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

**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: Phosphatidylinositol 3-Kinase; LPS: Lipopolysaccharide; 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 Isothiocyanate-Labelled Ovalbumin; CCD: Cytochalasin D; W: Wortmannin; HI: Heat-Inactivated.

**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
primary activation, and thus has an early, unique role in the gatekeeping 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 phagocytosed by monocytes/macrophages and DCs, in which it replicates triggers rapid tissue destruction and inflammation [22]. Apoptosis of macrophages in the liver occurs during systemic 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, P3, 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 5x10^6 per ml in RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1 (Gibco Life Technologies), 5 x 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 (Figure 1A), and a bacterial growth standard curve determined by spectrophotometry (Genesys 20, Thermo Scientific) (Figure 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 10⁸ 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 isothiocyanate-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, 10⁸ 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 [5μM] 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 was then determined using a BCA Protein Assay Kit (Thermo Scientific) and 45μg samples loaded on a 8-16% gradient Precise Protein Gel (Thermo Scientific), for SDS-PAGE. The proteins were then transferred to Immob-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 (US Biological), 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 monocytes

We demonstrated previously that 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 (Figure 2). However, the uptake of LY was enhanced significantly in the presence of W (Figure 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 (Figure 3). However, bovine APCs did not show significant increases in OVA endocytosis in the presence of W compared to antigen uptake at 37°C (Figure 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 (Figure 4). The addition of W promoted enhanced significantly phagocytosis of live and opsonized S. kentucky in bovine monocytes (Figure 4). Phagocytosis of HI bacterial cells was non-significantly increased in the presence of W [p = 0.06] (Figure 4).

Phosphatidylinositol 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 (Figure 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 (Figure 5). Expression levels of MAPK and β-actin proteins were unchanged in the presence of W in all three experimental groups (Figure 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
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 [5 μM]. 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.

of multiple innate immune responses, including IL-12, IL-1α 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,
demonstrating that TLRs are involved in bacterial, including 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 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, and W eliminated this increase in monocytes incubated with LY. Interestingly, decreased PI3K levels correlated with 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-dependent 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 macro pinocytosis 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.

ACKNOWLEDGEMENTS

We acknowledge the assistance of Ryan Lawrence (Lab Assistant) in the animal bleeding and PBMC separation procedures. We also thank Dr. John Harkness for editing this manuscript.

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