Cytosolic Phospholipase A$_2\alpha$ and Eicosanoids Regulate Expression of Genes in Macrophages Involved in Host Defense and Inflammation

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Abstract

The role of Group IVA cytosolic phospholipase A$_2$ (cPLA$_2\alpha$) activation in regulating macrophage transcriptional responses to Candida albicans infection was investigated. cPLA$_2\alpha$ releases arachidonic acid for the production of eicosanoids. In mouse resident peritoneal macrophages, prostacyclin, prostaglandin E$_2$ and leukotriene C$_4$ were produced within minutes of C. albicans addition before cyclooxygenase 2 expression. The production of TNFα was lower in C. albicans-stimulated cPLA$_2\alpha$$^{+/+}$ than cPLA$_2\alpha$$^{-/-}$ macrophages due to an autocrine effect of prostaglandins that increased cAMP to a greater extent in cPLA$_2\alpha$$^{+/+}$ than cPLA$_2\alpha$$^{-/-}$ macrophages. For global insight, differential gene expression in C. albicans-stimulated cPLA$_2\alpha$$^{+/+}$ and cPLA$_2\alpha$$^{-/-}$ macrophages (3 h) was compared by microarray. cPLA$_2\alpha$$^{+/-}$ macrophages expressed 86 genes at lower levels and 181 genes at higher levels than cPLA$_2\alpha$$^{-/-}$ macrophages (≥2-fold, p<0.05). Several pro-inflammatory genes were expressed at lower levels (Tnfa, Cx3c1f1, Cd40, Cd5, Csf1, Edn1, Cxcr7, Ifi1, Irf4, Akna, Ilfn, several IFNy-inducible GTPases). Genes that dampen inflammation (Socs3, Il10, Crem, Stat3, Thbd, Thbs1, Abca1) and genes involved in host defense (Gja1, Csf3, Trem1, Hdc) were expressed at higher levels in cPLA$_2\alpha$$^{+/-}$ macrophages. Representative genes expressed lower in cPLA$_2\alpha$$^{+/-}$ macrophages (Tnfa, Csf1) were increased by treatment with a prostacyclin receptor antagonist and protein kinase A inhibitor, whereas genes expressed at higher levels (Crem, Nr4a2, Il10, Csf3) were suppressed. The results suggest that C. albicans stimulates an autocrine loop in macrophages involving cPLA$_2\alpha$, cyclooxygenase 1-derived prostaglandins and increased cAMP that globally effects expression of genes involved in host defense and inflammation.

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Introduction

The oxygenated metabolites of arachidonic acid comprise a large family of bioactive lipids that have diverse roles in regulating homeostatic processes and in modulating inflammation and immune responses [1]. The production of eicosanoids is initiated by the release of arachidonic acid that is metabolized through the 5-lipoxygenase pathway to leukotrienes and by cyclooxygenases (COX) to prostanoids and thromboxane. Eicosanoids are secreted and act locally in an autocrine or paracrine fashion through interaction with specific G-protein coupled receptors (GPCR) to exert their biological effects [2–4]. Leukotrienes are pro-inflammatory mediators but prostaglandins (PG) have pro- and anti-inflammatory effects depending on the cell type-specific GPCR-dependent signal transduction pathways that are triggered [1]. Macrophages are an important source of eicosanoids that are produced rapidly in response to stimulation by bacterial and fungal pathogens [5–8]. Resident tissue macrophages are a first line of defense against invading microorganisms that are recognized by pattern recognition receptors that engage microbial surface structures. We have used resident mouse
peritoneal macrophages (RPM) to study the regulation of eicosanoid production in response to the model fungal agonist zymosan, cell wall particles of Saccharomyces cerevisiae [9–11]. Zymosan stimulates activation of the Group IVA cytosolic phospholipase A\(_2\) (cPLA\(_2\)), the first key regulatory enzyme in RPM that releases arachidonic acid for eicosanoid production [12]. To identify the pattern recognition receptors on RPM that mediate cPLA\(_2\) activation and eicosanoid production, the more medically relevant fungal pathogen Candida albicans was studied [13,14]. We found a role for dectin-1 and -2 that engage β-glucan and mannans on the C. albicans cell wall that, together with a MyD88-dependent pathway, promote cPLA\(_2\) activation and eicosanoid production [13,14]. Although C. albicans is a normal commensal organism, it is an opportunistic pathogen that is a leading cause of mycoses particularly in the immunocompromised and critically ill [15]. There has been considerable interest in elucidating the mechanisms regulating immune responses to C. albicans because of the prevalence of fungal infections [16].

Eicosanoids affect immune regulation by modulating cellular differentiation, phagocytic potential, migration and cytokine/chemokine production [5,17–19]. The types and balance of cytokines produced during the early responses of innate immune cells to infection influence the macrophage phenotype, differentiation of lymphocytes and adaptive immune responses [20–23]. In this study, we compared cPLA\(_2\)α\(^{++}\) and cPLA\(_2\)α\(^{-}\)-RPM to investigate the functional consequences of cPLA\(_2\)α activation and the effect of endogenously produced eicosanoids on gene expression in response to C. albicans. Our results demonstrate that C. albicans-stimulated cPLA\(_2\)α activation and the early production of prostanoids promotes an autocrine pathway in RPM that affects the expression of genes involved in host defense and to dampen inflammation.

Materials and Methods

Ethics Statement

The work with mice in this study was approved by the National Jewish Health Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with their guidelines.

Materials

DMEM was from Cambrex BioScience. FBS (Gemini Bioproducts) was heat inactivated at 56°C for 30 min before use. Human serum albumin was obtained from Intergen. Polyclonal antibodies to murine COX1 and COX2, the protein kinase A inhibitor H-89, the COX inhibitor NS-398, the IP receptor antagonist CAY10441, the IP receptor agonist iloprost and the EP2 receptor agonist butaprost were from Cayman Chemical Co. Antibodies to β-actin were from Cell Signaling. The stable cAMP analogue 8-Br-cAMP was from Santa Cruz Biotechnology, Inc. The mouse TNFα cytose ELISA kit was from Invitrogen. cAMP was quantified in macrophage lysates using the cAMP Biotrak EIA (non-acetylation protocol) from GE Healthcare according to the manufacturer’s protocol. RNA was isolated using the on-column DNase treatment with the RNeasy mini kit from Qiagen.

Mouse Strains

Pathogen-free Balb/c mice were obtained from Harlan Sprague Dawley. cPLA\(_2\)α\(^{++}\) mice were generated as previously described and backcrossed onto a Balb/c background for 10 generations [24]. The TLR4 mutant mouse strain C3H/HeJ and control strain C3H/HeOuJ were obtained from The Jackson Laboratory. TLR2\(^{-}\) (C57BL/6) and MyD88\(^{-}\) mice (C57BL/6/129) were generated as previously described [25]. MyD88\(^{-}\)-C57BL/6/129 mice were crossed to generate MyD88\(^{-}\)-C. albicans and MyD88\(^{-}\) littermate controls. C57BL/6 control mice were obtained from The Jackson Laboratory. Dectin-1\(^{-}\)-mice (129sv/ev) were produced as described previously [26], and age and strain matched controls obtained from Taconic. Mice were used for macrophage isolation at 7–12 wk of age.

C. albicans Strains and Culture

C. albicans (ATCC 10261) was used for experiments unless otherwise indicated. The C. albicans Capmrt1\(^{Δ}\) null mutant defective in glycosylation, the re-integrant strain (Capmrt1\(^{Δ}\)+CaPMR1) and parental wild-type control were generated as previously described [27]. C. albicans strains were grown on Sabouraud dextrose agar plates and maintained at 4°C.

RPM Infection

The day before the experiment, the strains were streaked onto fresh Sabouraud dextrose agar plates and incubated overnight at 37°C. C. albicans was scraped from the plate and washed twice in endotoxin-free PBS. Live C. albicans at a multiplicity of infection (moi) of 2 was used for all experiments.

RPM Isolation

RPM were obtained by peritoneal lavage as previously described [13]. Cells were plated at a density of 5 x 10\(^3\)/cm\(^2\) (48 well plate) and incubated for 2 h at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. After washing the cultures to remove non-adherent cells, the adherent macrophages were incubated in DMEM containing 10% heat inactivated FBS, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, 0.29 mg/ml glutamine for 16-18 h at 37°C. The cells were washed twice with serum-free DMEM containing 0.1% human serum albumin (stimulation medium) and then infected with C. albicans.

C. albicans Uptake and Killing assays

The ability of cPLA\(_2\)α\(^{++}\) and cPLA\(_2\)α\(^{-}\) RPM to bind and internalize C. albicans was compared using an in vitro recognition assay as described previously with modifications [26]. RPM were incubated for 30 min with Alex Fluor 488-labeled C. albicans (m.o.i. 10) prepared as described [28]. RPM were washed 3 times with stimulation media and incubated further for 1 h. Cells were lysed with 3% Triton X-100 and the fluorescence intensity was measured. The killing assay involved incubating cPLA\(_2\)α\(^{++}\) and cPLA\(_2\)α\(^{-}\) RPM with C. albicans (m.o.i. 5) for 30 min, followed by 3 washes and further incubation for 1 and 4 h. Cells were lysed with 3% Triton X-100 and the lysates streaked on Sabouraud dextrose agar plates to measure colony forming units (CFU).
Cytokine Measurement

The culture medium was removed at the indicated times after infection of RPM with C. albicans and stored at -80°C for cytokine measurement and eicosanoid analysis (see below). TNFs in the culture medium was quantified by ELISA and by Luminex assay, which gave similar results.

Mass Spectrometry Eicosanoid Analysis

The samples of culture media were thawed and mixed with an equal volume of cold methanol. Just prior to analysis they were diluted in water to a final methanol concentration of <15% and then extracted using a solid phase extraction cartridge (Strata Polymeric Reversed Phase 60 mg/ml, Phenomenex, Torrance, CA). The eluate (1 ml of methanol) was dried and reconstituted in 75 µl of HPLC solvent A (8.3 mM acetic acid buffered to pH 5.7 with NH4OH) and 25 µl of solvent B (acetonitrile/methanol, 65/35, v/v). An aliquot of each sample (50 µl) was injected into an HPLC and metabolites separated on a C18 column (Ascentis 15 mm x 2.1 mm, 5 µm, Supelco) eluted at a flow rate of 200 µl/min with a linear gradient from 25% to 75% solvent B in 13 min then increased to 98% in 2 min and held for 11 min. The HPLC system was directly interfaced into the electrospray ionization source of a triple quadrupole mass spectrometer (Sciex API 3000, PE-Sciex, Thornhill Ontario, Canada). Mass spectrometric analyses were performed in the negative ion mode using multiple reaction monitoring (MRM) for specific analytes. Deuterated internal standards were detected using the following transitions and limits of quantitation: PGE

Microarray Analysis

RPM cultured and stimulated with C. albicans for 3 h as described above were washed twice with endotoxin-free PBS and total RNA isolated. Template RNA quality was assessed with the Agilent Bioanalyzer 2100 and an Agilent Nano RNA 6000 kit per the Agilent protocol. RNA quality ranged from a RNA Integrity Number (RIN) of 8.1 to 10.0. An Agilent Quick Amp Labeling kit was used to generate Cy3 labeled RNA. Yields of 3.7-6.8 µg were obtained with specific activities of 7.5-9.4 pmol/µg. Fragmentation followed by hybridization was performed (Agilent Gene Expression Hybridization Kit) on Agilent Whole Mouse Genome kit 4x44 microarray slides at 65°C for 16 hr. Slides were washed according to the Agilent Quick Amp Labeling Kit protocol and scanned immediately on an Agilent G2505B scanner. The microarray results were log base 2 transformed and data normalization was applied using the 75% percentile shift method to adjust for experimental variability. Boxplots of resulting expression were examined for consistency and all quality control metrics were within acceptable ranges. Filtering was performed to exclude gene expression probes that did not reach a relative expression value of ≥35 across all groups. Microarray samples were grouped by unstimulated cPLA

Western Blots

To prepare lysates for western blots, cell monolayers were washed twice in ice cold PBS and then scraped in lysis buffer: 50 mM Heps, pH 7.4, 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 200 µM sodium vanadate, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 300 mM p-nitrophenyl phosphate, 1 mM phenylmethylsulfonylfuoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. After incubation on ice for 30 min, lysates were centrifuged at 15,000 rpm for 15 min and protein concentration in the supernatant determined by the bicinchoninic acid method. Lysates were boiled for 5 min after addition of Laemmli electrophoresis sample buffer, and then proteins were separated on 10% SDS-polyacrylamide gels. After transfer to nitrocellulose membrane, samples were incubated in blocking buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween (TTBS)) containing 5% nonfat milk for 1 h, and then incubated overnight at 4°C with primary antibodies in TTBS. The membranes were incubated with anti-rabbit IgG horseradish peroxidase antibody (1:5000) in TTBS for 30 min at room temperature. The immunoreactive proteins were detected using the Amersham ECL system.
StepOnePlus Real-Time PCR System (Applied Biosystems). PCR arrays in a 96-well format were used containing prevalidated primers tested for efficiency (SA Biosciences). The RT² Profiler PCR Array System included a reverse transcription control preloaded into the primer buffer of the RT² First Strand cDNA synthesis kit that measured the relative efficiency of the reverse transcription for all the samples. A genomic DNA control and a positive PCR control were also included in the system. The RT² Profiler PCR Array data were normalized to two housekeeping genes Gapdh and Hprt and the relative gene expression level (2^(-ΔCt)) was calculated using the formula ΔCt = Ct (gene of interest) - Ct (housekeeping gene). The data were analyzed on the PCR array data analysis SA Biosciences web portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Real-time PCR was also performed with cDNA synthesized with random hexamer primers (Superscript III polymerase, Invitrogen) using TaqMan fast universal PCR master mix. TaqMan assay probes were used:

- Csf1 (Mm00432686_m1)
- Csf3 (Mm00433335_g1)
- Tnf (Mm99999068_m1)
- l1l0 (Mm00439614_m1)
- Nr4a2 (Mm00443060_m1)
- Crem (Mm00516346_m1)
- Stat3 (Mm01219775_m1)

Gapdh (Mm99999915_g1). The housekeeping gene Gapdh and a calibrator containing mRNA from unstimulated cPLA₂⁺⁰⁺ and cPLA₂α⁻⁻ RPM were used for normalization. Threshold cycle values (Ct) were determined and used for ΔCt analysis of gene expression [32].

Results

The production of eicosanoids by RPM is initiated by the activation of cPLA₂, which occurs rapidly in response to C. albicans or zymosan due to post-translational processes [9–12]. The major arachidonic acid metabolites produced by RPM in response to C. albicans and zymosan are PGI₂, PGE₂, and LTC₄, and their production is dependent on cPLA₂ activation to provide arachidonic acid substrate [12–14]. As shown in Figure 1A, eicosanoids were produced mostly during the first 30 min after C. albicans addition. Prostaglandin production occurred before the increase in COX2 expression stimulated by C. albicans, which was detected 3 h after addition of C. albicans but not at 1 h (Figure 1B). In contrast, COX1 was constitutively expressed in RPM and was not affected by C. albicans infection. Microarray data also confirmed that COX2 expression was very low compared to COX1 in unstimulated cPLA₂⁺⁰⁺ RPM, but there was a significant increase in expression of COX2 (Ptgs2) but not COX1 (Ptgs1) in cPLA₂⁺⁰⁺ RPM treated with C. albicans for 3 h (Table 1). The results suggest that cPLA₂α-mediated release of arachidonic acid couples to COX1 for early production of prostaglandins.

Role of cPLA₂α in regulating TNFα production

The initial focus was to determine if cPLA₂α activation regulates TNFα production in C. albicans-stimulated RPM since prostaglandins can suppress production of this pro-inflammatory cytokine as we reported for L. monocytogenes-stimulated RPM [8,33,34]. First we investigated if TNFα production was mediated by similar PRRs that promote cPLA₂α activation in response to C. albicans. We reported that dectin-1 and MyD88, but not TLR2 or TLR4, play a role in the activation of cPLA₂α in response to C. albicans [13,14]. We found that production of TNFα 6 h after addition of C. albicans was reduced in dectin-1⁻⁻ and MyD88⁻⁻ RPM compared to dectin-1⁺⁺ and MyD88⁺⁺ RPM (Figure 2A and 2B). The requirement for MyD88 suggested a role for TLRs. A comparison of RPM from TLR2⁻⁻ and TLR2⁻⁻ mice showed that TNFα production was not mediated by TLR2 (data not shown). However, TLR4 partially contributed to C. albicans-mediated TNFα production, which was reduced by approximately 50% in TLR4⁻⁻ RPM (Figure 2C). Since mannans of C. albicans cell wall engage TLR4 we tested the ability of the C. albicans glycosylation mutant (Capmr1Δ null mutant), which is devoid of phosphomannans and has defective N- and O-linked mannans, to stimulate TNFα production in TLR4⁺⁺ and TLR4⁻⁻ RPM [27,35]. Compared to TLR4⁺⁺ RPM treated with wild type C. albicans, TNFα production in TLR4⁺⁺ RPM treated with Capmr1Δ null mutant was reduced by about 50% similar to the level observed in TLR4⁻⁻ RPM stimulated with wild type C. albicans (Figure 2C). TNFα production by TLR4⁺⁺ RPM was restored when the CaPMR1 gene was reintegrated into the mutant strain (Capmr1Δ+CaPMR1). Therefore PRRs on RPM that engage cell wall mannans and β-glucans contribute to TNFα production. Since cPLA₂α⁺⁺ and cPLA₂α⁻⁻ RPM were used to determine the role of cPLA₂α in regulating gene expression in response to C. albicans (as described below), we compared their levels of expression of PRRs involved in C. albicans recognition. Microarray data showed that cPLA₂α⁺⁺ and cPLA₂α⁻⁻ RPM express similar levels of PRRs Clec7a (dectin-1), Clec4n (dectin-2), Tlr4 and Tlr2 (Gene Expression Omnibus, www.ncbi.nlm.nih.gov.geol, GSE46533). We also compared the ability of cPLA₂α⁺⁺ and cPLA₂α⁻⁻ RPM to bind and internalize C. albicans. Results of a recognition assay demonstrated no differences in the uptake of Alex Fluor-labeled C. albicans by cPLA₂α⁺⁺ and cPLA₂α⁻⁻ RPM (data not shown). A C. albicans killing assay was also carried out by incubating RPM with C. albicans and then measuring the recovery of CFU from RPM after further incubation for 1 and 4 h. There were no differences in CFU recovered at 1 h in WT and cPLA₂α⁻⁻ RPM. However at 4 h there was a small but significantly higher level of C. albicans CFU recovered from cPLA₂α⁻⁻ RPM. In three independent experiments the CFU in cPLA₂α⁻⁻ RPM was 172%±32%, p<0.002 compared to cPLA₂α⁺⁺ RPM (100%). The results suggest that the cPLA₂α⁻⁻ RPM have a slightly greater ability to kill internalized C. albicans. The role of cPLA₂α activation and prostaglandin production in regulating the production of TNFα in response to C. albicans was investigated by comparing RPM from cPLA₂α⁺⁺ and cPLA₂α⁻⁻ mice, and by treating the macrophages with a cyclooxygenase inhibitor NS398 (Figure 3A). The production of
**Figure 1.** Time course of *C. albicans*-stimulated eicosanoid production. (A) RPM were incubated with *C. albicans* for the indicated times. The culture medium from unstimulated (open squares) or *C. albicans*-stimulated (closed squares) RPM was analyzed for eicosanoids by mass spectrometry. The data are the average of triplicate samples (±S.D.) from a representative experiment. (B) Cell lysates from unstimulated RPM (US) or RPM stimulated with *C. albicans* (CA) for 1, 3 and 6 h were analyzed for COX1 and COX2 expression by Western blotting. Sample loading was evaluated by probing with antibodies to β-actin.

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**Figure 2.** Role of PRRs in regulating *C. albicans*-stimulated TNFα production. Wild type (open bars) and Dectin-1−/− (A), MyD88−/− (B) and TLR4−/− (C) RPM (shaded bars) were incubated with *C. albicans* for 6 h. In panel C, RPM were stimulated with the parental wild type *C. albicans* (WT), the Capmr1Δ null mutant and the re-integrant strain (Capmr1Δ+CaPMR1). The data are the average of 3 experiments ±S.E. (*p<0.05). Levels of TNFα in the culture medium were determined by ELISA.

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TNFα was lower in cPLA\textsubscript{2}α\textsuperscript{+/+} RPM compared to cPLA\textsubscript{2}α\textsuperscript{-/-} RPM measured 6 h after \textit{C. albicans} addition. NS398 treatment enhanced TNFα production in cPLA\textsubscript{2}α\textsuperscript{+/+} but not in \textit{C. albicans}-stimulated cPLA\textsubscript{2}α\textsuperscript{-/-} RPM suggesting that prostanoids suppress TNFα expression. NS398 completely blocked production of PGE\textsubscript{2} and PGI\textsubscript{2} in RPM stimulated with \textit{C. albicans} for 6 h (data not shown), and at the concentration used (10 µM) inhibits both murine COX1 and COX2 [36]. To further investigate the role of prostanoids in the autocrine regulation of TNFα production, RPM were treated with agonists for the PGE\textsubscript{2} receptor EP\textsubscript{2} (butaprost) and the PGI\textsubscript{2} receptor IP (iloprost) (Figure 3B).

Microarray data showed that RPM express the IP receptor (Ptgir), the EP2 (Ptger2) and EP4 (Ptger4) receptors (Table 1). The agonists had no effect on the levels of TNFα produced by cPLA\textsubscript{2}α\textsuperscript{+/+} RPM that produce endogenous prostaglandins in response to \textit{C. albicans} (Figure 3B). However, the higher level of TNFα produced by \textit{C. albicans}-stimulated cPLA\textsubscript{2}α\textsuperscript{-/-} RPM, which do not produce endogenous prostaglandins, was reduced by the receptor agonists to the level produced by cPLA\textsubscript{2}α\textsuperscript{+/+} RPM. The data suggest that prostaglandins acting through the EP2 and IP receptors suppress TNFα production since it is enhanced by inhibiting prostaglandin production in \textit{C. albicans}-stimulated cPLA\textsubscript{2}α\textsuperscript{+/+} RPM and suppressed by prostaglandin receptor agonists in cPLA\textsubscript{2}α\textsuperscript{-/-} RPM.

The EP\textsubscript{2} and IP receptors mediate increases in cAMP, which is implicated in regulating \textit{Tnfα} gene expression [37,38]. As shown in Figure 4A, the stable cAMP analogue 8-Br-cAMP suppressed \textit{C. albicans}-stimulated TNFα production in cPLA\textsubscript{2}α\textsuperscript{-/-} RPM, as observed for the prostanoid receptor agonists, but had no effect on the lower level of TNFα produced by cPLA\textsubscript{2}α\textsuperscript{+/+} RPM. The results suggest that prostaglandins produced by \textit{C. albicans}-stimulated cPLA\textsubscript{2}α\textsuperscript{+/+} RPM act in an autocrine manner through prostaglandin receptors that increase cAMP to suppress TNFα production.

Figure 3. Role of prostaglandins in regulating \textit{C. albicans}-stimulated TNFα production. cPLA\textsubscript{2}α\textsuperscript{+/+} and cPLA\textsubscript{2}α\textsuperscript{-/-} RPM were incubated with (A) NS-398 (10 µM), or (B) iloprost (1 µM) or butaprost (10 µM) for 30 min followed by incubation with \textit{C. albicans} for 6 h. Levels of TNFα in the culture medium were determined by ELISA. The data are the average of 3 experiments ±S.E. (*p<0.05). doi: 10.1371/journal.pone.0069002.g003

Table 1. Relative expression values of cyclooxygenases and prostaglandin receptors in RPM.

| Official Symbol | Entrez_Gene_ID | Unstimulated Mean Expression | C. albicans-treated Mean Expression |
|-----------------|----------------|-------------------------------|-----------------------------------|
| Ptgs2*          | 19225          | 67 ± 14                       | 11243 ± 2938                     |
| Ptgs1           | 19224          | 4694 ± 2731                   | 2027 ± 655                       |
| Ptger2          | 19217          | 204 ± 18                      | 177 ± 65                         |
| Ptger4          | 19219          | 257 ± 24                      | 248 ± 50                         |
| Ptgir*          | 19222          | 589 ± 217                     | 1168 ± 179                       |

cPLA\textsubscript{2}α\textsuperscript{-/-} RPM were stimulated with \textit{C. albicans} for 3 h and gene expression determined by microarray analysis. The * denotes a significant (p<0.05) increase in expression by \textit{C. albicans} treatment.

Effect of \textit{C. albicans} on gene expression in RPM

We next determined the effect of \textit{C. albicans} on global gene expression in RPM by microarray and then evaluated how cPLA\textsubscript{2}α activation modulates the transcriptional response. \textit{C. albicans} stimulated an increase in expression of 427 genes (≥4.0-fold, p<0.05, n=3) in cPLA\textsubscript{2}α\textsuperscript{-/-} Balb/c RPM at 3 h. Relative expression levels for these genes and the fold change in response to \textit{C. albicans} are shown in Table S1A. Many of the genes that increase in response to \textit{C. albicans} represent
the common host-response that is induced in many cell types by a variety of infectious agents [39]. The data were analyzed using the DAVID bioinformatics resource to evaluate the functional clustering of genes that were increased in RPM in response to C. albicans [31]. The most highly enriched clusters contained genes in apoptosis, cytokines, wound and inflammatory responses, regulation of phosphorylation and protein kinase activity, cell motion, vascular development, regulation of cytokine production, MAP kinase phosphatase activity, regulation of transcription and growth factor activity (Table 2). Csf3 was the most highly induced gene by C. albicans (>600-fold) (Table S1A). The cytokine CSF3 regulates the production and function of neutrophils and is important for host defense against C. albicans [40,41]. As discussed below, the expression of Csf3 was regulated by cPLA2 activation. There were 110 genes down-regulated in RPM at 3 h by C. albicans (≥4-fold, p<0.05, n=3) (Table S1B). The clusters for the down-regulated genes had very low enrichment scores compared to the up-regulated genes when subject to DAVID analysis (data not shown).

**Genes expressed at lower levels in C. albicans-stimulated cPLA2α knockout than cPLA2α wild-type RPM**

Differential gene expression was compared in cPLA2α wild-type and cPLA2α knockout RPM treated with C. albicans for 3 h. We chose to study the effect of C. albicans infection on gene expression at 3 h in order to evaluate the role of cPLA2α activation and eicosanoids in regulating early responses during the acute phase of infection. The regulation of gene expression at later times becomes more complicated due to autocrine effects of the products of early response genes that promote induction of a second wave of gene induction. In cPLA2α wild-type RPM, 86 genes were expressed at lower levels and 181 genes at higher levels than cPLA2α knockout RPM (≥2-fold, p<0.05, n=3) (Tables S2A and S2B, respectively). When genes expressed at lower levels in cPLA2α wild-type RPM were analyzed using DAVID, they grouped into functional clusters involving GTP binding, regulation of cytokine production/cytokine receptor interaction and regulation of proliferation (Table 3). The expression of genes for GTP binding proteins included several IFN γ-inducible GTPases (guanylate binding proteins (Gbp) 1, 2, 3, 5, 6 and 7; immunity-related GTPase family M members (Irgm) 1 and 2; IFN γ-inducible protein (Ifi) 47 and IFN γ-inducible GTPase (Iigp) 1). Some of these genes regulate host defense to microbial infection although their function is poorly understood [42–45]. Several genes expressed lower in C. albicans-stimulated cPLA2α knockout than cPLA2α wild-type RPM in the cytokine cluster (Table 3) are pro-inflammatory such as the chemokine Cx3cl1 (fractalkine), Cd40, Tnfα and Ifnγ [46–48]. The lower expression of Ifnγ in cPLA2α wild-type RPM correlated with the reduced expression of the IFNγ regulated GTPases, although its level of expression in RPM was very low (Table S2A). Genes for the transcription factors, interferon regulatory factors (Irf) 1 and Irf4 (Cytokine cluster), and the AT-hook transcription factors...
Table 2. Functional annotation clusters of genes induced in *C. albicans*-stimulated RPM.

| Annotation Clusters                        | Official Symbol |
|--------------------------------------------|----------------|
| Apoptosis                                  | Bcl2, Cdf1, Cxcl1, Ddit4, Egr3, Epha2, Rypb, Traf1, Traf5, Ahr, F2r, Camp1, Fen1b, G2mb, Gadd45b, Gadd45g, Id1, I6, Jmyj6, Maff1, Myc, Nias1, Nkb1, Nra4a2, Osm, Phd1a, Bnip3, Bicam, Ppp1r15a, Srgn, Slah2, Mof1, Trb3, Trim69, Trf, Trfraf12a, Trfip3, Uroc5b, Zc3h12a |
| Cytokines, Response to wounding, and       | Areg, Cdf2a, Kdm6b, Bmp2, Bmp6, Ct2, Ct3, Ct4, Cc27, Ccr1, Cxcl1, Cxcl2, Cxcl3, Csf2, Csf3, F2r, F3, Gata15, Gata1, Hbegf, Idf1, I1a, Irb1, Irf1, Irf10, I23a, I68, Nfkbi, Olr1, Osm, Plaur, Plek, Procr, Prot, Scl7a2, Spk1, Trf, Trfraf9 |
| Inflammatory response                      | Cxcl2, Cxcl3, Cxcl4, Cxcl10, Cxcl11, Cxcr2, Epha2, Epha4, Gja1, Gja3, Hbegf, Idf1, Irf1, Irf4, Irf6, Lrp8, Lpar1, Ng1, Osm, Rac1, Rac3, Rac5, Rac7, Rac8, Rap1, Tnfaip3, Unc5b, Zc3h12a |
| Regulation of phosphorylation and protein kinase activity | C2d24a, Eph4a, Alcam, Ccr1, Cxcl2, Cxcl3, Eg2, Gata1, Hbegf, I1f5, I6p, I5g1, I4a2, Plau, Ppdm, Pvr, Ranix, Runx3, Zfand5, Tes, Trf, Trfraf12a, Vegfa |
| Cell motion                                | Eph4a, Junb, Smad7, Edn1, Ebi2, Ereg, Gata1, Hbegf, Idf1, Iqg1v, I6f8, Jmpf6, Pdpon, Prok2, Zfand5, Spk1, Socs3, Tgmx2, Trfraf12a, Vegfa, Zc3h12a, Zfp361 |
| Vascular development                       | Cxcl2, Cxcl3, Cxcl4, Cxcl8, Adora2a, Adora2b, F2r, Edn1, Ereg, Fst, Inbbn, Irf4, Iuba, I1b, I2b, I6, Nkb1, Prok2, Rel, Srgn, Spk1, Trf |
| Regulation of cytokine production          | Dusp1, Dusp2, Dusp4, Dusp8, Dusp10, Dusp14, Dusp16 |
| MAP kinase phosphatase activity            | Efl1, Kdm6b, Mx1, Pou3f1, Rypb, Skil, Itfl4, Ahr, Bmp2, Camp1, Eg1, Egr2, Fos1, Hes1, Ihnba, Id1, Id2, Idf3, Idf4, Irf6, Jarid2, Med13, Myc, Nkb1, Nufp1, Nra4a1, Nra4a2, Nra4a3, Osm, Plag1, Ptx1, Sap30, Trf, Vegfa |
| Growth factor activity                     | Areg, Bmp2, Bmp6, Cxcl1, Cxcl2, Cxcl3, Egr4, Gata15, Hbegf, Ihnba, Ihnbb, Itfl1, I68, Vegfa |
| Genes expressed at higher levels (427 genes, ≥2-fold, <0.05) in *C. albicans*-stimulated cPLAα−/− RPM were analyzed using DAVID bioinformatics resource. |

factor (Akna) were also expressed at lower levels in cPLAα−/− than cPLAα+/− RPM (Table S2A). AKNA promotes C40 expression suggesting a correlation between low expression of Akna and C40 in cPLAα−/− RPM [49]. AKNA functions in inflammation and cancer [50]. There was also a correlation with the lower expression of genes for guanylate binding proteins (Gbp) and Tnfa in cPLAα−/− RPM and their transcriptional regulator Irf1 [51]. IRF transcription factors play important roles in host defense and regulating immune responses [52].

cPLAα−/− RPM expressed lower mRNA levels of the chemokine Ccl5 (Cytokine cluster), which promotes the trafficking of cells to sites of inflammation [53]. PGE2 suppresses CCL5 production in macrophages and dendritic cells thus dampening inflammation and immune responses [54]. Colony stimulating factor 1 (Csfl, Cytokine cluster) was induced to a greater extent in cPLAα−/− RPM (10-fold) than cPLAα+/− RPM (3-fold) (Table S2A). It promotes macrophage-lineage development but also recruits myeloid cells during inflammation and infection, and promotes their survival [55]. Another pro-inflammatory gene expressed at lower levels in cPLAα−/− RPM was the vasoactive peptide endothelin 1 (Edn1, Regulation of proliferation cluster), which stimulates myeloid and mast cells at sites of inflammation [56] (Table S2A). Overall the results implicate cPLAα activation and eicosanoid production in suppressing the expression of pro-inflammatory genes, and transcription factors that regulate their expression.

We corroborated the microarray results by real-time PCR for representative genes expressed lower in cPLAα−/− than cPLAα+/− RPM. Their expression was preferentially enhanced by C. albicans in cPLAα−/− RPM compared to cPLAα−/− RPM suggesting that products of cPLAα activation suppress their expression (Figure 5). Results of real time PCR showed that expression of these genes was transient in cPLAα−/− RPM occurring maximally 3 h after stimulation with C. albicans.

Genes expressed at higher levels in *C. albicans*-stimulated cPLAα−/− than cPLAα+/− RPM

A larger number of genes were expressed at higher levels in cPLAα−/− RPM than cPLAα−/− RPM (181 genes, ≥2-fold, p<0.05, n=3) (Table S2B). From DAVID analysis, genes clustered in functional groups involving vascular development, embryonic morphogenesis, sexual reproduction, response to wounding, inflammatory and defense responses, growth factors and growth factor activity, DNA binding and transcription regulation, and disulfide bond (Table 4). Several genes in these clusters are associated with cancer development consistent with a role for prostaglandins in promoting carcinogenesis [57,58]. These include the Eph receptor A2 tyrosine kinase (Epha2, Vascular development cluster), the epidermal growth factor receptor (EGFR) ligands epiregulin (Ereg) and amphiregulin (Areg) (Growth factor cluster), the transmembrane glycoprotein podoplanin (Pdpn) and its transcriptional regulator the homeobox protein Prox1 (Vascular development cluster), the chemokine receptor 7 (Cxcr7, Disulide bond cluster), matrix metalloproteinase 13 (Mmp13, Embryonic morphogenesis cluster) and its transcriptional regulators Runx2 and nuclear receptor subfamily 4, group A, member 2 (Nr4a2). These genes expressed at higher levels in

Table 3. Functional annotation clusters of genes expressed at lower levels in *C. albicans*-stimulated cPLAα−/− than cPLAα+/− RPM.

| Annotation Clusters                        | Official Symbol |
|--------------------------------------------|----------------|
| GTP binding                               | Rab33A, Rass2d, Gbg1, Gbg2, Gbg3, Gbg5, Gbg6, Gbg7, Igmg1, Ifi47, Igmg1, Akr4 |
| Regulation of cytokine production, Cytokine receptor interaction | Cc40, Cc5, Cx30d, Csf1, Iinf, Ifk1, Ifk4, Ifk15a, Tcam2, I20rb, Itrfn14, Trf |
| Regulation of proliferation               | Cc40, Adm, Csf1, Edn1, Igf1, Iinf, I20rb, Lst1, Plau, Smo, Trfraf14, Trf |

Genes expressed at lower levels (86 genes, ≥2-fold, <0.05) in cPLAα−/− than cPLAα−/− RPM stimulated for 3 h with C. albicans were analyzed using DAVID bioinformatics resource.
cPLA₂α and cPLA₂α⁺/+ RPM promote angiogenesis, tumor growth and invasion, and are regulated by prostaglandins and cAMP [59–66]. Of particular interest were the large number of genes expressed at higher levels in C. albicans-stimulated cPLA₂α⁺/+ RPM that function to dampen inflammation. C. albicans induced high expression of suppressor of cytokine signaling 3 (Socs3, Vascular development and Embryonic morphogenesis clusters) in cPLA₂α⁺/+ RPM (16-fold) and to a lesser extent in cPLA₂α⁻/- RPM (6-fold) (Table 4, Table S2B). SOCS proteins
**Table 4. Functional annotation clusters of genes expressed at higher levels in C. albicans-stimulated cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM.**

| Annotation Clusters          | Official Symbol |
|-----------------------------|-----------------|
| Vascular development        | Eph2, Chx7, Ereg, Foxc1, Gja1, Ifgav, Lepr, Nus1, Polp, Prox1, S1pr1, Socs3, Zp3681 |
| Embryonic                    | Eph2, Chx7, Chst11, Foxc1, Hess1, Il10, Mmp13, Pbx1, morphogenesis | Prox1, Socs3, Spry2, Jag2 |
| Sexual reproduction          | Bcl6, Bcl2Bf1, Crem, Calca, Cadm1, Ereg, Fat, Foxc1, Jag2, Lepr, Pw13, Rgs2, Stat3 |
| Response to wounding         | Bmp6, Cd14, Calca, Ddash2, Entpd1, Gja1, Hdac3, Il11b6, Il10, Saa1, Saa2, Stat3, Thbd, Thbs1 |
| Inflammatory and Defense     | Areg, Bmp6, Chst11, Cdf3, Ereg, Foxc1, Gja1, Hgf, Inhbβ, Jag2 |
| activity                     | Anit3b, Bcl6, Bach2, Gata2, Lhx5, Msd1, Msx1, Setdbp1, Thap2, Crem, Chx7, Cdx2, Foxc1, Hess1, Hdac5, Lmp1, Nr4a2, Pbx1, Prox1, Runx2, Stat3, Fos2, Snbo2, Tats3, Tie1, Mafb, Zp36, Zp3681 |
| DNA binding                  | Nil53, Abca1, Cd14, Cdx80, Edn3, Eph2, Gpr35, Areg, Antrv2, Bmp6, Calca, Cacna1d, Cadm1, Cbln3, Cxcr7, Cxcr8, Entpd1, Ereg, Fat, Gja1, Havcr2, Hgf, Inhbβ, Ilegav, Il10, Jag2, Lepr, Ltf, Mansta, Mmp13, Mmp3, Mpz1l, Nia3c, Pila1a, Pnv3, Piger2, Lpar6, Ramp3, Sema6d, Tnafp6, Thbd, Thbs1, Trem1, Tnfrsf10a |
| Transcription regulation     | Disulfide bond  |

Genes expressed at higher levels (181 genes, ≥2-fold, <0.05) in cPLA₂α<sup>−/−</sup> RPM stimulated for 3 h with C. albicans were analyzed using DAVID bioinformatics resource.

function as negative feedback inhibitory pathways to control immune cell activation and inflammation [67]. Socs3 expression is also regulated by STAT3 (Table 4, Sexual reproduction and response to wounding clusters), which was induced 4-fold in C. albicans-stimulated cPLA₂α<sup>−/−</sup> RPM but not significantly affected in cPLA₂α<sup>+/+</sup> RPM (Table S2B). One of the most differentially expressed genes was Il10 (Embryonic morphogenesis cluster) that was induced 78-fold by C. albicans cPLA₂α<sup>−/−</sup> RPM and 7-fold in cPLA₂α<sup>+/+</sup> RPM (Table 4, Table S2B). The expression of Il10 is regulated in macrophages by the transcription factor PBX1 [68], also expressed at higher levels in C. albicans-stimulated cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM. The anti-inflammatory response (AIR) in macrophages induced by IL10 is mediated by STAT3 through induction of the helicase family co-repressor, Strawberry notch homologue 2 (Sbno2) [69–71]. Expression of Sbno2 (Table 4, DNA binding, Transcription regulation cluster) was increased in C. albicans-stimulated cPLA₂α<sup>−/−</sup> RPM but not in cPLA₂α<sup>+/+</sup> RPM (Table S2B).

Several genes implicated in suppressing Tnfα expression were expressed at higher levels in cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM. One of these genes, the zinc finger protein 36, C3H type-like 1 (Zfp361, DNA binding cluster), was increased by C. albicans in cPLA₂α<sup>−/−</sup> but not cPLA₂α<sup>+/+</sup> RPM (Table 4, Table S2B), and inhibits TNFα production in macrophages by destabilizing its mRNA [72]. The cAMP responsive element modulator (Crem, also called Icer) (Table 4, Sexual reproduction and DNA binding cluster), was highly induced in cPLA₂α<sup>−/−</sup> RPM (16-fold) in response to C. albicans but not significantly affected in cPLA₂α<sup>+/+</sup> RPM (Table S2B). CREM suppresses expression of pro-inflammatory genes including Tnfα [73]. The anti-inflammatory and immunosuppressive neuropeptide calcitonin gene-related peptide (Calc), Sexual reproduction cluster) that is higher in cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM suppresses Tnfα through induction of Crem [74,75]. C. albicans also induces expression of the cAMP-regulated nuclear receptor Nr4a2 to a greater extent in cPLA₂α<sup>−/−</sup> RPM (81-fold) than in cPLA₂α<sup>+/+</sup> RPM (10-fold) (Table S2B). NR4A2 suppresses Tnfα expression in macroglia and astrocytes [76].

Several other genes that are expressed at higher levels in cPLA₂α<sup>−/−</sup> RPM than cPLA₂α<sup>+/+</sup> RPM have diverse functions but also act to dampen inflammation (Table 4). Follistatin (Fst) (Sexual reproduction cluster) curbs inflammation by inactivating the inflammatory actions of activin [77]. Expression of the anti-inflammatory genes Thbs1 and Thbd are also expressed higher in C. albicans-stimulated cPLA₂α<sup>−/−</sup> RPM than cPLA₂α<sup>+/+</sup> RPM (Table S2B) [78,79].

Several genes expressed higher in cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM are involved in host defense such as the gap junction protein, alpha 1 (Gja1, Vascular development cluster) (Table 4, Table S2B). GJA1 promotes phagocytosis and host survival to bacterial infection [80]. Cs3 (Growth factor cluster) is highly upregulated in response to C. albicans in cPLA₂α<sup>−/−</sup> RPM (640-fold) but to a lesser extent in cPLA₂α<sup>+/+</sup> RPM (140-fold) (Table S2B). The orphan receptor triggering receptor expression on myeloid cells (Trem1, Disulfide bond cluster) is upregulated to a greater extent in C. albicans-stimulated cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM (2-fold) (Table S2B). TREM1 couples with the signaling adaptor DAP12 and has complex effects to enhance or dampen responses to TLR activation [81]. Histidine decarboxylase (Hdc), the encodes the rate-limiting enzyme for histamine synthesis, is another highly differentially expressed gene that is 20-fold higher in cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM (Table S2B). Hdc is transcriptionally induced in myeloid cells in response to cytokines and TLR agonists leading to immediate secretion of newly synthesized histamine [82]. Prostaglandins induce Hdc expression and also greatly potentiate the vasoactive effects of histamine [83–85].

The microarray results were corroborated by real-time PCR analysis for several representative genes expressed at higher levels in cPLA₂α<sup>−/−</sup> than cPLA₂α<sup>+/+</sup> RPM (Figure 6). When analyzed 1–6 h after C. albicans addition, several early response genes (Crem, Nr4a2, Cxcr7) showed highest expression in cPLA₂α<sup>−/−</sup> RPM at 1 h. The early induction of the transcriptional regulators Crem and Nr4a2 due to cPLA₂α activation suggests that they play a role in regulating gene expression to increases in cAMP. The expression of most genes peaked 3 h after C. albicans addition with some decreasing to near baseline by 6 h (Hdc, Map4k4, Il10, Stat3, Thbs1, Trem1) while others remained elevated (Cs3, Adamts9, Gja1).
Role of the IP receptor and PKA in regulating gene expression

We investigated the role of prostacyclin production (the prostanoid produced at the highest level in RPM) and PKA, the downstream mediator of cAMP, in regulating gene expression by treating cPLA\(_2\)\(\alpha^{+/+}\) RPM with the IP receptor antagonist CAY10441 and the PKA inhibitor H89 (Figure 7).

Representative genes expressed at lower levels in C. albicans-stimulated cPLA\(_2\)\(\alpha^{+/+}\) than cPLA\(_2\)\(\alpha^{-/-}\) RPM (Tnf\(\alpha\) and Csf1) were enhanced by blocking the action of PGI\(_2\) and inhibiting PKA. In contrast, genes expressed at higher levels in cPLA\(_2\)\(\alpha^{+/+}\) than cPLA\(_2\)\(\alpha^{-/-}\) RPM (Crem, Il10, Csf3, Nr4a2) were suppressed by the IP receptor antagonist and by the PKA inhibitor. The results suggest that cPLA\(_2\)\(\alpha\)-mediated prostaglandin production promotes an autocrine loop to increase cAMP and PKA activation for regulating expression of these genes.

Discussion

In this study we describe the changes in gene expression that occur in RPM during infection with C. albicans, and how gene expression is influenced by the activation of cPLA\(_2\alpha\) and...
endogenously produced lipid mediators. Resident tissue macrophages are sentinel cells that are important in first sensing and responding to microbial invasion. Therefore our study investigates how cPLA$_2^\alpha$ activation modulates macrophage responses during the initial stages of infection to affect the balance of host defense and inflammation. The production of eicosanoids in RPM is dependent on cPLA$_2^\alpha$ activation to provide arachidonic acid [12,14]. They are released within minutes of activation by $C.\ albicans$ to rapidly engage eicosanoid receptors for regulating transcriptional responses. Although there have been a number of studies investigating the effect of adding exogenous eicosanoids to cells, by comparing cPLA$_2^{\alpha+}$ and cPLA$_2^{\alpha-}$ RPM we are probing the primary mechanism for production of eicosanoids in macrophages at levels expected to occur locally in tissues in response to microbial infection. Our analysis provides global insight into the extensive changes in gene expression that are initiated by activation of cPLA$_2^\alpha$ and endogenously produced eicosanoids in resident tissue macrophages early in response to microbial infection.

The recognition of $C.\ albicans$ by macrophages is complex since the fungal cell wall contains several chemical components that differentially engage a number of receptors including a variety of TLRs and lectins [86]. These receptors promote unique signaling pathways that preferentially induce distinct cellular responses. In RPM $C.\ albicans$ triggers rapid

**Figure 7. Effect of IP receptor antagonist and PKA inhibitor on gene expression.** cPLA$_2^\alpha$ RPM were incubated with the IP receptor antagonist CAY10441 (1 µM) (light gray bars) and the PKA inhibitor H89 (10 µM) (black bars) for 30 min followed by stimulation with $C.\ albicans$ for 3 h. RNA was isolated and gene expression determined by real-time PCR. Gene expression values are presented as the % of control values (set at 100%), which is $C.\ albicans$-stimulated RPM not treated with CAY10441 or H89. The results are the average of 3 experiments ±S.E. (*p<0.05).

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activation of mitogen-activated protein kinases and calcium mobilization necessary for cPLA₂ activation through dectin-1, dectin-2 and MyD88 pathways [13, 14]. The results of this study suggest that the differential expression of many genes observed in cPLA₂⁺⁻ and cPLA₂⁺⁺ RPM is due to an autocrine loop involving cPLA₂, prostaglandins and increased cAMP production, which is significantly higher in C. albicans-stimulated cPLA₂⁺⁺ compared to C. albicans-stimulated cPLA₂⁺⁺ RPM. This is illustrated by results showing that TNFα production is suppressed by prostaglandins through increases in cAMP. Expression of TNFα occurs in part through dectin-1 and TLR4 in RPM that activate NF-κB and transcription [86]. In RPM the rapid production of prostanooids, particularly PGI₂ that acts through the IP receptor, increases cAMP and PKA activation that suppresses transcription by mechanisms that are not fully understood. In addition to TNFα we observed differential expression of several genes previously reported to be regulated by prostaglandins and increases in cAMP in a variety of cell types. These include Ccl5, Socs3, Il10, Gja1, Crem, Thbd, Aca1, Csf3, Trem1 [33, 69, 73, 87–93]. Similar to our results in C. albicans-stimulated RPM, an autocrine loop pathway involving cPLA₂, prostacyclin and cAMP has been shown to enhance expression of Areg, Ereg and Fst, Cre-dependent genes involved in vascular remodeling and angiogenesis [94]. This autocrine loop involving prostaglandins and cAMP is triggered in many cell types in response to a variety of agonists indicating that it is an important, widely used pathway for regulating gene expression.

The rapid increase in cAMP that occurs in C. albicans-stimulated cPLA₂⁺⁺ RPM is consistent with functional coupling of cPLA₂ activation and metabolism of arachidonic acid to prostanooids by constitutively expressed COX1 since the response occurs before the expression of COX2. A role for COX1 in mediating prostaglandin production in LPS-stimulated RPM has previously been reported [34], COX1 provides prostaglandins that regulate normal physiological processes and can regulate the early phases of inflammation [17]. cAMP expression the EP2, EP4 and IP receptors that mediate increases in cAMP, and our results show that EP2 or IP receptor agonists suppress TNFα production. It is likely that PGJ₂ and PGE₂ both contribute to the regulation of transcription through increases in cAMP. However, PGJ₂ is produced at higher amounts than PGE₂ during the first 15-30 min after activation by C. albicans. We were not successful in testing the EP2 receptor antagonist due to adverse effects on RPM. Although not addressed in this study, other eicosanoids such as LTC₄ and arachidonic acid itself released by RPM in response to C. albicans could also influence macrophage activation. Arachidonic acid has been shown to suppress the expression of the complement receptor immunoglobulin (CRIg) during maturation of human monocytes to macrophages resulting in a decrease in the phagocytosis of opsonized C. albicans [95]. LTC₄ could act through the CYSLT1 and CYSLT2 receptors expressed on RPM. For example these receptors promote calcium mobilization that may influence transcriptional responses due to cross-talk with cAMP signaling, and by potentiating cPLA₂α activation [96]. Leukotrienes have been shown to promote uptake of C. albicans by alveolar macrophages and to enhance fungicidal activity [97]. It is possible that leukotrienes contribute to the enhanced C. albicans killing we observed in cPLA₂⁺⁺ RPM compared to cPLA₂⁺⁺ RPM.

Microbial pathogens engage PRRs on macrophages that induce extensive effects on gene expression as we observed in C. albicans-stimulated RPM [39]. A characteristic of the "common host response" is increased expression of a large number of pro-inflammatory cytokines and chemokines that is important for the recruitment and activation of myeloid cells during infection [39]. Pro-inflammatory host defense responses are balanced by the activation of negative feedback loops that are important in dampening inflammation and potential tissue damage [21]. Our data suggest that cPLA₂α activation and lipid mediator production represents one of the negative feedback loops since cPLA₂α⁺⁺ RPM exhibit lower expression of select pro-inflammatory genes such as Tnfa, Csf1, Ccl5, Cd40, Cx3cl1, Edn, Ifny and several IFNγ regulated GTP binding proteins, and higher expression of anti-inflammatory genes such as Ifi10, Socs3, Stat1, Fst, Thbd, Tfshp1, Calca and Cxcr7 than cPLA₂⁻⁻ RPM. Historically there has been an emphasis on the role of prostaglandins in mediating the cardinal signs of inflammation that is supported by the clinical effects of non-steroidal anti-inflammatory drugs. However, prostaglandins play an important role in suppressing inflammation and immune responses by acting through prostanooid receptors that increase cAMP resulting in PTK activation as supported by our results [18]. This pathway has immunosuppressive effects by inhibiting the differentiation of antigen presenting cells, lymphocyte activation and production of Th1 cytokines.

Our results show that the activation of cPLA₂α and coupling to COX1 is an early response to C. albicans infection of RPM that can regulate the amplitude and timing of inflammation and host defense mechanisms as exemplified by the decrease in expression of Tnfa and increase of Ifi10. ERK activation and calcium mobilization are the signaling cascades activated by PRRs that are important for promoting IL10 production [69, 98]. These are the signals required for optimal cPLA₂α activation and eicosanoid production [99]. This cytokine signature is also a characteristic of resolution phase macrophages that contribute to restoration of normal tissue function by dampening inflammatory signals and the clearance of apoptotic neutrophils [100, 101]. Resolution phase macrophages are characterized by the expression of COX2, decreased TNFα and increased IL10 production controlled by cAMP production. Prostaglandins and eicosanoids in cAMP contribute to the resolution phase by enhancing the ability of macrophages to phagocytose apoptotic neutrophils [102, 103]. Activated and apoptotic neutrophils produce lyso-phosphatidylserine that acts through the macrophage G2A receptor to trigger an autocrine loop involving cPLA₂α activation, PGE₂ production, EP2 receptor-dependent increases in cAMP and PKA activation to enhance efferocytosis [102, 104]. Therefore cPLA₂α activation and prostaglandin production play a role in balancing host defense responses and the extent of inflammation in both the initiation and resolution phases of infection.

The results also indicate that cPLA₂α-mediated prostaglandin production enhances the expression of certain pro-inflammatory genes, such as Csf3, that are important for host
defense against *C. albicans* infection by promoting neutrophil function [40,41]. Prostaglandins also contribute to Candidiasis protection by promoting the Th17 response [105,106]. IL17 regulates neutrophil recruitment and is important for host defense to mucocutaneous Candidiasis [107–110]. However if pro-inflammatory responses go unchecked prostaglandins contribute to chronic inflammation that is characteristic of cancer, and vascular and autoimmune diseases [111]. The ability of prostaglandins to promote the development of Th17 differentiation and production of IL17 contributes to chronic inflammation associated with autoimmune diseases [111,112]. COX2 is overexpressed in cancers and prostaglandins promote cancer development by regulating angiogenesis, cell migration, adhesion and invasiveness in part through promoting receptor specific increases in cAMP [57,58]. Several of the genes that are differentially expressed in cPLA\(_{\alpha}^{-}\) RPM and cPLA\(_{\alpha}^{+}\) RPM (i.e. Gdf15, Eph2, Ereg, Areg, Lepr, Nr4a2, Runx2, Mmp13, Cxcr7, Pdpn, Proxl) are positively or negatively regulated in cancers compared to normal tissue as a result of prostaglandins [59–63,113]. Therefore, eicosanoids have complex biological effects depending on the tissue context, the specific receptors expressed on cells in the local environment and the timing of their production contributing to both anti- and pro-inflammation responses. Results from this study support an important role for cPLA\(_{\alpha}\) activation early in response to microbial infection in resident tissue macrophages that helps to balance the expression of genes important for host defense and genes that contribute to inflammation.

### Supporting Information

**Table S1.** S1A and S1B Genes (A) increased and (B) decreased by *C. albicans* in wild type RPM. (DOC)

**Table S2.** S2A and S2B Genes expressed at (A) lower and (B) higher levels in *C. albicans*-stimulated cPLA\(_{\alpha}^{-}\) than cPLA\(_{\alpha}^{+}\) RPM. (DOC)

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### Author Contributions

Conceived and designed the experiments: CCL SS. Performed the experiments: SS SM. Analyzed the data: CCL SS LJ RCM. Contributed reagents/materials/analysis tools: GDB JVB DLW NARG RCM. Wrote the manuscript: CCL SS DLB.

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