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Use of a pH-Sensitive Probe to Study the Role of PKC-epsilon in Phagocytosis of Pathogens

Rebekah Marie Pierce
University at Albany, State University of New York

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Student Name: Rebekah Pierce Student ID 001090681

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Honors Thesis Research Advisor: Dr. Michelle Lennartz — Print name — Signature — Date 29Apr2015

Honors Thesis Committee Member: Dr. Caro-Beth Stewart — Print name — Signature — Date 30Apr2015

Honors Comm. 2nd Member: (optional) — Print name — Signature — Date

CHOOSE ONE: ☒ **Department Chair** OR ☒ Director of Department Honors Committee

Biology Honors Committee Director & Department Chair RICHARD S. ZITOMER — Print name — Signature — Date 5/12/15

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“Use of a pH-Sensitive Probe to Study the Role of PKC-epsilon in Phagocytosis of Pathogens”

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Rebekah Marie Pierce

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Abstract

The main force behind innate immunity, our primary defense against infection, is the clearance of pathogens, or phagocytosis. Protein Kinase C-epsilon (PKC-ε) is necessary for efficient phagocytosis of antibody (IgG)-coated particles. Previous studies used IgG-coated glass beads, which are larger and more rigid than bacteria and lack their complex surface protein expression. To test the hypothesis that PKC-ε is necessary for phagocytosis of pathogens, we compared the rate of uptake of common pathogens by macrophages from wild type and PKC- ε knock out mice. We tested two targets, a bacteria (*E. coli*) and a yeast (zymosan), both physiologically relevant phagocytic targets of different sizes. To link these studies with our previous work, we included IgG-opsonized *E. coli*, to determine the involvement of PKC-ε in uptake of bacteria through the Fcγ receptor, a macrophage surface receptor that targets the immune complex IgG. Furthermore, to determine if differentiation environment impacted the results, we tested two populations of macrophages, *in vivo*-differentiated (ie. thioglycollate-elicited) peritoneal macrophages (PMacs) and *in vitro* differentiated bone marrow-derived macrophages (BMDMs).

The *E. coli* and zymosan used were labeled with the pH-sensitive dye pHrodo™, which fluoresces in the acidic environment of the phagosome but not in the neutral pH of the extracellular buffer, allowing us to specifically detect internalized particles. By utilizing flow cytometry (FACS) as a sensitive and objective measure of pathogen uptake, we determined not only the percentage of cells that internalized at least one target, but also the level of fluorescence/cell as a relative readout of the number of particles ingested. By plotting the mean fluorescence intensity (MFI) against time we were able to calculate the rate of internalization (slope of the MFI vs time line) and compare the results from two or more macrophage populations. We found that PKC-ε knockout macrophages had significantly lower rates of internalization for both unopsonized and IgG-opsonized pHrodo® *E. coli* particles, as well as a decrease in the rate of internalization of unopsonized zymosan. This suggests that the phagocytic defect in PKC-ε null macrophages is not restricted to one or a small subset of receptors, as the defect was seen across organism and target size.

Acknowledgments

A very big thank you to Dr. Michelle Lennartz for allowing me to work in her lab, and for helping me at all steps of the process of scientific research, from writing an abstract, keeping an experiment journal, conducting experiments, analyzing data, presenting data, and writing scientific papers, as well as helping me during experiments. Thank you to everyone in the Lennartz lab for helping me in general, from developing protocols to sharing solutions, and especially thank you to the lab manager, Cheryl Hanes, for all of the support and teaching given; without the help my experiments would have literally fallen apart many times over.
Thank you to the Center for Cell Biology and Cancer Research at Albany Medical College for allowing me to volunteer as part of the Lennartz lab, and use the machines and resources from the center.

Thank you to Dr. Zitomer, Dr. Osuna, and Dr. Cunningham, as well as Joanne Baronner, Blanche Feck, and Neketa Roberts in the Department of Biological Sciences at the University at Albany for support throughout the two years that led to this thesis. Especially thank you to Dr. Stewart, for reading this thesis and providing invaluable advice and suggestions.

Finally, thank you to my friends and family for listening to me go on and on about strange topics with terms that were completely foreign, but supporting and encouraging me nonetheless.

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Introduction

Innate immunity relies on the ability to recognize, internalize, and kill pathogens. Protein Kinase C (PKC) is a serine/threonine kinase that plays an important role in the immune response to pathogens, with several isoforms regulating specific processes. The isoform PKC-epsilon (PKC-ε) is involved in several responses to phagocytosis mediated by the Fc gamma receptors (FcγR), a specific class of Fc receptors found in macrophages that bind specifically to IgG, a specific immune complex within the immunoglobin superfamily (Fridman, 1991). The FcγR is responsible for promoting phagocytosis of pathogens coated with IgG; it is then further responsible for mediating the uptake of targets, generation of the microbicidal respiratory burst, and induction of inflammatory genes (Larsen et al., 2002). Previous studies have shown that PKC-ε localizes at both the forming (small arrow) and nascent phagosome (large arrow) (see Figure 1 below). Both locations are involved in the internalization of pathogens through the FcγR.

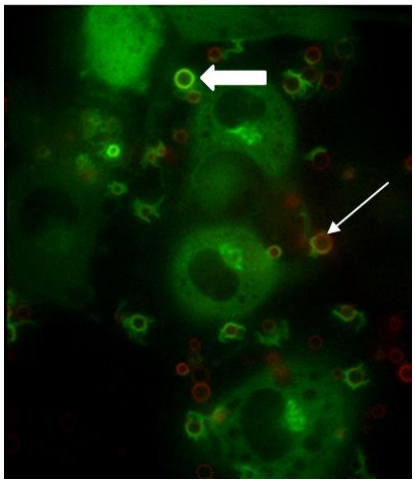

Figure 1: PKC-ε (labeled with GFP) localizes at both the forming (small arrow) and nascent phagosome (large arrow) (Wood, Chow et al, 2013).

Studies performed *in vivo* using live pathogens found that PKC-ε knockout mice are more sensitive to bacterial infection, dying at inoculums of bacteria that are cleared by wild type mice

(Castrillo et al, 2001). Previous studies examining the presence and role of PKC-ε during phagocytosis have used IgG-coated beads to elicit an inflammatory response by targeting the FcγR of macrophages. However, these artificial particles are larger than typical bacteria, and lack the complex mix of surface protein expression found on bacteria. The lab has reported (Larsen et al., 2002) that PKC-ε regulates the rate of IgG-mediated phagocytosis. To elucidate the mechanism by which PKC-ε promotes phagocytosis and pathogen uptake, we tested the hypothesis that PKC-ε is necessary for efficient internalization of pathogens. We tested the ability of macrophages to internalize *E. coli* and yeast cells (zymosan) conjugated with pHrodo™, a fluorescent pH dye that allowed us to follow uptake with flow cytometry. Additionally, we followed internalization of IgG-opsonized *E. coli*, to determine the involvement of PKC-ε in uptake of bacteria through the Fc-gamma receptor (FcγR) and relate these findings to previous experiments performed with beads opsonized with IgG.

Materials

ACK (Ammonium Chloride Potassium) Lysis Buffer: 155 mM Ammonium Chloride, 10 mM Potassium Bicarbonate, 0.01 mM EDTA.

Bone marrow differentiation media: DMEM containing 10% fetal bovine serum (FBS), 20% conditioned L-cell media, and sodium bicarbonate.

HBSS++ (Hank's Balanced Salt Solution): 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 5.55 mM glucose, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 1.5mM $CaCl_2$ and 1.5mM $MgCl_2$

BioParticles® Opsonizing Reagent *(Escherichia coli* and zymosan): Derived from highly purified rabbit polyclonal IgG antibodies and specifically designed to enhance the uptake of the *Escherichia coli* or zymosan BioParticles® conjugates, respectively. (From product sheet supplied by munfacurer, Molecular Probes, Life Technologies Corporation, Carlsbad, CA)

Methods

Cells

To measure the effect of Protein Kinase C-ε (PKC-ε), we compared macrophages from wild type and PKC-ε knockout mice. We further compared macrophages that were differentiated *in vivo* with those differentiated *in vitro*. For *in vivo* macrophage differentiation, we used thioglycollate-elicited peritoneal macrophages (PMacs). Thioglycollate is a sterile irritant that stimulates cell recruitment, allowing the harvesting of differentiated macrophages from the peritoneal cavity without causing illness. A matched set of wild type and PKC-ε null mice were injected with thioglycollate; after 72 hours (the optimal time for obtaining relatively pure macrophages), the cells from the peritoneum of each mouse were collected, and either plated overnight or immediately lysed with ACK lysing buffer to remove contaminating red blood cells. The cells that had been plated were lysed with ACK lysing buffer after 24 hours. After being lysed, the cells were resuspended in HBSS++, and used immediately.

Bone marrow-derived macrophages (BMDMs) were used to compare wild type and PKC-ε knockout cells that were differentiated *in vitro*. To collect BMDMs, the cells from the bone marrow of the femurs of the mice were extruded, and differentiated using bone marrow differentiation media containing L-cell derived growth factors. As stem cells require 7 days for differentiation into macrophages, cells were used 7-10 days after harvesting.

Targets

We used pHrodo® Green Conjugates (Life Technologies Corporation, Carlsbad, CA), particles to which a pH-sensitive dye has been conjugated. The particles fluoresce green in the

acidic environment of the phagosome but not in the neutral pH of the extracellular media. We used pHrodo® Green *E. coli* BioParticles®, and zymosan BioParticles®. Targets arrived desiccated and were resuspended in $HBSS^{++}$ to a final volume that corresponded with a multiplicity of infection, or MOI, as calculated by the manufacturer for a set number of targets per cell.

We also used targets that were opsonized with IgG, to compare opsonized targets with non-opsonized targets for wild type and knockout macrophages. Noting differences between unopsonized and IgG-opsonized targets would allow us to relate our data to previous experiments that used non-pathogen targets opsonized with IgG. Both *E.coli* and zymosan BioParticles® were opsonized using rabbit IgG opsonizing reagent and following the instructions provided by the manufacturer.

Phagocytosis.

To minimize cell loss due to adherence, phagocytosis time-courses were run in polypropylene tubes pre-coated with 0.5% BSA (bovine serum albumin). To synchronize ingestion, cells and tubes were cooled on ice prior to initiation of the experiment. Experimental tubes contained 2.5 x 10^5 cells in 200 µl $HBSS^{++}$. Controls included 5.0×10^5 cells with no added pathogen and 2.5 x 10^5 cells with targets that were maintained on ice for the duration of the timecourse (no internalization, background control). The pHrodo® E.coli were added at a multiplicity of infection (MOI) of 100, or 100 targets per macrophage, for both PMacs and BMDMs. The larger pHrodo® zymosan were added at a MOI of 10, and were only tested with PMacs. After the addition of targets, the tubes were centrifuged (200 x g, 5 min, 4°C) to maximize the contact between cells and targets.

After centrifugation, phagocytosis was initiated by transferring all experimental tubes to a 37°C water bath. At 2.5, 5, 7.5, 10, and 15 minutes, the tubes were removed, the cells were resuspended by scraping the tubes, and the cells were fixed with paraformaldehyde (PFA). A volume of 8% PFA equal to the volume already in the tube was added for a final concentration of 4% PFA. The 0 timepoint was maintained on ice at all times. Initial experiments also included 30 and 60 minute time points, but these were eliminated in favor of earlier timepoints when it was determined that a plateau had been reached by 15 minutes.

After fixing in PFA on ice for at least 15 minutes, the contents of each tube were transferred to polystyrene tubes compatible with the flow cytometer ("flow tubes"). The original polypropylene tubes were washed with 0.5% BSA in PBS, and the wash was also added to the "flow" tube. The cells were pelleted in the centrifuge (200 x g, 5 min, 4°C) and resuspended in 0.5% BSA in PBS for flow cytometry. Due to restrictions on the use of the flow cytometer, Dr. Lennartz performed the flow cytometry and provided me with the files.

Flow cytometry gating.

The flow data was analyzed on FlowJo. (FlowJo LLC, Ashland, OR) The cells were first gated for single cells, then gated for live cells. The geometric mean fluorescence intensity (MFI) was determined for each time point. This value represents the relative number of targets taken up by the macrophages at each time point. By plotting MFI against time, we were able to calculate the rate of pathogen internalization.

Results and Discussion

Designing gates for FACS data analysis

For this experiment, we wanted to make sure we were analyzing data from flow cytometry (FACS) exclusively for live, single macrophages. Using FloJo we gated first for single cells, excluding groups of cells and debris, by graphing the data by size and granularity. Granularity was determined by how the light from the flow cytometer scattered after hitting the particle. Forward scatter width was plotted against forward scatter area, and the cells were gated to exclude the more granular debris (example in Figure 1). We then gated for live cells, excluding dead, nonfunctioning macrophages. We plotted forward scatter against side scatter, and gated for the live cells, excluding the on axis signal (example in Figure 2). The gates were determined using the control containing no pathogens at each experiment, for both cell derivation types and both genotypes, and applied to the specific cell type for that experiment.

Optimizing phagocytosis of pHrodo®-conjugated targets.

The protocol used in this experiment was developed with bone marrow-derived macrophages, (BMDM) and was later applied to peritoneal macrophages (PMacs) to compare how *in vitro* (BMDMs), and *in vivo* (PMacs) differentiation affected the function of the wild type and PKC-ε null macrophages. The experiments were initially run up to 60 minutes to determine when pathogen internalization plateaued. For PMacs treated with unopsonized pHrodo® *E. coli* particles a "plateau" was observed in the MFI, the value representing number of pathogens internalized, at around 15 minutes. (Figure 3) Thus, further experiments with pHrodo® *E. coli*, both unopsonized and IgG-opsonized, time points were carried out until a final time point at 30 minutes, with earlier time points added to better analyze the initial rate of internalization. For

rate calculations, we used data from time points up to 10 minutes as this represented the initial rate of internalization without complications due to the limitations of cell size and particle availability.

As zymosan is larger than *E. coli*, to optimize the time course for pHrodo® zymosan, initial experiments were carried out to 90 minutes. A plateau in number of pathogens internalized was observed around 60 minutes. (Figure 4) However, for the sake of consistency between *E. coli* and zymosan, further experiments with both pathogens were run for 30 minutes, with rate analysis including data up to 10 minutes. Because all conditions were run at the same time it was possible to make multiple comparisons.

PKC-ε KO PMacs have reduced pathogen phagocytosis.

One model of human sepsis is instillation of *E. coli* into the peritoneum of mice. Thus, comparisons were made between wild type and PKC-ε knockout PMacs with unopsonized and IgG-opsonized pHrodo® *E. coli.* The rates are displayed in Table 1, taken from the slopes of the lines created by plotting the MFI against the time points up to 10 minutes, unless otherwise indicated. Due to the day-to-day variability in the assay (possible differences in MOI, biological variability in animals, etc), the Student's paired t-test was the most appropriate statistical test. Compared to PMacs from WT animals, internalization of unopsonized and IgG-opsonized *E. coli*, as well as unopsonized zymosan, was significantly slower in PKC-ε KO PMacs. This suggests that there is an intrinsic defect in the ability of PKC-ε KO macrophages to internalize targets and that the defect is not a function of the size of the particle. While it is interesting that uptake of zymosan, but not IgG-zymosan, was slower in PKC-ε KO cells, uptake of IgG-zymosan was lower in KO cells in 3 of 4 trials. More experiments are necessary to determine if

there are differences in uptake of IgG-zymosan. Overall, the lower rate of uptake is consistent with the susceptibility of PKC-ε KO mice to infection.

Opsonization impacts uptake by PKC-ε knock out, but not wild type, PMACs

The data from Table I was reanalyzed to ask if opsonization impacts target uptake. Table II shows the uptake rates from Table I, rearranged by genotype, to compare uptake of unopsonized and IgG-opsonized *E. coli*. A Student's paired t-test was run to determine if directing bacterial uptake to FcγR altered the rate of internalization. Although we found no significant difference in the rates (Table II), the uptake of IgG-opsonized *E. coli* was higher in 7 of 12 comparisons. Due to the biological variability among the mice from which the macrophages were harvested, as well as the week-to-week variability in the results, more experiments need to be performed, preferably running multiple series of macrophages of each genotype at the same time. As IgG-opsonized *E. coli* was higher in 3 of 6 WT and 4 of 6 PKC-ε KO, we may find, with a larger sample size, that internalization through the FcγR is more rapid than uptake through the receptors (whose identity remain to be determined) mediating phagocytosis of unopsonized *E. coli*.

Bone marrow-derived macrophages and peritoneal macrophages are not significantly different in their rate of pathogen internalization.

Phagocytosis studies with primary macrophages often use BMDMs. The advantage is the high numbers of relatively pure macrophages that are obtained by *in vitro* differentiation of bone marrow with growth factors. Thus, we compared uptake of *E. coli* in BMDM *vs* PMacs. As expected, there was no difference in uptake between the two cell populations (Table III).

However, the comparison between WT *vs* KO PMacs in this limited dataset also did not reach significance (p=0.29, not shown in table), although these 4 values are included in the larger dataset in Table I, where there was significance (p=0.031). This highlights the variability in the assay and the need for a high number of samples and/or running multiple samples simultaneously.

In conclusion, we have developed a flow-based assay to calculate rates of phagocytosis of physiologically relevant targets (*E. coli* and yeast). Although the assay is variable, efficient uptake of pathogens requires the expression of PKC-ε as PKC-ε KO PMacs have significantly lower internalization of large (yeast) and small (*E. coli*) targets. This significance extends to uptake of unopsonized *E. coli* and uptake of IgG-opsonized *E. coli* via Fcγ receptors. More work is necessary to validate these preliminary results.

Data

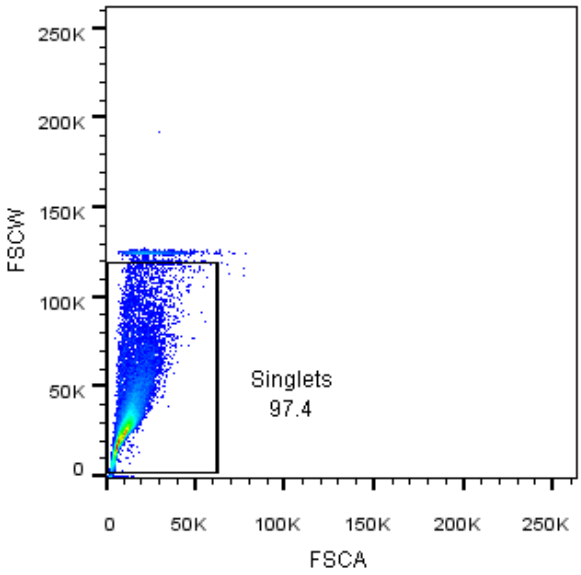

Figure 1:
Gating wild type PMacs for singlets by plotting forward scatter area (FSCA) *vs* forward scatter width (FSCW). The gated population excluded groups of macrophages and debris.

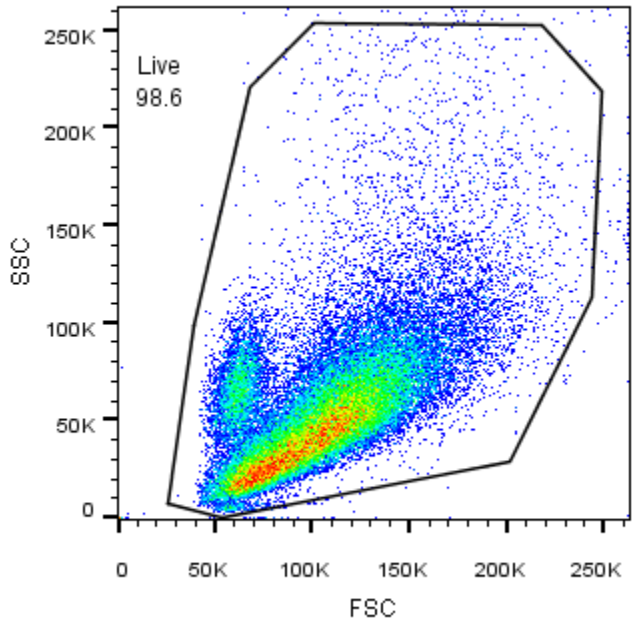

Figure 2:
Gating wild type PMacs for live cells by plotting forward scatter (FSC) *vs* side scatter (SSC). The gated population excluded dead macrophages.

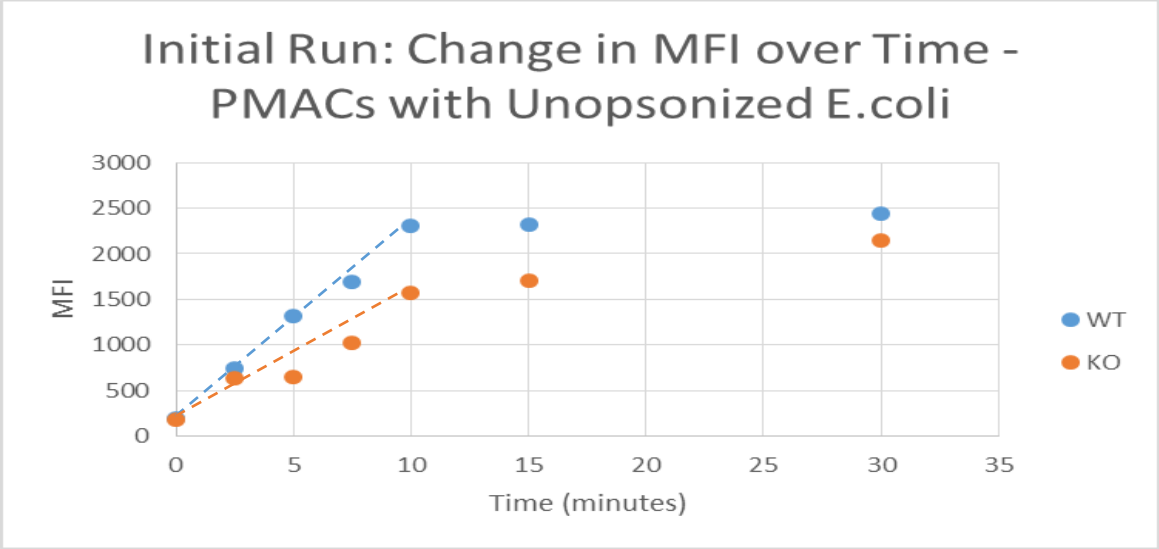

Figure 3:
Initial run of unopsonized pHrodo® *E.coli* with PMacs shows a plateau of uptake around 15 min. Initial relative internalization rates were calculated up to 10 min (indicated by dashed lines).

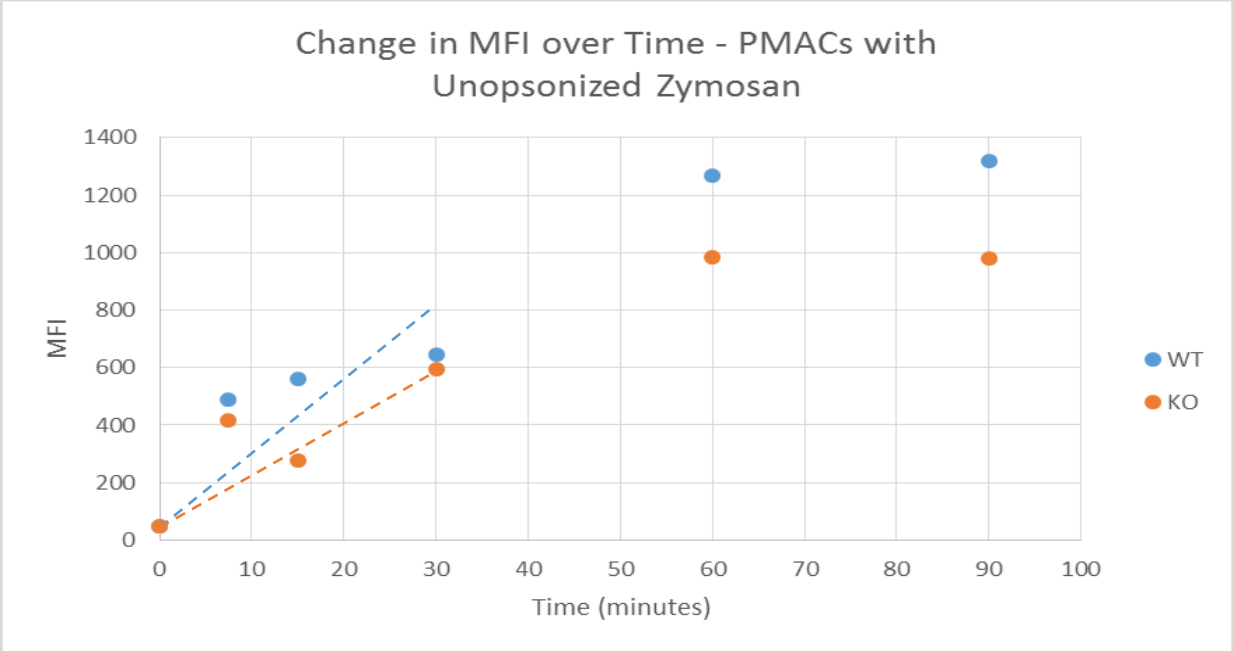

Figure 4:
Initial run of unopsonized pHrodo® zymosan with PMacs shows a plateau of uptake around 60 min. Initial relative internalization rates were calculated up to 30 min (indicated by dashed lines). For consistency, future experiments calculated initial rate of internalization up to 10 min.

Table I: Comparison of WT PMacs and PKC-ε KO PMacs. Relative internalization rates taken from slopes from plots of time vs MFI. KO macrophages have significant reduced internalization of *E. coli*, IgG-opsonized *E. coli*, and zymosan.

	E. coli		***E. coli*-IgG**		**Zymosan**		**Zymosan-IgG**	
Time Points (min)	**WT**	**KO**	**WT**	**KO**	**WT**	**KO**	**WT**	**KO**
(0-10 E. coli, 0-30 zymosan)	167.00	122.00	151.00	144.00	17.31	15.36	38.53	22.40
(0-10)	394.00	228.00	349.00	219.00	21.64	14.36	19.32	12.52
(0-10)	155.28	117.64	212.44	117.64	15.28	4.88	15.84	19.36
(0-10)	69.92	56.80	169.56	92.60	14.36	4.60	14.00	13.40
(0-10)	92.68	103.40	238.52	132.00	-	-	-	-
(0-10)	154.00	81.28	130.28	91.72	-	-	-	-
(0-10)	64.24	19.36	-	-	-	-	-	-
(0-10)	180.08	94.40	-	-	-	-	-	-
(0-10)	36.48	48.28	-	-	-	-	-	-
(0-10)	48.20	51.64	-	-	-	-	-	-
	n = 10		n = 6		n = 4		n = 4	
Average:	136.19	92.28	208.47	132.83	17.15	9.80	21.92	16.92
St. dev:	99.58	55.10	72.57	42.99	2.80	5.07	9.78	4.12
Paired t-test:		0.03		0.01		0.03		0.33

Table II: Comparison of Unopsonized (Unop.) and IgG-opsonized (IgG-op) *E. coli*. Relative internalization rates taken from slopes from plots of time vs MFI. Opsonization does not affect internalization in WT PMac. There is no significant difference in KO PMac, but IgG-opsonized *E. coli* displays increased internalization in 4 of 6 trials. Significance may occur with a larger sample.

	WT PMAC - *E.coli*		**KO PMAC - *E.coli***	
Time Points (min)	**Unop.**	**IgG-op**	**Unop.**	**IgG-op**
(0-10)	167.00	151.00	122.00	144.00
(0-10)	394.00	349.00	228.00	219.00
(0-10)	155.28	212.44	117.64	117.64
(0-10)	69.92	169.56	56.80	92.60
(0-10)	92.68	238.52	103.40	132.00
(0-10)	154.00	130.28	81.28	91.72
	n = 6		n = 6	
Average:	172.15	208.47	118.19	132.83
St. dev:	105.38	72.57	53.89	42.99
Paired t-test:		0.29		0.09

Table III: Comparison of PMac and BMDM particle uptake rates. Relative internalization rates taken from slopes from plots of time *vs* MFI. No significant difference in either WT or KO macrophages. However, KO BMDM show higher internalization than KO PMac in each case. The difference may become significant with a larger sample.

	WT - E.coli		**KO - E.coli**	
Time Points (min)	**PMAC**	**BMDM**	**PMAC**	**BMDM**
0-10	64.24	10.60	19.36	23.44
0-10	180.08	172.80	94.40	239.56
0-10	36.48	95.96	48.28	144.32
0-10	48.20	305.56	51.64	276.48
	n = 4		n = 4	
Average:	82.25	146.23	53.42	170.95
St. dev:	57.34	108.41	26.78	97.87
Paired t-test:		0.42		0.08

Conclusions

Our hypothesis that particle uptake is dependent on the expression of PKC-ε is supported by the data. For both unopsonized and IgG-opsonized pHrodo® *E. coli* particles, PKC-ε knockout peritoneal macrophages had a significantly slower rate of internalization. There was also a decrease in the rate of uptake for unopsonized zymosan in the knockout macrophages, arguing that the defect is not restricted to one or a small subset of receptors. While there was no significant difference between rates of uptake between wild type and knock out macrophages with IgG-opsonised zymosan particles, for three out of four experiments the knock out rate was lower than the wild type rate of uptake, suggesting that a larger sample is necessary to confidently assess significance.

Based on the data collected, it seems that opsonization with IgG does not affect particle uptake in a consistent pattern. However, for both WT and KO cells, there is a trend toward an increase in internalization rate for IgG-opsonized particles. It is likely that IgG opsonization directs targets to high affinity macrophage FcγR, thus promoting uptake, which does not occur with unopsonized targets. This is consistent with the hypothesis that PKC-ε is necessary for pathogen uptake; without the aid of IgG, the rate of uptake was lower in knockout macrophages.

While uptake rates are very different between wild type macrophages differentiated *in vivo* (PMacs) and *in vitro* (BMDMs), there is no consistent pattern. In knock out macrophages, however, uptake rates are higher in macrophages differentiated *in vitro* (BMDMs) than *in vivo* (PMacs). This implies that PKC-ε knockout macrophages may be more sensitive to differences in the environment causing differentiation. With a higher rate of uptake in the bone marrow-derived macrophages, it is possible that macrophages differentiated *in vitro* may be more efficient at pathogen uptake and phagocytosis than macrophages differentiated *in vivo*. Further

testing can be done to expand the sample size to see if the pattern remains, and to look for possible factors that macrophages differentiated *in vitro* are exposed to that could facilitate more efficient pathogen uptake and phagocytosis than those differentiated *in vivo*.

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