



Hyperactive Rac stimulates cannibalism of living target cells and enhances CAR-M-mediated cancer cell killing

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The 21kD GTPase Rac is an evolutionarily ancient regulator of cell shape and behavior. Rac2 is predominantly expressed in hematopoietic cells where it is essential for survival and motility. The hyperactivating mutation Rac2^{E62K} also causes human immunodeficiency, although the mechanism remains unexplained. Here, we report that in *Drosophila*, hyperactivating Rac stimulates ovarian cells to cannibalize neighboring cells, destroying the tissue. We then show that hyperactive Rac2^{E62K} stimulates human HL60-derived macrophage-like cells to engulf and kill living T cell leukemia cells. Primary mouse Rac2^{+E62K} bone-marrow-derived macrophages also cannibalize primary Rac2^{+E62K} T cells due to a combination of macrophage hyperactivity and T cell hypersensitivity to engulfment. Additionally, Rac2^{+E62K} macrophages non-autonomously stimulate wild-type macrophages to engulf T cells. Rac2^{E62K} also enhances engulfment of target cancer cells by chimeric antigen receptor-expressing macrophages (CAR-M) in a CAR-dependent manner. We propose that Rac-mediated cell cannibalism may contribute to Rac2^{+E62K} human immunodeficiency and enhance CAR-M cancer immunotherapy.

Rac GTPase | phagocytosis | macrophage | lymphopenia | *Drosophila*

The Rho family GTPase Rac is a key node in the signaling and cytoskeletal networks that regulate cell shape and movement. The classic study by Ridley et al. (1) demonstrated that constitutively active Rac (Rac1^{G12V}), expressed in serum-starved Swiss 3T3 fibroblasts causes peripheral actin polymerization, membrane ruffling, and macropinocytosis. Rac proteins are deeply conserved in evolution, likely present in the earliest eukaryotes (2). Human and *Drosophila* Rac proteins are nearly identical, and human Rac can function in *Drosophila* (3).

Drosophila border cells have long served as an *in vivo* model for elucidating Rac functions. The requirement for Rac in cell motility was demonstrated in this group of 6 to 10 follicle cells that migrate ~150 μm over ~4 h during *Drosophila* oogenesis (4). It is now clear that Rac is a nearly universal regulator of cell migration (5) and is required for individually migrating cells such as neutrophils and macrophages as well as collectively migrating cells like border cells and neural crest cells (6–12). Rac is also essential for wound healing, neuronal migration and pathfinding, angiogenesis, and tumor metastasis (13–15).

The human genome encodes three Rac proteins, Rac1, Rac2, and Rac3, which are >95% identical but differ in their expression patterns: Whereas Rac1 is ubiquitously expressed, Rac2 is expressed predominantly in hematopoietic cells, and Rac3 is enriched in neural tissue. Rac2 represents 90% of the Rac protein in immune cells where Rac1 and Rac2 have both redundant and nonredundant functions (16, 17). Rac activity is essential for immune cell motility, which is in turn required for B and T progenitor cells to leave the bone marrow, circulate, and extravasate into the spleen and thymus where B and T cells mature and differentiate, respectively. Cell migration is also essential for cells to move from one compartment to another within the spleen and thymus, to intravasate and re-enter the blood after undergoing positive and negative selection, and then to infiltrate tissues in response to injury or infection. Rac activity is also essential for monocytes, neutrophils, and macrophages to undergo chemotaxis (18, 19). Some of the evidence for these varied roles of Rac in the immune system comes from analysis of the phenotypes of mice with dominant-negative or recessive loss of function mutations in Rac1 and/or Rac2 (20), as well as human patients with mutations in Rac2 (21).

Human patients with dominant-negative or recessive loss-of-function mutations in Rac2 have health problems consistent with the requirement for Rac2 for immune cell migration and survival (17). Paradoxically though, patients with activating mutations in Rac2 also have immune deficiencies. For example, patients with a dominant Rac2^{+E62K} mutation present with recurrent lung infections and lymphopenia (22). When expressed in cultured cells, Rac2^{E62K} causes ~1.5-fold increase in Rac activity (22). Rac2^{+E62K} patient-derived neutrophils exhibit increased H₂O₂ production and macropinocytosis

Significance

The small GTPase Rac2 is a key regulator of immune cell shape and function. Hyperactivating Rac2 mutations cause rare human immunodeficiencies though the underlying mechanisms are unclear. Based on fundamental cell biology in the fruit fly, we found that hyperactive Rac causes cells to eat other cells alive. We show that activated Rac2 also drives mouse and human macrophages to cannibalize activated T cells and that active Rac enhances engulfment of cancer cell targets by chimeric antigen receptor macrophages (CAR-M). These results suggest that Rac2^{E62K}-stimulated cannibalism may contribute to Rac2^{+E62K} human immunodeficiency and enhance CAR-M cancer immunotherapy.

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Competing interest statement: The University of California, Santa Barbara has filed a patent application on the basis of these findings.

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(22), consistent with hyperactivity for known Rac2 functions. $Rac2^{+/E62K}$ mice exhibit similar defects including lymphopenia; however, no defects in T cell trafficking from the bone marrow to the thymus or maturation within the thymus were found. Therefore, the patients' lymphopenia remains unexplained (22).

Border cells require Rac activity for F-actin-mediated protrusions that drive cell motility (4). Focal activation of Rac in a single cell using photoactivatable Rac causes local F-actin polymerization and can steer the whole cluster (3). However, sustained and uniform expression of constitutively activated Rac impairs border cell motility (23), similar to T cells (24) and neutrophils (22).

Here, we report that high levels of hyperactive Rac in the border cells cause them to engulf living neighbors and destroy the entire egg chamber tissue. We further show that expressing $Rac2^{E62K}$ in human macrophage-like cells is sufficient to cause them to cannibalize Jurkat T cell leukemia cells. Primary macrophages from $Rac2^{+/E62K}$ mice engulf and kill living primary T cells. Interestingly, $Rac2^{E62K}$ expression also renders T cells hypersensitive to engulfment. $Rac2^{+/E62K}$ macrophages also non-autonomously enhance whole T-cell engulfment by $Rac2^{+/+}$ macrophages. We further show that $Rac2^{E62K}$ expression in CAR (chimeric antigen receptor)-macrophages (CAR-M, aka CAR-P) enhances receptor-dependent engulfment and killing of target cancer cells. We propose cannibalism as a possible mechanism contributing to the unexplained $Rac2^{+/E62K}$ patient T cell lymphopenia. The data also suggest hyperactive Rac may enhance CAR-M cancer immunotherapy.

Results

Expression of Active Rac in a Few Cells Is Sufficient to Cause Tissue Destruction. Border cells are a group of 4 to 8 migratory cells that originate within an epithelium of ~850 epithelial follicle cells that surround 16 germ cells in a structure called an egg chamber (Fig. 1A). At developmental stage 9, the border cells

delaminate from the epithelium and migrate ~150 μm , squeezing in between polyploid nurse cells until they reach the oocyte by stage 10 (Fig. 1B and Movie S1). Inhibiting all three fly Rac proteins prevents migration (4, 23) (Fig. 1C) by blocking both lead cell protrusion (Fig. 1D and E) and follower cell crawling (25). Constitutively active Rac (Rac^{G12V}) also blocks migration (Fig. 1F and G) (23), presumably by preventing the asymmetry in Rac activity that is required for polarized protrusion.

Border cells normally surround and carry two non-migratory polar cells (Fig. 1D). In contrast, Rac^{G12V} -expressing border cells exhibited complex morphological abnormalities including apparent fragmentation of polar cells (Fig. 1H and I). Surprisingly, increasing the temperature from 18 to 25 $^{\circ}\text{C}$, which increases the Rac^{G12V} expression level, resulted in non-autonomous destruction of the entire egg chamber (Fig. 1J and K). Overexpression of wild-type Rac (Rac^{WT}) caused a similar phenotype, albeit at lower frequency (SI Appendix, Fig. S1). This effect was perplexing because Rac activity is generally not lethal to cells, and intentionally killing border cells by expressing the pro-apoptotic gene Reaper (23) or by laser ablation (26) does not cause egg chamber death but rather permits development to stage 14.

Rac^{G12V} -Stimulated Tissue Destruction Requires the Engulfment Receptor Draper. In addition to promoting cell migration, Rac is one of hundreds of components of the phagocytic cup (27). Follicle cells normally engulf and kill nurse cells near the end of *Drosophila* oogenesis (28) via the engulfment receptor Draper (Drpr; Fig. 2A). So could Rac^{G12V} -expressing border cells be killing the nurse cells by inappropriately engulfing them?

To test this idea, we expressed Rac^{G12V} in border cells in *drpr* homozygous mutants. In well-fed control flies, egg chamber death during mid oogenesis is rare (Fig. 2B). In contrast, the majority of *slboGal4;UAS-Rac^{G12V}*-expressing egg chambers contained dying nurse cells with condensed and fragmented nuclei and overall

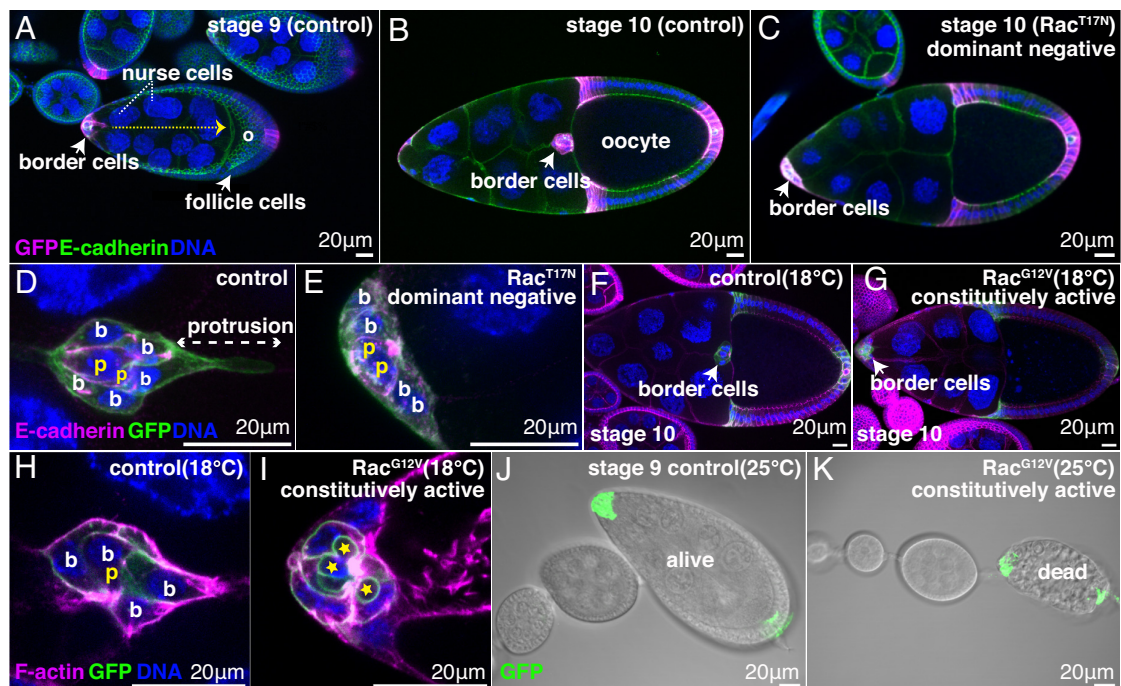


Fig. 1. Constitutively active Rac (Rac^{G12V}) in a subset of follicle cells causes wholesale tissue destruction. *slbo-Gal4; UAS-PLC δ 1-PH-GFP* egg chambers of the indicated stages and genotypes. (A–I) Confocal micrographs of (A) *UAS-lacZ* control showing border cell migration path (dotted arrow). Oocyte (o). (B) Stage 10 *UAS-lacZ* control showing completed migration. (C) Stage 10, *UAS-Rac^{T17N}* showing failed migration. (D) Control *UAS-lacZ* cluster. Border cells (b) surround and carry non-motile polar cells (p). The lead border cell protrudes, initiating migration. (E) *UAS-Rac^{T17N}* clusters lack protrusions. (F) Completed migration in control, *UAS-lacZ*. (G) Failed border cell migration in *UAS-Rac^{G12V}*. (H) Normal cluster morphology of *UAS-lacZ* control, (I) Abnormal cluster morphology in *UAS-Rac^{G12V}*. Phalloidin labels F-actin (magenta in H and I). (J and K) DIC imaging of (J) normal egg chamber morphology in a *UAS-lacZ* control and (K) dead *UAS-Rac^{G12V}* egg chamber. Anterior is on the Left.

abnormal egg chamber morphology (Fig. 2C). However, when homozygous mutant for *drpr*, the majority of *slboGal4;UAS-Rac^{G12V}* egg chambers exhibited normal morphology (Fig. 2D and E), except that border cells still failed to migrate (Fig. 2D). We conclude that active Rac kills the germline in a process that depends on Drpr-mediated target recognition.

Control border cells develop within the follicle cell epithelium in response to a cytokine secreted by the polar cells (29) (Fig. 2F and G). High magnification imaging revealed that ~75% of *slboGal4;UAS-Rac^{G12V}* border cell clusters engulfed at least one neighboring polar cell (Fig. 2G and G'), which is never observed in controls (Fig. 2F). Together, these results demonstrate that expression of active Rac in border cells causes them to engulf living neighbors.

To determine how many Rac^{G12V}-expressing cells would be sufficient to engulf another living cell, we generated clones of various sizes using the Flpout technique. Control border cell clones expressing GFP and lacZ exhibited normal morphologies, extended outward-directed protrusions, and carried rather than engulfed polar cells (Fig. 2H and H' and Movie S2). By contrast, even a single border cell expressing Rac^{G12V} and GFP could engulf a polar cell (Fig. 2I and I' and Movie S3).

Active Rac Is Sufficient to Cause Human Macrophages to Engulf Living Cells.

Rac mediates phagocytosis in organisms ranging from single-celled amoebae to human macrophages (30–32). So we wondered whether activated Rac would be sufficient to cause a human macrophage to eat a living cell. To test this idea, we transduced HL60 cells with a lentivirus expressing either a membrane-associated GFP (lck-GFP) alone as a negative control, or lck-GFP together with Rac2^{E62K}. We differentiated the cells into macrophage-like cells using an established protocol (33, 34) (SI Appendix, Fig. S2) and co-cultured them with human Jurkat T cell leukemia cells (Fig. 3A). We then counted the fraction of macrophages that engulfed at least one Jurkat T cell. Engulfment events were relatively rare in control lck-GFP-expressing cells (Fig. 3B), whereas expression of Rac2^{E62K} increased the fraction of macrophages that contained Jurkat cell fragments (Fig. 3C) from ~10 to ~70% (Fig. 3D). We measured a 2.5-fold increase in RacGTP in Rac2^{E62K}-expressing cells compared to controls (Fig. 3E), consistent with Hsu et al. (22). Overexpressing Rac^{WT} or Rac^{G12R}, which is another activating mutation, also stimulated Jurkat cell engulfment (Fig. 3F–I), although Rac2^{E62K} had the strongest effect. We conclude that, as in *Drosophila*, hyperactive Rac also promotes cannibalistic behavior in human cells.

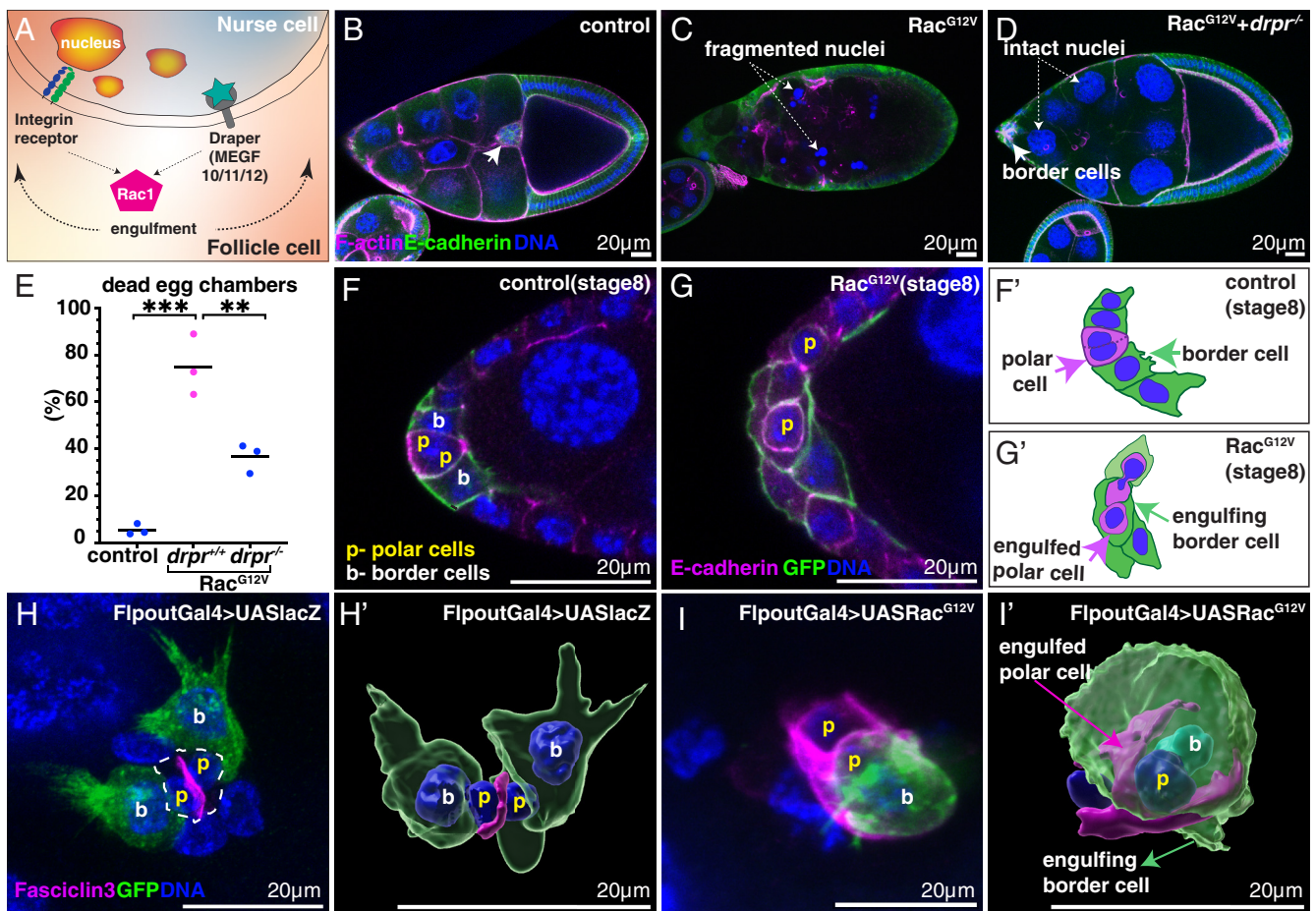


Fig. 2. Constitutively active Rac (Rac^{G12V}) causes germline (nurse cells and oocyte) death and polar cell engulfment. (A) Schematic showing Draper-receptor-mediated engulfment of nurse cells by follicle cells. (B–G) Confocal micrographs of *slbo-Gal4* egg chambers labeled with the indicated markers. Anterior is Left. (B) *UAS-lacZ* control exhibits normal morphology and completed border cell migration at stage 10. (C) Dying *UAS-Rac1^{G12V}* egg chamber. (D) The homozygous *drpr* mutation rescues normal egg chamber morphology but not border cell migration (arrow) in *UAS-Rac1^{G12V}*. (E) Quantification of dead egg chambers with or without *UAS-Rac^{G12V}* in the wildtype or *drpr* mutants. All data were analyzed by one-way ANOVA with Tukey's multiple comparisons test. ** indicates $P < 0.005$ and *** $P < 0.0005$. $N = 87$ (control), 53 (Rac^{G12V}) and 66 ($Rac^{G12V}; drpr^{-/-}$) egg chambers from three independent experiments (dots). (F and G) Stage 8 *slbo-Gal4; UAS-PLCδ1-PH-GFP* egg chambers showing (F) border cells (b) adjacent to polar cells (p) and (G) *UAS-Rac1^{G12V}* border cells engulf polar cells (p). (F' and G') Schematic drawings of F and G. (H) Clonal expression of either *UAS-lacZ* (control, H and H') or *UAS-Rac1^{G12V}* (I and I') in the GFP-positive subset of border cells (green). Fasciclin 3 (magenta in H–I) is restricted to the interface between two polar cells in controls (H and H') whereas border cells expressing Rac^{G12V} (I and I') engulf polar cells, delocalizing Fasciclin 3. H' and I' are 3D Imapris surface renderings of H and I.

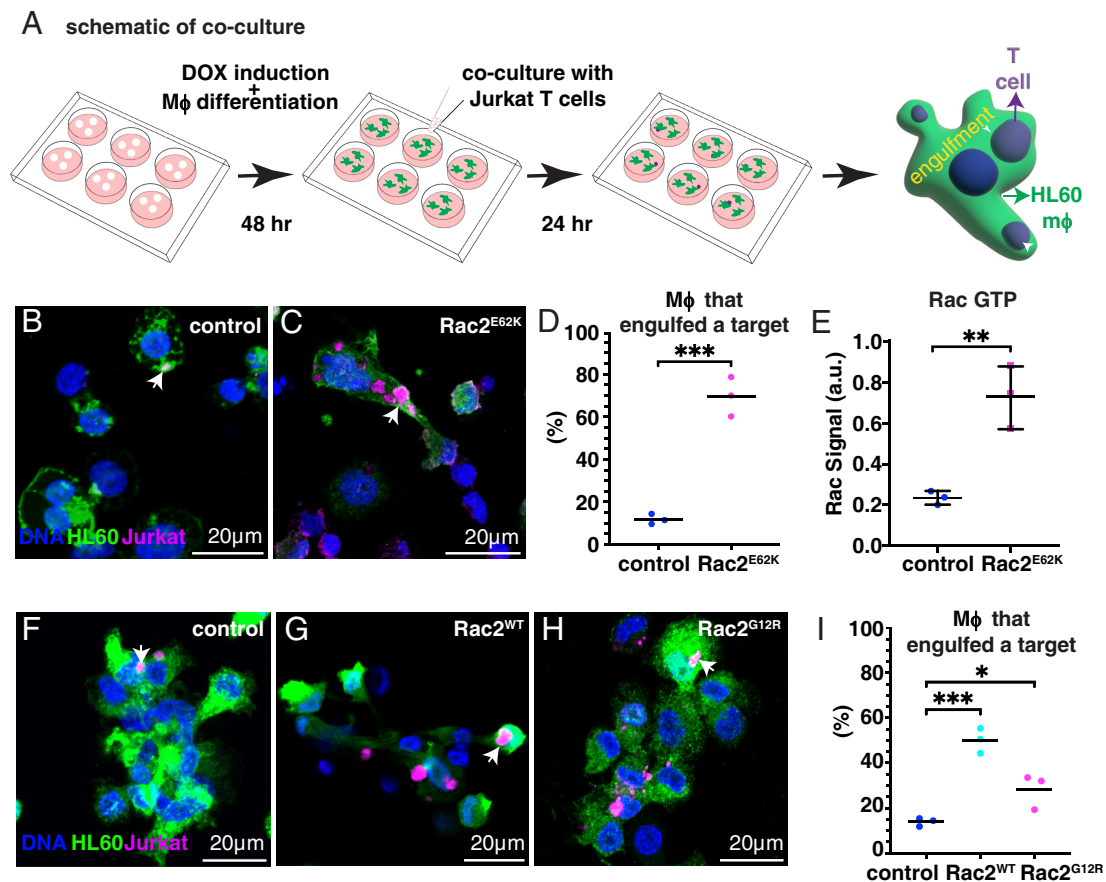


Fig. 3. Constitutively active Rac2^{E62K} in HL60-derived macrophage-like cells enhances engulfment and killing of leukemic Jurkat T cells. (A) Schematic of experimental design. (B–I) HL60-derived macrophage-like cells expressing Lck-GFP (green) and the indicated Rac2 variant co-cultured with mCherry-labeled (magenta) Jurkat T cells. DNA (labeled with Hoechst, blue) (B) control (Lck-GFP without Rac2 variant) (C) Lck-GFP with Rac2^{E62K} (D) Quantification of N = 614 (control) and 220 (Rac2^{E62K}) cells from three independent experiments (dots). (E) GTP-bound (active) Rac measured from differentiated HL60 macrophage lysates using the G-LISA Rac 1,2,3 Activation Assay Kit (Cytoskeleton, Inc.). Each dot is an average of two technical replicates. Statistics: Unpaired *t* test. ***P* < 0.005 and ****P* < 0.0005. (F) Control (Lck-GFP alone), (G) Rac2^{WT}, (H) Rac2^{G12R}, (I) Quantification of engulfment. N = 1,140 (control), 223 (Rac2^{WT}), and 653 (Rac2^{G12R}) samples from three independent experiments (dots). Engulfment data (F–H) were analyzed by one-way ANOVA with Tukey’s multiple comparisons test. * indicates *P* < 0.05 and ****P* < 0.0005.

Rac2^{E62K} Alters the Macrophage Transcriptional State. Together with Cdc42 and Rac1, Rac2 localizes to the phagocytic cup and promotes phagocytosis by stimulating local F-actin polymerization (30). So Rac2^{E62K} likely stimulates hyper-phagocytosis directly. In addition, Rac2^{E62K} might increase phagocytic activity by changing the cell state. To test whether Rac2^{E62K} causes substantial changes in the macrophage transcriptome, we compared RNAseq profiles for control and Rac2^{E62K} expressing HL60-derived macrophage-like cells. >1,000 genes were differentially expressed between these two cell populations (Fig. 4A). 1,551 genes were upregulated and 790 were downregulated $\geq 1.5X$. The most significantly upregulated pathways were inflammatory, including TNF- α signaling via NF- κ B (Fig. 4B). Gene ontology (GO) analysis showed significant enrichment of some genes associated with the cellular process of phagocytosis including receptors that mediate migration and phagocytosis, such as CD302, AXL, and TYRO3, as well as signaling and cytoskeletal regulators such as MYO1G and SH3BP1 (Fig. 4C). Antibody staining confirmed upregulation of CD302 protein (Fig. 4D and E). Enriched molecular functions included lipid metabolism and ion transporters (SI Appendix, Fig. S3). We conclude that Rac2^{E62K} expression alters the macrophage transcriptional state.

Rac2^{+E62K} Bone Marrow-Derived Macrophages Engulf Living Rac2^{+E62K} T Cells. Hsu et al. established a Rac2^{+E62K} mouse model (22), which recapitulates the human syndrome, including reduced circulating T cells and hyperactivated neutrophils. Neither

T cell trafficking from the bone marrow to the thymus nor T cell maturation within the thymus is impaired in Rac2^{+E62K} mice, and therefore, the peripheral lymphopenia remains unexplained. We also counted viable and dead CD3+ T cells from the thymus and found no measurable difference between Rac2^{+/+} and Rac2^{+E62K} mice (SI Appendix, Fig. S4).

Our results in *Drosophila* egg chambers and human macrophage-like cells hinted at hyperengulfment of lymphocytes by macrophages as a possible explanation. To test this idea, we obtained the Rac2^{+E62K} mice, isolated bone marrow-derived monocytes, and differentiated them into macrophages. We isolated splenic T cells from another animal and labeled them with both CellTrace (Far Red) and pHrodo (Red), which is a dye that fluoresces only within the acidic environment of the lysosome. We stimulated the macrophages with interferon-gamma (IFN- γ), co-cultured them with T cells (Fig. 5A), and compared engulfment in Rac2^{+/+} and Rac2^{+E62K} cells by fixed (Fig. 5B and C) and live imaging (Fig. 5D and E and Movies S4 and S5). Rac2^{+E62K} macrophages engulfed more Rac2^{+E62K} T cells than wild-type controls. This effect was significant whether we measured pHrodo intensity (Fig. 5D–F), the depletion of T cell targets after 10 h of co-culture (Fig. 5G), or the percentage of macrophages that engulfed a target (Fig. 5H).

We then tested whether the phenotype was autonomous to the macrophages, to the T cells, or both. Intriguingly, we found that the enhanced engulfment was due to a combination of increased

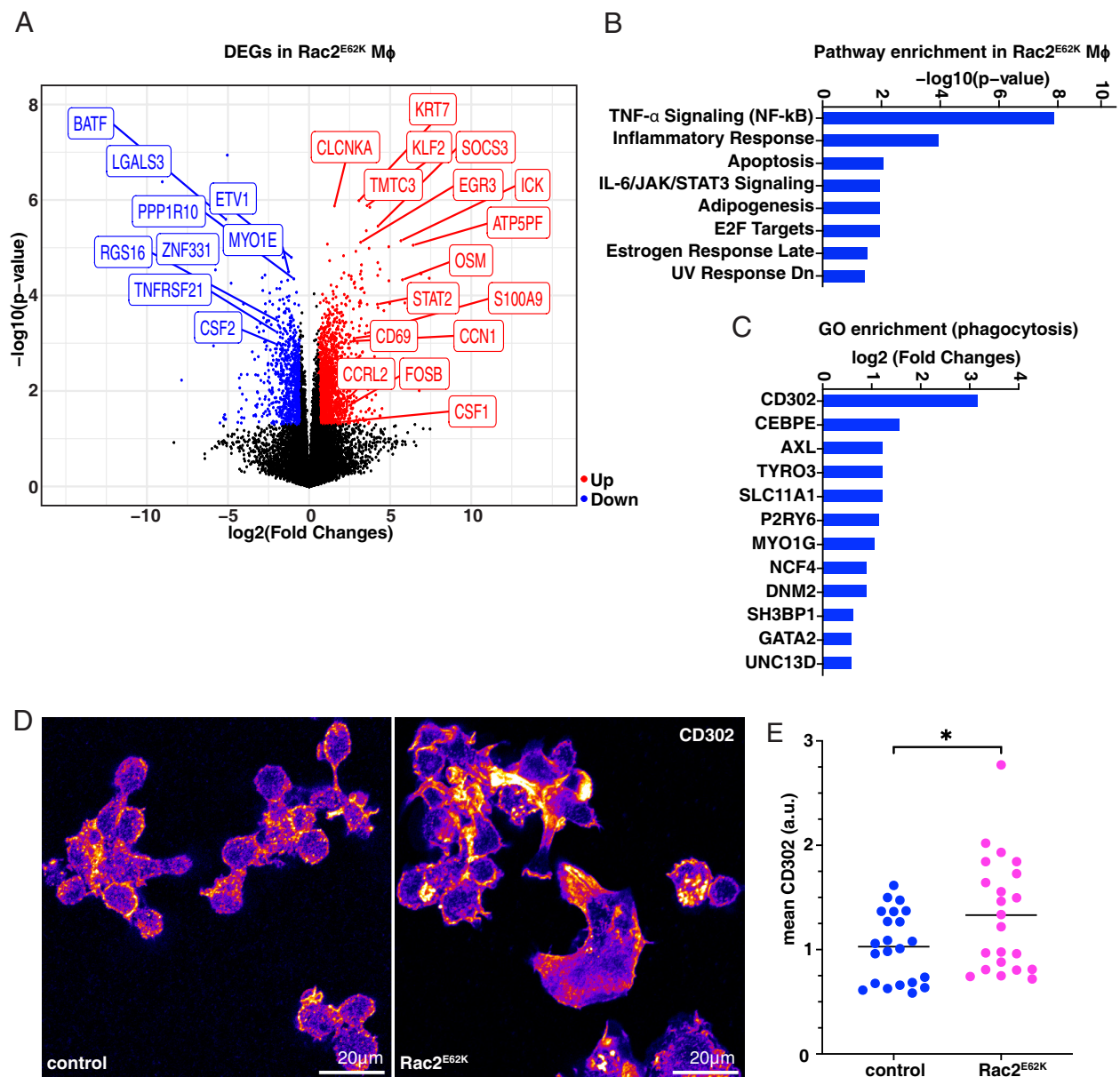


Fig. 4. Effect of $Rac2^{E62K}$ on gene expression in HL60-derived macrophage-like cells. (A) Volcano plot representation of DEGs. Red dots (upregulated $\geq 1.5X$), blue dots (downregulated $\geq 1.5X$), and black dots (mRNAs changed $< 1.5X$ and insignificant p-value) in $Rac2^{E62K}$ -expressing cells. Data were mapped, analyzed, and extracted from the Subio Platform [v1.24 (Subio, Inc., Japan)] and plotted in R. Select DEGs are labeled. (B) Pathway enrichment analysis was performed in Enrichr (Ma'ayan laboratory) on upregulated genes with a fold change (FC) > 3 and the Molecular Signatures Database (MSigDB) Hallmark 2020 output data were plotted. (C) GO term "phagocytosis" in DEGs and upregulated genes with FC > 1.5 and P -value < 0.05 . (D) CD302 expression in (control) HL60-LckGFP or $Rac2^{E62K}$ -Lck-GFP macrophages. (E) Mean CD302 fluorescence in control and $Rac2^{E62K}$ -expressing macrophages. Statistics: Unpaired t test, * indicates $P < 0.05$.

phagocytosis by $Rac2^{+/E62K}$ macrophages and increased susceptibility to engulfment by $Rac2^{+/E62K}$ T cells, with the greatest engulfment observed when both cell types were $Rac2^{+/E62K}$ (Fig. 5I).

Macrophages engulf activated T cells to limit inflammatory responses (35) and promote tolerance (36). Rac is activated downstream of the T cell receptor (37), so the $Rac2^{E62K}$ mutation might mimic chronic activation. To test whether activating T cells renders them more susceptible to engulfment, we treated wild-type and $Rac2^{+/E62K}$ T cells with anti-CD3 and anti-CD28 antibodies (38) for 3 d, collected the cells and washed them to remove any remaining antibody, and then co-cultured them with macrophages. We found that activated $Rac2^{+/+}$ T cells were engulfed to about the same extent as $Rac2^{+/E62K}$ T cells (Fig. 5J). Furthermore, anti-CD3 and anti-CD28 pretreatment only slightly increased engulfment of $Rac2^{+/E62K}$ T cells (Fig. 5J), consistent with an interpretation that they are already activated. We conclude that

activation sensitizes T cells to engulfment to a similar degree as the $Rac2^{+/E62K}$ mutation.

We next tested whether mixing $Rac2^{+/E62K}$ BMDMs with wild-type BMDMs would alter their behavior. Co-culture with $Rac2^{+/E62K}$ BMDMs increased T cell engulfment by wild-type BMDMs (Fig. 5J).

$Rac2^{+/E62K}$ Macrophages Are in a Unique State. Macrophages have classically been described as M1 (inflammatory) and M2 (anti-inflammatory) though there are substates, possibly intermediate states, and the states may be dynamic (39–41). To characterize the $Rac2^{+/E62K}$ BMDMs, we first measured Rac^{GTP} and found \sim two-fold increase relative to $Rac2^{+/+}$ cells (Fig. 6A), similar to HL60-derived macrophage-like cells (Fig. 3E) and $Rac2^{E62K}$ -transfected COS cells (22). We then isolated BMDMs from $Rac2^{+/+}$ mice and either did not stimulate them or induced them to a classic

A

bone marrow-derived macrophage (BMDM) engulfment assay

Day 7: Confirm differentiation by flow cytometry analysis and stimulate BMDMs with IFN- γ

Day 8: Coculture overnight
75K BMDMs:375K T cells

Day 1: Harvest femurs and differentiate whole bone marrow cells into BMDMs

Fixed Imaging
Time lapse

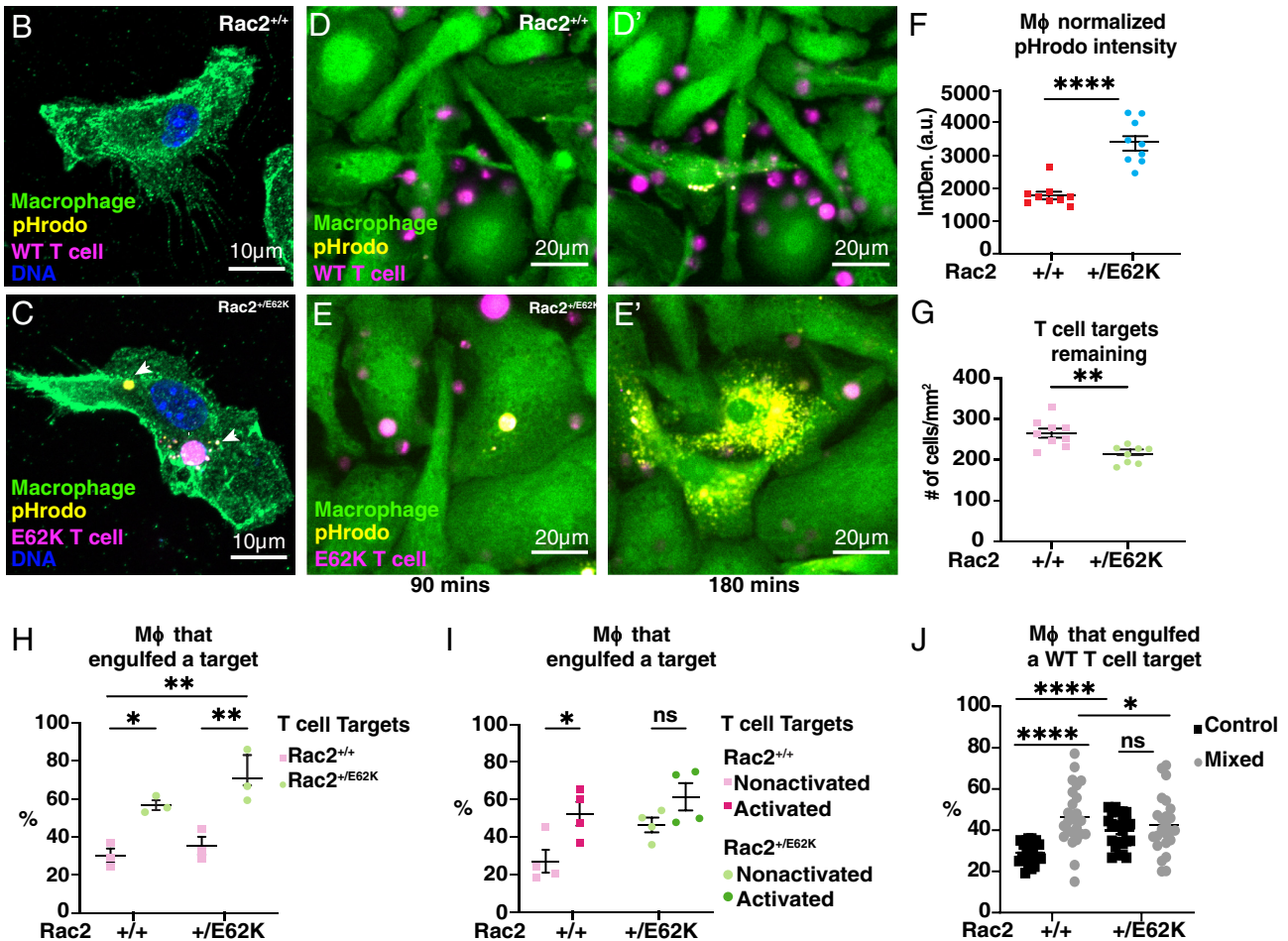
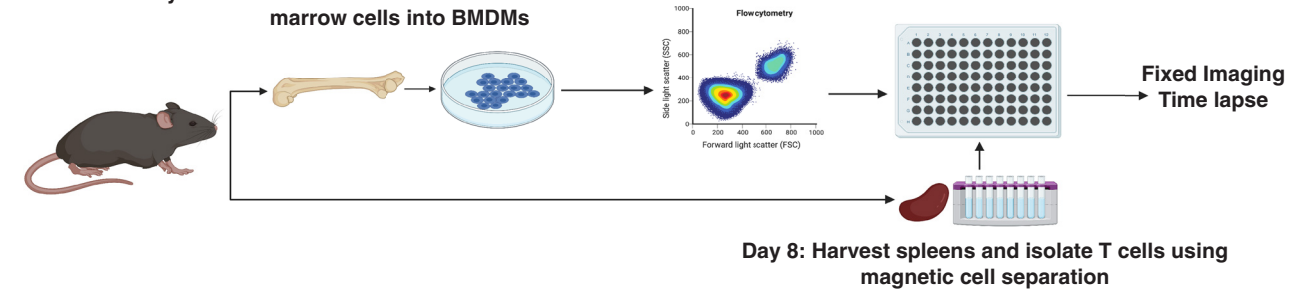


Fig. 5. Rac2^{E62K} enhances phagocytosis of T cells by macrophages and sensitizes T cells to engulfment. (A) Schematic of the phagocytosis assay. Rac2^{+/+} (WT) and Rac2^{+/E62K} BMDMs were incubated overnight with T cells labeled with CellTrace Far Red and pHrodo Red (B and C). Representative confocal images of BMDMs. (D–E) 10-h time-lapse imaging of macrophage (green) and T cell (magenta) coculture. pHrodo (yellow). Images correspond to *Movies S4* and *S5*. (F) pHrodo intensity as measured by integrated density (a.u.) and normalized to the number of macrophages in the field of view. Each point represents a measurement from one field of view. (G) Total number of T cell targets remaining at the end of a 10-h engulfment time-lapse experiment. Each point represents a measurement from one field of view. (H) Percentage of macrophages that engulfed at least one T cell target. Each point represents the average from two wells. (I) Percentage of macrophages that engulfed at least one T cell target (activated or non-activated). Each point represents the average from two wells. (J) Percentage of macrophages that engulfed at least one WT T cell in control or mixed macrophage conditions. Each point represents a technical replicate. n = 3 biological replicates for experiments (H–J). *, **, and **** indicate $P < 0.05$, 0.005, and 0.00005 respectively by either an unpaired t test or a two-way ANOVA followed by Sidak’s multiple comparison test. Error bars indicate mean \pm SEM.

M1 state using IFN- γ and LPS or to an M2 state with IL-4. We also isolated primary BMDMs from Rac2^{+/E62K} mice. We then carried out RNAseq and identified differentially expressed genes (DEGs) characteristic of M1, M2, and/or Rac2^{+/E62K}.

Remarkably, a ~two-fold increase in Rac2 activity was sufficient to cause differential expression of 2,259 genes (930 up and 1,329 down) (Fig. 6B), indicating a significant change in cell state. GO enrichment analysis with search terms “phagocytosis” and “positive

regulation of phagocytosis” returned *Ticam2*, *Rap1gap*, *Mfge8*, *Tlr4*, and *Pik3ca* as top hits (Fig. 6C). Similar to the HL60 cells, $\text{TNF-}\alpha$ signaling and inflammatory responses were top enriched pathways (Fig. 6D). The twofold increase in *Rac2* activity also caused gene expression changes that significantly overlapped with cytokine stimulation. A total of 1087 of the $\text{Rac2}^{+/E62K}$ DEGs overlapped with those differentially expressed in the M1 state, while 75 overlapped with the M2 state specifically, and 485 DEGs were common to all three states compared to unstimulated control BMDMs (Fig. 6E). Common upregulated GO enrichment terms (molecular function) between M1 and $\text{Rac2}^{+/E62K}$ included genes associated with membrane transporter activity (*SI Appendix, Fig. S5*). We conclude that *Rac* hyperactivation causes macrophages to adopt a uniquely stimulated state.

Rac2^{E62K} Enhances CAR-M Phagocytosis of Target Cancer Cells. CAR therapies are promising cancer immunotherapies (42). In CAR-T therapy, T cells are removed from a cancer patient, engineered ex vivo to express a CAR that recognizes a tumor

antigen, and then re-infused into the patient. While there have been exciting successes, there are limitations, including physical exclusion of T cells from solid tumors, antigen escape, and cytokine storms (42, 43). A newer cousin is CAR-M in which patient monocytes and/or macrophages are engineered to express a CAR (44–46). Potential advantages include the unrivaled ability of macrophages to infiltrate the tumor microenvironment, removal of tumor cells by engulfment, and the possibility of initiating a broader adaptive immune response via antigen presentation. A limitation of CAR-M however is that CAR macrophages tend to nibble on cells rather than engulfing and killing them, in a process known as trogocytosis (44). Therefore, enhancing whole target cell engulfment and killing is an important goal.

To test whether expressing Rac2^{E62K} in CAR-M cells could enhance their ability to engulf and kill target cells, we isolated BMDMs from $\text{Rac2}^{+/E62K}$ mice and from littermate controls, and used lentiviral infection to express an anti-CD19 CAR or a GFP-CAAX control (Fig. 7A). We then co-cultured the cells with CD19^+ Raji B cell lymphoma cells and carried out live imaging.

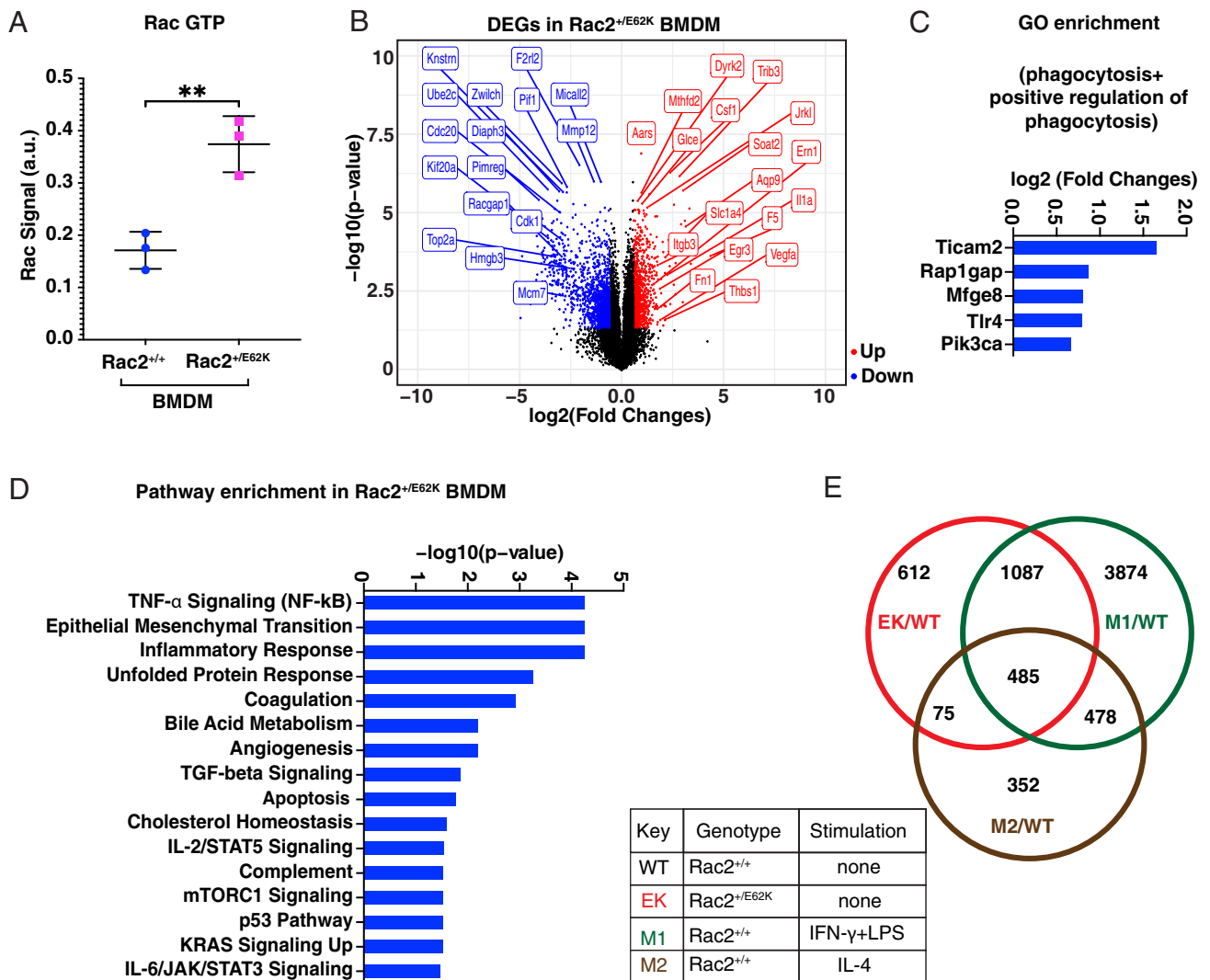


Fig. 6. Effect of $\text{Rac2}^{+/E62K}$ on Rac activity and gene expression in primary BMDMs. (A) GTP-bound (active) Rac was measured from $\text{Rac2}^{+/+}$ and $\text{Rac2}^{+/E62K}$ BMDM lysates using the G-LISA Rac 1,2,3 Activation Assay Kit (Cytoskeleton, Inc.). Data were analyzed by unpaired *t* test. ** indicates $P < 0.005$. (B) Volcano plot representation of differential expression analysis of genes in $\text{Rac2}^{+/+}$ and $\text{Rac2}^{+/E62K}$ macrophages. Data was mapped, analyzed, and extracted from the Subio Platform (v1.24) and plotted in R. Red dots (upregulated genes), blue dots (downregulated genes) and black dots (insignificant genes). Selected DEGs are labeled. (C) GO term phagocytosis and positive regulation of phagocytosis in DEGs and upregulated genes with (FC > 1.5) and P -value < 0.05. (D) Pathway enrichment analysis on DEGs was performed in Enrichr (Ma'ayan laboratory) on upregulated genes with (FC > 3) and the Molecular Signatures Database (MSigDB) Hallmark 2020 output data were plotted. (E) DEGs in groups EK/WT, M1/WT, and M2/WT were co-analyzed to assess the inflammatory profile of $\text{Rac2}^{+/E62K}$ BMDMs.

Little to no engulfment was observed in GFP-CAAX expressing macrophages in the absence of a CAR, regardless of whether they were Rac2^{+/+} (Fig. 7B and Movie S6) or Rac2^{+E62K} (Fig. 7C and Movie S7). Expression of the CAR significantly enhanced whole cell engulfment (Fig. 7D and Movie S8), as previously described (44, 45). Expression of the CAR in Rac2^{+E62K} macrophages further stimulated whole cell engulfment (Fig. 7E and F and Movie S9). We conclude that Rac activity specifically enhances CAR-dependent engulfment of target cells.

We further confirmed this result using flow cytometry (Fig. 7G and H), which detects both trogocytosis and whole cell engulfment. Rac2^{+E62K} or Rac2^{+/+} macrophages were infected with lentivirus expressing the GFP-CAAX control or the CAR-M-GFP. Target Raji cells were labeled with CellTrace Violet and co-cultured with macrophages. Both the frequency of CellTrace-positive macrophages (Fig. 7I) and the intensity of the CellTrace signal in macrophages (Fig. 7J) were highest in macrophages expressing both the CAR and Rac2^{E62K}.

Discussion

The results reported here leverage insights gleaned from studying a perplexing non-cell-autonomous egg-chamber-death phenotype in the *Drosophila* ovary to offer a possible explanation for a rare human immunodeficiency and then to enhance CAR-M-mediated engulfment and killing of cancer cells. These findings demonstrate the power of basic science in an experimental organism like *Drosophila* to lead to insights of potential clinical significance.

From Flies to Humans, Activated Rac Enhances Engulfment of Whole, Living Cells. In migrating cells, Rac proteins generally function downstream of a variety of receptors to promote actin polymerization, protrusion dynamics, and motility (1, 4, 47). Rac has also long been known to stimulate macropinocytosis, which is fluid engulfment (1), as well as phagocytosis (30, 48, 49). Proteomic studies have identified hundreds of different proteins within phagosomes from multiple species but surprisingly little overlap from one cell type to the next (32). Interestingly, the most conserved components are actin and Rac. Yutin et al. (32) even propose that horizontal gene transfer of an early form of Rac from a bacterium to an archeon could have conferred phagocytic ability, enabling engulfment of a bacterial symbiont, generating the first eukaryotic cell. What is clear is that Rac-driven actin polymerization is a fundamental feature of the engulfment machinery. Here, we show that hyperactive Rac drives cannibalistic behavior in diverse metazoan cell types and organisms.

In the context of the fly egg chamber and CAR-M, engulfment of living cells that is stimulated by hyperactive Rac is directed to specific targets by receptor-mediated recognition. For example, in the fly ovary, mutation of the engulfment receptor Drpr significantly reduces germ cell engulfment and egg chamber killing by border cells expressing activated Rac. Drpr normally mediates follicle cell recognition and engulfment of living nurse cells at the end of oogenesis (50). This raises the question as to how constitutively active Rac, which functions downstream of receptors, actually still requires the “upstream” engulfment receptor. There are at least two possible explanations. One is that active Rac amplifies the signal through feedback but still requires an initiating signal, for example, in a feedforward loop with phosphoinositide 3-kinase (7, 51, 52) or through upregulated expression of phagocytic receptors. Another possibility, which is not mutually exclusive, is that Rac functions downstream of a second receptor and that activation of both parallel pathways is essential for engulfment. For example, in both flies and in human macrophages,

integrin adhesion receptor activation is required in parallel to engulfment receptor activation. During engulfment, Rac may normally be activated downstream of integrins, as it is in cells migrating on integrin ligands (53).

Acute membrane recruitment of Rac and a phosphatidylserine receptor is sufficient to cause HeLa cells, which are not usually phagocytic, to engulf dead cells (54). Consistent with our results, neither active Rac nor the receptor alone is sufficient in that context either. Active Rac also stimulates engulfment of dead cells in *Caenorhabditis elegans* (55) and enhances mammalian cell phagocytosis of dead cells, beads (56), and opsonized red blood cells (57). Here, we show that hyperactive Rac stimulates engulfment and killing of unadulterated living cells.

The hyperactivating mutation Rac1^{P29S} is a driver mutation in melanoma (58). Cancer cells in general, and metastatic melanoma in particular, have been reported to cannibalize immune cells as well as other cancer cells (59). Cannibalism is defined as the internalization of one living cell by another (60), and this behavior may fuel cancer growth and suppress immune responses (61). When it has been examined, cell cannibalism and other cell internalization processes require Rac activity in the engulfing cell (62, 63). Intriguingly, melanoma is an aggressive cancer that is highly cannibalistic (59), though a possible connection between the Rac1^{P29S} driver mutation and cannibalism in metastatic melanoma has not been explored.

Rac1 overexpression is implicated in metastatic behavior in numerous additional cancers (64, 65). Furthermore, elevated Rac expression and/or activity correlate with poor prognosis in most cancers (66). The mechanism by which Rac is generally thought to drive metastasis is through its effects on migration, invasion, and survival, whereas its known effects on phagocytosis have been mostly overlooked (15, 64, 66). Based on the results presented here and the existing literature on Rac-mediated engulfment, it seems possible that Rac hyperactivity causes tumor cells to engulf and kill other living cells including anti-tumor T cells. Studies of the functional relationships between Rac overexpression and hyperactivity, cannibalistic behavior, and poor prognosis in cancer are warranted.

Rac2^{E62K} Lymphopenia May Result from Hyperactivation of Both Phagocytes and Lymphocytes. Rac GTPases cycle between active, guanosine triphosphate (GTP) bound and inactive, guanosine diphosphate (GDP) bound forms. Guanine nucleotide exchange factors (GEFs) activate Rac by replacing GDP with GTP. GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of the Rac enzymes, thus inactivating the proteins by hydrolyzing GTP to GDP. Mutations that damage the catalytic activity such as Rac^{G12V}, Rac^{G12R}, and Rac^{Q61L} are strongly activating as they lock the protein in the GTP-bound state. By contrast, Rac2^{E62K} disrupts interaction with specific regulatory proteins, including the GEF Tiam1 as well as a GAP. The net effect is a mild 1.5 to 2.5 fold increase in Rac-GTP (this study and ref. 22). Rac^{G12V} strongly impairs cell migration (24) and causes T cell apoptosis in the thymus (67), and Rac1^{Q61L} transgene expression in T cells drives their negative selection in the mouse thymus (68). These strongly activating mutations may mimic a highly autoreactive state because Rac functions downstream of the TCR, and strong TCR activation causes negative selection (aka deletion) of autoreactive T cells (69, 70). Patients with Rac2^{G12R} mutations exhibit severe combined immune deficiency and hypocellularity in the thymus (21), which may be due to a failure of innate and adaptive immune cell precursors to migrate out of the bone marrow combined with apoptotic death of T cells that do develop.

By contrast, Hsu et al. (22) reported normal lymphocyte development in the bone marrow and normal cellularity in the thymus in Rac2^{+E62K} mice despite a profound reduction in the number of

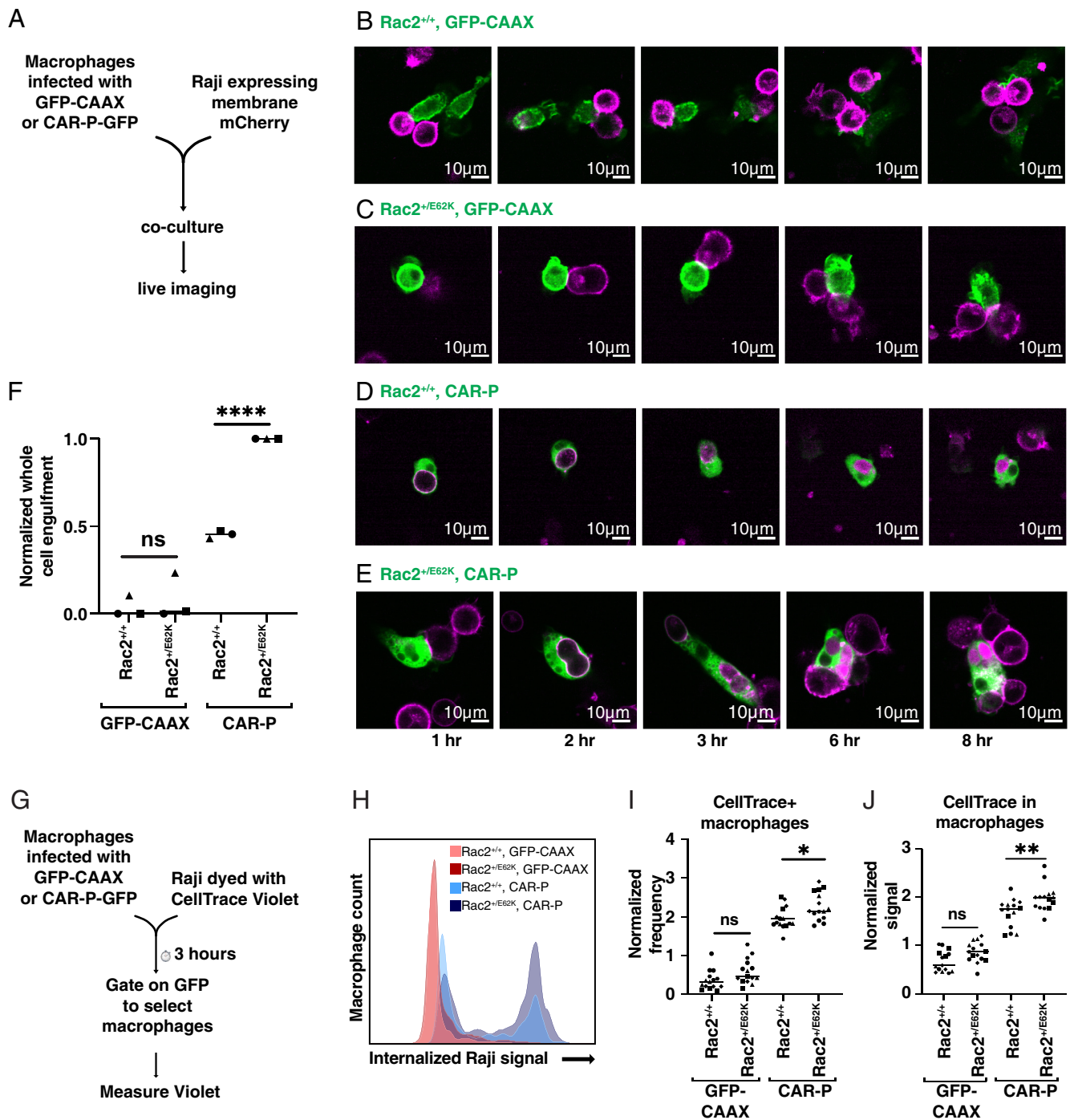


Fig. 7. $Rac2^{+/E62K}$ enhances whole cell phagocytosis of cancer cells by CAR macrophages. (A) Schematic of the experiment in (B–F). $Rac2^{+/+}$ and $Rac2^{+/E62K}$ BMDMs were infected with lentivirus encoding membrane-tethered GFP (GFP-CAAX) or a CAR that recognizes the B cell surface antigen CD19 and signals through the Fc Receptor intracellular signaling domain. (B–E) To distinguish between trogocytosis or whole cell phagocytosis, control GFP-CAAX (B and C) or CAR-GFP (D and E) BMDMs (green) were mixed with mCherry-CAAX-Raji B cell lymphoma cells (magenta) and imaged via spinning disc confocal. Images correspond to [Movies S6–S9](#). (F) Graph indicates the number of whole Raji cells eaten per macrophage in 8 h, normalized to the maximum observed on that day. ****, and **** indicate $P < 0.05$, 0.005, and 0.00005 respectively by an ordinary one-way ANOVA followed by Sidak's multiple comparison test. In graphs, each point represents data from one well and wells collected on the same day share a symbol. The line indicates the mean of each condition. (G) A schematic of the flow cytometry phagocytosis assay in (H and I). (H) Histogram shows representative flow cytometry data of CellTrace Violet signal within macrophage (GFP+) population. (I) Graph depicts the fraction of macrophages that were CellTrace+, normalized to the average of the day. (J) Graph depicts the mean CellTrace Violet intensity of GFP+ macrophages, normalized to the daily average.

circulating T cells. So the T cell lymphopenia, which likely underlies the chronic infections that $Rac2^{+/E62K}$ patients suffer, remained unexplained. We find that $Rac2^{+/E62K}$ T cells and activated T cells are more likely to be engulfed by the wildtype as well as $Rac2^{+/E62K}$ macrophages. These results suggest that mild activation of T cells insufficient to cause apoptosis may nevertheless sensitize them to

removal by phagocytosis. Our results also suggest that the combination of hyperactive phagocytosis by $Rac2^{+/E62K}$ macrophages and hypersensitivity to engulfment of $Rac2^{+/E62K}$ T cells may contribute to the dramatic reduction in circulating T cells in $Rac2^{+/E62K}$ mice and in human patients. However, additional experiments will be required to establish the relative contributions of cannibalism and

other mechanisms—such as trafficking defects and negative selection—to the *in vivo* phenotype.

Rac-Enhanced CAR (RaceCAR) Therapy. Although clearly detrimental as a germline mutation, our results suggest that it may be possible to harness the receptor-dependent hyperactivity that Rac2^{E62K} confers to macrophages to enhance CAR-M therapy. It is encouraging that Rac2^{E62K} enhanced the specific CD19/CAR-dependent engulfment with little to no detectable effect on receptor-independent engulfment. The observation that mixing Rac2^{+/+} and Rac2^{+/E62K} macrophages increased phagocytosis by the wild-type macrophages is intriguing and raises the possibility that RaceCAR macrophages might be able to inflame tumor-associated macrophages and thereby enhance the anti-tumor immune response both non-autonomously as well as autonomously. Cancer patients to be treated with CAR-M or RaceCAR-M would not have the Rac2^{E62K} mutation in their lymphocytes. So, we would not expect lymphopenia as a side effect of RaceCAR-M therapy. The viability of human patients with germline Rac2^{+/E62K} mutations also implies that engineered RaceCAR macrophages would be unlikely to engulf cell types other than the intended targets. Together, these results suggest that expressing Rac2^{E62K} together with a CAR may enhance the efficacy of CAR-M cancer immunotherapy without compromising safety.

Materials and Methods

Detailed materials and methods are available in *SI Appendix*. Briefly, *Drosophila* stocks and crosses were maintained under standard conditions and ovaries dissected, fixed, stained, and imaged as described (25). HL60

cells were infected with lentiviruses to express Rac constructs and Lck-GFP to identify infected cells. Lentivirus constructs were transfected into HEK293T cells and supernatants prepared as described (44). HL60 cells were differentiated into macrophage-like cells as described (33). Mouse BMDMs were prepared as described (44). RNA-seq library preparation and sequencing was performed by Genewiz (Azenta Life Sciences) and data was trimmed, filtered, and aligned to the reference genome to perform differential gene expression analysis on the Subio platform. RaceCAR-M/P BMDMs were generated by lentivirus infection, and phagocytosis was assessed by time-lapse imaging and flow cytometry as described (44).

Data, Materials, and Software Availability. Data including all raw image files in this study will be made available upon request. RNA-seq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number [GSE247106](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE247106) (71). All reagents generated in this study will be available upon request.

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