LETTER TO THE EDITOR

The highly selective Bruton tyrosine kinase inhibitor acalabrutinib leaves macrophage phagocytosis intact

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Figure S1

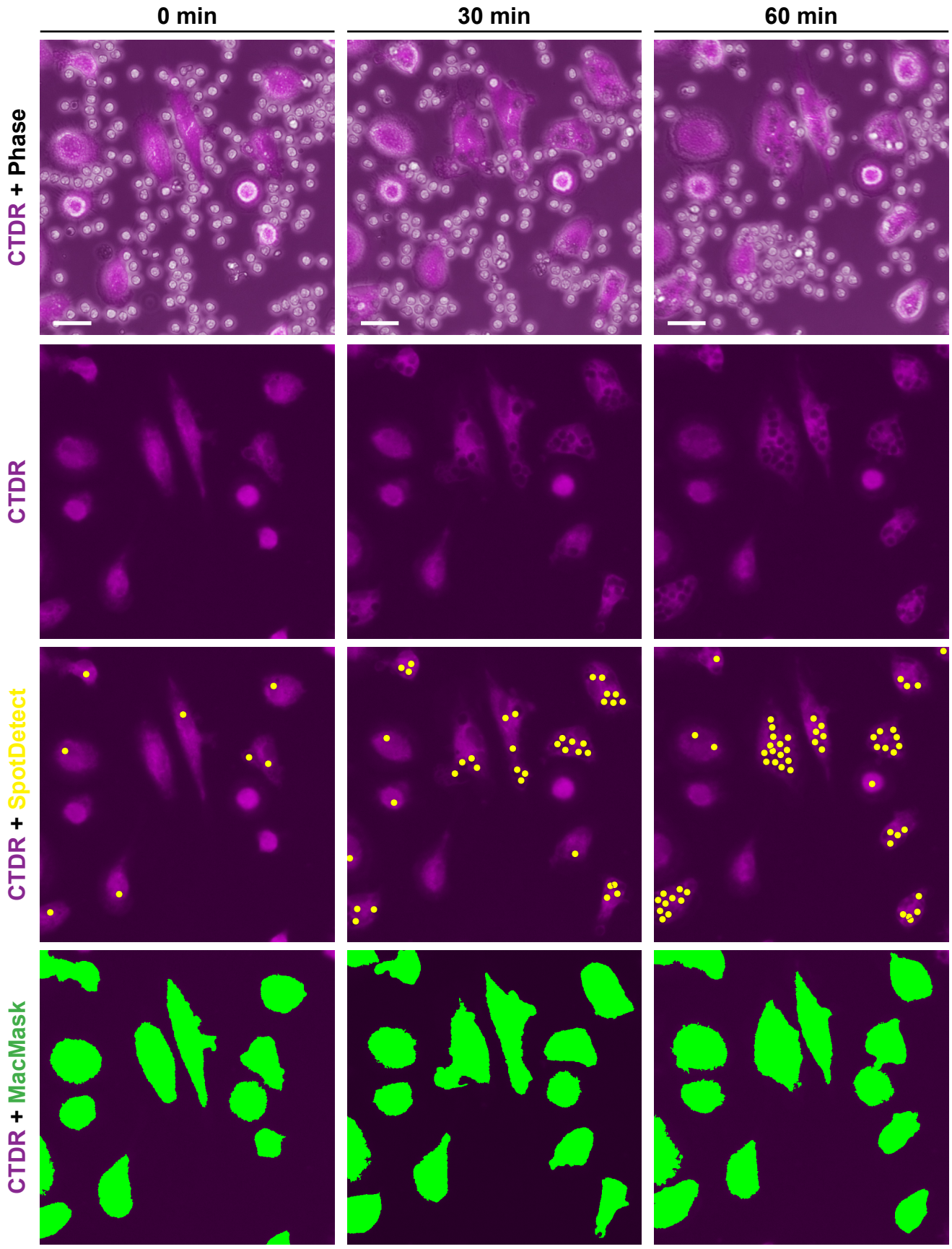
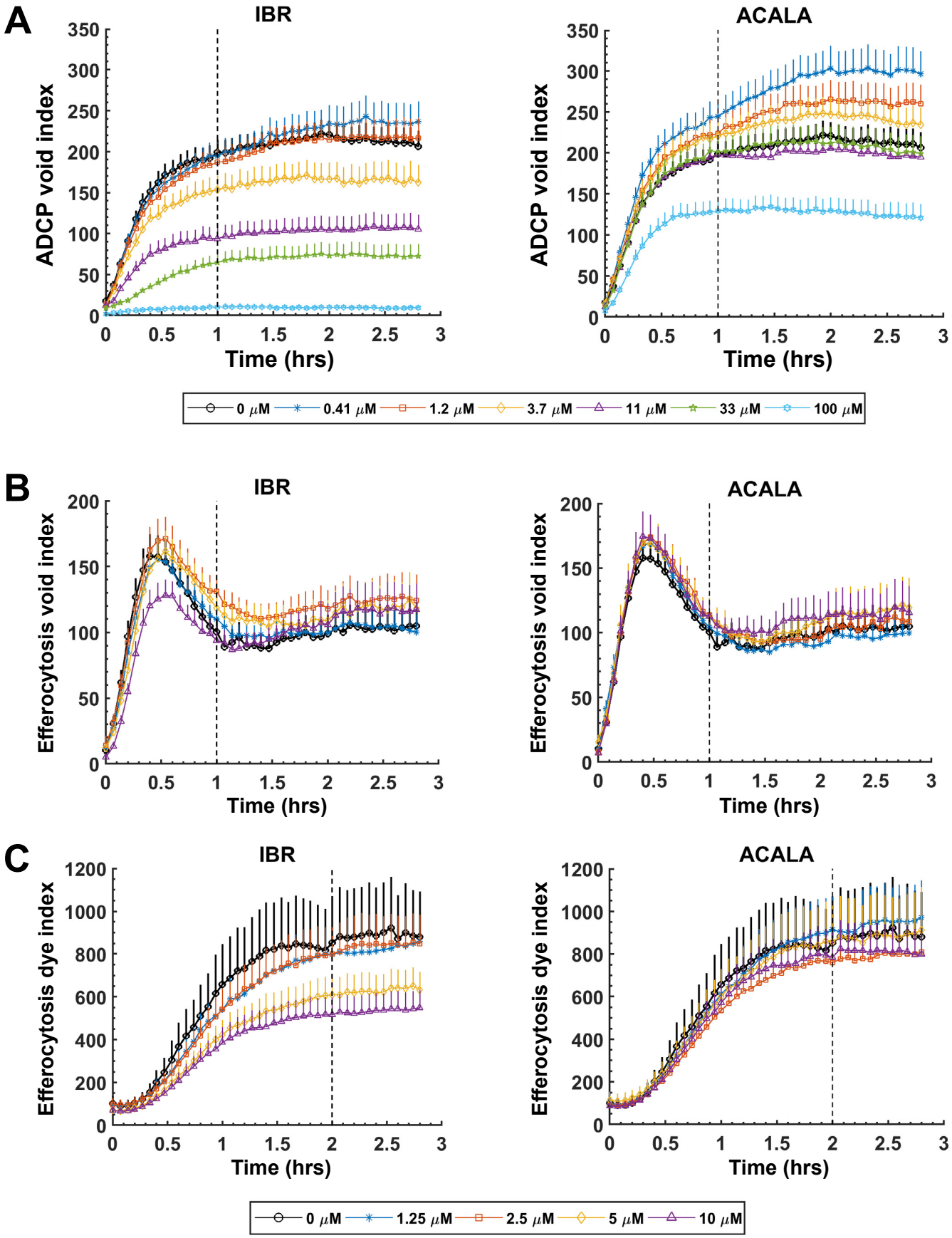

0 min
30 min
60 min
CTDR + Phase
CTDR
CTDR + SpotDetect
CTDR + MacMask

Figure S2


A
IBR
ACALA
ADCP void index
Time (hrs)
0 μM
0.41 μM
1.2 μM
3.7 μM
11 μM
33 μM
100 μM
B
IBR
ACALA
Efferocytosis void index
Time (hrs)
C
IBR
ACALA
Efferocytosis dye index
Time (hrs)
0 μM
1.25 μM
2.5 μM
5 μM
10 μM

Supplementary Figure Legends

Figure S1. Phagocytic quantitation method.

Live cell time-lapse video imaging of phagocytosis continuously measures actual engulfments as compared to previous measurements of single timepoints by pH sensitive-dye intensity changes after phagolysosomal processing or by loss of target cells.[1] $CD14^+$ peripheral blood mononuclear cells from anonymous healthy blood donors (American Red Cross) were matured into human monocyte-derived macrophages (hMDM) with 10 ng/ml human macrophage colony stimulating factor (M-CSF, PeproTech)[2] in 96-well black cyclic olefin microplates with optically clear cyclic olefin foil bottoms (CellCarrier-96 Ultra, PerkinElmer) and labeled in situ with Cell Tacker Deep Red (CTDR, Thermo Fisher Scientific).[1] An XY position in each well was imaged initially for three video frames after target cells were added. For antibody-dependent cellular phagocytosis (ADCP), anti-CD20 monoclonal antibody (mAb) was added and images were captured for the remaining 2.8 h in phase contrast and CTDR channels (**Video DS1**). Representative images of a ~8% subsection of the full field of view are shown for 0 min (left column), 30 min (middle column), and 60 min (right column) to illustrate ADCP void quantitation. Target internalization is difficult to assess when the phase and CTDR channels are shown together (top row) (**Video DS1**), but the visualization of voids makes assessment much easier when the CTDR channel is shown alone (second row) (**Video DS2**). Using NIS Elements AR 4.50 software (Nikon), we created a SpotDetect binary layer to count individual voids (yellow, third row) (**Video DS3**) and a MacMask binary layer to count the number of macrophages (green, fourth row) (**Video DS4**). These counts are used to calculate a phagocytic void index as a normalized measure of phagocytic engulfment events.[1, 3] For efferocytosis, pHrodo iFL Red STP ester (pHrodo Red, Thermo Fisher Scientific)-labeled apoptotic CLL target cells were added and an additional pHrodo Red color channel is collected during video imaging for measurement of changes in dye intensity with low pH. The pHrodo Red dye intensity is measured within the MacMask for each frame and used to calculate the dye intensity index.[1, 3] Scale bars = 25 μM.

Figure S2. Full length quantitation of acquired time-lapse images of phagocytosis after treatment with ibrutinib or acalabrutinib.

(A) Ibrutinib inhibits ADCP to a greater extent than acalabrutinib. Live cell time-lapse video imaging of rituximab-mediated ADCP of CLL cells by hMDM either untreated or treated with IBR or ACALA was collected as in **Figure 1B, Figure S1, and Figure DS1**. At each time point, phagocytic void and macrophage counts were converted to a void index as a measure of ADCP. ADCP time course over 2.8 h is shown for IBR (left) and ACALA (right) with drug concentrations indicated. The mean of 18 experiments for each time-point measured in duplicate or triplicate is shown. Only positive standard error bars shown for clarity. Subsequent AUC measurements use data from 0-1 h (vertical dotted line, **Figure 1C**). **(B)** Ibrutinib or acalabrutinib had little effect on kinetics of efferocytosis as measured by void index. Live cell time-lapse video imaging of apoptotic CLL cells (prepared by treatment with 1 μM venetoclax for 4h (~90% apoptotic, **Figure DS2**) by hMDM either untreated or treated with IBR or ACALA was

collected as in **Figure 2B, Figure S1, and Figure DS3**. At each time point, phagocytic void and macrophage counts were converted to a void index. Efferocytosis void index time course over 2.8 h is shown for IBR (left) and ACALA (right) with drug concentrations indicated. The mean of 7 experiments for each time-point measured in duplicate or triplicate is shown. Only positive standard error bars are shown for clarity. Subsequent AUC void index measurements use data from 0-1 h (vertical dotted line, **Figure 2C**). **(C)** Ibrutinib modestly inhibits phagolysosomal processing during efferocytosis as compared to acalabrutinib or untreated. Live cell time-lapse high-content microscopy imaging data was collected as described in **Figure 3 and Figure DS4** to assess phagolysosomal processing during efferocytosis. The dye intensity index time course over 2.8 h is shown for IBR (left) and ACALA (right) with drug concentrations indicated. An increase in dye intensity is a measure of increased acidity that is found in the phagolysosomal compartment. The mean of 6 experiments for each time-point measured in duplicate or triplicate is shown. Only positive standard error bars are shown for clarity. Subsequent AUC dye intensity measurements use data from 0-2 h (vertical dotted line, **Figure 3B**).

Supplementary Figure References

1. Chu CC, Pinney JJ, Whitehead HE, et al. High-resolution quantification of discrete phagocytic events by live cell time-lapse high-content microscopy imaging. J Cell Sci. 2020 Mar 5;133(5):jcs237883.
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3. Pinney JJ, Rivera-Escalera F, Chu CC, et al. Macrophage hypophagia as a mechanism of innate immune exhaustion in mAb-induced cell clearance. Blood. 2020 Jun 17;136(18):2065-2079.