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Communication

μMap-Interface: Temporal Photoproximity Labeling Identifies F11R as a Functional Member of the Transient Phagocytic Surfaceome

Sean W. Huth, Jacob B. Geri, James V. Oakley, and David W. C. MacMillan*

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ABSTRACT: Phagocytosis is usually carried out by professional phagocytic cells in the context of pathogen response or wound healing. The transient surface proteins that regulate phagocytosis pose a challenging proteomics target; knowledge thereof could lead to new therapeutic insights. Herein, we describe a novel photocatalytic proximity labeling method: "μMap-Interface", allowing for spatiotemporal mapping of phagocytosis. Utilizing photocatalyst-conjugated IGG-opsonized beads and initiating phagocytosis in a synchronized manner, we capture phagocytic interactome "snapshots" at the interface of the phagocyte and its target. This allows profiling of the dynamic surface proteome of human macrophages during the engulfment process. We reveal previously known phagocytic mediators as well as potential novel interactors and validate their presence with super-resolution microscopy. This includes F11R, an important cancer target yet to be investigated in the context of phagocytosis. Further, we demonstrate that knocking down F11R leads to an increased degree of phagocytosis; this insight could contribute to explaining its oncogenic activity. Lastly, we show capture of orthogonal phagocytic surfaceomes across different cells, using a neutrophil-like model. We believe this method will enable new insights into phagocytic processes in a variety of contexts.

Phagocytosis, the mechanism by which cells such as macrophages engulf and destroy pathogens and cancer cells (Figure 1A), is often subject to dysregualtion,[1] an aberrant pathway that underlies a host of disease states.[2,3] For example, the suppression of macrophage phagocytic activity in tumor microenvironments (TMEs) is known to enable tumor growth.[4] For this reason, numerous clinical efforts exist to regulate phagocytosis to better fight these disease states.[5] Despite these studies, a significant knowledge gap exists regarding the dynamic proteome of phagocytosis, especially at the phagocytotic cup.[6] These transient interaction networks are challenging to capture with current proteomics methods, with most studies primarily focused on isolation of phagosomes, or global protein profiling.[7,8] While these studies have been informative as to the nature of phagocytic proteomes, none have thus far captured the actively engulfing phagocytic cup. Herein, we describe a novel method for elucidating the protein interaction network involved in dynamic FC-mediated phagocytosis: μMap-Interface. Using this method, we not only temporally profile the phagocytic surfaceome in several contexts, but also discover a new functionally relevant phagocytic mediator: F11R. We believe these insights will lead to further understanding of phagocytosis that can inform next-generation therapeutic strategies.

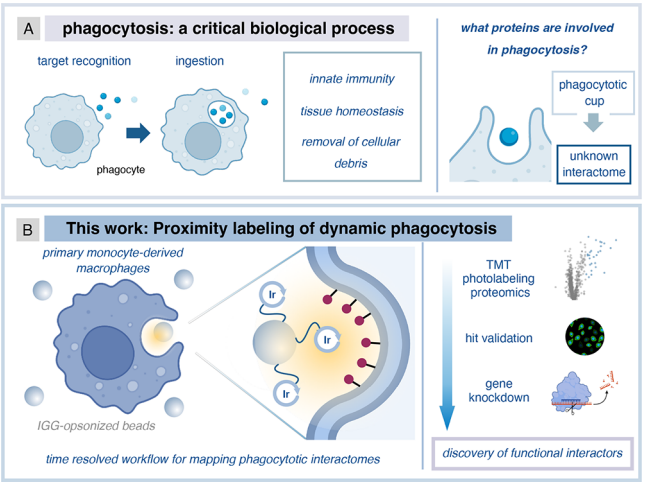

Figure 1. Overview of phagocytosis importance and the new photocatalytic μMap method to probe the phagocytic surface proteome.

It is generally accepted that phagocytosis occurs in four key steps: (i) detection of the target; (ii) activation of internalization; (iii) phagosomal cup formation; and (iv) lysosomal fusion to the phagosome and degradation.[9] Though methods have been described to profile and isolate phagosomes, as well as to probe surface proteomes of professional phagocytes, we were interested in capturing interactome changes during early phagocytosis, in proximity to the target−phagocyte interface.

Our laboratory has recently introduced μMap, a novel photocatalytic proximity labeling technology which can label cell-surface protein−protein interactions with high-resolu-

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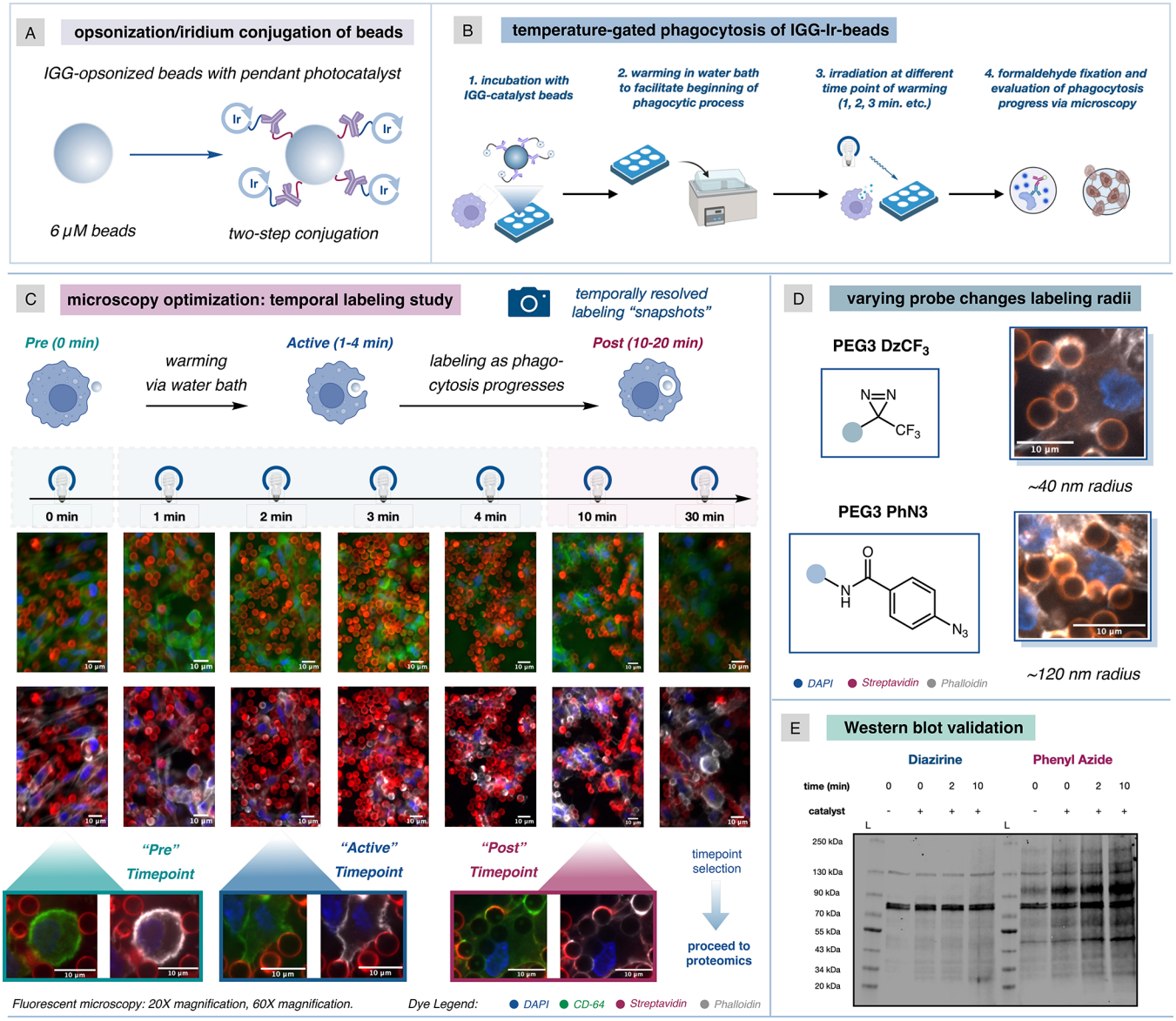

Figure 2. (a) Schematic for functionalization of carboxy beads, first with human IGG antibody for opsonization, then with iridium-photocatalyst for labeling experiments. (b) Temperature-gated activation of phagocytosis of macrophages in a synchronized manner; description of temporal optimization workflow. (c) Microscopy images showcasing time point optimization of phagocytosis labeling, with 20× wide-field images captured in 1 min intervals at 1−4 min, highlighting precise temporal control, and then at 10 and 30 min. The selected "before", "during", and "after" stages of active phagocytosis and their corresponding time points are highlighted on the bottom at 60× magnification. (d) Streptavidin staining via microscopy reveals a larger cross-section of phagocytic cup labeling consistent with a larger radius probe by microscopy. (e) Western blot labeling validation of labeling for both probes.

tion.[10] We reasoned that this unique high resolution proximity labeling would permit capture of proteins at the active phagocytic cup interface. Furthermore, μMap allows for precise temporal control, a necessary feature given that the macrophage-mediated engulfment of particles occurs within minutes.[11] Other novel photocatalytic strategies have also been recently described which have demonstrated the utility of this approach to capture "surfaceomes", or cell-surface interaction networks on living cells.[12,13] On this basis, we sought to expand our previously delineated μMap technology for phagocytic interactome capture. One robust existing method of studying FC-dependent phagocytosis involves the use of IGG-functionalized micron-beads that mimic opsonization.[14,15] These beads are then engulfed by macrophages in a FC-receptor dependent manner. This approach has allowed for imaging of the recognition, engulfment, and postengulfment process.[16] Thus, we conjugated polystyrene beads with human IGG, followed by iridium catalyst conjugation directly to the IGG (Figure 2A). This process creates opsonized beads that are capable of generating reactive intermediates. Due to its light-gated nature, this method, which we term "μMap-Interface", offers unique insight into the surface proteome or "surfaceome" of the phagocytic cup. (Figure 1B).

To capture the transient phagocytic cup via proteomics, we require a high degree of synchronicity. Lower temperatures (~4 °C) inhibit cytoskeletal remodeling events, including phagocytosis.[17,18] Thus, a temperature-gated approach could control FC-mediated phagocytosis, allowing surface proteome assessment at pre, active, and post phagocytosis time points. To test this strategy, we isolated human monocytes from

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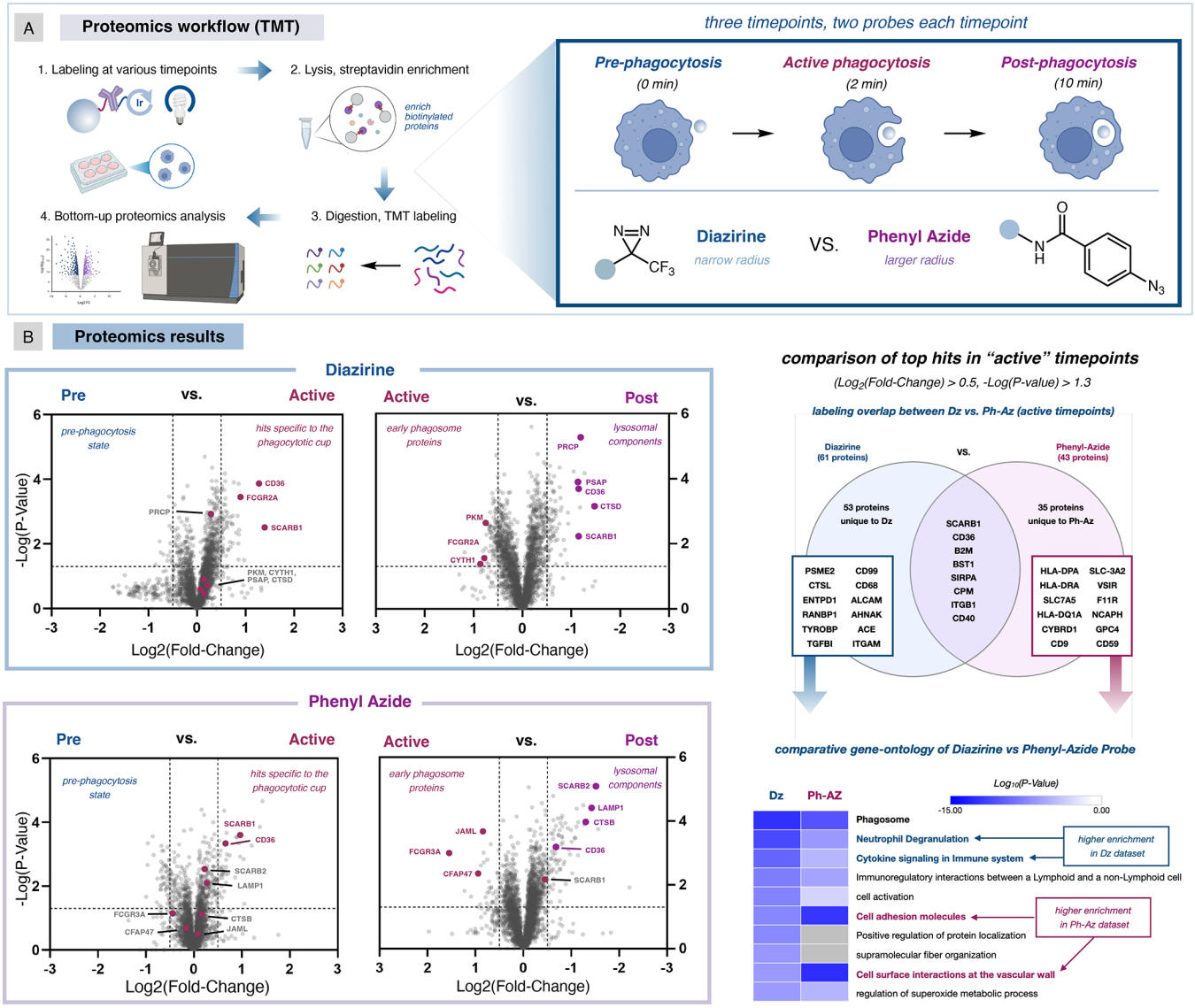

Figure 3. (a) Overview of TMT-based proteomics workflow: 1) labeling, 2) lysis and labeled protein enrichment, 3) peptide digestion/labeling, and 4) bottom-up TMT proteomics analysis. Time points analyzed are described as well as the two probes used for each time point. (b) Proteomics data comparing of both pre- vs active phagocytosis states and active vs postphagocytosis states. Data sets were generated for both the diazirine (narrow radius probe) and phenyl-azide (wider radius probe). Prephagocytosis states contained few enriched proteins, active phagocytosis states contained proteins important to early phagocytosis (FC receptor, etc.), and postphagocytosis data contained lysosomal components (as expected for the phagolysosome) Probe comparison reveals 8 shared proteins among the top hits, most of which are phagocytosis/macrophage associated. Comparative gene ontology between the two probes in their "active" time points reveals that diazirine leads to higher direct phagosome enrichment, as well as other processes such as cytokine signaling, while phenyl-azide data show a higher enrichment for cell-adhesion-related processes.

PBMCs via magnetic separation (Figure S1). These monocytes were then plated and polarized via GM-CSF to M-0 phenotype macrophages in accordance with previous methods.[19,20]

Comparative proteomics of pre, active, and postengulfment states would require knowledge of warming kinetics. Due to the unique temporal control of μMap, we captured "snapshots", performing labeling across warming time points of 1 min intervals and determining phagocytosis progress via microscopy (Figure 2B, 2C). Following M-0 macrophage cooling to 4 °C, media was exchanged for fresh media containing IGG-Iridium conjugated beads. Cells were centrifuged at 4 °C to adhere beads to the bottom of wells, then excess beads were gently washed away with fresh media. Finally, media was replaced with fresh media containing 500 μM diazirine-PEG3-biotin. At this point, macrophage-containing plates were warmed in a 37 °C water bath via direct floatation for varying time points, followed by irradiation with blue light. Immediately after irradiation, cells were fixed with paraformaldehyde and prepared for microscopy.

Confocal analysis was performed upon fixed cells, using DAPI as a nuclear stain, CD64 as a cell surface marker, streptavidin for biotinylation, and phalloidin to better see the phagocytic cup.[21] Time point zero was used as a control for a pre-engulfment state, where macrophages could recognize IGG-coated beads but not remodel their cytoskeleton to perform phagocytosis. By scanning time points in 1 min intervals via microscopy (20× magnification), we observed that phagocytosis began as early as 1 min and continued over the

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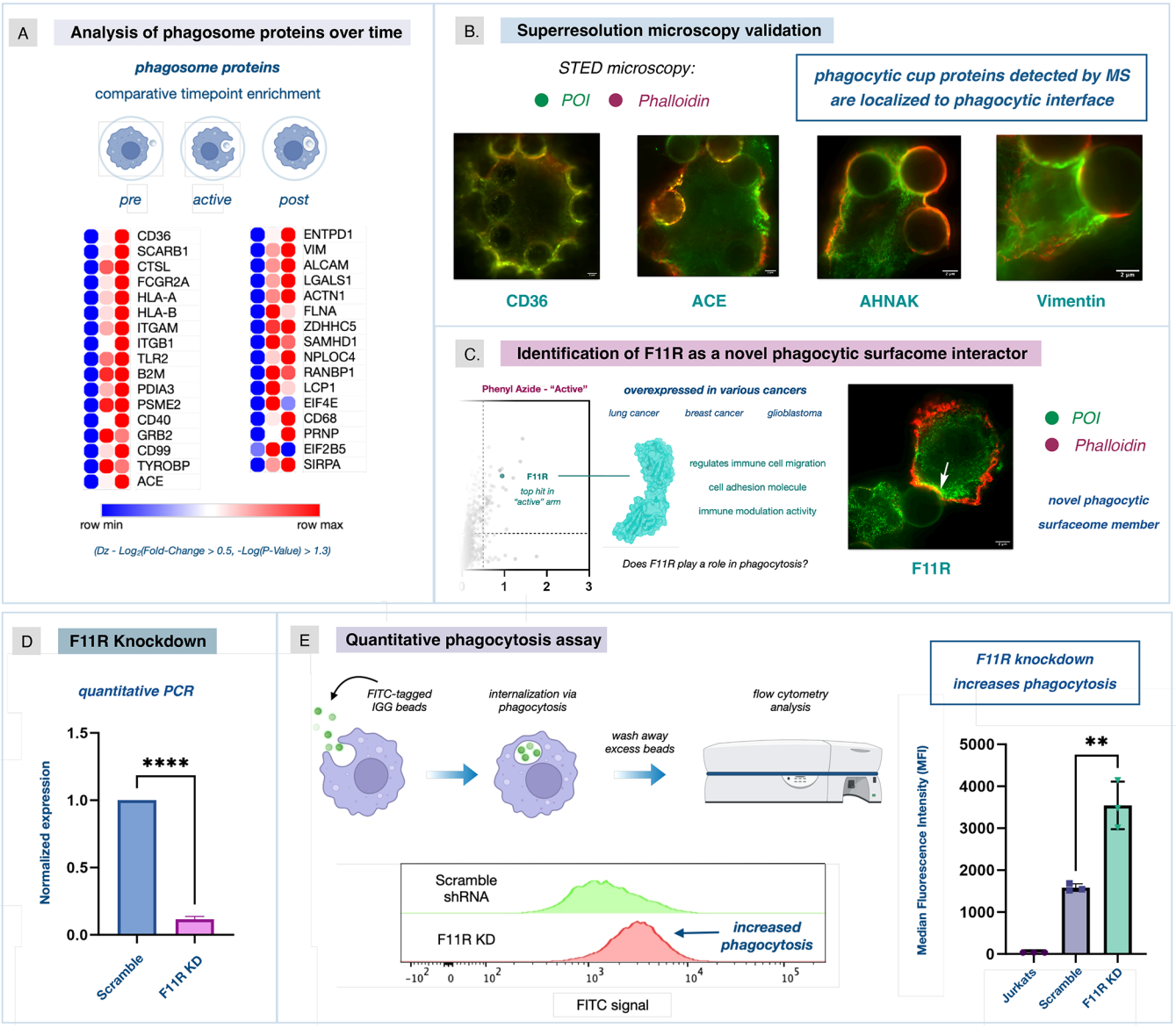

Figure 4. (a) Identification of phagosome proteins via gene ontology reveals an average protein intensity increase across time points (shown in the heatmap), consistent with capture of the "growing phagosome". (b) Super-resolution microscopy reveals the presence of proteins of interest in the phagocytic cup region (bead−cell interface). CD36, ACE, Vimentin, and AHNAK are validated as phagocytic surfaceome members detected via STED. (c) Among the top enriched hits in the phenyl-azide data is F11R, an adhesion protein with known important roles in cancer but not connected with phagocytic processes. Validation of F11R in the surfaceome via STED. (d) Confirmation of F11R knockdown via qPCR to conduct functional studies. (e) Overview of the quantitative flow cytometry phagocytosis assay. Analysis reveals an increase in fluorescence upon F11R knockdown, consistent with increased phagocytosis. This confirms the functional role of F11R in FC-mediated phagocytosis. Jurkat cells were used as a negative control.

span of the next 3 min. We selected 2 min as the "active" time point, since it resided in the middle of our determined engulfment time point range. (Figures 2C, S2). Finally, 10 min was selected as the "after" time point, as most beads were now internalized by the macrophages. Due to the small radius of our standard diazirine probe (~40 nm), it excels at capturing direct bead-cell contacts. To complement this data, we sought to use an additional probe with a larger radius to identify interactors extending further from the bead onto the phagocytic surface.[22] We hypothesized that more distal interactors could play an important role in phagocytic modulation, but would not be captured using our traditional carbene-intermediate based approach. To this end, we performed analogous labeling experiments with a phenyl-azide probe (~120 nm radius); pleasingly we were able to visualize via microscopy a larger portion of the phagocytic cup labeled, corresponding to a larger radius (Figure 2D). Labeling with both probes across time points was further confirmed via Western blotting (Figure 2E). Increased labeling at longer warming times was consistent with a higher degree of engulfment.

Having observed temporally resolved labeling of the phagocytic cup via microscopy, we profiled the corresponding proteomes using TMT-based proteomics. Macrophages were plated into large single-well plates, and three biological replicates each were used for the 0, 2, and 10 min time points, corresponding to pre-, active-, and postphagocytic periods, respectively, for both diazirine and phenyl-azide

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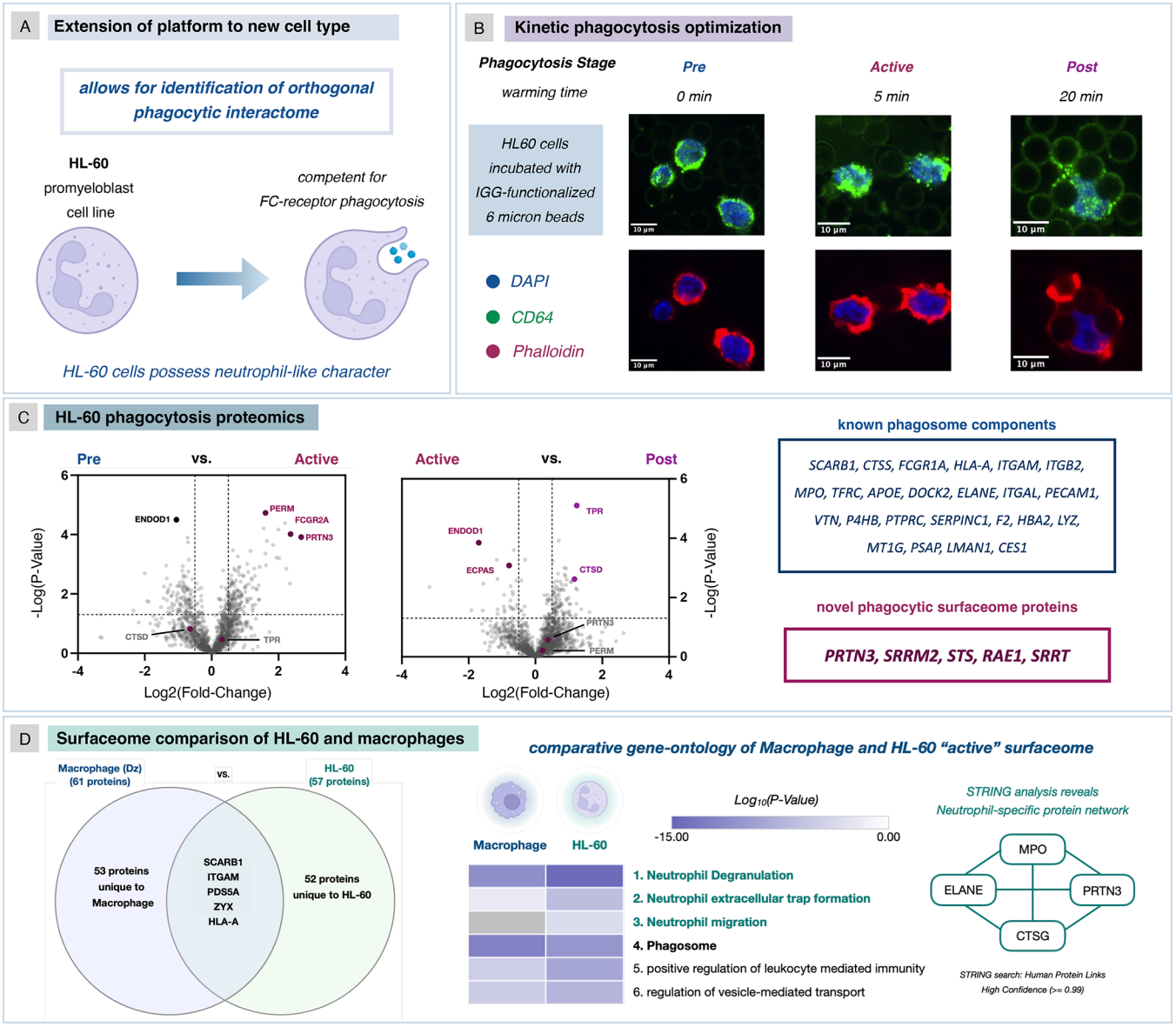

Figure 5. (a) Overview of HL60 cells as a neutrophil-like cell line to probe phagocytosis within another type of professional phagocytes. (b) Kinetics microscopy studies conducted as described previously revealed slightly slower phagocytosis, with active engulfment at 5 min and postphagocytosis at 20 min. (c) Proteomics results on HL-60 cells reveal known phagocytosis proteins enriched in the "active" time point, as well as neutrophil-specific and novel proteins. Postphagocytosis time point shows enrichment of lysosomal components but also TPR, which is a known neutrophil-specific protein involved in pathogen killing via super oxides. Known top-hit phagosome proteins, as well as novel ones, are tabulated. (d) Comparative analysis between macrophage and HL-60 surfaceomes reveals 5 common interactors. Comparative gene ontology reveals enrichment of neutrophil-characteristic processes in the HL-60 data, consistent with their neutrophil-like character. STRING analysis of HL-60 data unveils a network of 4 proteins found enriched (MPO, PRTN3, CTSG, ELANE), which are associated with neutrophils.

probes. After labeling, cells were lysed and subjected to affinity-pulldown via streptavidin beads. Enriched proteins were then cleaned up via standard TMT protocols and submitted for LCMS/MS shotgun proteomics (Figure 3A). Enrichment cutoffs were set at a 0.5 log-fold change, which allow for inclusion of subtle but meaningful changes in the surfaceome-enriched proteins. Excitingly, comparison of pre- and active phagocytosis time points for both probes showed strong enrichment of proteins associated with phagocytosis: integrins (ITGAM, ITGB1, ITGA6), other adhesion molecules (ALCAM, FLNA), and known macrophage receptors (SCARB1, SIRPA), including the FC-receptor.[23−26] In comparing post- to active phagocytosis, strong enrichment of lysosomal proteins was observed (CTSD, LAMP1, PRCP), consistent with formation of the phago-lysosome.[27,28] (Figure 3B). Analyzing the top-enriched "active" proteins between probes revealed some that were consistently enriched (CD36, SCARB1, SIRPA), but also many orthogonal interactors. Comparative gene ontology between the probes revealed a higher enrichment of the phagosome by the diazirine probe. The phenyl-azide probe in comparison showed higher enrichment of cell-adhesion processes. (Figure 3B, S3, S4). We hypothesize this difference partially arises from the narrow radius of the diazirine probe (higher phagosome enrichment), while the larger radius phenyl-azide probe captures more distal cell-adhesion molecules. While previous studies have demonstrated the relatively broad amino acid reactivity of both aryl azide and diazirine based probes, it is worth noting that

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orthogonal reactive species are generated.[22,29] In particular, aryl-azides are known to undergo several pathways to generate nitrenes, ketinimies, and benzazirines. This leads to slightly less efficient labeling, as well as differing amino acid selectivity, contributing to the orthogonality of our enriched interactors.[30,31]

We sought to analyze phagosome enrichment over time to confirm the temporally resolved nature of our method. This analysis revealed an increasing enrichment over time of phagosome-related proteins (Figure 4A, S5). Next, we sought to validate the localization and functional role of potentially novel proteins involved in phagocytosis. First, to validate phagocytic interactors, we performed stimulated emission-depletion microscopy to obtain super-resolution images of phagocytic cups. We employed THP-1 cells, which can be differentiated to macrophages via PMA stimulation and have previously been established as a model system for probing phagocytic function in macrophages.[32,33] After generating a list of interactors of interest, we fixed actively phagocytosing cells for STED analysis. Overlap between the actin stain and the protein of interest at the cell-bead interface was considered positive confirmation of its presence in the phagocytic cup. Four of the proteins enriched via MS—CD36, Vimentin, AHNAK, and ACE—have known or hypothesized roles in the phagocytic activity of macrophages.[34−37] Upon performing STED microscopy, we were pleased to see colocalization of these MS-identified hits (Figure 4B, S6).

Upon analyzing the "active" data time points for novel interactors, we found that F11R appeared as a top hit (Figure 4C). F11R, a junctional adhesion molecule, plays roles in adhesion modulation in cell-to-cell contacts.[38,39] Notably, F11R has also seen considerable interest as a cancer target, due to its role in lung cancer, gastric cancer, and glioblastoma.[40] Glioblastoma severity correlates with overexpression of F11R, and monocytes/macrophages in particular upregulate said protein.[41,42] We thus sought to investigate if F11R played a functional role in phagocytic capability of macrophages. Utilizing STED, we validated phagocytic cup localization of F11R (Figure S7). Utilizing THP-1 cells, we performed stable shRNA knockdowns of F11R; successful knockdown was validated via qPCR (Figure 4D) and Western blot (Figure S8). Using a previously established quantitative flow-cytometry experiment, we measured the impact of gene knockdown on phagocytic efficiency.[43,44] Following polarization, macrophages were incubated with FITC-tagged IGG opsonized beads, washed to remove excess beads, and then quenched with trypan blue. Dead cells were excluded via DAPI staining, and flow cytometry was performed to assay cell fluorescence corresponding to increased phagocytic ability.

Analysis of the data comparing a scramble shRNA control cell line revealed a significant increase in the fluorescence of F11R-knockdown macrophages, consistent with an increase in phagocytic ability (Figure 4E). This finding suggests a functional role of F11R in FC-mediated phagocytosis. We next performed targeted proximity labeling directly on F11R on the surface of THP-1 cells, which revealed enrichment of G-beta protein related processes previously associated with phagocytic activity.[45] Further studies will be necessary to obtain a full mechanistic picture, and determine F11R's role in phagocytosis in cancer contexts.

Having demonstrated the utility of our platform in macrophages, we sought to study other phagocytic cell types. HL-60 are promyelocytic cells capable of phagocytosis, and exhibit neutrophil-like properties, which we hoped to identify via proteomics (Figure 5A).[46] After confirming HL-60 phagocytotic activity (Figure S10), microscopy experiments revealed that HL-60 cells have slower phagocytosis kinetics than macrophages, reaching their "active" state at around 5 min and completing most phagocytosis by 20 min (Figure 5B). Upon validating the time points, we proceeded to perform proteomics.

Gratifyingly, we obtained high-quality proteomics data of HL-60 phagocytosis. Hits enriched in the "active" comparison include the FC-receptor and SCARB1, while the "post" comparison yielded lysosomal components and TPR, part of the neutrophil cytosolic factor 2[47,48] (Figure 5C). Excitingly, multiple proteins were found enriched in the "active" time point which are not currently known to be neutrophil phagocytic interactors. In particular, PRTN3, a serine protease, is associated with neutrophil granulocytes, but has not been linked directly to early phagocytic processes.[49] Further novel proteins identified include RNA-related proteins, such as SRMM2, an RNA splicing factor involved in cell−cell adhesion, as well as RAE1, which is thought to mediate attachment of messenger RNPs to the cytoskeleton.[50,51] Upon comparing proteins enriched in the "active" time point, we noted 5 proteins that overlapped, as well as a large orthogonal surfaceome. Comparative gene ontology analysis highlighted various neutrophil-related processes enriched higher in HL-60 cells, while STRING analysis revealed a neutrophil interaction network (MPO, ELANE, PRTN3, CTSG),[52−56] showcasing our method's ability to differentiate orthogonal phagocytic surfaceomes (Figure 5D, S11).

In conclusion, we describe herein the development of a novel method for capturing the dynamic surface proteome of phagocytosis. Using this method, we discovered F11R as a novel interactor and demonstrated functional relevance to phagocytic activity. We envision this method will constitute a valuable tool for the continued study of dynamic interactome changes during phagocytosis.

■ ASSOCIATED CONTENT

Data Availability Statement

Proteomics raw data has been uploaded to the MASSive database: MSV000095040.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.4c11058.

Experimental procedures and supplementary figures (PDF)

Tabulated proteomics data for primary macrophage proteomics experiments (XLSX)

Tabulated proteomics data for HL-60 proteomics experiments (XLSX)

qPCR data and analysis (XLS)

F11R proteomics data table containing F11R targeted proteomics data, as well as metascape analysis of top hits (XLSX)

Principal component analysis data of proteins in the TMT data set (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

David W. C. MacMillan − *Merck Center for Catalysis at Princeton University, Princeton, New Jersey 08544, United*

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States; Department of Chemistry, Princeton University, Princeton, New Jersey 08544, United States; orcid.org/0000-0001-6447-0587; Email: dmacmill@princeton.edu

Authors

Sean W. Huth − *Merck Center for Catalysis at Princeton University, Princeton, New Jersey 08544, United States; Department of Chemistry, Princeton University, Princeton, New Jersey 08544, United States*

Jacob B. Geri − *Merck Center for Catalysis at Princeton University, Princeton, New Jersey 08544, United States; Department of Chemistry, Princeton University, Princeton, New Jersey 08544, United States;* orcid.org/0000-0002-9215-5610

James V. Oakley − *Merck Center for Catalysis at Princeton University, Princeton, New Jersey 08544, United States; Department of Chemistry, Princeton University, Princeton, New Jersey 08544, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.4c11058

Notes

The authors declare the following competing financial interest(s): DWCM declares an ownership interest in the company Dexterity Pharma LLC, which has commercialized materials used in this work.

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