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Group V Secretory Phospholipase A2 Modulates Phagosome Maturation and Regulates the Innate Immune Response against *Candida albicans*¹

Barbara Balestrieri,*² Akiko Maekawa,* Wei Xing,* Michael H. Gelb,† Howard R. Katz,* and Jonathan P. Arm*‡

Phospholipase A₂ (PLA₂) hydrolyzes the sn-2 position of cell membrane phospholipids to release fatty acids and lysophospholipids. We have previously reported that group V secretory PLA₂ (sPLA₂) translocates from the Golgi and recycling endosomes of mouse peritoneal macrophages to newly formed phagosomes and regulates the phagocytosis of zymosan, suggesting a role in innate immunity. Here we report that in macrophages lacking group V sPLA₂, phagosome maturation was reduced 50–60% at early time points while the binding of zymosan was unimpaired. The ability of group V sPLA₂ to regulate phagocytosis extended to phagocytosis of IgG- and complement-sonicated sheep RBC. Moreover, macrophages lacking group V sPLA₂ had delays in phagocytosis, phagosome maturation, and killing of *Candida albicans*. Cytokine production and eicosanoid generation were not impaired by the lack of group V sPLA₂. Furthermore, in a model of systemic candidiasis, mice lacking group V sPLA₂ had an increased fungal burden in the kidney, liver, and spleen at day 7 postinfection and increased mortality. Thus, group V sPLA₂ regulates phagocytosis through major phagocytic receptors and contributes to the innate immune response against *C. albicans* by regulating phagocytosis and killing through a mechanism that is likely dependent on phagolysosomal fusion. *The Journal of Immunology*, 2009, 182: 4891–4898.

Phagocytosis, the process whereby phagocytic cells internalize large particles, is a key component of innate immunity (1). The mechanisms of phagocytosis depend on several variables, including the type of phagocytic cell, the nature of the stimulus, the number and types of receptors involved, and the ability of certain microorganisms to subvert the phagocytic process. Nevertheless, whatever the stimulus, phagocytosis involves three well-characterized steps, namely, binding of targets, formation of a phagocytic cup with subsequent particle uptake, and maturation of the phagosome by fusion with endosomal and lysosomal components to form the phagolysosome responsible for killing of pathogens (2).

Zymosan is a particle derived from the cell wall of *Saccharomyces cerevisiae* that has long been used as a model system to study phagocytosis. Zymosan is composed of carbohydrate polymers such as mannans and β-glucans and can bind a variety of receptors, such as dectin-1, specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1), complement receptor 3 (CR3), and the mannose receptor (3, 4). Dectin-1 is the main β-glucan receptor (5) and has been identified in many cell types, including monocytes/macrophages, neutrophils, and dendritic cells (3, 6). In macrophages, dectin-1 is essential for binding and uptake of zymosan (7) and for reactive oxygen species (ROS)⁵ generation (8, 9), while zymosan-induced cytokine production needs the cooperative action of dectin-1 and TLR2 (10).

*Candida albicans* is an opportunistic pathogen that shares many signaling events with zymosan (8, 11). *C. albicans* is phagocytosed through opsonin and nonopsonin receptors. Upon internalization, it is killed by activation of the respiratory burst and by activation of lysosomal proteins, an oxygen-independent mechanism (12). Its ability to establish a disseminated infection depends on down-regulation of both innate and acquired immune responses (13). Nevertheless, resistance to *C. albicans* infection is determined primarily by the ability of professional phagocytes to ingest and kill *C. albicans* (14).

Phospholipase A₂ (PLA₂) hydrolyzes the ester bond in the sn-2 position of cell membrane phospholipids to generate free fatty acids and lysophospholipids. When the free fatty acid is arachidonic acid, it serves as substrate for generation of eicosanoids, potent mediators of inflammation. There are >20 mammalian species of PLA₂. Of these, cytosolic PLA₂α (cPLA₂α) is absolutely required for arachidonic acid release and subsequent eicosanoid biosynthesis in response to various stimuli (15) such as zymosan (16). We

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3 Abbreviations used in this paper: ROS, reactive oxygen species; PLA₂α, phospholipase A₂α; cPLA₂α, cytosolic phospholipase A₂α; sPLA₂, secretory phospholipase A₂; sRBC, sheep RBC; Ptia2/e, the gene encoding group V sPLA₂; ppc, particles per cell; nos, multiplicity of infection; Cys-LT, cysteinyl leukotriene.

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have previously shown that group V secretory PLA₂ (sPLA₂) augments cPLA₂-dependent eicosanoid generation in freshly isolated mouse peritoneal macrophages after phagocytosis of zymosan (17). Using cultured peritoneal macrophages we showed that group V sPLA₂ translates to the forming phagosomes during phagocytosis of zymosan and regulates phagocytosis. This function was not shared with cPLA₂α or with a related member of the sPLA₂ family, group IIA sPLA₂ (18).

Here we investigate the mechanism through which group V sPLA₂ regulates phagocytosis, its contribution to phagocytosis and killing of C. albicans, and its role in host defense against systemic candidiasis.

Materials and Methods

**Materials**

Zymosan A, paraformaldehyde, complement C5-deficient human serum, gelatin veronal buffer, and BSA were from Sigma-Aldrich. Fund-1 (2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide) and FITC-zymosan were from Molecular Probes. Sabouraud dextrose agar plates were from BD Diagnostic Systems. Other reagents were rat monoclonal IgG2b anti-dectin-1 (clone 2A11; Serotec), rat monoclonal IgG2a against lysosome-associated membrane protein (Lamp)-1 (BD Pharmingen), goat polyclonal IgG against cathepsin D and control goat IgG (Santa Cruz Biotechnology), sheep RBC (sRBC) and rabbit IgG anti-sRBC (MP Biomedical), rabbit IgM anti-sRBC (Fitzgerald Industries International), and Cy3-conjugated donkey anti-rabbit IgG and rabbit IgG (Jackson ImmunoResearch Laboratories). Mouse recombinant group V sPLA₂ was described previously (19).

**Cell culture:** peritoneal macrophages

Mice with disruption of the gene encoding group V sPLA₂ (Pla2g5) (17) and wild-type littermates, bred to a C57BL/6 background for 11 generations, were 4–6 mo old. The use of mice for this study was reviewed and approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA). Peritoneal cavities of mice were flushed with 5 ml of ice-cold RPMI 1640 (18). Cells (1 × 10⁷) were plated on sterile glass coverslips in 24-well tissue culture plates in culture medium (RPMI 1640, 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% nonessential amino acids, 10% HEPES, 10% sodium pyruvate, 50 µM 2-ME, 10 µg/ml gentamicin) and incubated overnight at 37°C with 5% CO₂. After washing nonadherent cells, macrophages were cultured for an additional 24 h (for a total of 48 h of culture) and then washed and stimulated.

**Binding assay and expression of dectin-1**

Peritoneal macrophages were preincubated for 30 min with 4 µM cytochalasin D (Sigma-Aldrich) and then stimulated with zymosan at 10 particles per cell (p/c) in culture medium containing 4 µM cytochalasin D for 15 min to 1 h. Cells were washed extensively to remove unbound zymosan, stained with Diff-Quik (Dade Behring), and visualized by light microscopy (18). The binding index was obtained by dividing the number of bound particles by the total number of cells in the field, multiplied by the percentage of cells binding at least two particles.

To analyze dectin-1 expression, peritoneal macrophages were cultured for 48 h on petri dishes and harvested by incubation with PBS containing 4 mg/ml lidocaine/5 mM EDTA. Cells were fixed with 4% paraformaldehyde and blocked in HBA (HBSS without Mg²⁺ or Ca²⁺ (HBSS-) containing 0.1% BSA) containing 10% normal donkey serum (blocking buffer) for 30 min. Macrophages were incubated with rat anti-dectin-1 (20 µg/ml) or control rat IgG (Jackson ImmunoResearch) in blocking buffer for 1 h, washed, and incubated for 1 h with allophycocyanin-conjugated donkey anti-rat IgG (1/200) (Jackson ImmunoResearch). All the steps were performed at room temperature. Alternatively, cells were stained with allophycocyanin-conjugated rat anti-mouse CD11b (BD Pharmingen) or allophycocyanin-conjugated IgG2b and FITC-conjugated rat anti-mouse F4/80 or FITC-conjugated IgG2a (eBioience) (5 µg/ml). Flow cytometric analysis was performed on a FACSCanto flow cytometer, and data were analyzed using FlowJo software.

**Assessment of phagolysosome fusion**

Peritoneal macrophages were stimulated with 2.5 pps FITC-zymosan for 10 min to 1 h, fixed with 2% paraformaldehyde in PBS for 15 min, and permeabilized with 0.025% saponin in PBS for 10 min. Cells were washed, blocked in HBA containing 5% normal donkey serum (blocking buffer) for 1 h, and incubated with rat mAb anti-Lamp-1 (2.5 µg/ml) or goat polyclonal anti-cathepsin D (10 µg/ml) in blocking buffer for 2 h. Negative control cells were incubated with rat IgG or goat IgG, respectively. After washing, cells were incubated for 1 h with Cy3-conjugated donkey anti-rat IgG (1/400) or FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories), washed, and mounted in Vectashield mounting medium (Vector Laboratories) (18). All of the steps were performed at room temperature. In selected experiments macrophages were preincubated with C. albicans, stained for Lamp-1, and counterstained with Calcofluor White M2R (Sigma-Aldrich) (25 µM), a fluorochrome that binds the cell walls of fungi and can be visualized under UV light (20). Cells were imaged using a Nikon C1 confocal system combined with an Eclipse TE2000-U inverted microscope with ×60 oil PlanApo NA 1.4 objective lens. Eight to 10 Z-stack images were acquired through a small pinhole, and each image (0.05 µm) was saved as a series of independent sections and as a merged stack image using Nikon EZ-C Gold version 3.40 build 691. Conventional Nomarski differential contrast images and epifluorescence images were taken using a SPOT-RT digital camera, and data were analyzed using Photoshop software. The fusion index was obtained by analyzing each section individually and as merged images or epifluorescence images for accumulation of Lamp-1 (or cathepsin D) around the phagosomes (21) and by dividing the number of Lamp-1-surrounded phagosomes by the total number of cells in the field, multiplied by the percentage of cells showing at least one phagosome whose membrane was completely stained with Lamp-1.

**Erythrocyte phagocytosis assay**

For IgG opsonization, sRBC (10% suspension) were washed in gelatin veronal buffer, incubated for 20 min at 37°C with a subagglutinating concentration of rabbit IgG anti-sRBC (10 µg/ml), and washed again. Alternatively, to generate iC3b-coated erythrocytes, sRBC were incubated with rabbit IgM anti-sRBC (10 µg/ml) for 20 min at 37°C, washed, and resuspended in gelatin veronal buffer containing 10% C5-depleted human serum for 20 min at 37°C. During this incubation, C3b binds IgM and is then converted to iC3b, the ligand of CR3 (22). For iC3b-opsonized sRBC, peritoneal macrophages were preincubated for 15 min with RPMI 1640 containing 150 mM PMA without FBS to activate CR3 on the surface of the macrophages (1, 2). The medium was then substituted with culture medium. IgG- or iC3b-opsonized sRBC (~10 sRBC/cell) were gently pelleted onto macrophages (30 × g for 2 min) and incubated at 37°C in culture medium (23). At the indicated time (5 min to 1 h) glass coverslips were taken off the plate, washed, and noningested sRBC were lysed with 0.2% NaCl followed by 1.6% NaCl. Coverslips were dried, fixed, and stained with DAPI. Phagosomes containing sRBC were counted by optical microscopy. The phagocytic index was calculated by dividing the number of phagosomes by the total number of cells in a field, which was multiplied by the percentage of phagocytosing cells (18).

**Phagocytosis and killing of C. albicans**

* C. albicans (American Type Culture Collection 10231) was streaked on Sabouraud agar plates and incubated for 48 h at 37°C. The plates were maintained at 4°C, and the day before the experiment one colony was streaked on a fresh plate and incubated at 37°C. C. albicans were scraped from the plate, washed in endotoxin-free PBS (Sigma-Aldrich), and counted. * C. albicans (multiplicity of infection (moi) of 5) was gently pelleted (30 × g) onto peritoneal macrophages and incubated for 5 min to 1 h at 37°C in 5% CO₂. After washing to remove nonphagocytosed microorganisms, internalized yeast were counted as described (18). In selected experiments yeasts were stained with FUN-1 (8) in glucose buffer (RPMI 1640 containing 2% glucose, 10 mM HEPES, 10 µM FUN-1) to allow active metabolism. Macrophages stimulated with C. albicans were fixed with 2% paraformaldehyde for 15 min, washed, and mounted in Vectashield mounting medium.To distinguish internalized yeast, we used Nomarski images, and to visualize dead organisms we used fluorescence microscopy (488 nm output) (see Fig. 3B). In another set of experiments, time-dependent stimulation with C. albicans was followed by staining with DAPI and visualization by optical microscopy (18). In addition to using FUN-1, we measured killing of C. albicans using a serial dilution technique (24). Macrophages were challenged with live microorganisms for 15 min as described above, and washed to remove noningested C. albicans. Before incubation and after 1, 2, and 3 h of incubation at 37°C in 5% CO₂ (20, 24, 25), the wells were scraped with a plastic pipette and macrophages resuspended with 1 ml of endotoxin-free RPMI/H9262 (Sigma-Aldrich) (25). To quantify the number of viable C. albicans, serially diluted samples (100 µl) were spread on Sabouraud plates and incubated at 37°C for 24 h. The percentage of yeast killed by the macrophages...
was determined as follows: \([1 - \frac{(CFU after incubation/CFU recovered at the start of incubation)}{100}]\).

**Synchronized phagocytosis**

In selected experiments to synchronize phagocytosis, peritoneal macrophages were washed in cold RPMI 1640 and cooled on ice for 5 min before the addition of *C. albicans* for 15 min on ice. Unbound *C. albicans* was removed by washing cells once with warm medium. Cells were then incubated at 37°C for the indicated periods of time, stained for Lamp-1 and cathepsin D, and counterstained with Calcein Fluor White M2R.

**Cytokine production and cysteinyl leukotriene (Cys-LT) generation**

Peritoneal macrophages were stimulated with *C. albicans* (moi of 0.1–10) or zymosan (10 ppc) for 3 h. Supernatants were then harvested and assayed for TNF-α and Cys-LTs by commercial immunoassays (TNF-α from R&D Systems, Cys-LTs from GE Healthcare).

**In vivo models of systemic candidiasis**

Two hundred microliters of 0.9% NaCl containing \(5 \times 10^5\) CFU of *C. albicans* (to evaluate fungal burden) or 1 \(10^6\) (for the survival assay) was injected i.v. in *Pla2g5*-null and wild-type mice. Mice were checked daily for 33 days. Subgroups of three to five mice were sacrificed 1, 3, and 7 days after injection. Heart, lungs, liver, spleen, and left kidney were removed and weighed, weighed, and homogenized in 1 ml of endotoxin-free PBS in a tissue grinder. Serially diluted suspensions (100 μl) were then plated on Sabouraud plates. After 24 h growth at 37°C, numbers of *C. albicans* colonies were counted and expressed as log CFU per gram of tissue (25).

The right kidney was fixed with 4% formalin and embedded in paraffin, and serial sections were examined microscopically after staining with a combination of periodic acid-Schiff and H&E (20).

**Statistics**

We performed two-way ANOVA using the GraphPad Prism 5 software to compare time-dependent phagocytosis between populations of peritoneal macrophages derived from *Pla2g5*-null mice and their wild-type littermates. Differences in *C. albicans* colonization of tissues were analyzed by Mann-Whitney U test and survival by the log-rank test; otherwise, differences in outcomes were analyzed by Student’s t test.

**Results**

**Reduced phagocytosis in Pla2g5-null macrophages is not due to decreased binding of zymosan**

The first step in phagocytosis is the binding of the ligand to its cognate receptor. We first confirmed our previous finding (18) that *Pla2g5*-null macrophages have a defect in phagocytosis of zymosan (Fig. 1A). To assess whether the decrease in phagocytosis by *Pla2g5*-null macrophages was due to reduced binding of zymosan particles, we preincubated macrophages with cytochalasin D to inhibit phagocytosis and then stimulated the cells with zymosan. Fig. 1B shows that *Pla2g5*-null macrophages bind zymosan as efficiently as do wild-type macrophages. Because the main phagocytic receptor for zymosan is dectin-1 (3, 7), we also examined dectin-1 expression by flow cytometry and found that *Pla2g5*-null and wild-type peritoneal macrophages, identified by expression of F4/80 and CD11b, have similar expression of dectin-1 (Fig. 1C).

**Critical role of group V sPLA₂ in phagosomal maturation**

We have previously reported that in mouse peritoneal macrophages group V sPLA₂ translocates to the phagocytic cup and newly formed phagosomes within minutes of phagocytosis of zymosan (18). These data suggest that group V sPLA₂ may influence phagosome formation and maturation. To assess this hypothesis we studied the maturation of phagosomes containing FITC-zymosan by their ability to fuse over a period of 1 h with Lamp-1, a marker of late endosomes and lysosomes (26, 27). Fusion was quantified by counting zymosan particles fully surrounded by Lamp-1 that was visualized by staining with Cy3-conjugated Ab. Phagosomes containing FITC-zymosan merged with lysosomes in wild-type peritoneal macrophages more efficiently than in *Pla2g5*-null macrophages (Fig. 2A). Formal quantification of phagolysosome fusion over 1 h confirmed that *Pla2g5*-null macrophages had a 50–60% reduction in maturation of phagosomes (Fig. 2B; \(n = 3\), \(p < 0.03\) by ANOVA).

*Pla2g5*-null peritoneal macrophages are defective in phagocytosis of opsonized targets

Opsonization of phagocytic targets with IgG or complement potentiates phagocytosis and contributes to clearance of pathogens. Therefore, we investigated the ability of group V sPLA₂ to regulate phagocytosis mediated by two major phagocytic receptors, Fcy receptors and complement receptor 3 (CR3). The kinetics of phagocytosis of IgG-opsonized sRBC was more rapid than that of zymosan (18) or complement-coated targets, perhaps reflecting utilization of different signaling molecules (2), and was attenuated in *Pla2g5*-null peritoneal macrophages compared with wild-type macrophages (Fig. 3A; \(n = 3\), \(p < 0.01\) by ANOVA). In *Pla2g5*-null peritoneal macrophages, phagocytosis of iC3b-opsonized sRBC was attenuated ~60% compared with wild-type macrophages at each time point analyzed (Fig. 3B; \(n = 3\), \(p = 0.02\) by ANOVA). Thus, the ability of group V sPLA₂ to modulate phagocytosis extends to other major phagocytic receptors essential for innate and acquired immune responses against pathogens.
Group V sPLA2 is required for efficient phagocytosis and killing of C. albicans

To examine whether group V sPLA2 modulates phagocytosis of live fungi, we analyzed phagocytosis of C. albicans by peritoneal macrophages from 5 min to 1 h, the time of peak phagocytosis. Phagocytosis of C. albicans was reduced by 50% in Pla2g5-null peritoneal macrophages compared with wild-type macrophages at all time points studied (Fig. 4A; n = 3, **p < 0.02 and *p < 0.05 by t test). Phagocytosis of C. albicans by professional phagocytes is followed by killing (14). Therefore, we also examined killing of C. albicans by Pla2g5-null macrophages. FUN-1 is a vital dye that stains nucleic acids. Live C. albicans has a diffuse green cytoplasmic fluorescence, while dead cells exhibit an extremely bright, diffuse, green-yellow fluorescence (28). In wild-type macrophages starting at 1 h, the ingested C. albicans exhibited extremely bright green-yellow fluorescence indicative of death, which was not observed in Pla2g5-null peritoneal macrophages (Fig. 4B). Using a conventional serial dilution technique,
we confirmed that Pla2g5-null peritoneal macrophages had a decreased ability to kill C. albicans at 1 h (Fig. 4C; n = 4, p < 0.03 by t test). Nevertheless, killing reached wild-type levels by 2 and 3 h. Thus, Pla2g5-null macrophages are impaired in their ability to phagocytose C. albicans and have a delay in killing of C. albicans.

**Group V sPLA2 regulates phagolysosome fusion but not TNF-α or Cys-LTs production in response to fungal particles**

Various lines of evidence have shown that oxygen-independent mechanisms, such as lysosomal enzymes of macrophages (14), are important in killing of C. albicans. Because we found a delay in phagolysosome fusion in Pla2g5-null macrophages after phagocytosis of zymosan, we analyzed fusion of C. albicans-containing phagosomes with lysosomes stained by Lamp-1. We found that, as for zymosan, Pla2g5-null macrophages have a defect in phagolysosome fusion after ingestion of C. albicans that was marked at 30 min (Fig. 4, D and E; n = 2, p < 0.05 by t test).

To more clearly elucidate the delay in phagolysosome fusion in Pla2g5-null macrophages, we synchronized phagocytosis of C. albicans by allowing particles to bind at 4°C, washing away excess unbound particles, and then transferring cells to 37°C to initiate phagocytosis. Macrophages were then immunostained for Lamp-1.

**FIGURE 5.** Delayed phagosome-lysosome fusion in Pla2g5-null peritoneal macrophages. Peritoneal macrophages isolated from wild-type (filled bars) and Pla2g5-null (open bars) mice were incubated with C. albicans from 30 min to 2 h (moi of 2). To synchronize phagocytosis, particles were first allowed to bind at 4°C, excess unbound particles were washed away, and then cells were transferred to 37°C. Indirect immunofluorescence microscopy was used to visualize the distribution of Lamp-1 (A) or cathepsin D (B). The fusion index (A and B) and phagocytic index (C) were calculated as described in Materials and Methods. Data are means ± SEM of two experiments. *, p < 0.05 and **, p < 0.02 by t test.

**Table I.** Production of TNF-α and Cys-LTs by peritoneal macrophages stimulated with C. albicans or zymosan

<table>
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<tr>
<th>Mediator</th>
<th>Mouse Strain</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>Zymosan (10 ppc)</th>
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<tbody>
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<td>TNF-α, ng/ml</td>
<td>Wild type</td>
<td>1.58 ± 0.05</td>
<td>7.33 ± 0.43</td>
<td>9.63 ± 0.09</td>
<td>15.61 ± 0.22</td>
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<tr>
<td></td>
<td>Pla2g5-null</td>
<td>1.58 ± 0.21</td>
<td>8.46 ± 0.68</td>
<td>10.90 ± 0.42</td>
<td>19.07 ± 2.39</td>
</tr>
<tr>
<td>Cys-LTs, ng/10⁶ cells</td>
<td>Wild type</td>
<td>3.62 ± 14.11</td>
<td>19.87 ± 27.15</td>
<td>19.57 ± 0.00</td>
<td>4.24 ± 32.56</td>
</tr>
<tr>
<td></td>
<td>Pla2g5-null</td>
<td>4.85 ± 56.77</td>
<td>22.01 ± 20.37</td>
<td>19.88 ± 38.04</td>
<td>3.79 ± 11.97</td>
</tr>
</tbody>
</table>

Data represent means ± SEM of two individual mice per strain.

Pla2g5-null mice are more susceptible than wild-type mice to disseminated candidiasis

To examine the function of group V sPLA2 in vivo, we next infected mice i.v. with C. albicans as a model of systemic candidiasis and evaluated the fungal burden and survival of the mice. The kidney is the major target organ in systemic candidiasis (31), and hence the fungal burden in the kidney was 10- to 100-fold greater than that seen in the liver and spleen (Fig. 6). One and 3 days after i.v. injection of C. albicans (5 × 10⁶ CFU/mouse) the numbers of CFU recovered from the kidney, liver, and spleen were similar in wild-type and Pla2g5-null mice. Although at day 7 the increased
Whitney's spleen was significantly greater in 0.03, respectively, by Mann-Whitney lower than from other organs (800 CFU/g and 11021/H11021/H11021). To assess outgrowth of the pathogen and its ability to invade organs, subgroups of three to five Pla2g5-null (C) and wild-type (B) mice were sacrificed 1, 3, and 7 days after infection. The outgrowth of *C. albicans* was measured by serial dilution and expressed as CFU per gram of tissue. Individual data points are shown for kidney (A), liver (B), spleen (C); means are indicated by horizontal bars. D, Histopathology of the kidney of *C. albicans*-infected mice. Histological sections of representative wild-type and Pla2g5-null kidneys 7 days after infection are stained with periodic acid-Schiff. Arrow points to pseudohyphae. Magnification of 40 for both panels. **, p < 0.02 (B) and *, p < 0.05 (C) by Mann-Whitney U test.

![FIGURE 6](http://www.jimmunol.org/)

FIGURE 6. Pla2g5-null mice are more susceptible than wild-type mice to live *C. albicans* infection. Mice were infected with *C. albicans* by the i.v. injection of 200 µl of 0.9% NaCl containing 5 x 10^6 CFU. To assess outgrowth of the microorganism and its ability to invade organs, subgroups of three to five Pla2g5-null (C) and wild-type (B) mice were sacrificed 1, 3, and 7 days after injection. The outgrowth of *C. albicans* was measured by serial dilution and expressed as CFU per gram of tissue. Individual data points are shown for kidney (A), liver (B), and spleen (C); means are indicated by horizontal bars. D, Histopathology of the kidney of *C. albicans*-infected mice. Histological sections of representative wild-type and Pla2g5-null kidneys 7 days after infection are stained with periodic acid-Schiff. Arrow points to pseudohyphae. Magnification of 40 for both panels. **, p < 0.02 (B) and *, p < 0.05 (C) by Mann-Whitney U test.

The number of CFU recovered from the kidney of Pla2g5-null mice did not achieve statistical significance (Fig. 6A; p < 0.07 by Mann-Whitney U test), the number of CFU recovered from the liver and spleen was significantly greater in Pla2g5-null mice compared with wild-type littermates (Fig. 6B and C; **, p < 0.02 and *, p < 0.03, respectively, by Mann-Whitney U test). *C. albicans* recovered from the heart and lung only 24 h after i.v. infection was lower than from other organs (<800 CFU/g and <300 CFU/g, respectively), was not significantly different in Pla2g5-null and wild-type mice, and had been cleared by day 3.

Because the kidney is the major target organ in systemic candidiasis, we examined the microscopic appearance of the kidneys. Kidneys of both wild-type and Pla2g5-null mice showed scattered foci of neutrophils infiltrating the renal cortex and medulla on days 1 and 3 after infection with *C. albicans*. Kidneys of wild-type mice were infiltrated with small numbers of yeast and hyphae, which appeared to be more extensive in kidneys of Pla2g5-null mice with formation of hyphae and pseudohyphae (data not shown). On day 7 after infection, the kidneys of the wild-type mice appeared to be healing with regeneration of interstitial tissue and diffuse infiltration of macrophages, fibroblasts, and fibrocytes; fewer neutrophils were present and there were occasional yeasts, but there was no hyphal formation (Fig. 6D). In contrast, on day 7 in the kidneys of Pla2g5-null mice (Fig. 6D) there were dense foci of inflammation, with *C. albicans* yeast and hyphae still present within neutrophilic infiltrates.

To further assess the susceptibility of Pla2g5-null mice to *C. albicans* infection, mice were injected i.v. with 1 x 10^6 *C. albicans* CFU/mouse. Over the subsequent month, 50% of the Pla2g5-null mice died compared with 12.5% of the wild-type mice (Fig. 7; p < 0.1 by log-rank test), suggesting that defective clearance of *C. albicans* in Pla2g5-null mice leads to increased mortality.

**Discussion**

There are >20 mammalian phospholipases A2 (32). However, it is likely, given the diversity of their biochemical properties and cellular and subcellular distribution, that PLA2 enzymes serve a diversity of biological functions independent of their ability to generate eicosanoids. We have previously reported that group V PLA2 regulates phagocytosis of zymosan and translocates to the forming phagosomes (18). In the present study we demonstrated that the defect in phagocytosis by Pla2g5-null macrophages is not due to impaired expression of dectin-1 or to impaired binding of zymosan. However, phagolysosome fusion was defective in Pla2g5-null macrophages. Thus, group V sPLA2 regulates phagocytosis by influencing the rate of maturation of the phagosomes with functional consequences for phagocytosis and killing of live pathogens.

Zymosan and *C. albicans* share similar signaling pathways, including activation of dectin-1 and TLRs on phagocytes (11, 33). Indeed, Pla2g5-null macrophages were impaired in their ability to engulf *C. albicans* (Fig. 4A) as well as zymosan (Ref. 18 and Fig. 1A). Pla2g5-null macrophages also had a delayed ability to kill *C. albicans* (Fig. 4, B and C). Indeed, while 1 h after ingestion wild-type macrophages were able to kill ~50% of the ingested *C. albicans*, Pla2g5-null macrophages were able to kill only ~15% of the yeast (Fig. 4C), and by 2 h there was no difference. It has been previously reported that among sPLA2s, group IIA, group V, and group X sPLA2 have antibacterial activity (34, 35), although there are no data on their ability to kill fungi. Addition of mouse recombinant group V sPLA2 directly to *C. albicans* did not affect the survival of the yeast (data not shown), suggesting that the delay in killing in Pla2g5-null macrophages is not due to direct antifungal actions of the enzyme.

Macrophages are essential in protection against candidiasis (12), relying on both nonoxidative and oxidative mechanisms to kill the organism (14). The nonoxidative mechanisms of killing used by
macrophages include lysosomal enzymes activated when the phagosome matures to form a microbicidal organelle (12). The defect in phagocytosis of C. albicans and the delay in killing of C. albicans showed by Pla2g5-null macrophages suggest a role for the enzyme in maturation of phagosomes containing C. albicans. Indeed, using C. albicans stained with Calcofluor White we showed impaired fusion of Lamp-1-positive organelles with phagosomes in Pla2g5-null macrophages (Fig. 4D), a defect similar to that seen in zymosan-containing phagosomes. For this set of experiments we used centrifugation to synchronize the onset of phagocytosis. This protocol did not remove unbound targets, thereby allowing ongoing phagocytosis as occurs during the course of infection, but potentially masking the impact of the lack of group V sPLA2 on the rate of phagocytosis and phagolysosome fusion. We therefore used a temperature-dependent technique to allow binding of targets to the macrophage at 4°C, washing to remove unbound particles, and then initiating phagocytosis by rapidly bringing the temperature to 37°C. Using this protocol to synchronize the onset of phagocytosis, we showed a very marked defect (~70–80%) in acquisition by the phagosome of two lysosomal markers, Lamp-1 (a late endosome and lysosome marker) and cathepsin D (a lysosome marker), in Pla2g5-null macrophages at 30 min, an effect that was less marked at 1 h and 2 h. We also showed that the absence of group V sPLA2 slows the phagocytic process (Fig. 5). The oxidative mechanisms of killing include generation of reactive nitrogen and oxygen species and have been shown to be mainly dependent on dectin-1 (8). Because resident peritoneal macrophages are poor producers of ROS (12), we used thioglycolate-elicited macrophages to investigate production of ROS but found that they did not produce a significant amount after stimulation with C. albicans. However, Pla2g5-null thioglycolate-elicited macrophages stimulated with zymosan, a more potent stimulus for ROS production, showed a diminished capacity to generate ROS compared with wild-type macrophages (data not shown). Hence, our major finding is the delay in phagosome fusion in Pla2g5-null macrophages in response to C. albicans, and any defect in oxidative mechanisms remains to be determined.

Proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IFN-γ activate professional phagocytes to phagocytose and kill the yeast. In particular, TNF-α production is critical for the host response against the fungus, as demonstrated by increased mortality of mice lacking cytokines of the TNF family (25, 36). Lack of group V sPLA2 had no effect on TNF-α generation after phagocytosis of zymosan or C. albicans (Table I). Thus, group V sPLA2 does not regulate phagocytosis and killing of C. albicans through regulation of TNF-α production. This is consistent with previous data reporting that binding of zymosan in the absence of phagocytosis is sufficient for proinflammatory cytokine production (37). Therefore, a defect in phagocytosis and phagosome maturation would not necessarily lead to diminished generation of TNF-α.

It has been previously been shown that zymosan and C. albicans induce leukotriene generation by inflammatory cells (16, 17, 29). However, lack of group V sPLA2 did not affect leukotriene generation in response to zymosan or C. albicans (Table I). These data may seem in conflict with the previously reported role of group V sPLA2 in regulating leukotriene generation after phagocytosis of zymosan (17). However, the peritoneal macrophages used in the present study were cultured for 2 days, whereas in our previous study macrophages were cultured for just a few hours. Indeed the ability of macrophages to produce eicosanoids depends on the state of activation/maturation of the macrophages (38, 39), and prolonged culture gives rise to a different phenotype of macrophage with attenuated ability to produce leukotrienes compared with freshly isolated peritoneal macrophages: 5 ng per 10⁶ cells (Table I) compared with 42 ng per 10⁶ cells (17), respectively, after phagocytosis of zymosan. Our present data also support the hypothesis that the role of group V sPLA2 in regulating phagocytosis is independent of its role in eicosanoid generation and is consistent with the observation that phagocytosis of zymosan is intact in cPLA2α-deficient macrophages (18) and is not impaired by cPLA2α inhibitors (40). Furthermore, it has been previously shown that although binding of β-glucan induces cPLA2α activation and arachidonic acid release (16), the internalization of yeast particles was not essential for cPLA2α activation, although arachidonic acid release was enhanced after C. albicans internalization (16). These data emphasize the concept of nonredundant functions of different types of PLAs (41) and support the hypothesis that the ability of group V sPLA2 to modulate phagocytosis of yeast particles is related to the regulation of phagocytic events that require formation and/or maturation of the phagosome as opposed to cellular responses triggered only by binding to specific receptors.

Our finding that group V sPLA2 modulates phagocytosis and killing of C. albicans prompted us to extend the study to in vivo model of systemic candidiasis (42). While the early fungal burden up to day 3 was equivalent in wild-type and Pla2g5-null mice, by day 7 these mice showed an impaired ability to clear C. albicans from spleen and liver and kidney (Fig. 6) (31). Depending on the dose and strain of C. albicans, as well as the strain of mice, lesions in the kidney may vary from white macroscopic abscesses to lesions with a diffuse inflammatory reaction containing polymorphonuclear cells and/or macrophages (42, 43). In our model, histological examination of the kidney of wild-type mice at day 7 (Fig. 6D) showed an inflammatory response with residual neutrophilic infiltration replaced by mononuclear cells, fibroblasts, and occasional yeast. However, kidneys from mice lacking group V sPLA2 (Fig. 6D) showed persistent neutrophilic infiltrates with yeast and hyphae, suggestive of an impaired ability to clear the infection and actively proliferating C. albicans. These data indicate that the distribution of C. albicans into the organs in the early phase of infection is similar between Pla2g5-null and wild-type mice. As the infection proceeds and C. albicans localizes in liver and spleen and increasingly in the kidney (42), Pla2g5-null mice demonstrate a defect in clearance of the fungal infection compared with wild-type mice, most likely due to impaired phagocytosis and killing. At a higher dose of C. albicans, mice lacking group V sPLA2 showed increased mortality (Fig. 7). Thus, our data are consistent with the essential role of phagocytosis in the host immune response to fungal pathogens and consistent with those of other groups showing that a defect in phagocytosis can lead to enhanced fungal dissemination and increased lethality in experimental animals (14, 44) and humans (45).

Although the resistance to C. albicans infection is determined initially by the innate immune system, the adaptive immune system also contributes to protection against fungal infections (13). Several studies have shown the protective role of Th1 rather than Th2 cytokines (46), while the function of Abs in fungal infection is still controversial (13). However, the role of opsonic receptors in mediating phagocytosis of C. albicans is well established, and therefore the modulation of phagocytosis through opsonin receptors by group V sPLA2 (Fig. 3) could also contribute to the decreased survival of Pla2g5-null mice.

The present study has extended our early data on the role of group V sPLA2 in regulating phagocytosis of zymosan to show that its role extends to regulation of ingestion and killing of C. albicans and phagolysome maturation. The ability of group V sPLA2 to modulate phagocytosis through several receptors renders this molecule a key regulator of signaling generated by multiple
ligands and likely multiple pathogens. These signals likely converge to regulate one or more fundamental steps in the phagocytic process, thereby contributing to an efficient innate and adaptive immune response. The relevance of the in vitro findings is indicated by the impaired clearance of \textit{C. albicans} in mice lacking group V sPLA$_2$ with associated higher mortality, providing the first demonstration that group V sPLA$_2$ plays an important role in the host response to fungal pathogens in vivo.

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References