Phosphatidylinositol 3-Kinase Regulates Macrophage Responses to Double-Stranded RNA and Encephalomyocarditis Virus

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Key Words

Inflammation • Innate immunity • Macrophages • Nitric oxide • Virus infection

Abstract

Virus infection of macrophages stimulates the expression of proinflammatory and antiviral genes interleukin-1 (IL-1), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). In this study, we show that phosphatidylinositol 3-kinase (PI3K) is required for the inflammatory response of macrophages to virus infection. When macrophages are infected with encephalomyocarditis virus (EMCV) there is a rapid and transient activation of PI3K and phosphorylation of its downstream target Akt. Inhibitors of PI3K attenuate EMCV- and double-stranded RNA-induced iNOS, COX-2 and IL-1 β expression in RAW264.7 cells and mouse peritoneal macrophages. The attenuation of inflammatory gene expression in response to PI3K inhibition correlates with the induction of macrophage apoptosis. The morphology of macrophages shifts from activation in response to EMCV infection to apoptosis in the cells treated with PI3K inhibitors and EMCV. These morphological changes are accompanied by the activation of caspase-3. These findings suggest that PI3K plays a central role in the regulation of macrophage responses to EMCV in-

fection. When PI3K is activated, it participates in the regulation of inflammatory gene expression; however, if PI3K is inhibited macrophages are unable to mount an inflammatory antiviral response and die by apoptosis.

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Introduction

Virus infection of macrophages results in the expression of proinflammatory and antiviral genes that include interleukin-1 (IL-1), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [1, 2]. The expression of iNOS and subsequent production of nitric oxide plays a primary role in the cellular response to virus infection. Nitric oxide attenuates virus infection by nitrosylation and inactivation of viral proteins required for replication [3, 4]. Virus infection of iNOS-deficient mice results in reduced viral clearance and higher mortality rates compared to wild-type controls [5, 6]. COX-2 catalyzes the oxidation of arachidonic acid to prostaglandin H₂, which is subsequently isomerized to various pros-

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tanoids. Prostaglandins generated by COX-2 participate in the cellular response to virus infection by modulating virus replication [7].

Recent studies have identified a number of pathways involved in the regulation of inflammatory gene expression in macrophages treated with doubled-stranded RNA (dsRNA) or encephalomyocarditis virus (EMCV). EMCV is a picornavirus that has been used as a prototype virus to study antiviral responses [8]. Nuclear factor (NF)- κ B plays a primary role in regulating macrophage expression of iNOS, COX-2 and IL-1 in response to EMCV and synthetic dsRNA (poly IC) [1, 2, 9]. NF- κ B is held in the cytoplasm in an inactive complex with inhibitory protein (I) κ B. Following phosphorylation by an I κ B kinase (IKK), I κ B is targeted for degradation, allowing NF- κ B to translocate to the nucleus to activate gene expression [10]. In addition to NF- κ B, the activation of a secondary signaling pathway that is selective for the target gene of interest is also required for EMCV- and poly IC-induced inflammatory gene expression by macrophages. These secondary signaling pathways include extracellular signal-regulated kinase (ERK) activation for IL-1 β expression [9], iPLA₂ activation for iNOS [11] and Jun N-terminal kinase (JNK) and p38 activation for COX-2 [12]. The regulation of inflammatory gene expression in response to EMCV and dsRNA appears to be independent of the dsRNA-dependent protein kinase (PKR), a dsRNA sensor that initiates various antiviral responses [9, 13, 14]. These studies suggest that virus-induced proinflammatory gene expression is dependent on the activation of NF- κ B and a second signaling cascade that is selective for the target gene of interest.

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase composed of an Src homology 2 domain containing a 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) [15]. PI3K is activated in response to growth factors, cytokines and other stimuli and regulates diverse cellular functions, including proliferation and survival [15]. Virus infection has been shown to activate PI3K [16–19] and this activation appears to favor viral replication by preventing host cell death, suggesting that viruses utilize host PI3K to enhance viral survival [20]. However, the role of PI3K in host cell defense is not well defined although PI3K has been shown to be required for nitric oxide production by activated macrophages [21]. Since nitric oxide plays a primary role in host defense against virus infection, we examined the role of PI3K in the regulation of virus-induced nitric oxide production by macrophages. We show that inhibition of PI3K attenuates dsRNA- and EMCV-induced iNOS expression and nitric oxide production. The regulatory role of PI3K does

not appear to be selective for iNOS, as inhibition of PI3K attenuates dsRNA- and EMCV-induced COX-2 and IL-1 expression as well. In addition to its role in the regulation of inflammatory genes, PI3K also exerts antiapoptotic functions by preventing macrophage apoptosis following virus infection. These findings suggest that virus-induced PI3K activity is required for the inflammatory response of macrophages to virus infection. Inhibition of PI3K signaling attenuates the inflammatory response and results in macrophage apoptosis.

Materials and Methods

Materials and Animals

RAW264.7 cells, L929 cells, RINm5F cells, Dulbecco's modified Eagle's medium (DMEM; containing 10% heat-inactivated fetal calf serum and 1 $\times$ L-glutamine) and RPMI medium (containing 10% heat-inactivated fetal calf serum, 1 $\times$ L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin) were all obtained from Washington University Tissue Culture Support Center (St. Louis, Mo., USA). C57BL/6J mice were purchased from Harlan (Indianapolis, Ind., USA). Mouse recombinant IFN- γ was obtained from R&D Systems (Minneapolis, Minn., USA). Poly IC was purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and prepared as previously described. LY294002 was purchased from Calbiochem (La Jolla, Calif., USA). Rabbit anti-iNOS antiserum was generously provided by Dr. Pam Manning (Pfizer, St. Louis, Mo., USA). Rabbit anti-COX-2 antiserum was obtained from Cayman Chemical (Ann Arbor, Mich., USA). 3ZD monoclonal mouse anti-IL-1 β was from Biological Resources Branch, DCTD at the NCI. Rabbit anti-phospho-Akt (Ser473) was obtained from BioSource (Camarillo, Calif., USA) and from Cell Signaling (Beverly, Mass., USA). Mouse anti-GAPDH was purchased from Ambion (Austin, Tex., USA). Horseradish-peroxidase-conjugated donkey anti-rabbit and donkey anti-mouse antibodies were obtained from Jackson ImmunoResearch (West Grove, Pa., USA). PCR primers for iNOS, IL-1 β , EMCV and GAPDH were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Wild-type and dominant negative p85 α have been previously described [22]. All other reagents were obtained from commercially available sources. The Saint Louis University Institutional Review Board has approved all animal studies contained within this report.

Peritoneal Macrophage Isolation and Cell Culture

Peritoneal exudate cells (PECs) were obtained from wild-type C57BL/6J mice by peritoneal lavage as described previously [23]. Following isolation, 4 $\times$ 10⁵ cells/400 μ l complete CMRL-1066 were incubated at 37°C under an atmosphere of 95% air and 5% CO₂. Cells were washed 3 times with complete CMRL-1066 to remove nonadherent cells before treating with poly IC or EMCV.

RAW264.7 cells were removed from growth flasks by treatment with 0.05% trypsin, 0.02% EDTA at 37°C, washed with DMEM and plated at the indicated concentration. Macrophages were cultured for a minimum of 2 h under an atmosphere of 95% air and 5% CO₂ prior to the initiation of experiments.

Virus Propagation and Infection

The B variant of EMCV was a generous gift from Dr. Ji-Won Yoon (University of Calgary, Calgary, Alta., Canada) and has been described previously [24]. EMCV was propagated in L929 cells, supernatants were clarified by centrifugation, and titers were determined by standard plaque assay. Cell monolayers were infected at a multiplicity of infection of 1 PFU/cell by the addition of EMCV to the culture medium at 37°C for the times indicated.

PI3K Activity Assay

The enzymatic activity of PI3K was measured as previously described [25]. Briefly, RAW264.7 cells were infected with EMCV for the indicated times, cell lysates were harvested and p85 immune complexes were enriched by immunoprecipitation. Immunoprecipitates were washed and subsequently incubated with phosphatidylinositol and [γ -³²P]ATP in reaction buffer for 10 min at 37°C. After termination of the reaction by the addition of HCl, lipids were extracted and resolved by thin-layer chromatography. Phosphatidylinositol-3-phosphate production was quantified using a PhosphorImager™ (Molecular Dynamics).

Nitrite and IL-1 Determination

Nitrite production was determined by the addition of 50 μ l Greiss reagent to 50 μ l macrophage cell culture supernatant [26]. Absorbance at 540 nm was measured and nitrite concentrations were quantified by comparison to a sodium nitrite standard curve. The release of bioactive IL-1 from macrophages was measured using the RINm5F cell bioassay as previously described [27]. Briefly, macrophage supernatants were diluted (1:50 to 1:200) in RPMI and 20 μ l was added to RINm5F cells (2×10^5 cells/180 μ l RPMI). Following a 24-hour culture, IL-1-induced nitrite production by RINm5F cells was determined using the Greiss assay as described above and IL-1 concentrations were calculated from an IL-1 standard curve.

Western Blot Analysis

Protein samples were separated under denaturing conditions by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose (GE Healthcare, Piscataway, N.J., USA) or PVDF (Pall Life Sciences, Pensacola, Fla., USA) membranes under semidry transfer conditions. Antibody dilutions were as follows: mouse anti-pro-IL-1 β , 1:2,000; rabbit anti-COX-2 1:2,000; mouse anti-GAPDH, 1:5,000; all other primary antibodies were used at 1:1,000. Horseradish-peroxidase-conjugated donkey anti-mouse and donkey anti-rabbit secondary antibodies were used at 1:5,000 and 1:7,000 dilution, respectively. Antigen was detected by chemoluminescence according to the manufacturer's specifications (GE Healthcare).

Real-Time PCR

Total RNA was isolated from macrophages using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, Calif, USA). First-strand cDNA synthesis was performed using oligo(dT) and a reverse transcriptase Superscript preamplification system (Invitrogen, Carlsbad, Calif., USA) following the manufacturer's recommendations. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, Calif., USA) and a Research DNA Engine Opticon II thermocycler with continuous fluorescence detection (MJ Research, Waltham, Mass., USA). The mRNA levels of dsRNA- and EMCV-induced genes

were normalized to GAPDH. PCR primer sequences for COX-2 were: forward primer 5'-TTT GTT GAG TCA TTC ACC AGA CAG AT-3' and reverse primer 5'-CAG TAT TGA GGA GAA CAG ATG GGA TT-3'. PCR primers for iNOS, IL-1, and GAPDH have all been described [28].

Transfection

RAW264.7 cells were transiently transfected using the Amaxa Nucleofector electroporator (Amaxa Biosystems, Gaithersburg, Md., USA). RAW264.7 cells were removed from growth flasks by treatment with 0.05% trypsin and 0.02% EDTA at 37°C, washed with DMEM, and incubated for 2 h at 37°C. 2×10^6 cells were resuspended in electroporation buffer and electroporated with 2 μ g of plasmid using program D-032. 4×10^5 cells/400 μ l DMEM were plated per condition. Six hours after transfection, the medium was replaced and the cells were cultured overnight at 37°C. The cells were washed twice with DMEM before initiation of experiments. Transfection efficiency of greater than 50%, as determined by electroporation with enhanced green fluorescent protein were routinely obtained using this methodology.

Caspase-3 Activity

RAW264.7 cells (2×10^5 /400 μ l DMEM) were pretreated with LY294002 for 1 h; EMCV, poly IC or camptothecin were added and the cells were cultured for an additional 24 h. Caspase-3 activity was determined using a caspase-3 fluorometric assay kit according to the manufacturer's instructions (R&D Systems). The relative fluorescence units were normalized to total protein content of each sample as determined by the BCA assay (Pierce, Rockford, Ill., USA). Data are presented as fold-increase over the untreated control.

Statistics

Statistical comparisons between groups were made using one-way ANOVA. Significant differences between groups ($p < 0.05$) were determined using a Newman-Keuls post hoc analysis.

Results

EMCV and dsRNA Activate PI3K in Macrophages

To evaluate the effects of EMCV infection and poly IC treatment on PI3K activation, the phosphorylation of Akt was examined by Western blot analysis. EMCV infection of RAW264.7 cells for 20 min results in a 3.7 ± 0.6 -fold increase in Akt phosphorylation compared to untreated controls (mean $\pm$ SEM of 4 independent experiments). The stimulatory actions of EMCV on Akt phosphorylation are transient, with phosphorylation persisting for 15 min to 2 h after infection (fig. 1a). PI3K activation returns to basal levels 3–4 h after infection as evidenced by the return of Akt to basal levels of phosphorylation. The synthetic dsRNA poly IC also stimulates Akt phosphorylation in a fashion similar to EMCV infection (fig. 1b). Inhibition of PI3K using LY294002 attenuates the stimulatory actions of EMCV or dsRNA on Akt phosphorylation (fig. 1b).

Fig. 1. EMCV infection of macrophages activates PI3K. RAW264.7 cells ($4 \times 10^5/400 \mu\text{l}$ DMEM) were treated for the indicated times with EMCV (**a**) or for 15 min with poly IC (50 $\mu\text{g/ml}$; **b**), the cells were harvested and Akt phosphorylation was determined by Western blot analysis. Total Akt (**a**) and a nonspecific band (**b**) are shown as a loading control and results were quantified using Im-

ageQuant software. RAW264.7 cells ($4 \times 10^5/400 \mu\text{l}$ DMEM) were infected with EMCV (1 MOI) for the times indicated and PI3K enzymatic activity was determined by the incorporation of radiolabeled phosphate into phosphatidylinositol-3,4,5-triphosphate (PIP3) (**c**). Incorporation was quantified by autoradiography. Results are representative of 3 independent experiments.

The ability of LY294002 to attenuate Akt Ser473 phosphorylation in response to EMCV infection and poly IC treatment may be indirectly mediated by the inhibition of PDK1 Thr308 phosphorylation of Akt [29]. Therefore, to confirm that Akt phosphorylation correlates with PI3K activation, an immuno-complex kinase assay for PI3K activation was performed on EMCV-infected RAW264.7 cells. Consistent with Akt phosphorylation, EMCV stimulates a rapid activation of PI3K that was first measured 30 min after infection (fig. 1c). PI3K activity returns to near basal levels 2 h after infection. This rapid activation is consistent with the rapid stimulation of PI3K activity 30 min after HCMV infection [17].

PI3K Inhibition Attenuates EMCV-Stimulated Nitrite Production and iNOS, IL-1 β and COX-2 Expression by Macrophages

To determine whether PI3K participates in the regulation of the inflammatory response of macrophages, the effects of PI3K inhibition on EMCV-induced iNOS, COX-2 and IL-1 β expression were examined. As shown in figure 2a, LY294002 attenuates EMCV-induced iNOS expression and nitrite formation by RAW264.7 cells in a concentration-dependent manner with maximal inhibition at 10 μM . Similar to iNOS, EMCV-induced IL-1 β and COX-2 expressions are also attenuated by this PI3K

inhibitor (fig. 2b, c). Primary naïve PECs require two pro-inflammatory signals for iNOS expression [13, 30]. As shown in figure 2d, in combination with IFN- γ , EMCV stimulates iNOS expression and nitrite production by PECs. Inhibition of PI3K using LY294002 attenuates EMCV + IFN- γ -stimulated iNOS expression and nitrite production by primary mouse macrophages.

The effects of LY294002 on EMCV-stimulated iNOS, COX-2 and IL-1 mRNA accumulation following 6 h incubation were evaluated by real-time PCR. Infection of RAW264.7 cells with EMCV stimulates the accumulation of iNOS, COX-2 and IL-1 β mRNA. At a concentration of 10 μM , LY294002 attenuates the stimulatory actions of EMCV on iNOS, COX-2, and IL-1 β mRNA accumulation by greater than 50% (fig. 3). These findings suggest that inflammatory gene expression in response to EMCV infection is mediated, in part, by PI3K activation.

PI3K Inhibition Attenuates dsRNA-Induced Nitrite Production and iNOS, IL-1 β and COX-2 Expression by Macrophages

In macrophages, EMCV and poly IC have been shown to stimulate inflammatory gene expression by similar mechanisms [1, 2]. Consistent with a role for PI3K in the regulation of inflammatory gene expression following

Fig. 2. PI3K inhibition attenuates EMCV-induced macrophage activation. RAW264.7 cells (2×10^5 or $4 \times 10^5/400 \mu\text{l}$ DMEM; **a, b**) or mouse PEC (4×10^5 cells/400 μl cCMRL-1066; **c, d**) were pre-treated with the indicated concentrations of LY294002 or vehicle control for 1 h at 37°C. The macrophages were infected with EMCV (1 MOI) and 24 h after infection, nitrite production in culture supernatants was quantified by Greiss assay (**a, d**), and iNOS (**a, d**), COX-2 (**b**), and IL-1 β (**c**) expression was determined by Western blot analysis. Results are the average $\pm$ SEM or representative of 3 independent experiments. * $p \leq 0.05$.

Fig. 3. PI3K inhibition attenuates EMCV-stimulated inflammatory gene expression. RAW264.7 cells ($2 \times 10^5/400 \mu\text{l}$ DMEM) were pretreated with the indicated concentrations of LY294002 for 1 h at 37°C followed by the addition of EMCV (1 MOI) and cultured for an additional 6 h. The cells were harvested, total RNA isolated, and iNOS, COX-2, and IL-1 β mRNA accumulation was determined by real-time PCR. mRNA accumulation was normalized to GAPDH. Results are the mean $\pm$ SEM for 3 independent experiments.

EMCV infection, LY294002 attenuates dsRNA-induced nitrite production and the release of biologically active IL-1 from RAW264.7 cells (fig. 4a, b). Inhibition of PI3K activity also attenuates poly-IC-stimulated RAW264.7 cell iNOS, COX-2 and IL-1 β mRNA accumulation following 6 h incubation (fig. 4c) and protein expression following 24 h incubation (fig. 4d).

Expression of dnPI3K Attenuates EMCV-Stimulated Inflammatory Gene Expression

To provide molecular confirmation for a role for PI3K in the regulation of inflammatory gene expression,

RAW264.7 cells were transiently transfected with a vector encoding a mutant of the p85 α subunit lacking a binding site for the catalytic subunit of PI3K [22]. Using COX-2 as a target inflammatory gene, we show that EMCV infection of RAW264.7 cells expressing the empty vector control results in expression of COX-2 (24 h) and phosphorylation of the Akt (15 min, fig. 5). RAW264.7 cells expressing the dominant negative p85 α mutant fail to express COX-2 in response to EMCV infection (24 h) and this effect correlates with attenuation of the stimulatory actions of 15 min infection on Akt phosphorylation (fig. 5b).

Fig. 4. PI3K inhibition attenuates dsRNA-induced nitrite production, IL-1 release, and iNOS, IL-1 β and COX-2 expression by macrophages. RAW264.7 cells ($4 \times 10^5/400 \mu\text{l}$ DMEM) were pretreated with the indicated concentrations of LY294002 for 1 h, poly IC (50 $\mu\text{g/ml}$) was added and cells were cultured for an additional 24 h (**a**, **b**, **d**). Culture supernatants were collected and analyzed for nitrite production (**a**) and the release of biologically active IL-1 (**b**). The cells were harvested, and iNOS, IL-1 β and COX-2 expression was determined by Western blot analysis (**d**). GAPDH is

shown as a loading control. The accumulation of iNOS, COX-2 and IL-1 β mRNA was determined by real-time PCR on total RNA isolated from RAW264.7 cells ($2 \times 10^5/400 \mu\text{l}$ DMEM) pretreated with or without the indicated concentrations of LY294002 for 1 h followed by a 6-hour incubation with poly IC (50 $\mu\text{g/ml}$) (**c**). Results are the average $\pm$ SEM (**a-c**) or representative (**d**) of three independent experiments. * Statistical significance was reported when $p \leq 0.05$.

Fig. 5. Expression of a dnPI3K mutant attenuates EMCV-induced inflammatory gene expression. RAW264.7 cells (2×10^6) were electroporated with 2 μg of dominant negative p85 α plasmid or with a vector control. Transfected cells ($4 \times 10^5/400 \mu\text{l}$ DMEM) were treated with EMCV (1 MOI) for 24 h (**a**) or for 15 min (**b**).

The cells were harvested and COX-2 (**a**) and phospho-Akt (**b**) expression was determined by Western blot analysis. GAPDH is shown as a loading control. Results are representative of 2 independent experiments.

Fig. 6. Inhibition of PI3K results in the death in EMCV-infected macrophages. RAW264.7 cells ($2 \times 10^5/400 \mu\text{l}$ DMEM) were incubated for 24 h with $10 \mu\text{M}$ LY294002 or EMCV (1 MOI) or pre-incubated for 1 h with LY294002 followed by the addition of EMCV and continued culture for 24 h. Macrophage morphology was examined by phase contrast microscopy (original magnifica-

tion $40\times$; **a**), or the cells were harvested and caspase-3 activity was determined (**b**). Caspase activity was normalized to total protein content and is presented as fold-increase over control. Results are representative of the average $\pm$ SEM of 3 independent experiments. * $p \leq 0.05$.

Effects of PI3K Inhibition on the Viability of EMCV-Infected Macrophages

Macrophage activation is associated with morphological changes that include rounding and ruffling of the cell surface and the formation of dendrite-like processes. As expected, the morphological changes observed in RAW264.7 cells 24 h after EMCV infection are consistent with robust macrophage activation (fig. 6a). Alone, inhibitors of PI3K, at concentrations that attenuate macrophage expression of inflammatory genes in response to EMCV infection (fig. 2), do not modify the quiescent morphology of untreated macrophages. In contrast, inhibition of PI3K (using LY294002) in macrophages infected with EMCV results in dramatic morphological changes that are consistent with induction of cell death by apoptosis. The formation of long dendrite-like processes and ruffled cell membranes is attenuated, and the cells condensate. PI3K is a critical regulator of cell survival in many cell types, including macrophages [31], that when active is known to prevent apoptotic cell death [15, 32]. Therefore we examined the effects of PI3K inhibition on caspase-3 activity in EMCV-infected macrophages. Cas-

pase-3 is an executioner caspase that is activated in both the intrinsic and extrinsic pathways of apoptosis [33]. Consistent with morphological changes associated with apoptosis, there is a 2-fold increase in caspase-3 activity in RAW264.7 cells pretreated for 1 h with LY294002 followed by 24 h infection with EMCV (fig. 6b). The measured fold-increase in caspase activity under these conditions is likely an underestimate of total caspase activation as $10 \mu\text{M}$ LY294002 attenuates but does not prevent EMCV-stimulated nitric oxide (fig. 4) and nitric oxide is a known inhibitor of caspase-3 activity [34]. Consistent with antiapoptotic actions of PI3K activation, EMCV infection of macrophages does not increase caspase-3 activity in a statistically significant manner. Treatment of RAW264.7 cells with camptothecin, a topoisomerase inhibitor and apoptosis inducer, was used as a positive control for apoptosis and resulted in a 3-fold increase in caspase-3 activity (not shown). These findings suggest that PI3K plays a role in regulating the responses of macrophages to EMCV infection, with the activation of an inflammatory response when active and the induction of cell death by apoptosis when inactive.

Discussion

The mechanisms responsible for determining the response of macrophages to virus infection have yet to be fully defined. The potential responses include the activation of inflammation, induction of classical antiviral responses and induction of cell death. We have shown that macrophages infected with EMCV, or treated with poly IC, respond by the rapid activation of signaling pathways that converge on the expression of inflammatory genes such as iNOS, COX-2, and IL-1 [2, 9, 11, 12, 14]. Chronologically, activation of inflammatory gene expression precedes induction of the classical type 1 IFN antiviral response that is associated the recognition of intracellular dsRNA [14, 35]. In cells that fail to respond with activation of antiviral activity (inflammation or the type 1 IFN response), apoptosis is a potential outcome as this form of death represents a mechanism to remove a virally infected cell. PI3K plays a primary role as a regulator of cell viability as an antiapoptotic factor and previous studies using mouse embryonic fibroblast lacking p85 α , a regulatory subunit of PI3K, have shown increased cell death following EMCV infection as compared to p85 α /+ cells [36]. Additional studies have also provided evidence for an antiapoptotic role for PI3K during Sendai virus infection [31].

In this study, the role that PI3K plays in regulating the response of macrophages to EMCV infection and poly IC treatment was examined. Using immuno-complex kinase assays and phosphorylation of the PI3K substrate Akt, we show that EMCV and poly IC stimulate the rapid activation of PI3K in macrophages. Chemical and molecular inhibition of PI3K results in an attenuation in the inflammatory response of macrophages to EMCV infection or treatment with dsRNA. The inflammatory genes inhibited include iNOS, COX-2 and IL-1 β . The inhibition of PI3K not only attenuates the expression of inflammatory genes, it also results in a change in the morphology of macrophages from activated to apoptotic. EMCV infection stimulates a macrophage morphology that is consistent with activation. This is evidenced by cell enlargement, formation of ruffled membranes and long dendritic-like processes (fig. 6). Alone, PI3K inhibitors do not modify the quiescent phenotype of naïve macrophages; however, in EMCV-infected macrophages PI3K inhibition is associated with an apoptotic morphology characterized by cell condensation and the loss of processes and ruffled membranes. The appearance of this apoptotic morphology is associated with a 2-fold increase in caspase-3 activity. These findings suggest that in the absence

of PI3K activation, EMCV infection stimulates macrophage apoptosis, and when activated, the induction of a PI3K-directed antiviral response of inflammatory gene expression takes place. These findings support a central role for PI3K in the determination of the macrophage response to virus infection.

Several viruses have previously been shown to activate PI3K [16–19, 31]. From the perspective of the invading virus, activation or utilization of host PI3K may be to prevent host cell death in an effort to favor viral replication. Consistent with this view, Thomas et al. [18] have shown that respiratory syncytial virus activates PI3K in airway epithelial cells and inhibition of PI3K results in apoptosis. In addition, Johnson et al. [17] have shown that human cytomegalovirus activates PI3K in fibroblasts and inhibition of PI3K prevents viral replication. The role of PI3K during virus infection from the perspective of the host cell is not well defined. Our findings suggest that PI3K plays a primary role in the induction of inflammatory gene expression that may serve as a host cell defense of macrophages during virus infection. Inhibition of PI3K attenuates dsRNA- or EMCV-induced iNOS, COX-2 and IL-1 expression, nitric oxide production and the release of biologically active IL-1. These findings are in agreement with a regulatory role of PI3K in the production of nitric oxide and release of IL-1 from macrophages and the enhanced levels of virus load in animals deficient in iNOS [21].

The third response of infected cells is activation of type 1 IFN antiviral activities. The antiviral response is characterized the recognition dsRNA by receptors such as PKR [37, 38], melanoma differentiation-associated gene-5 (mda5) [39], retinoid-acid-inducible gene (RIG-I) [40] and TLR3 [41], induction of type 1 IFN expression, and induction of antiviral genes by these IFNs. We have shown that the inflammatory gene response of iNOS, COX-2 and IL-1 occurs by pathways that appear to be independent of classical dsRNA receptors such as PKR [9, 13, 14], TLR3 [12] and mda5 [manuscript in review]. In these studies, inhibition of inflammatory gene expression in response to EMCV infection does not modify type 1 IFN production, and inhibition of classical dsRNA receptors such as TLR3 does not modify the inflammatory gene response [12]. The signaling receptor activated by EMCV infection is currently unknown. EMCV capsid void of virus RNA is capable of activating mitogen-activated protein kinase (MAPK) and NF- κ B signaling pathways and iNOS expression, suggesting that the antiviral pathway of inflammatory gene expression does not require viral RNA accumulation or viral protein expression [14]. These findings suggest that EMCV capsid protein

may activate MAPK and NF- κ B signaling pathways by interacting with a cell surface receptor. We are currently examining the potential role of G-protein-coupled receptors (GPCR) as potential regulators of inflammatory gene expression in response to EMCV infection. GPCR are known activators of PI3K and MAPK [15, 42] and, as shown in the current and our previous studies, PI3K and MAPK are required for inflammatory gene expression by macrophages in response to EMCV infection.

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