Figures and figure supplements

Chimeric antigen receptors that trigger phagocytosis

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Figure 1. Identification of intracellular signaling region for CAR-P. (A) Schematics show the structure of CAR-P constructs. An αCD19 (purple) or αCD22 (blue, center) scFv directs CAR specificity. Intracellular signaling domains from Megf10 or the indicated engulfment receptor (green) activate

**Figure 1 continued on next page**
engulfment. CAR-pGFP contains only GFP and no intracellular signaling domains (right). All constructs include a transmembrane domain from CD8 and a C-terminal GFP. (B) J774A.1 macrophages expressing αCD19 CAR-P with the indicated intracellular signaling domain (green) engulf 5 μm silica beads covered with a supported lipid bilayer containing His-tagged CD19 extracellular domain. The beads were visualized with atto390-labeled lipid incorporated into the supported lipid bilayer (magenta). Cells infected with the cell membrane marker, mCherry-CAAX, were used as a control (no CAR, top left). To the right of each image is a histogram depicting the frequency of cells engulfing the indicated number of beads. The average number of beads eaten per cell is quantified in (C). (D) Bone marrow derived macrophages were infected with CAR-FcRV or GFP-CAAX (green, left and center top; grey, center bottom) and incubated with CD19 beads (magenta) for 45 min. Images show an x-y plane through the center of the engulfed beads (left), or a cross section (center) of the z plane indicated in the inset panel (white line). The histogram depicts the number of cells engulfing the indicated number of beads. The scale bar indicates 5 μm and n = 78–163 cells per condition, collected during three separate experiments. Error bars denote 95% confidence intervals and *** indicates p<0.0001 compared to mCherry-CAAX control by Kruskal-Wallis test with Dunn’s multiple comparison correction.
Figure 1—figure supplement 1. Expression level of CAR-P constructs in macrophages. Images of macrophages infected with various αCD19 CAR-P-GFP constructs were acquired with identical acquisition settings and scaling to depict differences in expression levels. Fluorescent intensity at the cell cortex of 20 representative αCD19 CAR-P-GFP-infected macrophages was quantified using the mean intensity of a two pixel width linescan at the cell membrane, minus the mean intensity of a linescan immediately adjacent to the cell. The images are the same cells included in Figure 1B and fluorescent intensity was measured from the same macrophages assayed in Figure 1C. The dashed red line indicates the position of a bead in contact with the CAR-P-GFP macrophage. The scale bar indicates 5 μm.

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Figure 2. CAR-P expression drives specific engulfment of diverse beads. (A) Macrophages infected with the αCD19 (purple) or αCD22 (blue) CAR-p

Megf10 or mCherry-CAAX control were fed 5 μm beads ligated with either CD19 (left) or CD22 (right). Engulfment is quantified as the mean beads eaten per cell. The fraction of phagocytic cells is as follows: 31/144 GFP-CAAX cells engulfed CD19 beads, 87/149 αCD19 CAR-P

Megf10 engulfed CD19 beads, 20/142 αCD22 CAR-P

Megf10 engulfed CD19 beads, 28/140 GFP-CAAX cells engulfed CD22 beads, 18/151 αCD19 CAR-P

Megf10 engulfed CD22 beads, 103/148 αCD22 CAR-P

Megf10 engulfed CD22 beads (pooled data was collected during three separate experiments). Error bars denote 95% confidence intervals and *** indicates p<0.0001 compared to mCherry-CAAX control by Kruskal-Wallis test with Dunn’s multiple comparison correction. (B) J774A.1 macrophages expressing the αCD19 CAR-p

Megf10 (green) were fed beads of various sizes (magenta, diameter of bead indicated below image). The beads were covered in a supported lipid bilayer ligated to His-tagged CD19 extracellular domain and the number of beads engulfed per cell is reported below each image (magenta bars indicate CAR-p

Megf10 macrophages and pink bars indicate CAR-p

GFP). The αCD19 CAR-P

Megf10 macrophages were also incubated with 10 μm beads coated in phosphatidylserine (PS) and ICAM-1 (blue bar in graph, 51/390 cells engulfed a bead). The fraction of cells engulfing a CD19 bead is as follows: 135/169 CAR-P

Megf10 and 134/187 CAR-P

GFP cells engulfed a 2.5 μm bead, 126/395 CAR-P

Megf10 and 112/499 CAR-P

GFP cells engulfed a 5 μm bead, 48/377 CAR-P

Megf10 and 21/378 CAR-P

GFP cells engulfed a 10 μm bead, 120/706 CAR-P

Megf10 and 45/675 CAR-P

GFP cells engulfed a 15 μm bead, 194/760 CAR-P

Megf10 and 23/587 CAR-P

GFP cells engulfed a 20 μm bead (data is pooled from at least three separate experiments). Error bars denote 95% confidence intervals of the mean. *** indicates p<0.0001 respectively by Mann-Whitney test. All scale bars represent 5 μm.

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Figure 3. A phosphorylated ITAM at the cell-target synapse drives engulfment. (A) Macrophages expressing αCD19 CAR-P
Megf10 (green, top) or αCD19 CAR-P-GFP were incubated with CD19-ligated beads (position indicated with dotted line), fixed and stained for phosphotyrosine (magenta, top; greyscale, bottom). The fold enrichment of phosphotyrosine at the cell-bead synapse compared to the cell cortex is graphed on the right (n ≥ 11; each dot represents one cell-bead synapse, lines represent the mean ± one standard deviation). (B) Schematic shows the structure of CAR-P constructs in the plot at right. An αCD19 (purple) scFv directs CAR specificity. The intracellular signaling domains from CD3ζ activate engulfment. On the right is a histogram depicting the fraction of macrophages engulfing the indicated number of CD19-coated beads. (C) Comparison showing the average number of beads eaten per cell in J774A.1 macrophages expressing αCD19 CAR-Ps with the indicated intracellular signaling domain. 5 μm silica beads covered with a supported lipid bilayer containing His-tagged CD19 extracellular domain were used as an engulfment target (n = 156–167 cells per condition collected during three separate experiments). Error bars denote 95% confidence intervals and *** indicates p<0.0001 compared to CAR-P-GFP control by Kruskal-Wallis test with Dunn’s multiple comparison in correction. (D) Model of the liposome-based fluorescence quenching assay used to determine affinity between the Syk tSH2 domains and the receptor tails of CD3ζ and FcRγ, two intracellular signaling domains that promote engulfment. Binding between the Syk tSH2 reporter (Syk tSH2), green, and a receptor tail, purple, was detected by rhodamine quenching of BG505 dye on the reporter (see Materials and methods). Kd was determined by assessing mean fluorescence quenching for the last 20 timepoints collected −45 min after ATP addition over a receptor titration from 0 to 500 nM. Each point represents the mean ± SD from three independent experiments. Kd ± SE was calculated by nonlinear fit assuming one site specific binding.

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Figure 3—figure supplement 1. F-actin is enriched at the cell-target synapse. Phalloidin staining (magenta overlayed with brightfield and DAPI, left; heatmap, right) of F-actin in a CAR-P^FcR_Y expressing macrophage shows a 2.3 fold enrichment at the cell-bead synapse (standard deviation of 1.2). The graph depicts actin enrichment at 30 cell-bead synapses collected on three separate days. Each dot represents a cell-bead synapse. The box plot indicates the interquartile range. The cell is in contact with a second bead, but this site of contact has not initiated cup formation and no actin enrichment.

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Figure 4. CAR-P promotes trogocytosis and whole cell eating. (A) J774A.1 macrophages expressing the αCD19 CAR-P<sup>Megf10</sup> (top panel, green in merge, left; greyscale, center) engulf pieces of CD19<sup>+</sup>Raji B cells (labeled with mCherry-CAAX; magenta in merge, left; greyscale, right). The corresponding control αCD19 CAR-P<sup>GFP</sup>-infected cells are shown below. Arrows point to pieces of ingested Raji B cell. The proportion of CAR-P expressing macrophages internalizing one or more bite within 90 min is quantified on the right. Bites are defined as a fully internalized mCherry-positive vesicle >1 μm in diameter; n = 46 CAR-P<sup>Megf10</sup> macrophages, n = 39 CAR-P<sup>FcRv</sup> macrophages and 102 CAR-P<sup>GFP</sup> macrophages acquired during three separate experiments. (B) Time course of a J774A.1 macrophage expressing CAR-P<sup>FcRv</sup> (green) internalizing a whole Raji B cell labeled with mCherry-CAAX (magenta). These images correspond to frames from Figure 4—video 2. (C) Schematic shows the structure of CAR-P<sup>tandem</sup> construct, Figure 4 continued on next page
combining the intracellular signaling domain from FcRγ and the p85 recruitment domain from CD19. (D) Time course of a J774A.1 macrophage expressing CAR-P\textsuperscript{tandem} (green) internalizing a whole Raji B cell labeled with mCherry-CAAX (magenta). These images correspond to frames from Figure 4—video 3. (E) Macrophages and Raji B cells were incubated together at a 1:2 macrophage:Raji ratio, and the number of whole Raji B cells eaten per 100 macrophages during 4–8 hr of imaging is graphed. Graph depicts pooled data from four independent experiments; n = 921 CAR-p\textsuperscript{GFP}, n = 762 CAR-p\textsuperscript{p85γ}, n = 638 CAR-p\textsuperscript{p110αγ}, n = 555 CAR-p\textsuperscript{tandem} cells. Sample sizes were selected for their ability to detect a 5% difference between samples with 95% confidence. (F) 10,000 macrophages and 20,000 Raji B cells were incubated together for 44 hr. The number of Rajis was then quantified by FACS. 2–3 technical replicates were acquired each day on three separate days. The number of Rajis in each replicate was normalized to the average number present in the GFP-CAAX macrophage wells on that day. * indicates p<0.01, *** indicates p<0.0001 by two-tailed Fisher Exact Test (a and e) or by Ordinary one way ANOVA with Dunnet’s correction for multiple comparisons (f); error bars denote 95% confidence intervals.

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Figure 4—figure supplement 1. CAR-P localizes with pTyr at synapse with Raji B cell. Phosphotyrosine staining (teal) of macrophages expressing CAR-pMegf10 (green) in contact with Raji B cells (cell membrane visualized with mCherry-CAAX, magenta). Below, the enrichment at the synapse is quantified as the mean intensity of a five pixel width linescan at the synapse divided by the mean intensity at the adjacent cell cortex for at least 11 sites of contact. Each dot represents one cell-cell synapse, lines represent the mean ± one standard deviation, and the graph is the pooled results of three biological replicates. The scale bar indicates 5 μm.
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**Figure 4—figure supplement 2.** NIH 3T3 cells internalize Raji B cell bites. NIH 3T3 cells expressing the αCD19 CAR-\textit{p\textsuperscript{Megf10}} (green in merge, left; greyscale, center) engulf pieces of CD19\textsuperscript{+}Raji B cells (labeled with mCherry-CAAX, magenta in merge, left; greyscale, right). The control αCD19 CAR-\textit{p\textsuperscript{GFP}}-infected 3T3s are shown below. Arrows point to pieces of ingested Raji B cell. The proportion of cells taking at least one bite after 90 min co-incubation is graphed on the left (graphs show the pooled data of three separate experiments, n = 111 CAR-\textit{p\textsuperscript{Megf10}} 3T3 cells and 121 CAR-\textit{p\textsuperscript{GFP}} 3T3; *** indicates p<0.0001 by two-tailed Fisher Exact Test; error bars denote 95% confidence intervals). Bites are defined as a fully internalized piece of mCherry-labeled material >1 μm in diameter.

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Figure 4—figure supplement 3. Opsonization by an anti-CD47 antibody enhances whole cell internalization through CAR-P Macrophages expressing CAR-P<sup>FcR<sub>V</sub></sup> and Raji B cells were incubated together at a 1:2 macrophage:Raji ratio (20,000 macrophages and 40,000 Rajis) without antibody addition (No ab) or in the presence of anti-CD19 or anti-CD47 antibodies as indicated. The number of whole Raji B cells eaten per 100 macrophages during 4–8 hr of imaging is graphed. Graph depicts pooled data from three independent experiments; n = 232 with no antibody, n = 257 with anti-CD19 antibody, n = 347 with anti-CD47 antibody; * indicates p<0.05 by two-tailed Fisher Exact test.
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Figure 4—figure supplement 4, CAR-P promotes internalization of cancer antigen. (A) Schematic of antigen internalization and cross-presentation assay. CAR-P expressing Bone Marrow Derived Dendritic Cells (BMDC) were differentiated using GM-CS. CAR-P BMDC were incubated with Raji B cells

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expressing soluble ovalbumin (OVA). DC with OVA bites (internalized antigen) were then incubated with OTI T cells (OVA specific CD8+T cells) and OTI proliferation assessed as a measure of T cell stimulation. Results from each step of this assay are shown in sequence in (B), (C), and (D). (B) Ovalbumin staining (magenta) in Raji B cells infected with mCherry-CAAX-p2a-Ovalbumin lentivirus (OVA) and uninfected controls (uninfected) shows robust OVA expression in infected cells. At right the intracellular OVA signal is plotted as corrected total cell fluorescence (CTCF) for the ovalbumin channel. Each dot represents the CTCF of one cell; n = 26 cells OVA, n = 33 cells (uninfected); lines represent the mean ± one standard deviation, and the graph is the pooled results of three biological replicates. The scale bar indicates 5 μm. (C) Bone marrow-derived dendritic cells expressing the CAR-P<sup>FcR</sup>V (top panel, green in merge, left; greyscale, center) engulf pieces of CD19 +Raji B cells (labeled with mCherry-CAAX; magenta in merge, left; greyscale, right). The control aCD19 CAR-P<sup>GFP</sup>-infected dendritic cells are shown below. Arrows point to pieces of ingested Raji B cell. The proportion of cells taking at least one bite after 90 min co-incubation is graphed on the right of images. Graphs show the pooled data of two separate experiments; n = 28 CAR-P<sup>FcR</sup>V dendritic cells and n = 33 CAR-P<sup>GFP</sup> dendritic cells; *** indicates p<0.0001 by two-tailed Fisher Exact Test; error bars denote 95% confidence intervals. Bites are defined as a fully internalized piece of mCherry-labeled material >1 μm in diameter. (D) OTI T cell proliferation after 72 hr incubation with CAR-P transduced CD11c + dendritic cells. ±RAJI below the x-axis indicates whether Raji-OVA B cells were added to CAR-P transduced dendritic cells prior to OTI addition. To measure proliferation, T cells were uniformly stained with eFluor670 dye on day 0, and proliferation was measured by dilution of the cell-bound dye. Graphs show the mean ±SD of three independent biological replicates. Data points are values for individual wells of differentiated CD11c + dendritic cells. Boxed data indicate the mean % T cells dividing when dendritic cells were pulsed with SL8 (OVA) peptide, which directly binds to MHC without undergoing cross presentation. If dendritic cell differentiation was successful, the pulsed dendritic cells should be capable of inducing robust OTI proliferation. Sample sizes were selected to match previous studies that were able to detect robust T cell stimulation (Roberts et al., 2016).

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