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NEGATIVE REGULATORY EFFECTS OF PHOSPHATIDYLINOSITOL3-KINASE PATHWAY ON PHAGOCYTOSIS AND MACROPINOCYTOSIS IN BOVINE MONOCYTES

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Abstract

Recent studies have shown that monocytes and macrophages not only present antigens to effector T cells and stimulate and shape T cell-mediated immune responses, but they also prime naïve T cells, thus initiating adaptive immune responses. Phosphatidylinositol 3-kinase functions at an early phase of toll-like receptor signaling pathways, modulates the magnitude of the primary immune responses, and is involved in the reorganization of the actin cytoskeleton during macropinocytic and phagocytic antigen uptakes, important early steps in triggering adaptive immune responses.

We assessed by flow cytometry the endocytic capacities of bovine monocytes by using endocytic tracers and *Salmonella* transformed with a green fluorescence plasmid GFP to evaluate macropinocytosis, mannose receptor-mediated endocytosis, and phagocytosis in bovine professional antigen presenting cells, respectively. Our data reveal that wortmannin, an inhibitor of phosphatidylinositol 3-kinase signaling pathway, significantly increased macropinocytosis and phagocytosis but did not affect the mannose receptor-mediated antigen uptake in bovine monocytes. Protein expression data support these findings by showing decreased levels of phosphoinositide 3-kinase in the presence of wortmannin during macropinocytosis.

We expanded further the key role of phosphatidylinositol 3-kinase as an endogenous suppressor of primary immune responses, suggesting a novel mechanism of phosphatidylinositol 3-kinase

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Conflict of Interest

No financial and competing interests are declared

antigen uptake modulation that may provide a unique therapeutic target for controlling excessive inflammation.

Keywords

Phosphatidylinositol 3-Kinase Pathway; Wortmannin; Monocytes; Phagocytosis; Macropinocytosis; Receptor-Mediated Endocytosis; Antigen Uptake

Introduction

Recent studies show that monocytes and macrophages not only present antigens to effector T cells and stimulate and shape T cell-mediated immune responses, but they also prime naïve T cells, thus initiating adaptive immune responses [1–3]. In particular, monocytes are recruited rapidly to the site of infection, they give rise to macrophages and inflammatory dendritic cells (DCs), are recruited directly from the blood via high endothelial venules to the T cell areas of the lymph nodes and finally, are able to cross-present antigens derived from endocytosed pathogens to cytotoxic T cells [2]. Monocytes and macrophages express multiple phagocytic and signaling pattern recognition receptors (PRRs) that sense and bind pathogen-associated molecular patterns (PAMPs). Namely, C-type lectin mannose receptor (MR) and toll-like receptors (TLRs) are involved in bacterial pathogen uptake in the early phase of infection, whereas receptors that bind antigens opsonized with IgG, mostly Fc receptors, scavenger receptors, and activated complement factors are involved in later stages when monocytes differentiate into macrophages [4, 5]. The glycoprotein ovalbumin (OVA), which contains mannose residues, was reported to be endocytosed through the MR, that has been shown to be essential for pro- and anti- inflammatory cytokine production and is dependent on TLR-triggering by pathogens or synthetic ligands [6, 7].

Macropinocytosis is a potent non-selective mechanism of antigen uptake limited to immature DCs and their myeloid progenitors, monocytes and macrophages. Activated by exogenous stimuli, internalization of solutes via macropinocytosis provides a unique endocytic pathway to complement immune monitoring via PRRs [8, 9]. The internalization of solutes by macropinocytosis is much more effective than by other non-selective mechanisms [8, 9]. Phagocytosis also is an important early step in triggering the adaptive immune responses, which require processing of bacterial pathogens and presentation of their antigens to $CD4^+$ and $CD8^+$ T cells by professional antigen presenting cells (APCs) [2].

Macropinocytosis and phagocytosis depend on membrane-cytoskeleton interactions, thereby reflecting cortical actin dynamics and cell motility, and phosphatidylinositol 3-Kinase (PI3K) activation was demonstrated to be involved in the dramatic reorganization of the actin cytoskeleton [10]. One of the most potent inflammatory agents able to activate monocytes and macrophages is LPS, a component of the outer membrane of Gram-negative bacteria that binds to the surface protein CD14 and stimulates the TLR4-MD2 complex that results in rapid production of inflammatory mediators and cytokines in monocytes and macrophages. Namely, IL-1α, IL-1β, IL-6, IL-12 and TNF-α are produced mainly through the activation of the NF-kB and mitogen-activated protein kinase (MAPK) signaling pathway [11]. Previously, several reports showed the involvement of MAPK signaling

pathway in LPS/*E. coli* active phagocytosis in medfly hemocytes and in West Nile virus endocytosis, including phagocytosis, in a mosquito cell line [12–14].

The resultant inflammatory response is essential for the eradication of pathogens; however, excessive and prolonged activation of innate immunity is harmful, and, in some cases, even fatal to the host, owing to severe tissue damage and circulatory failure [15]. Several studies have shown that PI3K is an endogenous suppressor of IL-12 and IL-1β production triggered by TLR signaling and limits excessive Th1 type polarization [16–20]. Unlike other gate-keeping systems, IRAK–M (IL-1 receptor-associated kinase-M) and SOCS-1 (suppressor of cytokine signaling-1) induced by TLR signaling and function during the second or continuous exposure to stimulation, PI3K functions at the early phase of TLR signaling, modulates the magnitude of primary activation, and thus has an early, unique role in the gate-keeping system, preventing excessive innate immune responses [17].

Salmonella enterica, with over 2000 different serovars, is indigenous to the gastrointestinal tracts of many mammals, birds, and reptiles, usually at low levels [21]. *Salmonella* is hagocytosed by monocytes/macrophages and DCs, in which it replicates triggers rapid tissue destruction and inflammation [22]. Apoptosis of macrophages in the liver occurs during systematic *Salmonella* infection *in vivo. In vitro Salmonella* strains induced delayed apoptosis that requires activation of TLR4 on macrophages by the bacterial LPS [23]. NF-κB and MAPK are particularly important for the induction of anti-apoptotic factors [23]. *Salmonella* virulence proteins are essential for altering the balance in favor of apoptosis during intracellular infection, but mechanisms involved are not understood fully.

Despite numerous observations that have implicated PI3K signaling as a regulator of various biological functions, including the pro-inflammatory response to TLR signaling, PI3K effects on inflammatory response varies, depending on several factors that remain to be elucidated [24, 25].

In this study, we investigated the role of PI3K in the early stages of an immune response, antigen uptake mediated via macropinocytosis, MR-mediated endocytosis, and phagocytosis in bovine monocytes. We hypothesized that PI3K plays an important role as an endogenous regulator of the TLR-dependent and independent signaling cascades initiated during macropinocytosis and phagocytosis in bovine monocytes.

Materials and methods

Animals

Conventionally reared, healthy cows from a Holstein herd at the Mississippi State University Dairy Facility were used. The animals have been subjected to a comprehensive vaccination program, including Frontier 4 Plus Vaccine (IBR, BVD, PI3, RSV, Diamond Animal H, Inc). The Mississippi State University Institutional Animal Care and Use Committee approved all animal use (IACUC #09-039).

Cell Preparation

Bovine peripheral blood mononuclear cells (PBMCs) were separated as described elsewhere [33, 34]. Briefly, PBMCs were isolated on Histopaque gradients (1.077 g/ml, Sigma). Cells were resuspended to 5×10^6 per ml in RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1 (Gibco Life Technologies), 5×10^{-5} M 2-mercaptoethanol, 75 μg/ml gentamicin (Gibco Life Technologies).

To isolate monocytes, PBMCs were added to a tissue culture plate for 10–12 hours at 37° C. After removing non-adherent cells, the adherent cells (70–80% monocytes) were washed twice in PBS and incubated with endocytic tracers.

Salmonella preparation and infection

Salmonella kentucky strain was isolated from chickens and serotyped at Poultry Research and Diagnostics Laboratory (Jackson, CVM-MSU). This strain was transformed with GFP, a plasmid with green fluorescence, as described previously [49]. *Salmonella kentucky* was cultured overnight in LB with ampicillin at 37° C and centrifuged at 3000xg. GFP expression was confirmed by flow cytometry (Fig. 1A), and a bacterial growth standard curve determined by spectrophotometry [Genesys 20, Thermo Scientific] (Fig. 1B). To opsonize *S. kentucky*, the bacterial strain was cultured in the presence of 10% BSA. We used heat-inactivated (HI) *S. kentucky* to evaluate the scavenger-receptor-mediated phagocytosis in bovine monocytes, as described [50]. Briefly, *S. kentucky* at concentration 10^8 bacterial cells/ml was HI at 60° C for 30 min. To confirm that bacterial cultures were sterile, *S. kentucky* was cultured in LB with ampicillin overnight at 37° C.

Endocytosis Assays and Flow Cytometry

The endocytic capacities of monocytes were determined by using following endocytic tracers: Lucifer Yellow (LY) and fluorescein isothiocyanante-labeled ovalbumin (FITC-OVA) (both from Invitrogen) to evaluate macropinocytosis and MR-mediated endocytosis, respectively.

The ability of monocytes to endocytose FITC-OVA and LY was measured as described elsewhere [33, 34, 42]. Briefly, monocytes were treated with FITC-OVA or LY at final concentrations 100 μg/ml for 30 min at 37° C to measure active endocytosis or at 4° C to determine background levels of endocytosis (negative control). Monocytes were washed three times by centrifugation in cold PBS and analyzed using a FACSCalibur (Becton Dickinson) as follows. After setting a gate on large granular cells, the FITC-OVA or LY incorporation was measured and analyzed. To inhibit selectively various pathways involved in antigen uptake, bovine monocytes were incubated for 15 min in the presence of cytochalasin D [CCD] (2.5 μg/ml), latrunculin A [5μM] and wortmannin [5–20 μM] (all from Sigma) before the addition of FITC-OVA or LY.

To evaluate *S. kentucky* phagocytosis in bovine APCs, 10^8 live, HI and BSA-treated bacterial cells were added for 2 hours to monocyte cultures and incubated at 37° C. To determine background levels of phagocytosis (negative controls), cells were incubated in the

presence of *S. kentucky* at 4° C. Cells were washed three times by centrifugation in cold PBS and analyzed by flow cytometry.

Western blot

Kinase p85 expression levels were determined by Western blotting analysis. Adherent PBMC populations (70–80% pure monocytes) were treated with FITC-OVA or LY at final concentrations 100 μg/ml for 30 min at 37 °C. To inhibit selectively PI3K pathway involved in antigen uptake, cells were incubated for 15 min in the presence of wortmannin [5 μM] before addition of FITC-OVA or LY. Cells were washed three times by centrifugation in cold PBS, and monocyte protein extractions for all treatments were performed using RIPA Buffer (Thermo Scientific). The concentration of proteins were then determined using a BCA Protein Assay Kit (Thermo Scientific) and 45 μg samples loaded on a 8–16% gradient Precise Protein Gel (Thermo Scientific), for SDS-PAGE. The proteins were then transferred to Immun-Blot PVDF (BIO-RAD) for detection. The blots were probed with the following primary antibodies: anti-PI3 Kinase (LifeSpan BioSciences), anti-MAP Kinase 1,2 (USBiological), and the housekeeping protein anti-β-actin (Ambion). Anti-PI3 kinase (p85 regulatory subunit) and anti-β-actin antibodies were then labeled with goat anti-mouse IgG (H+L)-AP (Zymed) secondary antibody, and the MAP kinase 1,2 antibody was labeled with goat anti-rabbit IgG (H+L)-AP (Invitrogen) secondary antibody. The labeled proteins were detected with BCIP/NBT Phosphatase Substrate (KPL).

Data analysis

Data (2–7 independent experiments) were analyzed by analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison *post hoc* test and are presented as means + SD. The level of significance for all tests of effects was set as $P<0.05$.

Results

Effect of Wortmannin on the Endocytosis in Bovine Moncytes

We demonstrated previouslythat bovine monocytes cultured for up to 24 hrs had moderate capacity to uptake FITC-DX and low to moderate capacity to endocytose LY, suggesting that macropinocytosis did not play a significant role in active antigen uptake in bovine monocytes [26]. To determine if the early phases of selective and non-selective antigen uptake in monocytes are regulated by PI3K-dependant signaling pathways, we evaluated the effect of PI3K inhibitor, wortmannin (W), on antigen uptake by phagocytosis, MR-mediated antigen uptake and macropinocytosis in bovine monocytes.

In this study, bovine PBMCs were cultured for 10–12 hrs, and adherent cells, 70–80% pure monocytes, were collected to assess the cells ability to endocytosis with following endocytic tracers LY, FITC-OVA and live, opsonized and HI *S. kentucky.* To assess macropinocytosis in bovine APCs, we measured the uptake of LY in cells pre-incubated with the inhibitors of macropinocytosis, CCD, latrunculin A (LAT), the PI3K inhibitor, W, and control cells in medium only. To ensure active endocytosis in monocytes, their viability in the presence of the inhibitors was assessed by flow cytometry. Inhibitors, W and CCD, did not change the numbers, granularity, or size of monocytes in the designated region at all concentrations

used which varied from 72% to 79% of total PBMCs compared to the 37° C (74% of total PBMCs) and 4° C (70% of total PBMCs) controls (data not shown). However, LAT at concentration 100 μM promoted apoptosis in bovine monocytes as seen by significant changes in cell size and granularity and decreased numbers of APCs in the designated region to 12% of total PBMCs (data not shown). As expected, monocytes after 12 hrs culture expressed an insignificant capacity to uptake LY that decreased numerically in the presence of CCD and LAT (Fig. 2). However, the uptake of LY was enhanced significantly in the presence of W (Fig. 2). The significant increases in LY uptake were evident in the presence of W at all concentrations used; however, after 2 hrs of W exposure the uptake of LY was inhibited completely in bovine monocytes (data not shown).

To characterize the possible role of PI3K in receptor-mediated endocytosis in monocytes, the uptake of FITC-OVA in control cells and APCs pre-incubated in the presence of CCD and W has been analyzed. Bovine monocytes actively endocytosed FITC-OVA at 37° C, and this uptake was inhibited significantly in the presence of CCD (Fig. 3). However, bovine APCs did not show significant increases in OVA endocytosis in the presence of W compared to antigen uptake at 37° C (Fig. 3).

Finally, to investigate the role of the PI3K pathway inhibitor W in the phagocytic antigen uptake in bovine monocytes, we used as an antigen live, opsonized and inactivated by exposure to high temperature (HI) *S. kentucky*. Monocytes after 2 hrs incubation with live *S. kentucky* at 37° C did not express active phagocytic ability; however, consistent, non-significant increases in bacterial antigen uptake of opsonized and HI *S. kentucky* have been observed (Fig. 4). The addition of W promoted enhanced significantly phagocytosis of live and opsonized *S. kentucky* in bovine monocytes (Fig. 4). Phagocytosis of HI bacterial cells was non-significantly increased in the presence of W [$p = 0.06$] (Fig. 4).

Phosphoinositide 3-kinase protein expression in selective and non-selective endocytosis in bovine monocytes

To evaluate further the involvement of PI3K- and MAPK- mediated pathways in active endocytic antigen uptake in bovine monocytes, we assessed by Western blot analysis kinase proteins expression levels. Kinase protein data revealed that PI3K and MAPK are expressed in bovine monocytes in the absence of OVA and LY. The protein expression data show that the regulatory subunit of PI3K (p85) protein visual expression levels in bovine monocytes have been decreased in the presence of OVA and increased in monocytes incubated with LY compared to control cells (Fig. 5). The addition of W inhibited the PI3K expression in control monocytes and eliminated the effects of OVA and LY on the expression levels of PI3K in bovine monocytes (Fig. 5). Expression levels of MAPK and β-actin proteins were unchanged in the presence of W in all three experimental groups (Fig. 5).

Discussion

Investigations into the actin cytoskeleton regulatory signaling events that may be involved in the endocytic pathways are limited in professional APCs. PI3K has been implicated in the regulation of actin-dependent endocytosis, intracellular membrane traffic, and cell growth [27]. Similarities between the signaling and mechanical pathways used in phagocytosis and

macropinocytosis often imply that inhibitors block both pathways, as is the case with PI3K inhibitors, in particular W [27, 28]. Several earlier reports demonstrated that W blocks both macropinocytosis and FcR-mediated phagocytosis in murine bone marrow-derived macrophages, transformed fibroblasts and macropinocytosis in murine immature DCs by binding PI3K catalytic subunit and irreversibly inhibits the regulatory subunit [10, 27–30]. In contrast to previous observations in our study, W enhanced significantly macropinocytosis and the phagocytic uptake of live and opsonized *S. kentucky* in bovine monocytes, suggesting the negative regulatory role for PI3K in monocyte antigen uptake. Importantly, our data agree and contribute to the recent reports on the PI3K-mediated negative regulation of multiple innate immune responses, including IL-12, IL-1β production and Th1 polarization defining PI3K as a negative regulator in the early phase of the innate immune responses [17–20, 31, 32]. In this study, we show that the negative signaling regulation by PI3K is involved in the important professional APC function of monocytes, non-selective and selective antigen uptakes.

Differences From the earlier observations regarding W effects on the actin-mediated antigen uptake mechanisms in our study could be due to several reasons. Firstly, there are phenotypic and functional differences between monocytes as myeloid progenitor cell populations and fully differentiated cells such as macrophages, fibroblasts, and DCs. Secondly, there are some species-specific differences in the APCs functions [33, 34]. Finally, different experimental conditions and difficulties in studying the role of PI3K in TLR signaling [24] explain the differences in the effects of W on endocytic antigen uptake. Our results revealed that bovine monocytes incubated with W for 2 hrs and longer expressed significant dose-dependent decreases in non-selective, fluid phase uptake and phagocytosis in the presence of W, thus confirming previous reports on the inhibitory effects of W in the actin-mediated endocytic uptake [10, 27–30].

In our study, we demonstrated that W did not alter significantly active endocytosis of FITC-OVA in bovine monocytes. Active endocytosis of FITC-OVA in bovine monocytes was inhibited significantly in the presence of actin polymerization inhibitor CCD, suggesting that macropinocytosis was the major mechanism of the OVA uptake [8]. This finding does not contradict previous data on the inhibitory effects of W on both macropinocytosis and FcR-mediated phagocytosis [10, 27, 28, 35] because endocytosis of OVA is not FcR-mediated, not restricted exclusively to macropinocytosis, and is conducted through MR-mediated and/or macropinocytic mechanisms [4, 5, 36].

In contrast to significant OVA endocytosis, active antigen uptake of the live, opsonized or HI *S. kentucky* was not observed in bovine monocytes incubated in the presence of bacteria for 2 hrs. Our findings are in agreement with previous reports demonstrating that TLRs are involved in bacterial, including some highly pathogenic *Salmonella* strains, uptake in the early phase of infection, whereas receptors that bind opsonized antigens, mostly Fc receptors, are involved in later stage when monocytes differentiate into macrophages [4, 5, 23, 37].

In this study, protein expression levels of MAPK, the kinase was demonstrated to “share” the TLR-induced signaling pathways with PI3K [38], did not change visually in the presence

of OVA, LY and W in bovine monocytes, suggesting that the PI3K signaling was TLR-independent.

According to the earlier reports, the PI3K protein expression showed decreases at very low nanomolar concentrations of W, and the complete inhibition of PI3K was reached at higher concentrations of 0.1 to 10 μM in finally differentiated cells [10, 27, 28, 30, 35, 39]. Our data agree with previous observations that PI3K is expressed constitutively in innate immune cells [17, 31, 32, 40]. However, in contrast to the earlier reports that PI3K is activated rapidly by antigens, expression levels of PI3K have been decreased in the presence of OVA, suggesting that OVA antigen, unlike some pathogens, does not involve PI3K-dependant TLR signaling pathways [17, 31, 32, 40]. Interestingly, the decreased levels of PI3K in the presence of OVA correlate with significantly increased active OVA uptake in bovine monocytes, thus supporting our hypothesis on the role of PI3K as an endogenous regulator of antigen uptake in professional APCs. The addition of LY increased the expression levels of PI3K, and W eliminated this increase in monocytes incubated with LY. Interestingly, decreased PI3K levels correlated with the significantly enhanced macropinocytosis in bovine APCs in the presence of the PI3K inhibitor W. This finding suggests that PI3K is an endogenous suppressor of signaling events involved in macropinocytosis that is not receptor-mediated but not in the MR-mediated uptake of OVA in bovine monocytes.

Conclusion

In conclusion, we expanded further the key role of PI3K as an endogenous suppressor in the TLR and non- TLR-dependant signaling cascades during macropinocytosis and phagocytosis in bovine monocytes. In light of emerging evidence on the plasticity of monocytes responding to their environment by differentiating into a variety of macrophages and DC-like cells [41], the regulatory signaling events that control early antigen uptake mechanisms are especially important. Viruses and other pathogens have subverted macropinocytosis and phagocytosis by activating signaling pathways, including PI3K-dependent that trigger actin-mediated membrane ruffling and blebbing [27, 42–46], and some viruses use other endocytic mechanisms for entry but require macropinocytosis to promote penetration [47, 48]. Therefore, the aspect of emerging importance is to investigate further the molecular signaling control of lineage commitment in the mononuclear phagocyte system.

Collectively, the unique features of phosphoinositide 3-kinase as an endogenous suppressor of primary immune responses and its contributions to regulation of antigen uptake in bovine monocytes may provide a unique therapeutic target for controlling excessive inflammation. Thus, the better understanding of how immunity is regulated via PI3K, would allow us to manipulate those signals and in this way, develop strategies to modulate, prevent, and treat the multiple diseases in which this mechanism is implicated

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Abbreviations

DCs	dendritic cells
PRRs	pattern recognition receptors
PAMPs	pathogen-associated molecular patterns
MR	mannose receptor
TLRs	toll-like receptors
OVA	ovalbumin
APCs	antigen presenting cells
PI3K	Phosphatidilinositol 3-Kinase
LPS	lypopolysaccharide
MAPK	mitogen-activated kinase
IRAK-M	IL-1 receptor-associated kinase-M
SOCS-1	suppressor of cytokine signalling-1
PBMCs	peripheral blood mononuclear cells
S	*Salmonella*
GFP	green fluorescence plasmid
LY	Lucifer Yellow
FITC-OVA	fluorescein isothiocyanante-labelled ovalbumin
CCD	cytochalasin D
W	wortmannin
HI	heat-inactivated

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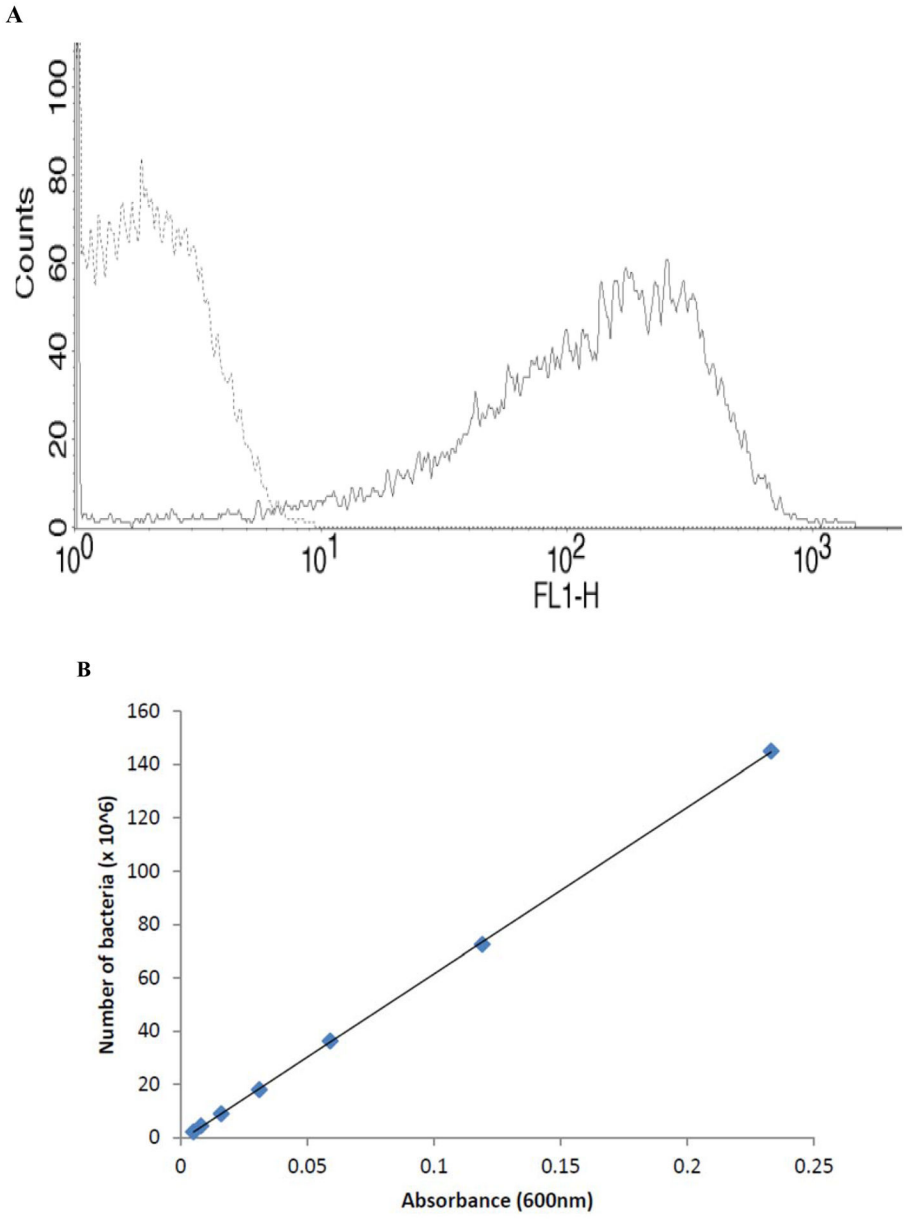

Figure 1.

Fluorescence intensity and bacterial growth of *S. kentucky*.

(A) - Fluorescence intensity of GFP-labeled (GFP+) *S. kentucky*.

GFP+ *S. kentucky* (bold solid line)

GFP− *S. kentucky* (dotted line)

(B) - *S. kentucky* bacterial growth standard curve by spectrophotometry.

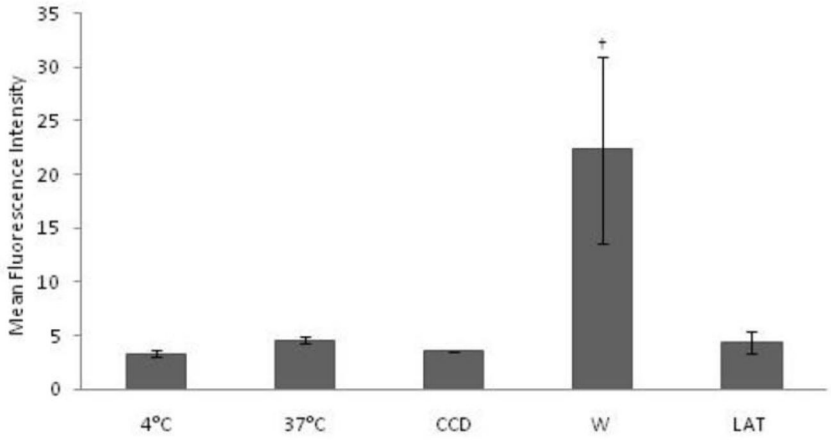

Figure 2.

Macropinocytosis of LY in bovine monocytes. LY uptake at 37° C was assessed in monocytes by flow cytometry in the presence or absence of the macropinocytosis inhibitors CCD [2.5 μg/ml], LAT [5μM] and W [5μM]. Macropinocytosis at 4° C was measured to determine background levels of LY uptake. Samples were analyzed in two representative experiments, and data are expressed as Mean Fluorescent Intensity (MFI). † Presence on the top of bars indicate treatment differences from bars without † designation ($P < 0.05$).

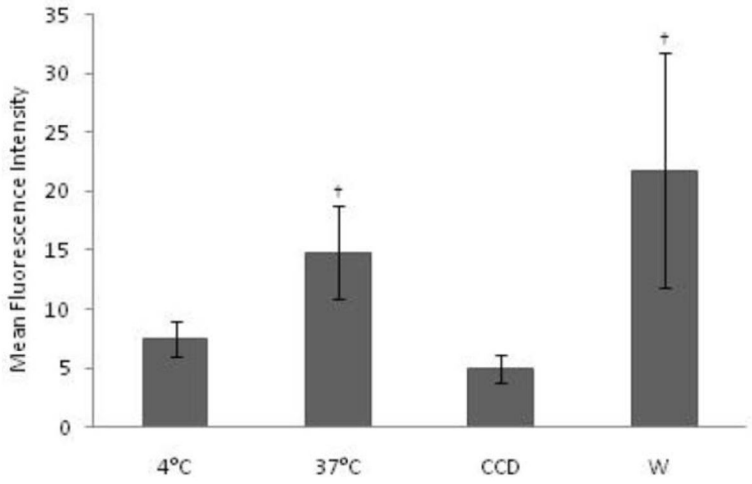

Figure 3.

Endocytosis of FITC-OVA in bovine monocytes. FITC-OVA endocytosis was assessed in bovine monocytes by flow cytometry in the presence or absence of endocytosis inhibitor CCD [2.5 μg/ml] and W [5μM]. Endocytosis at 4° C was measured to determine background levels of FITC-OVA uptake. Samples were analyzed in seven representative experiments, and data are expressed as Mean Fluorescence Intensity (MFI). † Presence on the top of bars indicate treatment differences from bars without † designation ($P < 0.05$).

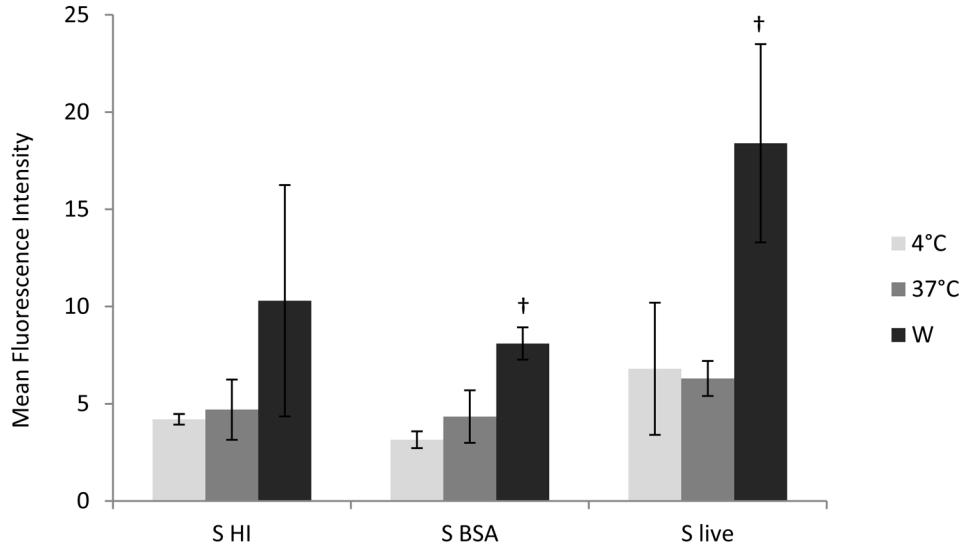

Figure 4.

Phagocytosis of GFP-labeled *Salmonella Kentucky* in bovine monocytes. Antigen uptake by phagocytosis was evaluated in bovine monocytes in the presence or absence of W [5μM]. Phagocytosis at 4° C was measured to determine background levels of *S. kentucky* uptake. Samples were analyzed in three (*S*. live), three (*S*. BSA or opsonized) and four (*S*. HI) representative experiments, and data are expressed as Mean Fluorescence Intensity (MFI). † Presence on the top of bars indicate treatment differences from bars without † designation ($P < 0.05$).

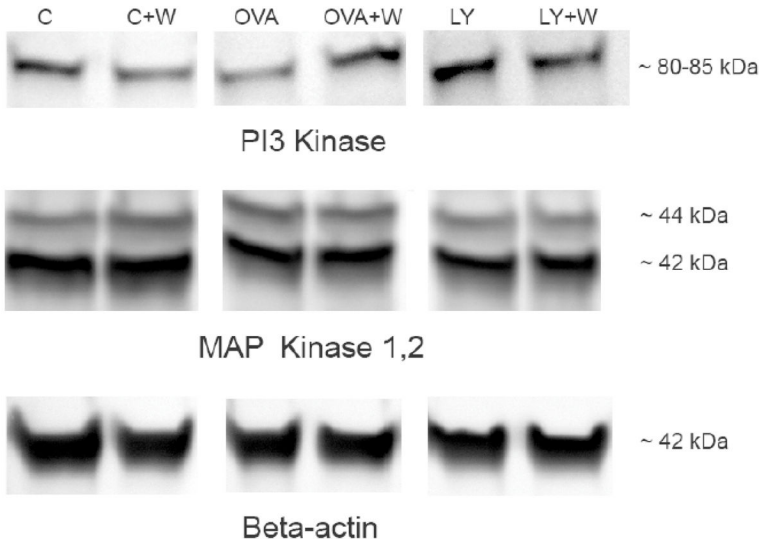

Figure 5.
Western blot analysis of PI3K and MAPK protein expression in the presence of PI3K inhibitor in bovine monocytes relative to that of control monocytes with β-actin as housekeeping protein. One of two representative experiments.