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The roles of complement receptor 3 and Fcγ receptors during *Leishmania* phagosome maturation

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ABSTRACT

Leishmania **are intracellular parasites adapted to surviving in macrophages, whose primary function is elimination of invading pathogens. *Leishmania* entry into host cells is receptor-mediated. These parasites are able to engage multiple host cell-surface receptors, including MR, TLRs, CR3, and FcγRs. Here, we investigated the role of CR3 and FcγR engagement on the maturation of *Leishmania*-containing phagosomes using CD11b−/− and FcγR−/− macrophages, and assessing EEA1 and lysosome-associated proteins is necessary for the phagosome maturation delay, characteristic of *Leishmania* infection. *Leishmania*-containing phagosomes do not fuse with lysosomes until 5 h postinfection in WT mice. Phagolysosome fusion occurs by 1 h in CD11b and FcγR common chain KO macrophages, although receptor deficiency does not influence *Leishmania* entry or viability. We also investigated the influence of serum components and their effects on phagosome maturation progression. Opsonization with normal mouse serum, complement-deficient serum, or serum from *Leishmania*-infected mice all influenced phagosome maturation progression. Our results indicate that opsonophagocytosis influences phagosomal trafficking of *Leishmania* without altering the intracellular fate. *J. Leukoc. Biol.* 93: 921–932; 2013.**

Introduction

Intracellular pathogens are highly specialized to reside inside cells, requiring them to avoid or resist host-killing mechanisms. *Leishmania* take advantage of the intracellular niche, surviving inside phagolysosomes of vertebrate macrophages and DCs. *Leishmania* infectious-stage parasites (metacyclic promastigotes) gain entry into host cell macrophages via receptor-mediated phagocytosis. Metacyclic promastigotes actively modulate normal phagosome progression by delaying fusion with lysosomes, theoretically allowing the parasites time to mature into a more antimicrobial-resistant amastigote stage. Amastigotes are eventually released from infected cells and invade neighboring cells, also through receptor-mediated phagocytosis.

Phagocytosis of a foreign body normally results in the development of an early phagosome characteristically marked by EEA1 and rab5, progressing to a late maturation stage marked by LAMP1 and rab7. The late phagosome fuses with lysosomes, allowing for degradation of phagosomal contents. *Leishmania* phagosomes mature at ~5 h, whereas heat-killed parasites traffic to a LAMP1-positive compartment by 1 h, typical of the maturation progression associated with phagocytosis of most microbes and nonmicrobial particles [1, 2]. The proposed mechanism of phagosome maturation delay is insertion of *Leishmania* LPG in to the phagosome membrane, causing the retention of actin, thus preventing fusion with lysosomes [2]. As LPG is a major parasite surface component, interacting with macrophage receptors, it seems likely that LPG engagement of host receptors and resulting signaling could influence phagosome maturation. The most well-characterized host cell receptors for *Leishmania* recognition are CR3, FcγRs, MR, scavenger receptor, and some TLRs [3, 4]. However, it is not clear whether engagement of specific macrophage surface receptors by *Leishmania* influences phagosome maturation and whether this event correlates with parasite survival.

Abbreviations: −/−=deficient, BMDM=bone marrow-derived macrophage, CR3=complement receptor 3, EEA1=early endosome antigen 1, KO=knockout, LAMP1=lysosome-associated membrane protein 1, LPG=lipophosphoglycan, MR=mannose receptor , PG=phosphoglycan, RPMI-C=RPMI, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, SLA=soluble *Leishmania* antigen

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The use of specific macrophage receptors by *Leishmania* remains an active field of study, as certain receptors have been characterized to potentially have different functions in cell signaling and disease outcome. Currently, it is believed that pathogen entry via CR3 does not elicit an oxidative burst, suggesting that use of this receptor for entry increases parasite survival [5]. Another receptor family thought to be important in pathogen recognition is FcRs, through opsonizing antibodies.

CR3 is a β_2 integrin that is found on leukocytes and is a versatile receptor with roles in immunity, adhesion, and phagocyte migration [6]. CR3 is composed of two chains: CD11b and CD18. CD11b is a type I transmembrane surface protein composed of extracellular, transmembrane, and cytoplasmic domains. The C-domain, or lectin-like domain, is thought to be responsible for recognition of pathogen surface molecules, such as *Mycobacterium tuberculosis* oligosaccharides and *Leishmania* LPG [7]. The binding of C3bi, fibrinogen, and bacterial antigens is facilitated by the 200-aa-long I domain near the N-terminal end [8]. These two domains explain the wide range of ligand binding exhibited by CR3. *Leishmania* LPG is a C3 acceptor and can bind CR3 directly [9]; thus, this receptor is thought to be a primary route of *Leishmania* invasion.

FcγRs are important receptors for recognition of antibodies and antibody-coated pathogens. Upon cell–cell spread within an established infection or reinfection of a previously infected host, IgG produced by the vertebrate host covers the surface of *Leishmania* amastigote parasites, making them more susceptible to phagocytosis through FcγRs [10]. There are several FcγR family members that can cause activation or inhibition of cell signaling, dependent on the motif attached to the cytoplasmic domain of the receptor. Of particular interest are FcγRI (CD64) and III (CD16), both of which share a common γ chain (FcγR) containing an ITAM in the cytoplasmic domain [11]. Ligation of FcγRs by IgG-opsonized *Leishmania* amastigotes modulates cytokine expression [12, 13], and FcγRs contribute to susceptibility in BALB/c mice [10, 14]. FcγRs are especially important during subsequent secondary infections, as the host has developed IgG that readily binds to parasite surfaces. However, naturally occurring antibodies—IgM and IgG isotypes found in uninfected individuals—also recognize and opsonize *Leishmania* promastigotes and activate the classical complement pathway [7, 15]. Most of the research concerning FcγRs and *Leishmania* has been performed, evaluating secondary infection using the amastigote life-cycle stage. However, evidence of naturally occurring antibodies makes investigation of the infective metacyclic stage extremely interesting in evaluating the use of FcγRs in parasite entry.

Previous research has implicated a role for CR3 and FcγRs in avoiding immune activation. Here, we explored the deficiencies in knowledge concerning CR3 and FcγR engagement and phagosome maturation during *Leishmania* infection, using CD11b−/− and FcγR−/− macrophages.

MATERIALS AND METHODS

Animal use

Six- to 8-week-old female WT C57Bl/6 and CD11b−/− C57Bl/6 mice were provided by Tanya Mayadas-Norton (Brigham and Women's Hospital and Harvard Medical School, Cambridge, MA, USA). FcγR−/− mice were purchased The Jackson Laboratory (Bar Harbor, ME, USA). C3−/− mice were a generous gift from Jeff Schorey (University of Notre Dame, Notre Dame, IN, USA) [16]; this strain originated on a C57Bl/6 × 129/J background and was backcrossed to C57Bl/6 mice for two generations. Breeding lines of WT and KO were generated from heterozygous matings for all strains. All mice were bred for multiple generations before use in the Freimann Life Science Center at the University of Notre Dame.

Parasite culture

Leishmania donovani 1S strain (MHOM/SD/62/1S), isolated from a visceral leishmaniasis patient in the Sudan, was used for the experiments presented in this study. LPG−/− *L. donovani* mutant R2D2 and PG−/− *L. donovani* C3PO [17] were generously provided by Dr. Salvatore Turco (University of Kentucky). All experiments were repeated at least one time with the *Leishmania major* Freidlin V1 (MHOM/IL/80/Friedlin) strain with similar results (data not shown). Parasites were cultured at 26°C without CO_2 in medium 199, supplemented with 20% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml biotin. Infective-stage metacyclic promastigotes were isolated from stationary cultures (4–5 days old) by using a uniform procedure based on a modification of a method of density-gradient purification described previously [18]. All parasites were tested for *Mycoplasma* contamination [PCR detection method (TaKaRa Bio, Shiga, Japan)] and endotoxin [*Limulus* amoebocyte lysate assay (Endosafe; Charles River Laboratories, Bar Harbor, ME, USA)]. Parasites were washed and unopsonized or opsonized with 5% normal C57Bl/6 mouse serum, C3−/− serum, *Leishmania*-infected serum, or C3−/− *Leishmania*-infected serum prior to infection. Metacyclic promastigotes were labeled with CFSE (Molecular Probes, Carlsbad, CA, USA) at a 5-μM concentration in PBS. After addition of CFSE, parasites were placed at 37°C for 5 min, shaking every minute, followed by quenching with FBS [19]. Parasites were centrifuged at 3500 RPM for 15 min and resuspended in PBS at 20:1 parasites/macrophage for infection. Unattatched parasites were washed out after 1 h postinfection.

Macrophage culture

Bone marrow cells were flushed from femurs and tibias of 6- to 8-week-old mice by standard techniques [20]. Progenitor cells were incubated at 37°C with 5% CO_2 in RPMI-C. L929 cell-conditioned medium, as a source of M-CSF, was added at a concentration of 25% and replaced on the 3rd day [21]. On the 6th day of culture, adherent BMDM cells were harvested, incubated overnight in RPMI-C, and infected on the 7th day.

Immunofluorescence staining

The immunofluorescence staining followed the protocol by Boshans et al. [22]. Briefly, infected macrophages were fixed in 2% PFA (Sigma, St. Louis, MO, USA) in PBS for 30 min at 37°C. Fixed cells were permeabilized with 0.02% Triton X-100 (Sigma), blocked with 0.2% BSA and 0.02% gelatin, and washed with 1% BSA in PBS. The anti-goat polyclonal EEA1 (200 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:20 dilution, and anti-rat monoclonal LAMP1 (50 μg/ml; Developmental Hybridoma Bank, University of Iowa, Iowa City, IA, USA) was used at 1:100 in 1% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 2 h at room temperature. Cy3-conjugated donkey anti-goat (1.5 mg/ml; Jackson ImmunoResearch Laboratories), used at 1:350, and Alexa Fluor 594 donkey anti-rat (2 mg/ml; Invitrogen Life Technologies, Carlsbad, CA, USA), used at 1:750, were used as secondary antibodies for EEA1 and LAMP1, respectively. Actin was stained using Alexa Flour 594-phalloidin (Invitrogen Life Technologies), diluted in PBS at 1:100 for 15 min at room temperature. For LysoTracker staining, BMDM were incubated overnight with 100 nM LysoTracker Red DND-99 (Molecular Probes) prior to infection. Stained cells were mounted using ProLong Gold (Invitrogen Life Technologies) and stored at 4°C until viewed. Imaging was performed using a MRC 1024 scanning confocal microscopy imaging system

(Bio-Rad Laboratories, Hercules, CA, USA). One hundred CFSE-positive (*Leishmania*-containing) phagosomes were assessed for other markers and reported as percent positive.

IgG subclass determination

Six- to 8-week-old mice were injected in the ear dermis with 10^5 *L. major* metacyclic promastigotes in a volume of 20 μl. Serum was harvested at 6–8 weeks postinfection. ELISA was performed using SLA-coated plates. SLA was produced by freeze-thawing a 4-day-old culture of *Leishmania*, and protein concentration was determined using a BCA assay (Pierce, Rockford, IL, USA). IgG subclass-specific secondary antibodies (SouthernBiotech, Birmingham, AL, USA) were used to determine IgG subclass levels. Absorbances were recorded using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). For each isotype, the absorbance for each normalizing sample was used to generate a mean absorbance.

Flow cytometry

Unopsonized and opsonized parasites were fixed in 2% PFA for 30 min and washed three times. Parasites were blocked in 10% normal donkey serum (Jackson ImmunoResearch Laboratories) in FACS buffer. IgG staining used Alexa Fluor 488 goat anti-mouse IgG (H+L; Invitrogen Life Technolgies), and IgM staining used Alexa Fluor 488, γ-chain, absorbed against human IgG1, IgG2, IgG3, IgG4, and IgA (Invitrogen Life Technologies), both at 5 μg/stain. Appropriate isotype controls were used as negative controls. Flow cytometry was performed using an FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with MXP analysis software (Beckman Coulter).

Model-fitting methods

To model the relationship of LAMP1 and time and EEA1 and time, we applied β regression using a logit link function [23]. The β distribution

$$f(y;\mu,\phi) = \frac{\Gamma(\phi)}{\Gamma(\mu\phi)\Gamma((1-\mu)\phi)} y^{\mu\phi-1}(1-y)^{(1-\mu)\phi-1},\ 0 < y < 1,$$

where the mean is μ, and ϕ is a measure of dispersion [24], allows for modeling the response variables—LAMP1 or EEA1—as a proportion. As both response variables change as a function of time, we can model the mean as a function of time using the logit link function, such that

$$\mu = \frac{\exp(\beta_0 + \beta_1 t)}{1 + \exp(\beta_0 + \beta_1 t)}$$

where $-\infty < \beta_i < \infty$, and t is measured in hours. The logit link function is flexible, robust, and allows the mean to vary between one and zero. The logit link function is a familiar characteristic in logistic regression [25]. Parameter values are obtained by maximizing the likelihood function

$$L(\beta_0, \beta_1, \phi \mid y, x) = \prod_{i=1}^{n} \frac{\Gamma(\phi)}{\Gamma\left(\frac{\exp(\beta_0+\beta_1 t_i)}{1+\exp(\beta_0+\beta_1 t_i)}\phi\right)\Gamma\left(\left(1-\frac{\exp(\beta_0+\beta_1 t_i)}{1+\exp(\beta_0+\beta_1 t_i)}\right)\phi\right)} y_i^{\left(\frac{\exp(\beta_0+\beta_1 t_i)}{1+\exp(\beta_0+\beta_1 t_i)}\right)\phi-1}(1-y_i)^{\left(1-\left(\frac{\exp(\beta_0+\beta_1 t_i)}{1+\exp(\beta_0+\beta_1 t_i)}\right)\right)\phi-1},$$

where n is the number of observations. For all experiments, the likelihood was maximized using the Simplex numerical search routine implemented in Mathematica 5.2. Maximums were verified graphically.

After finding the best-fit parameter estimates in each experiment for the LAMP1 and EEA1 response variables, we determined the intersection of the two logit functions in time. The intersection occurs when

$$\frac{\exp(\beta_{0,1} + \beta_{1,1} t)}{1 + \exp(\beta_{0,1} + \beta_{1,1} t)} = \frac{\exp(\beta_{0,2} + \beta_{1,2} t)}{1 + \exp(\beta_{0,2} + \beta_{1,2} t)}$$

where the second subscript of the β parameters identifies the estimates for LAMP1 ($\beta_{0,1}$ and $\beta_{1,1}$) and EEA1 ($\beta_{0,2}$ and $\beta_{1,2}$). All solutions were found in Mathematica 5.2.

RESULTS

As LPG is the primary component of the *Leishmania* surface that interacts with host cells and is essential for *Leishmania* parasites to delay phagosome maturation (ref. [1] and Supplemental Fig. 1), we hypothesized that host proteins involved in recognition of LPG may also play a role in delaying the maturation of *Leishmania* phagosomes. To ensure that LPG is essential to the phagosome delay characteristic of *Leishmania* infection, we infected WT macrophages with two different *L. donovani* mutants deficient in LPG biosynthesis. C3PO is a mutant deficient in the GDP-mannose transporter, necessary for biosynthesis of the repeating units in all PGs and therefore, is lacking secreted PG, proteo-PG, and LPG [26]. R2D2 is deficient in the glactosylfuranose, necessary for extending the nacent LPG chain [27]. BMDMs were infected with CFSE-labeled parasites at various time-points and stained for early (EEA1) and late (LAMP1) phagosome markers. As reported previously, phagosomes containing WT parasites do not lose EEA1 and gain LAMP1 reactivity until 6 h postinfection, and C3PO-containing phagosomes traffic to a LAMP1-positive compartment by 1 h postinfection (Supplemental Fig. 1A). Similarly, R2D2 parasites also do not delay the phagosome maturation process (Supplemental Fig. 1A).

To determine whether CR3 and FcγRs influenced phagosome maturation, we assessed this process in WT, CD11b−/−, and FcγR−/− macrophages via confocal microscopy. Uninfected macrophages from all strains stain equally for EEA1 and LAMP1 (**Fig. 1A**). The majority (95%) of unopsonized *Leishmania*-containing phagosomes is EEA1-positive in WT macrophages at 1 h postinfection (Fig. 1B and D), whereas only 9% of CD11b−/− (Fig. 1B) and 30% of FcγR−/− (Fig. 1D) macrophages are positive. Eight hours postinfection, unopsonized *Leishmania* phagosomes in WT (97%), CD11b−/− (98%), and FcγR−/− (97%) macrophages are positive for LAMP1 (Fig. 1C and E). Infection with living *Leishmania* is necessary for the delay in phagosome maturation, as heat-killed parasites lose EEA and gain LAMP1 reactivity by 1 h postinfection (Supplemental Fig. 1B). As with WT macrophages, heat-killed parasites in CD11b−/− macrophages traffic to a LAMP1-positive compartment faster than live parasites (Supplemental Fig. 1B).

To examine the effect of engaging, specific receptors on the maturation pattern, opsonization was used to "force" the parasites through CR3 or FcγRs; for example, opsonization with normal mouse serum presumably results in C3bi accumulation on parasite surfaces, thus targeting CR3 for internalization by macrophages. To confirm that our parasites were opsonized, we used flow cytometry to assess C3b or antibody deposition on the *Leishmania* surface. Only parasites opsonized with C3-sufficient serum (collected from uninfected or infected mice) positively stained for C3 compared with unopsonized parasites; parasites opsonized with serum collected from C3−/− mice exhibited staining similar to unopsonized parasites (Supplemental Fig. 2A). Unopsonized parasites did not stain for IgG or IgM on their surface (Supplemental Fig. 2B). Normal serum and C3−/− serum-opsonized *Leishmania* exhibited IgM (Supplemental Fig. 2B) and some IgG (Supplemental Fig. 2B) present on their surfaces, the amount varying between serum

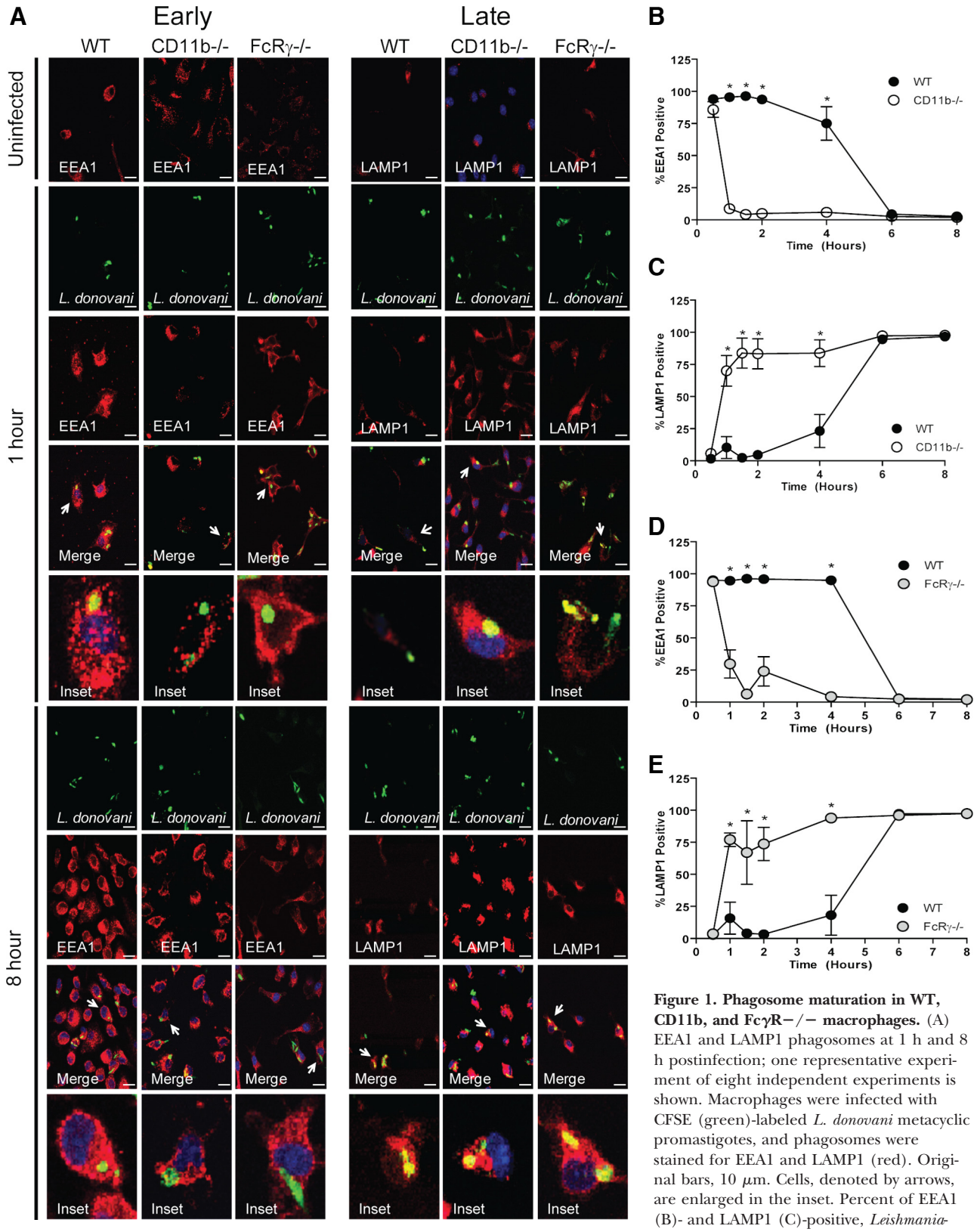

Figure 1. Phagosome maturation in WT, CD11b, and FcγR−/− macrophages. (A) EEA1 and LAMP1 phagosomes at 1 h and 8 h postinfection; one representative experiment of eight independent experiments is shown. Macrophages were infected with CFSE (green)-labeled *L. donovani* metacyclic promastigotes, and phagosomes were stained for EEA1 and LAMP1 (red). Original bars, 10 μm. Cells, denoted by arrows, are enlarged in the inset. Percent of EEA1 (B)- and LAMP1 (C)-positive, *Leishmania*-containing phagosomes (CFSE-positive) in CD11b−/− and WT macrophages was assessed over 8 h infection (n=9). Percent of EEA1 (D)- and LAMP1 (E)-positive, *Leishmania*-containing phagosomes in FcγR−/− and WT macrophages was assessed over 8 h infection (n=6). Closed circles represent WT, open circles represent CD11b−/−, and shaded circles represent FcRg−/−. One hundred CFSE-positive phagosomes/coverslip were counted in duplicate/time-point. Each data point represents the mean ± SEM. All comparisons were significant (P<0.001), determined by ANOVA; *significance by Bonferonni's post-test.

samples, indicating a potential role for antibodies available for promastigote entry. Opsonization of parasites with infected mouse serum from C3-sufficient and C3−/− mice had similar amounts of IgG and IgM on their surface.

Opsonized parasites were used to infect WT BMDMs, and the time of switching from an EEA1- to a LAMP1-positive compartment was assessed for *Leishmania* phagosomes (**Fig. 2A**). Our measure of phagosome maturation was the loss of EEA1 positivity and acquisition of LAMP1 in phagosomal membranes. Serum opsonization with C3-sufficient serum (C3+Ab−) resulted in an ~3-h *Leishmania* phagosome maturation pattern. Removal of C3 components by using C3−/− serum (C3−Ab−) impacts *Leishmania* phagosome maturation directly, as C3−/− opsonized parasites exhibited a 4- to 5-h phagosome maturation pattern, similar to unopsonized parasites. Opsonization with *Leishmania* immune serum (C3+Ab+), containing C3 and *Leishmania*-specific IgG (**Table 1** and Supplemental Fig. 2B), resulted in phagosomes maturing at ~2 h postinfection in WT macrophages. The presence of C3 did not influence the response when antibody is present as WT macrophages mature at ~2 h, as observed when normal mouse-infected serum was used.

To determine more accurately the exact time of loss of EEA1 and gain of LAMP1 on *Leishmania* phagosomes, we generated mathematical models to assess the switch time by calculating the intercept between the EEA1 and LAMP1 curves

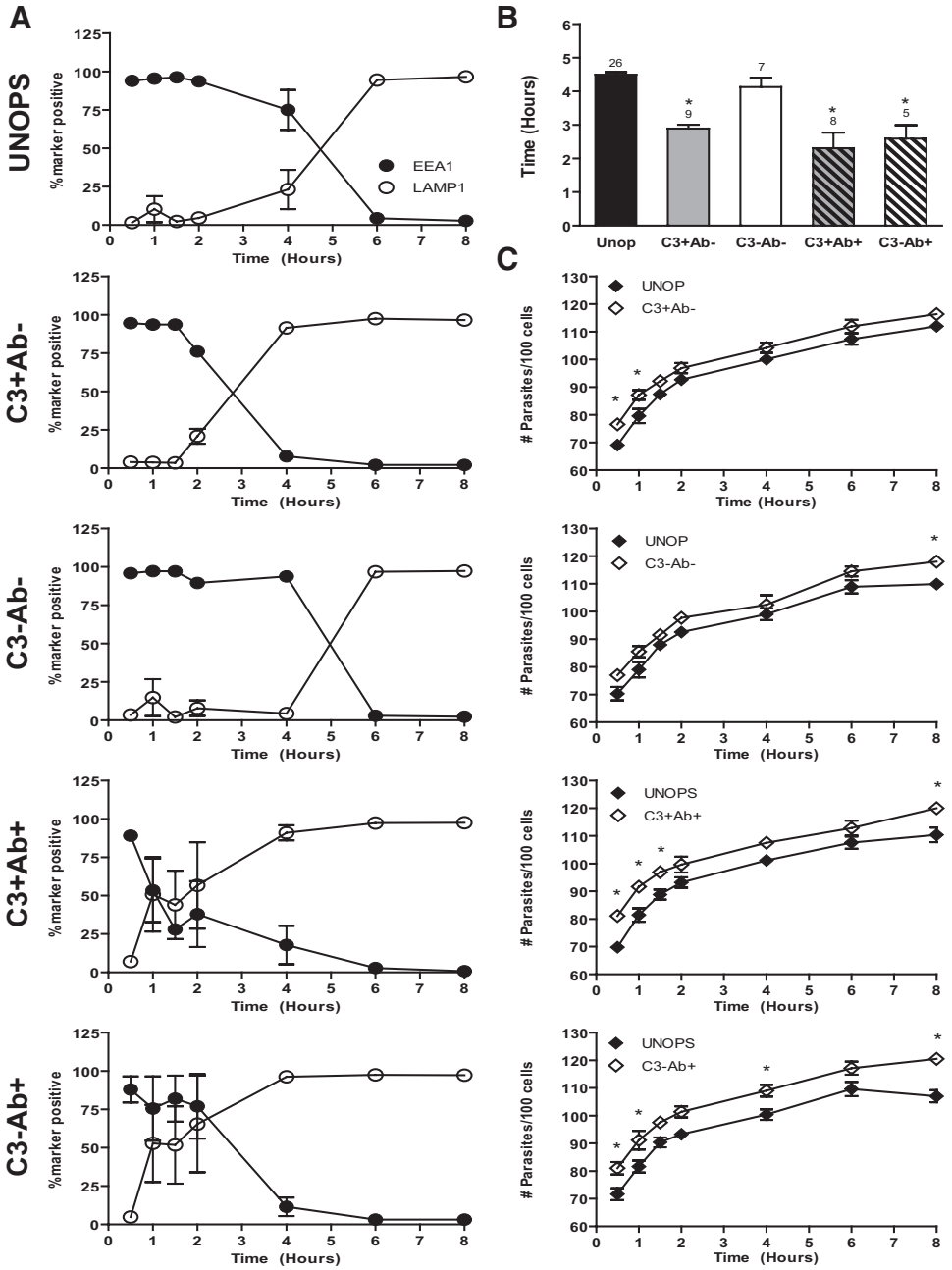

Figure 2. Opsonization affects phagosome maturation. (A) WT macrophages infected with *L. donovani*, unopsonized (UNOPS) or opsonized in normal mouse serum (C3+Ab−), C3−/− serum (C3−Ab−), infected mouse serum (C3+Ab+), or C3−/− infected serum (C3−Ab+). Parasites were labeled with CSFE, and phagosomes were assessed for EEA1 and LAMP1. Closed circles represent EEA1, and open circles represent LAMP1. One hundred CFSE phagosomes/coverslip were counted in duplicate/time-point. Each data point represents the mean ± SEM. (B) Mathematical modeling of WT macrophage-phagosome maturation times. Modeling was determined by Mathematica 5.2. Mean switch intercept times ± SEM for each condition were used to generate graphs. The number of successful models incorporated into each condition calculation is indicated above each bar. (C) The number of intracellular parasites/100 cells for each opsonization condition. Each data point represents the mean ± SEM. *$P \leq 0.05$ Bonferonni's multiple comparison test following ANOVA.

TABLE 1. *Leishmania*-specific antibody subclasses from *Leishmania*-infected mouse serum

Isotype	C3+/+	C3−/−
IgG1	2.64 ± 0.15	2.69 ± 0.39
IgG2a	2.52 ± 0.93	2.21 ± 0.35
IgG2b	3.2 ± 0.44	3.3 ± 0.00
IgG3	2.37 ± 0.07	1.46 ± 0.18
IgE	2.77 ± 0.39	1.94 ± 0.33
IgM	2.69 ± 0.25	2.52 ± 0.59
Total Ig	3.51 ± 0.19	3.54 ± 0.36

Isotypes determined by specific ELISA; data are represented as optical density.

(Supplemental Fig. 3). With the application of these models to data from WT macrophages, we determined that phagosome maturation with unopsonized parasites resulted in a 4.45 ± 0.075-h maturation pattern (Fig. 2B). Opsonization of *Leishmania* parasites certainly influences phagosome progression, as parasites opsonized with normal mouse serum (C3+Ab−) switch to a LAMP-positive compartment at 2.81 ± 0.35 h. Opsonization with *Leishmania*-specific IgG modified the maturation pattern as well; the switch times for C3+Ab+ and C3−Ab+ opsonized parasites were calculated at 2.31 ± 1.31 and 2.59 ± 0.40 h, respectively. Opsonization with C3 or infected serum slightly increased the rate of phagocytosis measured at early time-points, although it is evident that *Leishmania* parasites readily infect WT macrophages, even in the absence of opsonization (Fig. 2C).

All opsonization conditions in CD11b−/− macrophages resulted in ~1 h phagosome maturation, indicating a crucial role for CR3 in phagosome maturation induced by *Leishmania* infection (**Fig. 3**). Interestingly, the absence of CD11b did not influence the infection rate under any of the opsonization conditions (Fig. 3). In FcγR−/− macrophages, opsonization with C3, in the presence or absence of parasite-specific IgG, slightly changed the maturation pattern from WT macrophage inducing an earlier loss of EEA1 and LAMP1 acquisition (~2 h rather than 3 h; **Fig. 4**). Suprisingly, IgG opsonization-induced maturation in the absence of C3 was not significantly different between WT and FcγR−/− macrophages. Also of interest is the fact that all of the opsonization conditions, except for infected serum from WT mice (C3+Ab+), significantly altered EEA1 loss and LAMP1 acquisition in FcγR−/− compared with unopsonized parasites (C3+Ab−: EEA1, $P=0.0016$, and LAMP1, $P=0.0293$; C3−Ab−: EEA1, $P<0.0001$, and LAMP1, $P<0.001$; C3+Ab+: EEA1, $P=0.0719$, and LAMP1, $P=0.4029$; C3−Ab+: EEA1, $P<0.0001$, and LAMP1, $P=0.0003$). As with CD11b−/− macrophages, the absence of FcγR−/− did not influence parasite uptake (Fig. 4).

Phagosome maturation was assessed using LysoTracker Red staining to verify data generated by LAMP1 staining-indicated lysosomal fusion. This dye readily stains acidic compartments—a clear indicator of phagosome maturation. LysoTracker Red was loaded into macrophages overnight, prior to infection, to allow for complete marking of lysosomes. LAMP1 staining correlated very closely to LysoTracker Red in WT, CD11b−/−, and FcγR−/− macrophages. As observed in **Fig. 5**, WT macrophages, infected with unopsonized *Leishmania*, do not exhibit LAMP1 (5%) or LysoTracker Red (3%) at 1 h. FcγR−/− and CD11b−/− macrophages exhibit LAMP1 (95% and 92%, respectively) and LysoTracker Red (96% and 97%, respectively) at 1 h. Upon opsonization, the same maturation pattern is observed between LAMP1 and LysoTracker Red in macrophages from all mouse strains used (Fig. 5).

Presumably, early fusion of *Leishmania* phagosomes with lysosomes would result in damage of metacyclic promastigotes and eventual elimination of the parasites. To test this hypothesis, we infected WT, CD11b−/−, and FcγR−/− macrophages with *L. donovani*-unopsonized metacyclic promastigotes and assessed parasite burdens, 72 h postinfection. Parasite growth was not affected by the absence of CD11b or FcγR in the presence or absence of opsonization with normal mouse serum (**Fig. 6**).

Actin retention is believed to contribute to the phagosome maturation delay exhibited upon infection with *Leishmania* parasites; this recruitment surrounds the phagosome and prevents fusion with the lysosome, allowing the parasites enough time to change life-cycle stages [28–30]. Actin recruitment around phagosomes was observed in WT macrophages infected with unopsonized *Leishmania* at 1 h postinfection and disappeared by 4 h (**Fig. 7**), corresponding to LAMP1 positivity (Fig. 1). Few phagosomes in CD11b−/− and FcγR−/− macrophages at any time-point had actin accumulation upon infection with unopsonized *Leishmania* phagosomes (Fig. 7). Opsonization of *Leishmania* resulted in actin recruitment to the phagosomes in FcγR−/− macrophages, similar to WT cells. Parasites coated with C3−/− or C3-immune (C3-sufficient or C3−/−) serum caused actin recruitment to *Leishmania* phagosomes in CD11b macrophages (Fig. 7). If actin were recruited initially, actin disaggregation around *Leishmania* phagosomes was not influenced by opsonization, dissipating by 4 h in all conditions (Fig. 7). Notably, actin recruitment/retention did not correlate with the loss of EEA1 positivity and fusion of lysosomes with the parasite phagosomes, as actin was retained in many opsonized conditions (Fig. 7) where EEA1 is lost and LAMP1 acquired on phagosomal membranes (Figs. 2–4).

DISCUSSION

Recent literature has begun to shed light on the many mechanisms by which intracellular pathogens subvert host cell-killing mechanisms. In particular, the role of host surface receptors and their downstream signaling effects is an active area of investigation. CR3 and FcγRs are cell-surface receptors that are engaged by *Leishmania* and have been implicated in parasite entry. Our data demonstrate that both of these proteins are necessary for delaying phagosome maturation (Figs. 1 and 3–5) during *Leishmania* infection. Typically, *Leishmania* phagosome maturation takes 5 h [1]; however, unopsonized *Leishmania* infection of CD11b−/− and FcγR−/− macrophages exhibited a phagosome maturation pattern of 1 h or less (Fig. 1), typical of nonmicrobial particles. Our data indicate a clear role for these receptors in *Leishmania*-induced phagosome maturation delay.

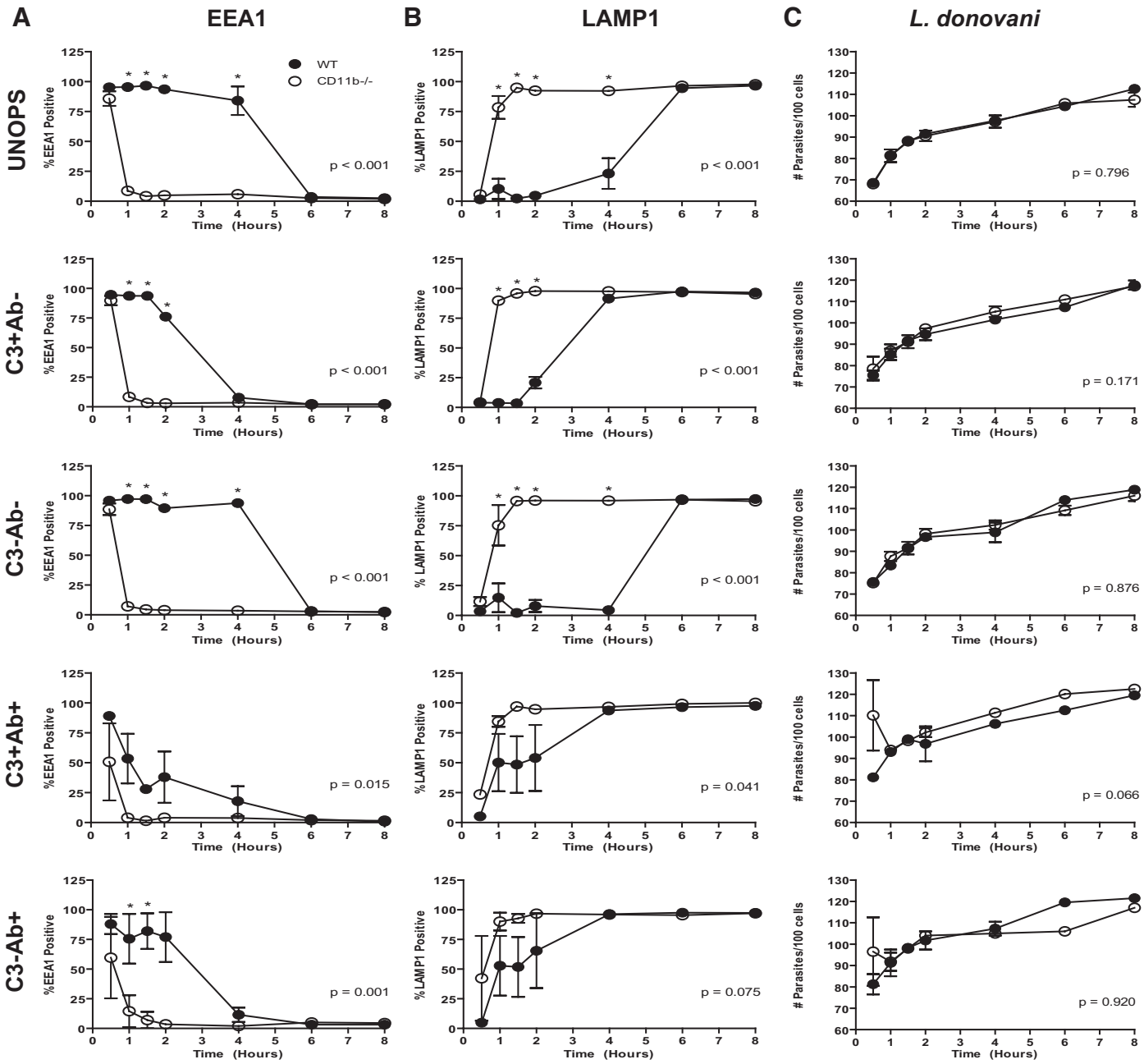

Figure 3. Opsonization does not affect phagosome maturation in CD11b−/− macrophages. WT (closed circles) and CD11b−/− (open circles) macrophages infected with *L. donovani*, unopsonized (n=7) or opsonized in normal mouse serum (C3+Ab−; n=4), C3−/− serum (C3−Ab−; n=4), infected mouse serum (C3+Ab+; n=3), or C3−/− infected serum (C3−Ab+; n=3). Parasites were labeled with CSFE, and phagosomes were stained for EEA1 (A) and LAMP1 (B). One hundred CFSE-positive phagosomes/coverslip were counted in duplicate/time-point. (C) Number of intracellular parasites/100 cells for each opsonization condition. Each data point represents the mean ± SEM. *$P \leq 0.05$ Bonferonni's multiple comparison test following ANOVA; ANOVA P values presented on graphs.

The primary role of CR3 is in recognition of C3bi, a component of the complement pathway naturally found in host serum. *Leishmania* LPG [4, 7] and GP63 [31, 32] are readily complement-opsonized and also bind CR3 directly, thus implicating CR3 as the main route of entry for the parasite. Through the use of different opsonization conditions, we were able to examine the outcome of conditions, allowing parasites to engage CR3 and/or FcγRs on the characteristics of parasite entry and phagosome maturation. Although foreign particles engulfed by WT macrophages normally traffic to LAMP1-positive compartments within 1 h postphagocytosis, *Leishmania*-containing phagosomes do not aquire LAMP1 until 4–5 h postinfection (Fig. 1). *Leishmania* coated in normal mouse serum (containing C3) resulted in an ~3-h phagosome matura-

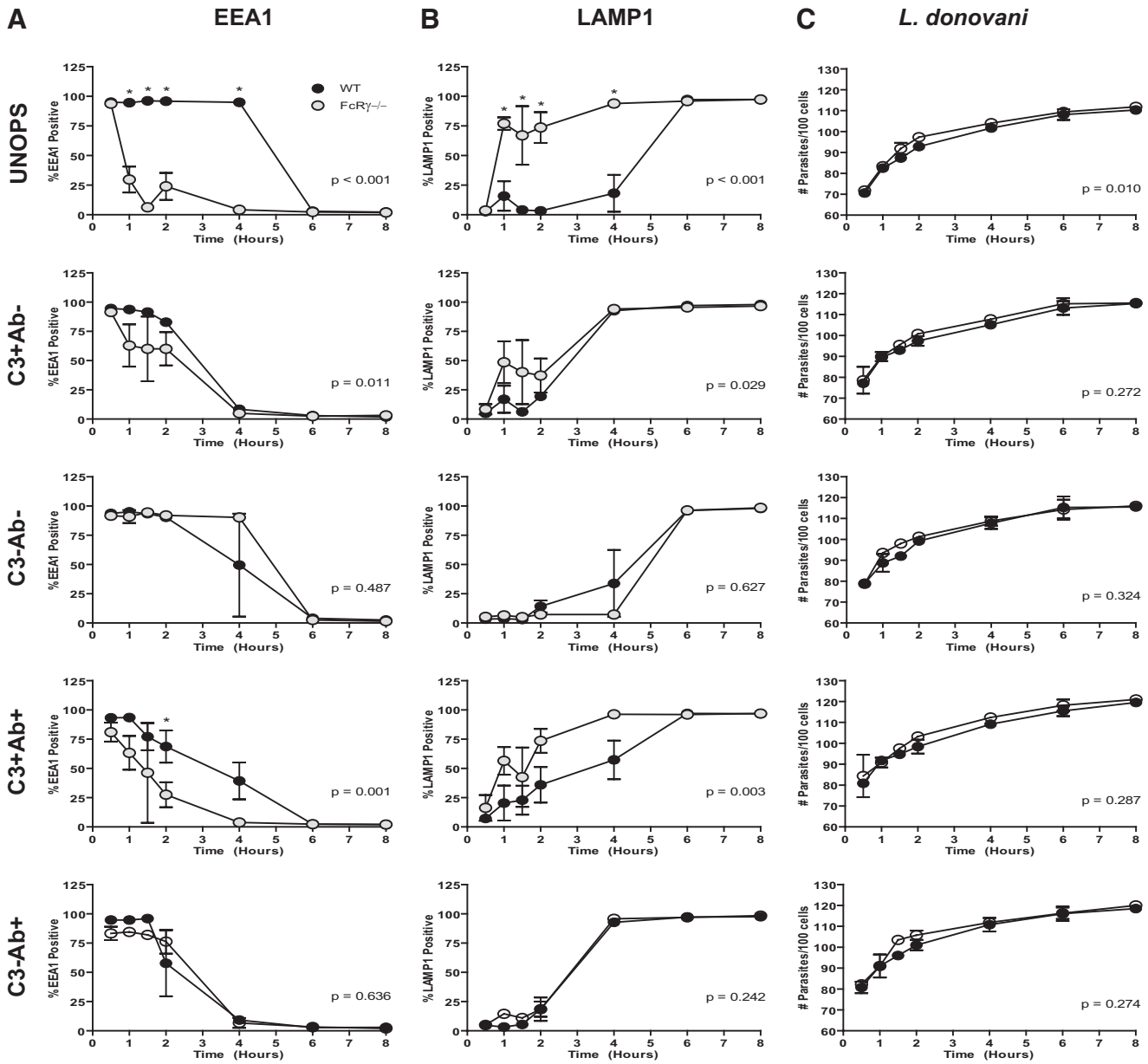

Figure 4. Opsonization effects on phagosome maturation in FcγR−/− macrophages. WT (closed circles) and FcγR−/− (shaded circles) macrophages infected with *L. donovani*, unopsonized (n=6) or opsonized in normal mouse serum (C3+Ab−; n=5), C3−/− serum (C3−Ab−; n=3), infected mouse serum (C3+Ab+; n=5), or C3−/− infected serum (C3−Ab+; n=2). Parasites were labeled with CSFE, and phagosomes were stained for EEA1 (A) and LAMP1 (B). One hundred CFSE-positive phagosomes/coverslip were counted in duplicate/time-point. (C) Number of intracellular parasites/100 cells for each opsonization condition. Each data point represents the mean ± SEM. *$P \leq 0.05$ Bonferonni's multiple comparison test following ANOVA; ANOVA P values presented on graphs.

tion delay (Fig. 2). However, *Leishmania*-containing phagosomes acquired LAMP1 by 1 h after phagocytosis in CD11b−/− macrophages under these conditions (Fig. 3). This result suggests that when CR3 is available, C3-opsonized parasites enter via CR3, and phagosome maturation takes ~3 h. When CR3 is not available, another receptor is used, which results in rapid phagosome maturation. Potential receptors include CR1 (CD35) or CR2 (CD21), which bind complement breakdown products [33]. CR1 assists in CR3 binding and internalization of opsonized parasites, and blocking CR1 and CR3 results in dramatic inhibition of *L. major* binding and phagocytosis by human macrophages [34–36]. Furthermore, CR4 (CD11c/CD18) also binds C3bi and has been implicated in phagocytosis of other pathogens [37, 38]. Whereas FcγR−/− macrophages also express CR3, our results indicated that CR3 engagement through opsonization of *Leishmania* parasites with normal mouse serum or C3-sufficient immune serum results in a slightly faster progression to a mature

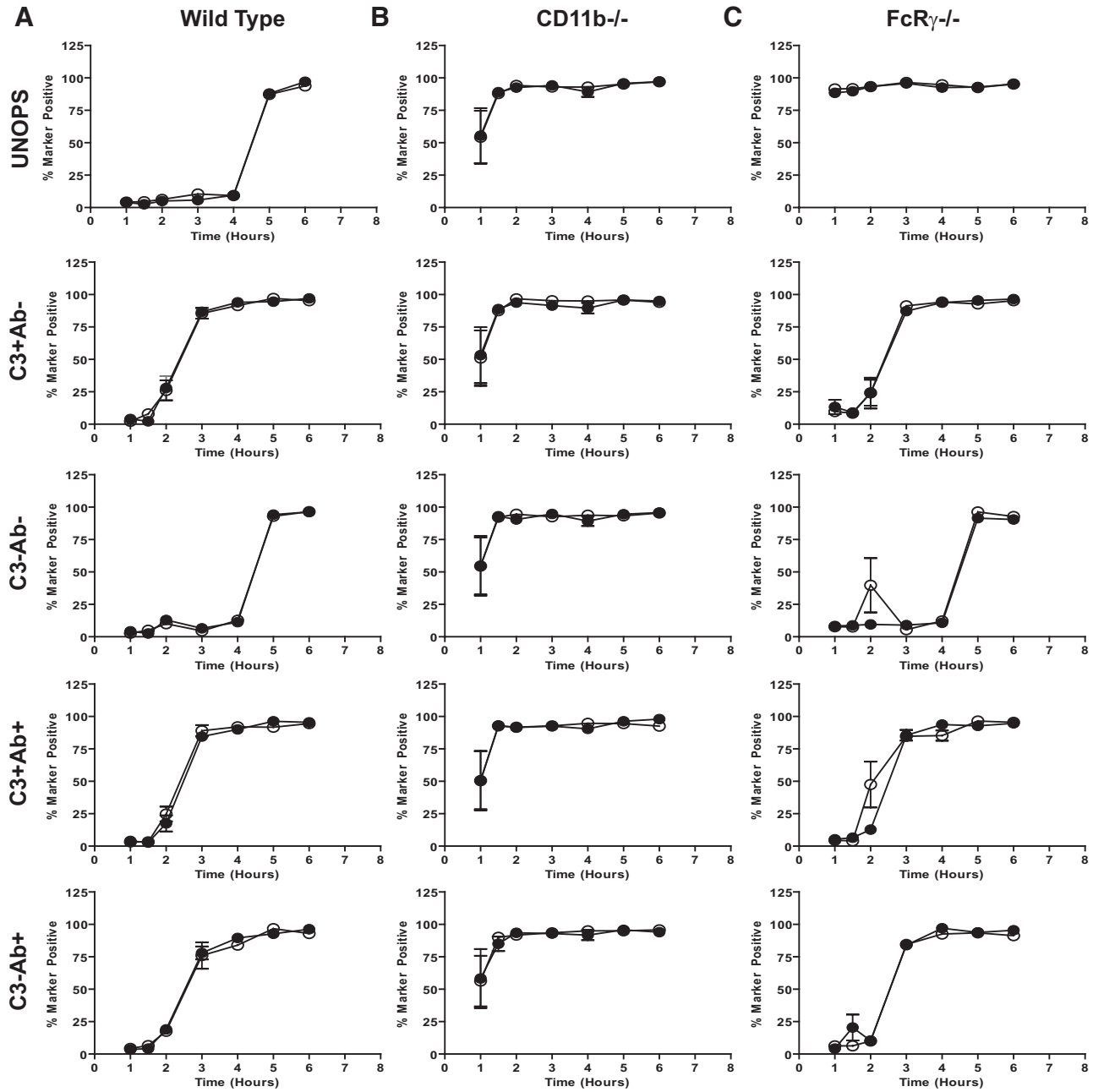

Figure 5. Phagosome maturation confirmation of LAMP1 by LysoTracker Red. WT (A), CD11b−/− (B), and FcγR−/− (C) macrophages, infected with unopsonized *L. donovani* or parasites opsonized in normal mouse serum (C3+Ab−), C3−/− serum (C3−Ab−), infected mouse serum (C3+Ab+), or C3−/− infected serum (C3−Ab+). Parasites were labeled with CSFE, and phagosomes were stained for LAMP1 (open circles) or by LysoTracker Red (closed circles). One hundred CFSE-positive phagosomes/coverslip were counted in duplicate/time-point. Each data point represents the mean ± SEM from pooled replicates of two independent experiments.

phagosome in FcγR−/− compared with WT macrophages. One possibility to explain this result is that FcγR-mediated signaling modulates the phagosome maturation pathway when parasites are coligated with CR3; a thesis that is not unprecedented as FcγRs and CR3 have been reported to cooperate in other systems [39–41].

To examine a role for C3 directly, we examined parasites opsonized in C3−/− serum, which resulted in a reversion back to a 4- to 5-h phagosome maturation pattern in WT and FcγR−/− macrophages compared with parasites opsonized in normal mouse serum. These results implicate C3 as the protein responsible for the shift to a 3-h phagosome maturation pattern. *Leishmania* phagosomes in CD11b−/− macrophages matured with the normal kinetics expected with nonmicrobial particles (i.e., acquired LAMP1 by 1 h) in all opsonization conditions, clearly demonstrating a necessity for CR3 during

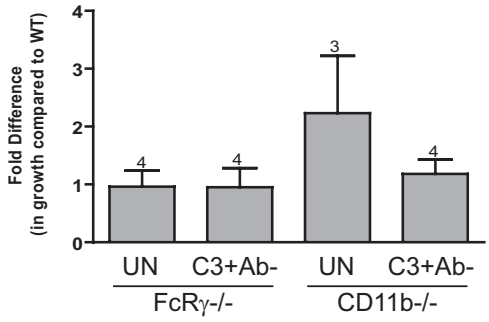

Figure 6. Parasite survival in WT, CD11b−/−, and FcγR−/− macrophages. WT, CD11b−/−, and FcγR−/− macrophages were infected with unopsonized (UN) or normal serum-opsonized (C3+Ab−) *L. donovani* for 1 h, washed to remove nonattached parasites, and incubated for an additional 71 h. The number of intracellular parasites/100 cells was assessed at 4 h and 72 h postinfection by Diff-Quick staining and light microscopy. Growth was determined by dividing the 72-h value by the 4-h value. Data are expressed as a fold difference between KO macrophages compared with WT control macrophages. Each data point represents the mean ± s[SCAP]D. No statistically significant differences were detected (Student's *t*-test).

Leishmania-induced maturation delay. The differential binding abilities of parasites to the two domains within CD11b may explain some differences observed. Unopsonized parasites bind CD11b through the lectin domain [7], whereas opsonized parasites bind through the I domain [8]. This differential signaling between the two domains may influence phagosome maturation.

Also of interest is the effect of antibodies and other serum components in *Leishmania* phagosome maturation. Intact C3 is important for production of IgG and for efficient removal of antibodies [40]. However, we did not detect notable differences in the amount of *Leishmania*-specific IgG production in C3−/− mice (Supplemental Fig. 2B and Table 1). The presence of *Leishmania*-specific IgG in C3-sufficient or C3−/− serum induced an ~2.5-h phagosome maturation pattern in WT macrophages, suggesting that IgG-mediated entry influences the rate of phagosome maturation directly. The precise role of FcγR in the response to IgG opsonization, however, is less clear, as the presence/absence of FcγR did not appear to modulate the timing of maturation much in the presence of IgG. The FcγR−/− mice used here are deficient in FcγRI and FcγRIII expression, both activating receptors. FcγR−/− macrophages do express FcγRIIb (CD32) [42], which unlike FcγRI and FcγRIII, has inhibitory signaling capabilities by expressing an ITIM motif in the cytoplasmic domain. Whereas most studies assessing FcRs during *Leishmania* infection are focused primarily on IgG, as a result of its role in secondary infection recognition, other Ig isotypes may also play a vital role in the immune response against *Leishmania*. Particularly interesting is the role of IgM, as a result of naturally occurring antibodies within the host. In addition to FcγRs, macrophages also express the Fcα/μ receptor, which is able to bind IgM efficiently, making this receptor a possible candidate for influencing phagosome maturation [43]. The potential cooperation between multiple receptors requires further investigation to tease out the interplay between CR3 and FcRs.

There are several other receptors and surface opsonins involved in the phagocytosis of *Leishmania* parasites. MR has

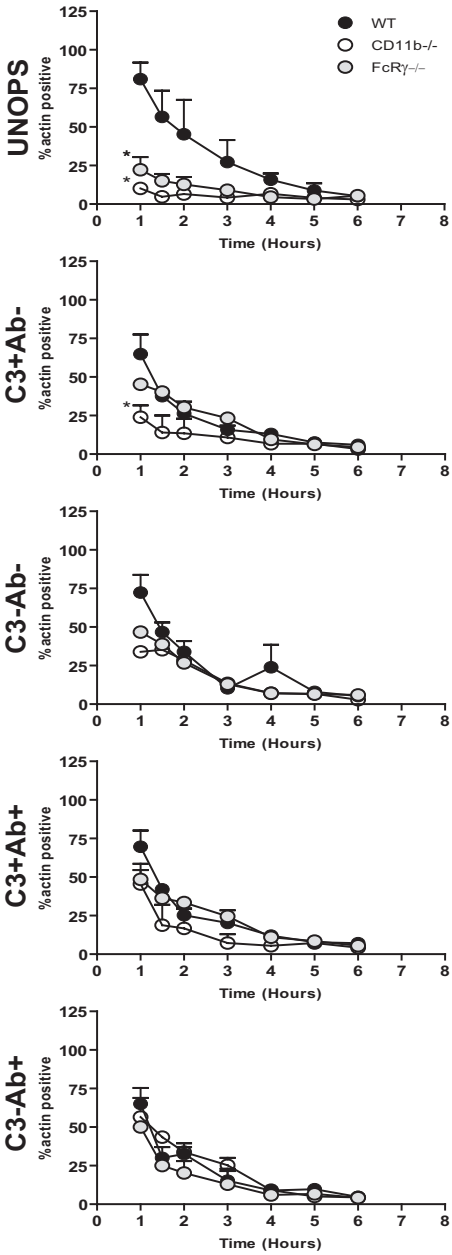

Figure 7. Actin recruitment around L. donovani phagosomes in WT (closed circles), CD11b−/− (open circles), and FcγR−/− (shaded circles) macrophages over 6 h infection. Macrophages infected with unopsonized *L. donovani* or parasites opsonized in normal mouse serum (C3+Ab−), C3−/− serum (C3−Ab−), infected mouse serum (C3+Ab+), or C3−/− infected serum (C3−Ab+). Parasites were labeled with CSFE, and phagosomes were stained with Alexa Fluor 594 phalloidin to mark actin. One hundred CFSE-positive phagosomes/coverslip were counted in duplicate/time-point. Each data point represents the mean ± SEM from two to four independent experiments. ANOVA was used with a subsequent Bonferonni's multiple comparison. *$P \leq 0.05$ compared with WT.

been implicated as collaborating with CR3 in *Leishmania* binding to macrophages and phagocytosis; these receptors have additive effects for binding and internalization of *L. donovani* to macrophages, either through cooperation or modulation of one another [44]. This possible receptor cooperation is substantiated further by the finding that both receptors need to be in close proximity to each other to bind to the parasite [45]. CR3 also cooperates with fibronectin receptors to bind the *L. major* surface protein, GP63, and facilitates phagocytosis [46].

In our experiments, opsonization of *Leishmania* with immune serum (C3+Ab+ and C3−Ab+) or C3-sufficient nonimmune serum (C3+Ab−) resulted in a slight increase in the initial rate of parasite uptake, observed at early time-points (Fig. 2C). This increase was not detected in CD11b−/− (Fig. 3) or FcγR−/− (Fig. 4) macrophages, further supporting the idea that CD11b and FcγR cooperate in uptake of opsonized parasites. Of interest is the fact that regardless of the opsonization status, host genotype, or phagosome phenotype, the parasites survived and proliferated (Figs. 3, 4, and 6). This result is contrary to reports suggesting that *Leishmania* promastigotes that traffic to a mature compartment are degraded [2, 47, 48]. Our results support the thesis of others that *Leishmania* promastigotes are adapted to survive in acidic compartments [49, 50].

The current mechanism proposed to explain the delay in phagosome maturation in *Leishmania*-infected cells is LPG insertion into the phagosome membrane, disrupting lipid microdomains and accumulation of F-actin [51]. As expected, actin recruitment was observed early during infection in WT macrophages but never observed in CD11b−/− or FcγR−/− macrophages infected with unopsonized *Leishmania*. Opsonization, with C3-sufficient or C3−/− serum, in the presence or absence of *Leishmania*-specific antibodies, did not influence actin retention in WT macrophages. Opsonization did, however, induce actin recruitment to *Leishmania*-containing phagosomes in the absence of CD11b or FcγR. We observe early actin retention, which fully dissipates by 4 h postinfection. Interestingly, the accumulation of LAMP1 does not always correlate with loss of actin in our experiments. In particular, phagosome maturation in CD11b−/− macrophages infected with parasites, opsonized in C3−Ab−, C3+Ab+, and C3−Ab+ serum, exhibited actin retention, while also acquiring LAMP1 on phagosomes. These conflicting results are most likely a result of effects of serum components (C3, IgG, and IgM) on other receptors being used for entry of *Leishmania* when CR3 is unavailable. These results are especially intriguing, as they point to other mechanisms being involved in prolonging the phagosome maturation process.

In our study, the kinetics of LAMP1 acquisition mirrored LysoTracker accumulation in *Leishmania*-containing phagosomes, extending our observations to another marker of phagosome maturation. A previous study reported that *L. donovani* excludes the vesicular proton-ATPase from phagosomes and prevents their acidification for up to 24 h [48]. In both studies, the acidiotropic dye LysoTracker Red was used as an indication of acidification; however, we preloaded the macrophages for 24 h prior to infection compared with only 2 h in the other study. Other possible differences to explain the discrepant results are that in our experiments, we assessed C57Bl/6 macrophages infected with purified metacyclic promastigotes, whereas the other researchers investigated BALB/c macrophages infected with stationary-phase *Leishmania* cultures, likely containing procyclic and metacyclic promastigotes.

Our data indicate a necessity for CD11b and FcγR in delaying phagosome maturation and actin recruitment upon phagocytosis of *Leishmania* spp. by murine macrophages. The absence of CD11b and FcγR results in a faster *Leishmania* phagosome maturation pattern in the absence of opsonization. Use of different opsonins has started to shed light on the importance of multiple receptors cooperating with each other during phagocytosis, phagosome maturation, and actin recruitment during infection of macrophages by this intracellular protozoan.

AUTHORSHIP

R.P. participated in experimental design, performed all infections and microscopy experiments, analyzed the data, and drafted the manuscript. M.A.M. conceived of and coordinated the project and participated in project design, data analysis, and manuscript drafting. C.R.C. and J.P.W. participated in *Leishmania* infections, generation of macrophages, and discussion of results. U.G.D. and M.E.W. participated in experimental design and drafting and editing the manuscript. B.J. aided with confocal microsopy, macrophage generation, and infections. W.B. and M.H. completed the flow cytometry experiments to assess opsonization of *Leishmania* parasites. C.L.J. generated the mathematical models.

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REFERENCES

1. Desjardins, M., Descoteaux, A. (1997) Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. *J. Exp. Med.* **185,** 2061–2068.
2. Scianimanico, S., Desrosiers, M., Dermine, J. F., Meresse, S., Descoteaux, A., Desjardins, M. (1999) Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes. *Cell. Microbiol.* **1,** 19–32.
3. Mosser, D. M., Rosenthal, L. A. (1993) *Leishmania*-macrophage interactions: multiple receptors, multiple ligands and diverse cellular responses. *Semin. Cell Biol.* **4,** 315–322.
4. Stafford, J. L., Neumann, N. F., Belosevic, M. (2002) Macrophage-mediated innate host defense against protozoan parasites. *Crit. Rev. Microbiol.* **28,** 187–248.
5. Schonlau, F., Scharffetter-Kochanek, K., Grabbe, S., Pietz, B., Sorg, C., Sunderkotter, C. (2000) In experimental leishmaniasis deficiency of CD18 results in parasite dissemination associated with altered macrophage functions and incomplete Th1 cell response. *Eur. J. Immunol.* **30,** 2729–2740.
6. Ross, G. D. (2002) Role of the lectin domain of Mac-1/CR3 (CD11b/CD18) in regulating intercellular adhesion. *Immunol. Res.* **25,** 219–227.

7. Dominguez, M., Torano, A. (1999) Immune adherence-mediated opsonophagocytosis: the mechanism of *Leishmania* infection. *J. Exp. Med.* **189,** 25–35.
8. Velasco-Velazquez, M. A., Barrera, D., Gonzalez-Arenas, A., Rosales, C., Agramonte-Hevia, J. (2003) Macrophage–*Mycobacterium tuberculosis* interactions: role of complement receptor 3. *Microb. Pathog.* **35,** 125–131.
9. Talamas-Rohana, P., Wright, S. D., Lennartz, M. R., Russell, D. G. (1990) Lipophosphoglycan from *Leishmania mexicana* promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocyte integrins. *J. Immunol.* **144,** 4817–4824.
10. Padigel, U. M., Farrell, J. P. (2005) Control of infection with *Leishmania major* in susceptible BALB/c mice lacking the common γ-chain for FcR is associated with reduced production of IL-10 and TGF-β by parasitized cells. *J. Immunol.* **174,** 6340–6345.
11. Ravetch, J. V., Bolland, S. (2001) IgG Fc receptors. *Annu. Rev. Immunol.* **19,** 275–290.
12. Kane, M. M., Mosser, D. M. (2001) The role of IL-10 in promoting disease progression in leishmaniasis. *J. Immunol.* **166,** 1141–1147.
13. Woelbing, F., Kostka, S. L., Moelle, K., Belkaid, Y., Sunderkoetter, C., Verbeek, S., Waisman, A., Nigg, A. P., Knop, J., Udey, M. C., von Stebut, E. (2006) Uptake of *Leishmania major* by dendritic cells is mediated by Fcγ receptors and facilitates acquisition of protective immunity. *J. Exp. Med.* **203,** 177–188.
14. Thomas, B. N., Buxbaum, L. U. (2008) FcγRIII mediates immunoglobulin G-induced interleukin-10 and is required for chronic *Leishmania mexicana* lesions. *Infect. Immun.* **76,** 623–631.
15. Dominguez, M., Torano, A. (2001) *Leishmania* immune adherence reaction in vertebrates. *Parasite Immunol.* **23,** 259–265.
16. Bohlson, S. S., Strasser, J. A., Bower, J. J., Schorey, J. S. (2001) Role of complement in *Mycobacterium avium* pathogenesis: in vivo and in vitro analyses of the host response to infection in the absence of complement component C3. *Infect. Immun.* **69,** 7729–7735.
17. King, D. L., Turco, S. J. (1988) A ricin agglutinin-resistant clone of *Leishmania donovani* deficient in lipophosphoglycan. *Mol. Biochem. Parasitol.* **28,** 285–293.
18. Spath, G. F., Beverley, S. M. (2001) A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. *Exp. Parasitol.* **99,** 97–103.
19. Kamau, S. W., Nunez, R., Grimm, F. (2001) Flow cytometry analysis of the effect of allopurinol and the dinitroaniline compound (Chloralin) on the viability and proliferation of *Leishmania infantum* promastigotes. *BMC Pharmacol.* **1,** 1.
20. Flamant, S., Lebastard, M., Pescher, P., Besmond, C., Milon, G., Marchal, G. (2003) Enhanced cloning efficiency of mouse bone marrow macrophage progenitors correlates with increased content of CSF-1 receptor of their progeny at low oxygen tension. *Microbes Infect.* **5,** 1064–1069.
21. Stanley, E. R., Heard, P. M. (1977) Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *J. Biol. Chem.* **252,** 4305–4312.
22. Boshans, R. L., Szanto, S., van Aelst, L., D'Souza-Schorey, C. (2000) ADP-ribosylation factor 6 regulates actin cytoskeleton remodeling in coordination with Rac1 and RhoA. *Mol. Cell. Biol.* **20,** 3685–3694.
23. Ferrari, S. L., Cribari-Neto, F. (2004) β Regression for modelling rates and proportions. *J. Appl. Stat.* **31,** 799–815.
24. Bain, L. J., Englehardt, M. (1987) *Introduction to Probability and Mathematical Statistics.* Duxbury, Belmont, CA, USA.
25. Hosmer, D. W., Lemeshow, S. (2000) *Applied Logistic Regression.* John Wiley and Sons, New York, NY, USA.
26. Descoteaux, A., Luo, Y., Turco, S. J., Beverley, S. M. (1995) A specialized pathway affecting virulence glycoconjugates of *Leishmania. Science* **269,** 1869–1872.
27. Ryan, K. A., Garraway, L. A., Descoteaux, A., Turco, S. J., Beverley, S. M. (1993) Isolation of virulence genes directing surface glycosyl- phosphatidylinositol synthesis by functional complementation of *Leishmania. Proc. Natl. Acad. Sci. USA* **90,** 8609–8613.
28. Descoteaux, A., Turco, S. J. (2002) Functional aspects of the *Leishmania donovani* lipophosphoglycan during macrophage infection. *Microbes Infect.* **4,** 975–981.
29. Lerm, M., Holm, A., Seiron, A., Sarndahl, E., Magnusson, K. E., Rasmusson, B. (2006) *Leishmania donovani* requires functional Cdc42 and Rac1 to prevent phagosomal maturation. *Infect. Immun.* **74,** 2613–2618.
30. Winberg, M. E., Holm, A., Sarndahl, E., Vinet, A. F., Descoteaux, A., Magnusson, K. E., Rasmusson, B., Lerm, M. (2009) *Leishmania donovani* lipophosphoglycan inhibits phagosomal maturation via action on membrane rafts. *Microbes Infect.* **11,** 215–222.
31. Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P., Mosser, D. M. (1995) Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *J. Immunol.* **155,** 3102–3111.
32. Russell, D. G. (1987) The macrophage-attachment glycoprotein gp63 is the predominant C3-acceptor site on *Leishmania mexicana* promastigotes. *Eur. J. Biochem.* **164,** 213–221.
33. Fearon, D. T., Carroll, M. C. (2000) Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu. Rev. Immunol.* **18,** 393–422.
34. Rosenthal, L. A., Sutterwala, F. S., Kehrli, M. E., Mosser, D. M. (1996) *Leishmania major*-human macrophage interactions: cooperation between Mac- 1 (CD11b/CD18) and complement receptor type 1 (CD35) in promastigote adhesion. *Infect. Immun.* **64,** 2206–2215.
35. Da Silva, R. P., Hall, B. F., Joiner, K. A., Sacks, D. L. (1989) CR1, the C3b receptor, mediates binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. *J. Immunol.* **143,** 617–622.
36. Sutterwala, F. S., Rosenthal, L. A., Mosser, D. M. (1996) Cooperation between CR1 (CD35) and CR3 (CD 11b/CD18) in the binding of complement-opsonized particles. *J. Leukoc. Biol.* **59,** 883–890.
37. Ben Nasr, A., Haithcoat, J., Masterson, J. E., Gunn, J. S., Eaves-Pyles, T., Klimpel, G. R. (2006) Critical role for serum opsonins and complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in phagocytosis of *Francisella tularensis* by human dendritic cells (DC): uptake of *Francisella* leads to activation of immature DC and intracellular survival of the bacteria. *J. Leukoc. Biol.* **80,** 774–786.
38. Schlesinger, L. S., Horwitz, M. A. (1991) Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-γ activation inhibits complement receptor function and phagocytosis of this bacterium. *J. Immunol.* **147,** 1983–1994.
39. Moalli, F., Doni, A., Deban, L., Zelante, T., Zagarella, S., Bottazzi, B., Romani, L., Mantovani, A., Garlanda, C. (2010) Role of complement and Fc{γ} receptors in the protective activity of the long pentraxin PTX3 against *Aspergillus fumigatus. Blood* **116,** 5170–5180.
40. Pishko, E. J., Kirimanjeswara, G. S., Pilione, M. R., Gopinathan, L., Kennett, M. J., Harvill, E. T. (2004) Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3. *Eur. J. Immunol.* **34,** 184–193.
41. Van Egmond, M., van Vuuren, A. J., Morton, H. C., van Spriel, A. B., Shen, L., Hofhuis, F. M., Saito, T., Mayadas, T. N., Verbeek, J. S., van de Winkel, J. G. (1999) Human immunoglobulin A receptor (FcαRI, CD89) function in transgenic mice requires both FcR γ chain and CR3 (CD11b/CD18). *Blood* **93,** 4387–4394.
42. Pleass, R. J., Woof, J. M. (2001) Fc receptors and immunity to parasites. *Trends Parasitol.* **17,** 545–551.
43. Shibuya, A., Sakamoto, N., Shimizu, Y., Shibuya, K., Osawa, M., Hiroyama, T., Eyre, H. J., Sutherland, G. R., Endo, Y., Fujita, T., Miyabayashi, T., Sakano, S., Tsuji, T., Nakayama, E., Phillips, J. H., Lanier, L. L., Nakauchi, H. (2000) Fc α/μ receptor mediates endocytosis of IgM-coated microbes. *Nat. Immunol.* **1,** 441–446.
44. Wilson, M. E., Pearson, R. D. (1988) Roles of CR3 and mannose receptors in the attachment and ingestion of *Leishmania donovani* by human mononuclear phagocytes. *Infect. Immun.* **56,** 363–369.
45. Blackwell, J. M., Ezekowitz, R. A., Roberts, M. B., Channon, J. Y., Sim, R. B., Gordon, S. (1985) Macrophage complement and lectin-like receptors bind *Leishmania* in the absence of serum. *J. Exp. Med.* **162,** 324–331.
46. Brittingham, A., Chen, G., McGwire, B. S., Chang, K. P., Mosser, D. M. (1999) Interaction of *Leishmania* gp63 with cellular receptors for fibronectin. *Infect. Immun.* **67,** 4477–4484.
47. Descoteaux, A., Turco, S. J., Sacks, D. L., Matlashewski, G. (1991) *Leishmania donovani* lipophosphoglycan selectively inhibits signal transduction in macrophages. *J. Immunol.* **146,** 2747–2753.
48. Vinet, A. F., Fukuda, M., Turco, S. J., Descoteaux, A. (2009) The *Leishmania donovani* lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the recruitment of synaptotagmin V. PLoS Pathog. **5,** e1000628.
49. Spath, G. F., Schlesinger, P., Schreiber, R., Beverley, S. M. (2009) A novel role for Stat1 in phagosome acidification and natural host resistance to intracellular infection by *Leishmania major. PLoS Pathog.* **5,** e1000381.
50. Zakai, H. A., Chance, M. L., Bates, P. A. (1998) In vitro stimulation of metacyclogenesis in *Leishmania braziliensis, L. donovani, L. major* and *L. mexicana. Parasitology* **116,** 305–309.
51. Lodge, R., Descoteaux, A. (2005) *Leishmania donovani* promastigotes induce periphagosomal F-actin accumulation through retention of the GTPase Cdc42. *Cell. Microbiol.* **7,** 1647–1658.

KEY WORDS:
CD11b · mannose receptor · deficient · LAMP1 · EEA1