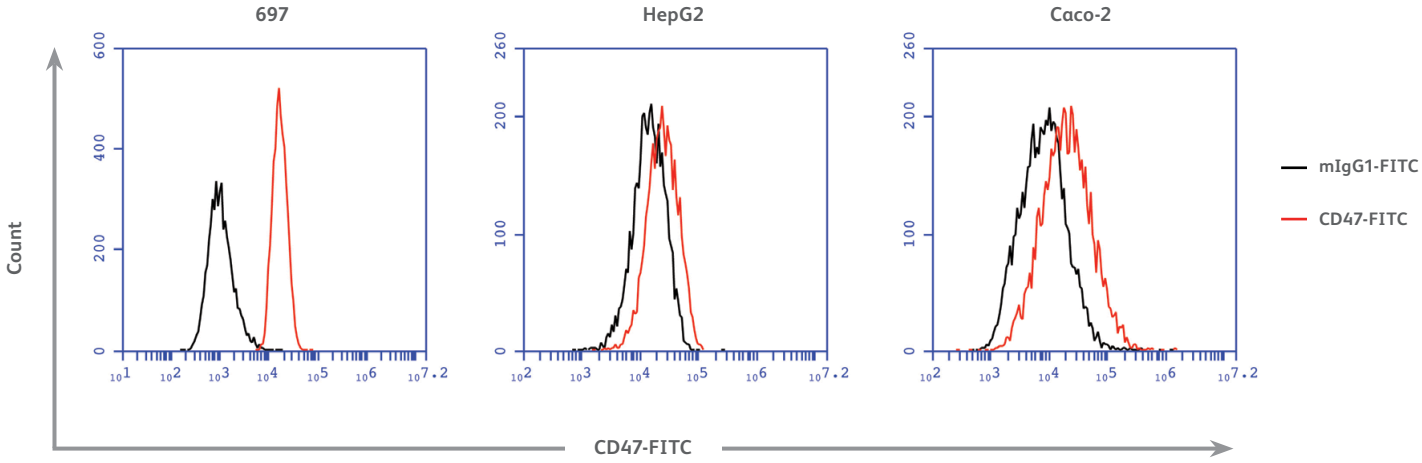

Figure 1. Relative expression of CD47 on selected cancer cell lines
Cancer cell lines were stained with either mIgG1 MOPC-21 FITC or anti-CD47 B6H12 FITC. The samples were acquired on a BD Accuri™ C6 Plus flow cytometer equipped with the BD CSampler™ Plus automated sampling accessory and the data was analyzed using the BD Accuri™ C6 Plus software. **A**. The signal-to-noise ratio was used to measure the relative levels of expression of CD47 on the tested cell lines. **B**. Sample histogram overlays show CD47 expression for 697, HepG2 and Caco-2 cell lines.

We selected three cell lines for further study based on the resulting CD47 expression. Cell line 697 (acute lymphoblastic leukemia, DSMZ ACC 42) expressed moderate levels of CD47 and is known to be susceptible to phagocytosis by monocyte-derived macrophages,[2] whereas the HepG2 (human liver cancer, ATCC HB-8065) and Caco-2 (human epithelial colorectal cancer, ATCC HTB-37) cell lines expressed extremely low levels of CD47 (Figure 1B).

Based on previous research, we selected 10 µg of anti-CD47 blocking antibody (purified NA/LE CD47) for the ensuing experiments. To confirm that this concentration sufficiently blocks CD47, we incubated the cells first with purified antibody followed by staining with the same antibody clone conjugated to FITC. The lack of FITC signal confirmed that all anti-CD47 binding sites were indeed occupied by the blocking antibody (Figure 2).

We derived macrophages from monocytes by culturing them for 8 days with macrophage colony-stimulating factor (M-CSF). The selected cancer cell lines were labeled with CFSE, co-cultured with the macrophages for 6 hours and analyzed on the BD Accuri C6 Plus. Phagocytosis was measured by quantifying $CFSE^+$ cells in the macrophage population, identified by their positive CD11b expression. Due to the brightness of CFSE, we used a 99% attenuation filter in FL1 to keep the signal on scale.

Figure 2

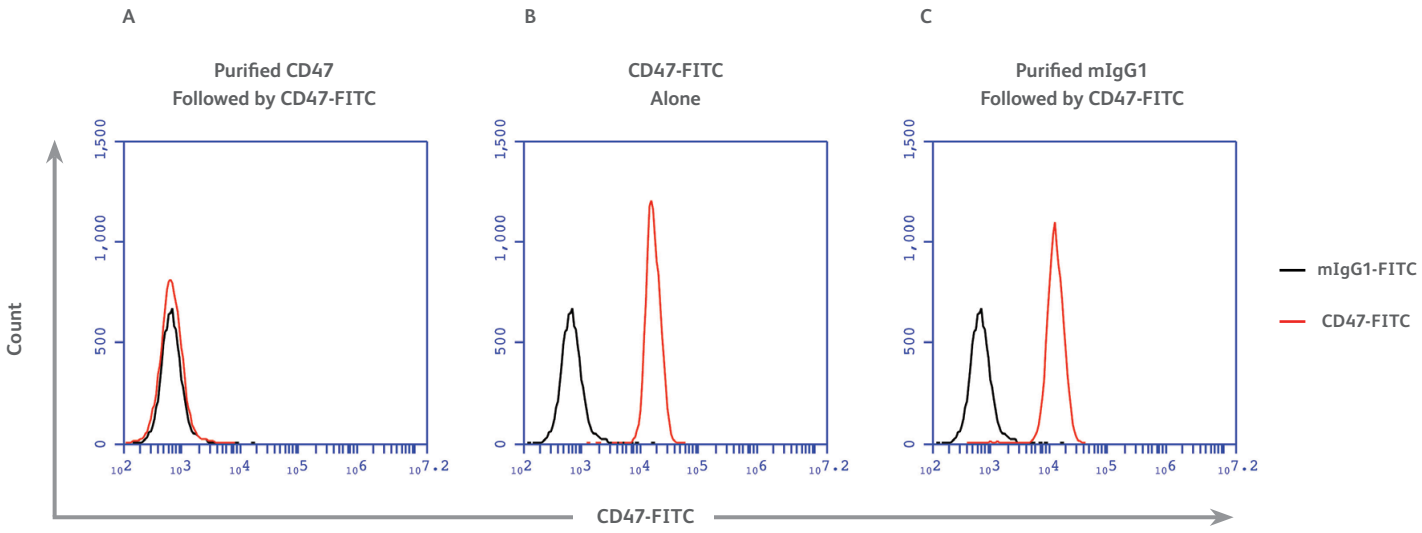

Figure 2. Validation of CD47 blocking
The 697 cells were incubated for 30 minutes at room temperature with 10 µg of BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD47, prior to staining with BD Pharmingen™ FITC Mouse Anti-Human CD47 (red, **A**). Cells stained with FITC anti-CD47 alone (**B**), or cells pre-incubated with BD Pharmingen™ Purified Mouse IgG1 (isotype control), followed by staining with FITC anti-CD47 (**C**), were used as controls. **Results:** Incubation with purified NA/LE anti-CD47 resulted in complete blocking of CD47 (**A**) as compared to controls (**B, C**).

The results are shown in Figure 3. Without CD47 blocking, the basal phagocytosis level (Q3-UR) for the 697 cells was lower than for the HepG2 and Caco-2 cells, likely due to higher expression of CD47 in 697 cells. Blocking CD47 in the 697 cells (Figure 3A, right) resulted in a tenfold increase in phagocytosis, confirming that CD47 inhibitory signals play a role in the inhibition of phagocytosis. For the HepG2 and Caco-2 cells, blocking with CD47 did not affect phagocytosis, as expected due to their low levels of CD47 expression. The overall low phagocytosis of these cells may imply that CD47:SIRP-α binding is not the only mechanism for inhibiting phagocytosis in these cell lines (Figures 3B and 3C).

These experiments demonstrate how flow cytometry assays of four or fewer colors on the BD Accuri C6 Plus can assess the expression levels of target antigens involved in inhibition of immune functions as well as measuring phagocytic activity of macrophages.

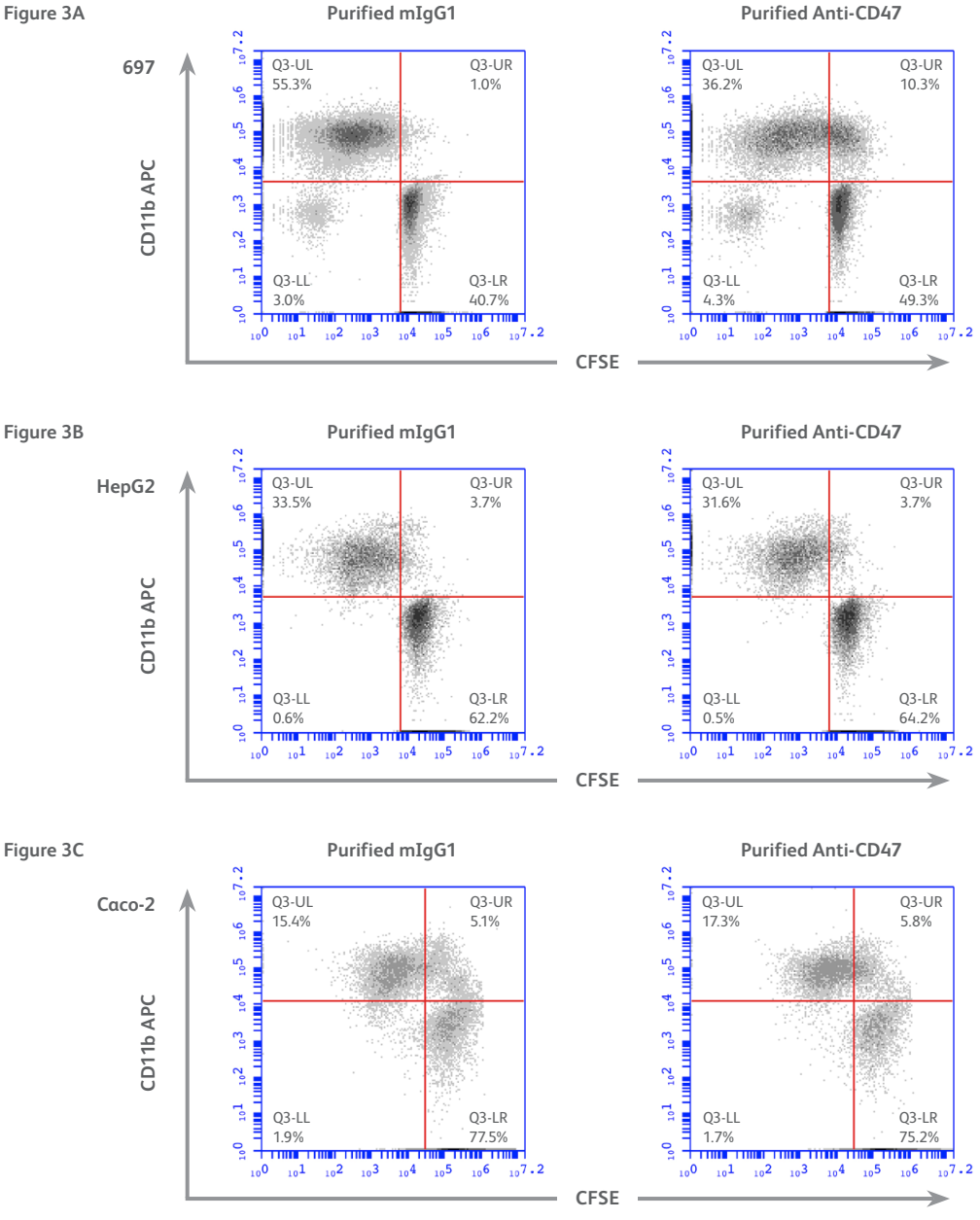

Figure 3. Effects of blocking CD47 on phagocytosis

Peripheral blood mononuclear cells were cultured for 4 hours in complete medium. Adhering monocytes were then cultured in the presence of M-CSF (50 ng/ml) for 8 days. Monocyte-derived macrophages were co-cultured with CFSE-labeled 697 (**A**), HepG2 (**B**) or Caco-2 (**C**) cells at a 1:1 ratio along with 10 µg of either purified mIgG1 (left plots) or purified NA/LE mouse anti-human CD47 (right plots) in a U-bottom 96-well plate. Cells were stained with CD11b APC to identify the macrophages. Cells were spun down at 300*xg* and co-cultured for 6 hours. Samples were acquired on the BD Accuri C6 Plus fitted with the BD Accuri™ FL1 99% Attenuation Filter. **Results:** Macrophages and target cancer cell lines can be discriminated based on the mutually exclusive expression of CD11b and CFSE, respectively. Phagocytosis can be measured as a function of the percentage of $CD11b^{+}CFSE^{+}$ cells, representing macrophages that have bound/ingested target cells. **A**. Co-culture of macrophages with 697 cells in the presence of mIgG1 (left plot) resulted in a minimal basal level of phagocytosis. Blocking with anti-CD47 (right plot) increased phagocytosis tenfold. **B, C**. The basal levels of HepG2 and Caco-2 cell phagocytosis were slightly higher as compared to 697 cells. Phagocytosis was not appreciably changed by blocking with anti-CD47, which was as expected considering the low levels of CD47 expression in these cell lines.

Ordering information

Systems and software	
Description	Cat. No.
BD Accuri™ C6 Plus Flow Cytometer System	660517
BD CSampler™ Plus Automated Sampling System (optional)	660519
BD Accuri™ FL1 99% Attenuation Filter	653172

Reagents	
Description	Cat. No.
BD Pharmingen™ APC Mouse Anti-Human CD11b/Mac-1	550019
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD14	562692
BD Pharmingen™ FITC Mouse Anti-Human CD47	556045
BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD47	556043
BD Pharmingen™ FITC Mouse IgG1	555748
BD Pharmingen™ Purified Mouse IgG1	555746
BD Horizon™ CFSE	565082

References

1. Barkal AA, Weiskopf K, Kao KS, et al. Engagement of MHC class I by the inhibitory receptor LILRB1 suppresses macrophages and is a target of cancer immunotherapy. *Nat Immunol*. 2018;19:76-84.
2. Métayer LE, Vilalata A, Amos Burke GA, Brown GC. Anti-CD47 antibodies induce phagocytosis of live, malignant B cells by macrophages via the Fc domain, resulting in cell death by phagoptosis. *Oncotarget*. 2017;8:60892-60903.

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