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Original Article

β-Arrestin 2: a Negative Regulator of Inflammatory Responses in Polymorphonuclear Leukocytes

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Abstract: Heterotrimeric Gi proteins have been previously implicated in signaling leading to inflammatory mediator production induced by bacterial lipopolysaccharide (LPS). β-arrestins are ubiquitously expressed proteins that alter G-protein-coupled receptors signaling. β-arrestin 2 plays a multifaceted role as a scaffold protein in regulating cellular inflammatory responses. Polymorphonuclear leukocytes (PMNs) activated by LPS induce inflammatory responses resulting in organ injury during sepsis. We hypothesized that β-arrestin 2 is a critical modulator of inflammatory responses in PMNs. To examine the potential role of β-arrestin 2 in LPS-induced cellular activation, we studied homozygous β-arrestin 2 (-/-), heterozygous (+/-), and wildtype (+/+) mice. PMNs were stimulated with LPS for 16h. There was increased basal TNFα and IL-6 production in the β-arrestin 2 (-/-) compared to both β-arrestin 2 (+/-) and (+/+) cells. LPS failed to stimulate TNFα production in the β-arrestin 2 (-/-) PMNs. However, LPS stimulated IL-6 production was increased in the β-arrestin 2 (-/-) cells compared to (+/+) cells. In subsequent studies, peritoneal PMN recruitment was increased 81% in the β-arrestin 2 (-/-) mice compared to (+/+) mice ($p<0.05$). β-arrestin 2 deficiency resulted in an augmented expression of CD18 and CD62L ($p<0.05$). In subsequent studies, β-arrestin 2 (-/-) and (+/+) mice were subjected to cecal ligation and puncture (CLP) and lung was collected and analyzed for myeloperoxidase activity (MPO) as index of PMNs infiltrate. CLP-induced MPO activity was significantly increased ($p<0.05$) in the β-arrestin 2 (-/-) compared to (+/+) mice. These studies demonstrate that β-arrestin 2 is a negative regulator of PMN activation and pulmomary leukosequestration in response to polymicrobial sepsis.

Key Words: Polymorphonuclear leukocytes (PMN), lipopolysaccharide (LPS), β-arrestin 2, adhesion receptors

Introduction

Polymorphonuclear leukocytes (PMNs) are critical cells involved in process of innate immunity [1, 2]. Activation of toll-like receptor (TLR)s on granulocytes result in induction of signaling pathways that produce chemokines, cytokines and other inflammatory mediators [3, 4]. Lipopolysaccharide (LPS) binds to TLR4 and leads to the secretion of pro-inflammatory molecules including TNF-α, IL-6 and chemokines. Fluorescence resonance energy transfer analysis has demonstrated that LPS binds initially to the membrane-bound CD14 and is transferred not only to TLR4 but to a cluster of receptors in lipid rafts which elicit the associated immune response [5]. Among these clustered receptors are G-protein coupled receptors (GPCRs). Previous studies have elucidated the involvement of post receptor heterotrimeric guanine nucleotide binding regulatory (Gi) proteins in LPS signal transduction [6-10]. In vitro kinase assays performed on human CD14 co-immunoprecipitated proteins demonstrated the presence of G$\alpha i2$ and G$\alpha i3$ proteins [7]. Our studies and others suggest that TLR4 signaling is, in part, Gi protein regulated [6, 9]. The importance of Gi proteins in regulating TLR activation also has been underscored by findings in G$\alpha i2$ KO mice. Although there are phenotype differences of inflammatory cell responses to TLR activation in G$\alpha i2$ KO mice, the in vivo pro-inflammatory response to

endotoxin is augmented suggesting that G*i2* KO mice signaling pathways predominantly down-regulate TLR activation [8].

β-Arrestins 1 and 2 are adaptor proteins that regulate Gi protein function by forming complexes with most GPCRs. This occurs following agonist binding and phosphorylation of receptors by G protein-coupled receptor kinases. β-arrestins play a central role in the processes of homologous desensitization and GPCR sequestration that leads to termination of G protein activation by endocytosis in clathrin-coated pits [11-14]. It has also been shown that β-arrestins 1 and 2 function as multifunctional scaffold/adaptor proteins for GPCR activation of signaling cascades [15-19]. Our studies and others have recently demonstrated that β-arrestins 1 and 2 also regulate TLR activation in specific cell lines and bone marrow macrophages from β-arrestins 2 KO mice [20-23].

PMNs are the most numerous type of white blood cell involved in the innate immune response. PMNs have a very short life span (hours), have phagocytic functions, and produce cytokines and chemokines that are critical in the innate immune response [1, 2]. However, the role of β-arrestins in the regulation of PMN innate immune activation has not been previously investigated. The availability of β-arrestin 2 KO mice provides an approach to evaluate the role of this β-arrestins isoform in innate immunity [24]. Therefore, we hypothesized that β-arrestins 2 regulates the inflammatory response in PMNs upon activation of TLR4 and chemotactic responses to an inflammation stimulus. Specifically, we examined the effect of β-arrestins 2 deficiency on: 1) oyster glycogen-induced recruitment of PMNs to the peritoneal cavity, 2) LPS-induced PMN pro-inflammatory mediator production, 3) LPS binding/uptake by PMNs, 4) the expression of specific surface adhesion receptors of PMNs, and 5) In a clinically relevant murine model, we examined the effect of β-arrestins 2 deficiency on pulmonary myeloperoxidase activity at 18h after cecal ligation and puncture (CLP) induced polymicrobial sepsis.

Materials and Methods

Mice

Male WT (+/+), heterozygous (+/-), and β-arrestin 2 knockout (-/-) C57BL/6 mice, 6-9 weeks of age, housed at the Medical University of South Carolina were used in this study. Mice were allowed access to food and water ad libitum and maintained on a 12-hr light/12-hr dark cycle. The investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and commenced with the approval of the Institutional Animal Care and Use Committee.

Reagents

Protein-free S. minnesota R595 LPS was provided by Dr. Ernst Reitschel, Borstel, Germany. Oyster glycogen, type II, was purchased from Sigma (St. Louis, MO). RPMI 1640 media was purchased from Gibco Invitrogen Corporation (Carlsbad, CA). DPBS, fetal bovine serum, and penicillin/ streptomycin were purchased from Cellgro Mediatech Inc. (Herndon, VA). TNF-α and IL-6 ELISA kits, flow cytometry staining buffer, and as anti-mouse FITC-labeled antibodies to CD45, CD11b, F4/80, CD18, and CD62L for flow cytometry were purchased from eBioscience (San Diego, CA).

Experimental methods

Mice were injected with 10 ml of 2% oyster glycogen in sterile DPBS to recruit PMNs to the peritoneal cavity, as previously described [25]. After 5 h, the peritoneal cells were lavaged and harvested using 10 ml per mouse of RPMI 1640 supplemented with 1% fetal bovine serum and 1% penicillin/streptomycin. The peritoneal exudate cells were counted and centrifuged in a Beckman GPR Centrifuge at 1500 rpm for 30 minutes. The supernatant was removed and the cells resuspended in 3 ml of media. Based upon CD45 expression and morphologic criteria, the cell population was >95% PMNs. The cells were counted and plated in a 24-well plate such that each well contained 1 ml of medium with 5 x 10^5 cells/ml. The samples in each well were treated with increasing concentrations of LPS (0, 10, 100, or 500 ng/mL) and allowed to incubate for 16 hr. The incubation time was selected based on previous studies that the cells produce measurable amounts of cytokines after 12-24h. Viability was greater than 90% after 16hs of LPS stimulation as

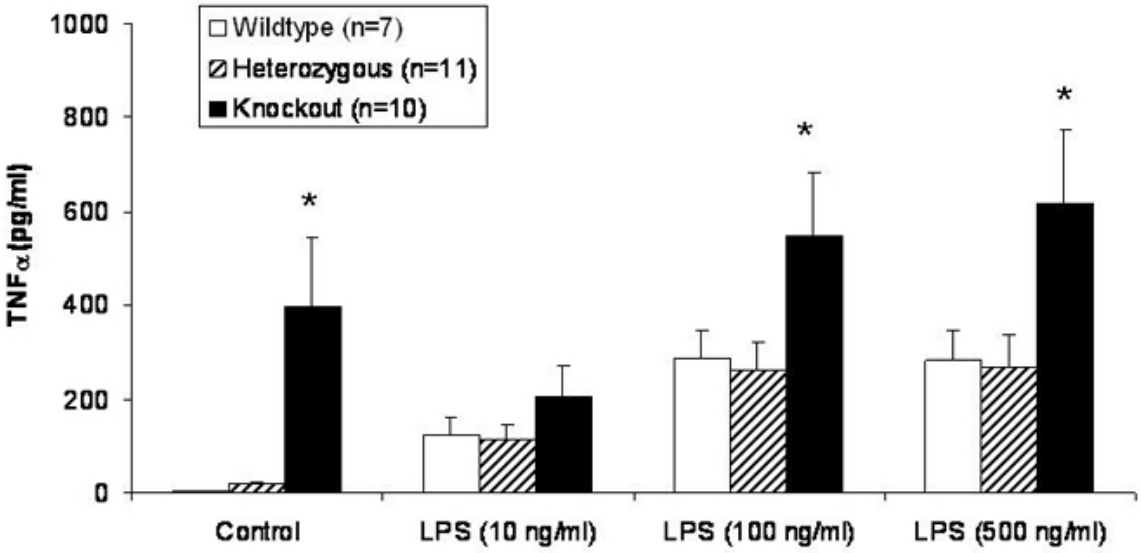

Figure 1. TNFα production by oyster glycogen elicited peritoneal cells. Murine PMNs were stimulated with LPS (0, 10, 100, or 500 ng/ml) for 16 hours. The supernatant was collected to test for TNF-α production using ELISA. Wildtype n=7; heterozygous n=11; knockout n=10. * = $p<0.05$ compared to wildtype.

determined by Trypan Blue. After incubation, the plate was centrifuged at 1500 rpm for 5 minutes. The supernatant was collected for ELISAs to measure TNF-α and IL-6 levels.

Flow cytometry

To study expression of surface molecules, specifically activation markers and adhesion molecules, cells were plated in a 96-well plate such that each well contained 5 x 105 cells. Antibodies used for flow cytometry were diluted 1:50, and 50 μL was added to each well, after which the samples were allowed to incubate at 4°C for at least 20 minutes. Samples were washed twice and then resuspended in flow cytometry staining buffer, after which the samples underwent analysis with a Becton Dickinson FACSCalibur analytical flow cytometer housed at the Analytical Flow Cytometry Facility at the Medical University of South Carolina.

To study LPS uptake, PMNs were plated in 6-well plates containing 5 x 106 cells. The cells were incubated with FITC-LPS (10 μg/ml) for various times. Cells were scraped into flow cytometry staining buffer and washed twice, after which the samples underwent flow cytometry analysis. Since LPS binding to the cell surface receptors and LPS uptaking into the cells were not differentiated, we refer to the response as binding/uptake.

Cecal ligation and puncture

Sepsis was induced by cecal ligation and puncture (CLP) as described previously [26]. Specifically, a midline incision was made below the diaphragm to expose the cecum. The cecum was ligated at the colon juncture with a 6-0 silk ligature suture without interrupting intestinal continuity and punctured twice with a 22-guage needle. The cecum was returned to the abdomen, and the incision was closed in layer with a 6-0 silk ligature suture. With this procedure, WT mice exhibit considerably prolonged survival times beyond 24 hs. Less than 10% mortality within 18 hours of CLP.

Measurement of myeloperoxidase activity

Myeloperoxidase activity was determined in lung as an index of neutrophil accumulation as previously described [27]. Tissues were

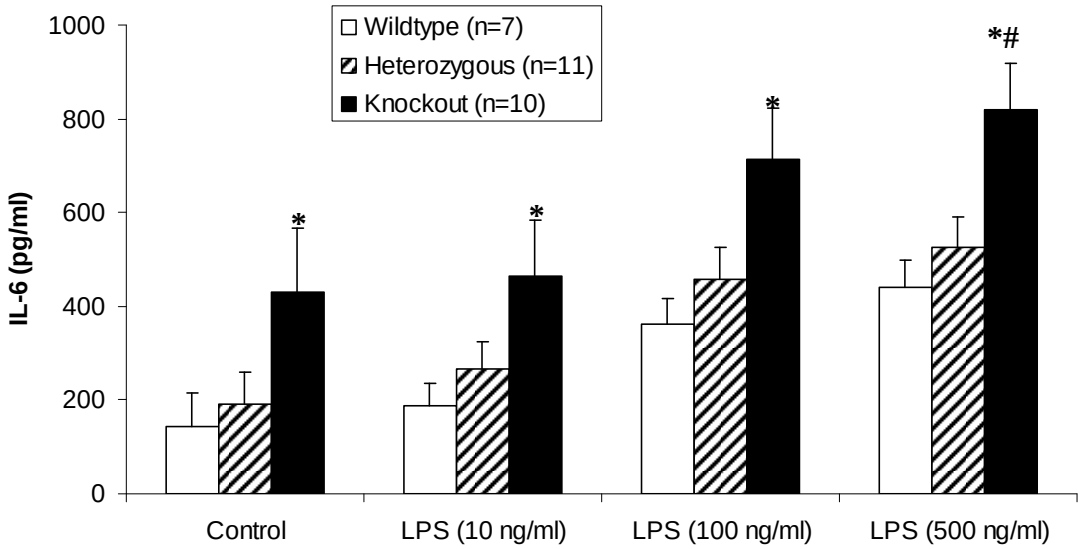

Figure 2. IL-6 production by oyster glycogen elicited peritoneal cells. Murine PMNs were stimulated with LPS (0, 10, 100, or 500 ng/mL) for 16 hours. The supernatant was collected to test for IL-6 production using ELISA. Wildtype n=7; heterozygous n=11; knockout n=10. * = $p<0.05$ compared to wildtype. # = $p<0.05$ compared to control.

homogenized in a solution containing 0.5% hexa-decyl-trimethylammonium bromide dissolved in 10mM potassium phosphate buffer (pH 7.0) and were centrifuged for 30 min at 20,000×g at 4°C. An aliquot of the supernatant was allowed to react with a solution of tera-methyl-benzidine (1.6mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol hydrogen peroxide/min at 37°C and was expressed in units per 100 mg of tissue.

Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined using ANOVA with Fisher's probable least-squares difference test using Microsoft Excel and Statview software (SAS Institute, Cary, NC). Nonparametric statistical analysis was performed using the Mann-Whitney Test for two-group comparisons with Statview software. $P < 0.05$ was considered significant.

Results

β-Arrestin 2 deficiency augments proinflammatory mediator production by PMNs

PMNs harvested from β-arrestin (-/-), (+/-) and (+/+) mice were stimulated with LPS. TNF-α and IL-6 production was determined. In the β-arrestin (-/-) there was a significant ($p<0.05$) increase in basal and stimulated TNF-α production compared to (+/-) or (+/+) extent at the lowest LPS concentration (10 ng/mL, **Figure 1**). Thus, oyster glycogen alone appeared to have maximally stimulated TNF-α production in the (-/-) cells.

There were significantly greater increases in IL-6 production in (-/-) cells in basal and at all concentrations of LPS compared to (+/-) or (+/+) PMNs (**Figure 2**). As with TNF-α oyster glycogen obscured further stimulation with LPS except at the highest LPS concentration. When compared to (+/+) mice, the (-/-) mice exhibited a 85±1% (376pg/ml) increase in IL-6 production when stimulated with LPS (500 ng/mL) ($p<0.05$).

β-Arrestin 2 deficiency does not affect LPS binding/uptake to PMNs

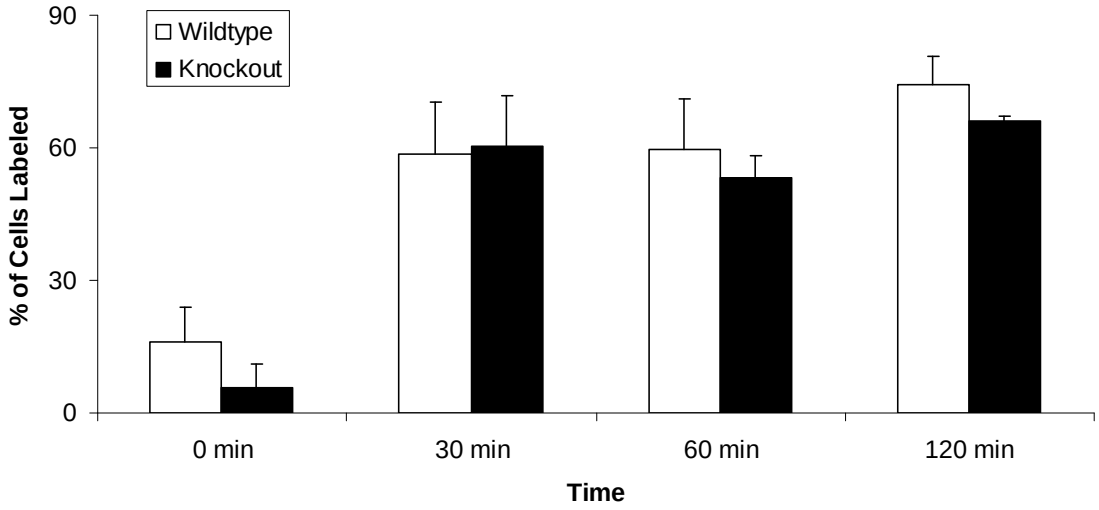

Figure 3. LPS uptake by oyster glycogen elicited peritoneal cells. Murine peritoneal exudate cells were incubated with FITC-labeled LPS, after which cells were washed and analyzed using flow cytometry to determine the percentage of labeled cells.

To determine if β-arrestin 2 deficiency alters the binding/uptake of LPS to PMNs, the uptake of FITC-labeled LPS by flow cytometry was examined at 30, 60, and 120 min in β-arrestin2 (-/-) PMNs and (+/+) cells. FITC-labeled LPS was significantly increased in β-arrestin 2 KO and WT PMNs at 30 minutes but no further increase occurred at 60 and 120 min of incubation (**Figure 3**). There were no significant differences between β-arrestin 2 (-/-) and (+/+) cells. Additionally, flow cytometry expression of TLR4 was not altered between β-arrestin 2 (-/-) and (+/+) cells (data not shown).

β-Arrestin 2 deficiency augments PMN chemotaxis

To analyze the chemotaxic response of PMNs after administration of oyster glycogen, peritoneal recruitment of PMNs was quantitated at 5h. A significant ($p<0.05$) increase (81± 1 % 4.2E+6 cells, $p<0.05$) in PMN recruitment was observed in the (-/-) mice as compared to both (+/+) and (+/-) mice (**Figure 4**).

β-Arrestin 2 deficiency augments expression of CD18 and CD62L by PMNs

The expression of selected activation markers and adhesion molecules known to be expressed on the surface of PMNs were examined. Flow cytometry was used to determine expression of CD45, a granulocyte/leukocyte marker used to confirm the purity of the harvested cells. In addition, expression of F4/80, a pan-macrophage marker; CD11b and CD18, adhesion receptors and CD62L, also known as L-selectin were determined. In β-arrestin 2 (-/-) cells, CD18 expression was elevated by 22% ($p<0.05$), and CD62L expression was elevated in the KO mice by 4.9 fold ($p<0.05$) compared to (+/+) cells (**Figure 5**).

β-Arrestin 2 deficiency augments cecal ligation and puncture-induced myeloperoxidase activity in lung

β-Arrestin 2 (-/-) and (+/+) mice were subjected to CLP. 18h after CLP lung was collected and myeloperoxidase activity (MPO) as index of PMNs infiltrate into lung were examined. CLP-induced MPO activity was significantly increased (2.8 fold, $p<0.05$) in the β-arrestin 2 (-/-) compared to (+/+) (**Figure 6**).

Discussion

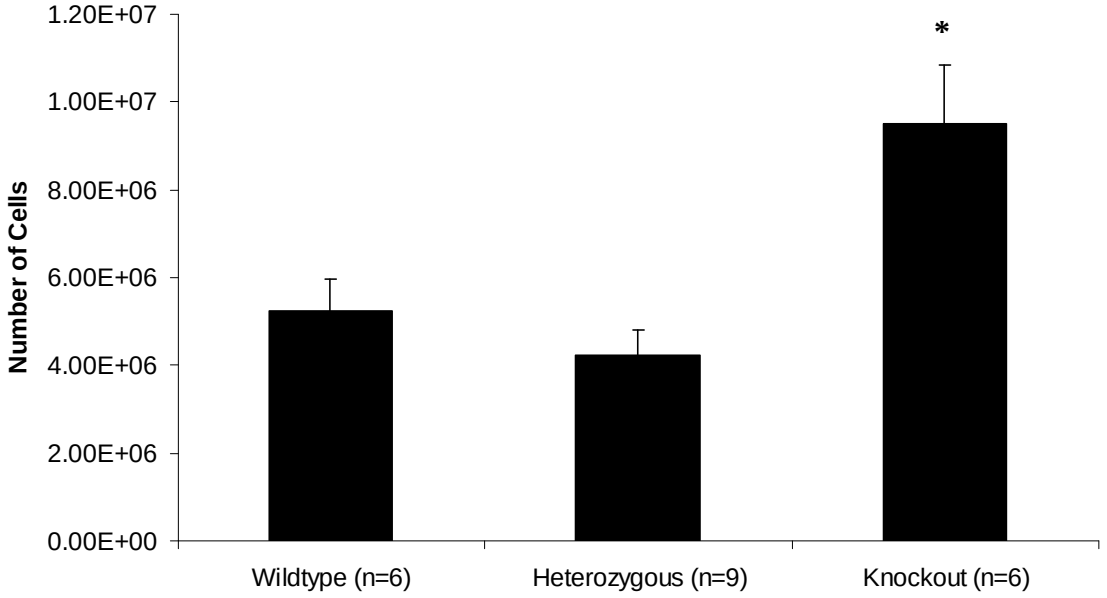

Figure 4. Absolute peritoneal cell counts. Murine peritoneal exudate cells were counted after harvesting using a hemacytometer. Wildtype n=6; heterozygous n=9; knockout n=6. * = $p<0.05$ compared to wildtype.

Our studies demonstrate that LPS-induced IL-6 production was significantly increased in PMNs harvested from β-arrestin 2 (-/-) mice compared to (+/+) mice. Thus, β-arrestin 2 is a negative regulator of pro-inflammatory mediator production in PMNs. β-arrestin 2 deficiency had no effect on LPS binding/uptake to PMNs or TLR4 expression

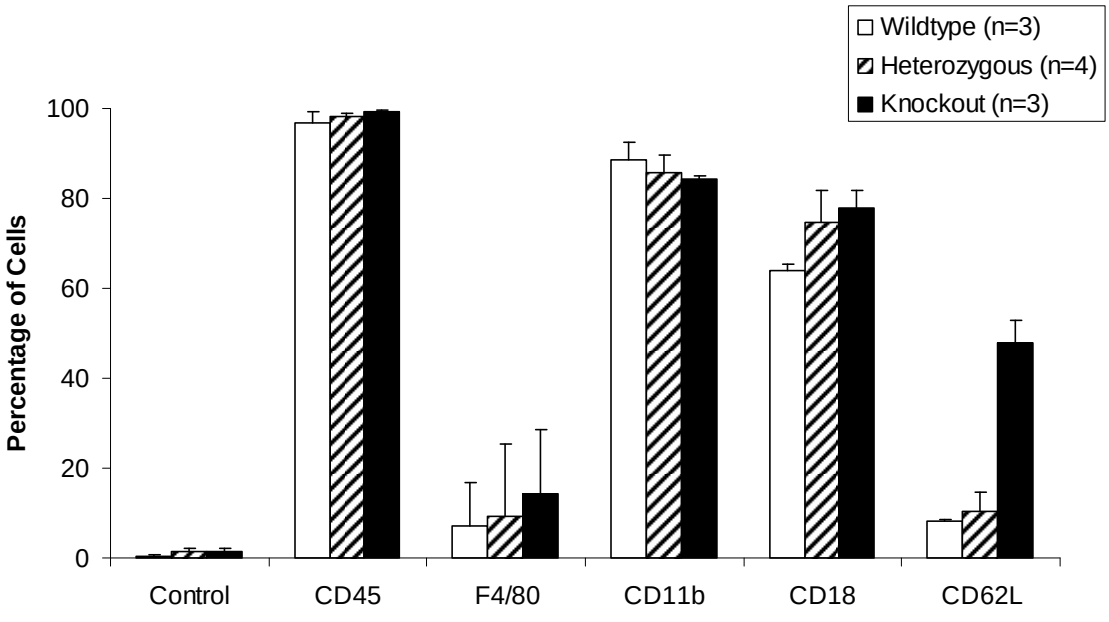

Figure 5. Adhesion molecular expression by oyster glycogen elicited peritoneal cells Murine peritoneal exudate cells were incubated with FITC-labeled antibodies to CD45, F4/80, CD11b, CD18, and CD62L, after which cells were washed and analyzed using flow cytometry to determine the percentage of labeled cells for each antibody. * = $p<0.05$ compared to wildtype. ** = $p<0.05$ compared to wildtype by nonparametric test.

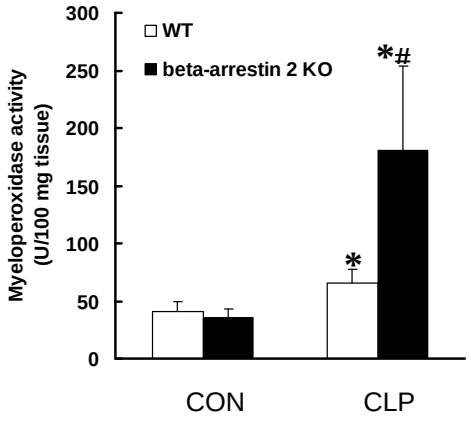

Figure 6. Cecal ligation and puncture-induced myeloperoxidase activity β-arrestin 2 (+/+) and (-/-) mice were subjected to CLP. 18h after CLP lung MPO were examined. *, $p<0.05$ compared to control. #, $p<0.05$ compared to (+/+) underwent CLP. N=5.

on the surface of PMNs. However, we found that PMN chemotaxis was greatly augmented in β-arrestin 2-deficient mice, suggesting that it may play a significant role in the mediation of PMN recruitment and activity at the site of inflammation. Expression of adhesion receptors CD18 and particularly CD62L were also found to be increased in β-arrestin 2-deficient mice, suggesting a role for the β-arrestin 2 in expression of these receptors at the cell surface. In our study with β-arrestin 2 we demonstrated a marked increase in pulmonary MPO activity relative to WT mice. These findings suggest that β-arrestin 2 negatively regulates PMN tissue infiltration during sepsis.

The measured basal +LPS stimulated production of TNFα and IL-6 in oyster glycogen recruited PMNs from β-arrestin 2-deficient mice suggest a predominant anti-inflammatory function of β-arrestin 2 in PMNs. Our recent studies showed that both β-arrestin 1 and 2 negatively regulate NFκB activation [20]. In HEK293 cells rendered LPS-responsive by stable transfections with CD14 and TLR4, we demonstrated by siRNA depletion of β-arrestin 1 and 2 augmented NFκB activation in response to LPS [20]. On the other hand, over-expression of WT β-arrestins 1 and 2 in these cells suppressed LPS-induced NFκB activation [20]. These findings agree with studies that β-arrestin 2 directly interacts with IκBα, thus preventing the phosphorylation and degradation of IκBα [21, 28]. Recent studies have demonstrated that β-arrestins 1 and 2 directly interact with TRAF6 following TLR or IL-6 activation [29]. The complexes of β-arrestins and TRAF6 prevented its auto-ubiquitination and activation of NFκB [29]. These studies further support an inhibitory role for β-arrestins in the regulation of LPS signaling. Since cytokine production was also increased in oyster glycogen recruited PMNs, it is probable that β-arrestin 2 may negatively regulate other inflammatory stimuli through similar mechanisms.

In addition to studying the effect of β-arrestin 2 on the production of pro-inflammatory cytokines, we demonstrated an increase in chemotaxis of PMNs induced by oyster glycogen in the peritoneal cavity of β-arrestin 2 deficient mice as compared to (+/+) mice. These findings are in accordance with other studies implicating β-arrestins as negative regulators of chemotaxis. The interactions between β-arrestins and Gi protein-coupled receptors involved in regulation of PMN chemotaxis, specifically in neutrophils, are pronounced in the CXC subfamily of receptors, including CXCR1 and CXCR2 [29]. Exposure to increased concentrations of chemokines, e.g. IL-8 can cause neutrophils to become unresponsive to further stimulation by other inflammatory cytokines; therefore, desensitization and internalization of CXC receptors is necessary for proper neutrophil functioning during inflammation. Barlic et al. showed that CXCR1 internalization is decreased in HEK293 cells with low β-arrestin 2 expression, and that such internalization is increased with additional expression of β-arrestin 1 or 2 [30, 31]. Su et al. showed that neutrophil recruitment with the binding of the chemokine CXCR1 to the CXCR2 receptor was increased in β-arrestin 2 deficient mice, and that although increased neutrophil activity in the form of calcium mobilization and superoxide anion production were increased in KO mice, receptor internalization was markedly decreased [32]. These two studies suggest that β-arrestin 2 may be negatively regulate chemotaxic activity of neutrophils by mediating chemokine receptor internalization and ultimately terminating chemokine signaling. However, such an interpretation may be an over simplification of the response.

One concept of chemotaxis is that desensitization and recycling of chemotactic receptors are essential for maintaining cellular polarity that promotes chemotaxis [29]. This concept is, in part, based upon in vitro studies where β-arrestin 2 deficiency actually suppresses chemotaxis [29, 32]. Thus, β-arrestin regulation of PMN recruitment in vivo likely reflects responses to other signals at the site of inflammation that are not present in transwell filter assays. Also, in contrast to CRCR1 and CXCR2, β-arrestin 2 appears to positively regulate CXCR4 in vivo lymphocyte chemotaxis [33]. Therefore, the effect of β-arrestins in regulation of chemotaxis in vivo depends upon the chemotactic receptor activation.

We also observed an increase in CD18 and CD62L (L-selectin) expression in the KO mice as compared to WT, in the recruited PMNs. This suggests that β-arrestin 2 is involved in the inhibition of signaling by these two integrins in PMNs. Mulligan et al. [25] examined the role of adhesion molecule expression in recruitment of neutrophils and found that blocking the selectins reduced the accumulation of neutrophils in the peritoneal cavity after oyster glycogen-induced peritonitis. A similar trend was seen after the use of blocking antibodies for CD11a, CD11b, and CD18 [32], indicating that these adhesion molecules are involved in the recruitment of neutrophils and the subsequent release of cytokines. β-arrestin may thus reduce the relative expression of these adhesion molecules and thus reduce PMNs to endothelial surfaces.

The CLP model is accepted as a clinically relevant sepsis model. CLP-induced pulmonary MPO activity was significantly increased in β-arresin 2 KO compared to WT mice demonstrating that β-arrestin 2 negatively regulates pulmonary leukosequestration. In WT mice, CLP-induced small but statistically significant increase of pulmonary MPO activity suggesting that the strain of mice has low response to sepsis induced inflammation. However, the β-arrestin 2 (-/-) mice with same background exhibited a more severe response suggesting that β-arrestin 2 negatively regulates tissue PMN infiltration in sepsis. Whether this is a result of altered PMN or endothelial adhesion receptor expression and/or altered chemokine production is currently under investigation. However this finding highlights the translational significance of β-arrestin 2 in polymicrobial sepsis.

Our studies demonstrate an effect of β-arrestin 2 on PMN inflammatory responses as reflected by altered cytokine production, PMN recruitment, adhesion molecule expression. and pulmonary leukosequtration in response to CLP. The extent that such responses may affect other pathogenesic events of sepsis remains to be determined.

Acknowledgments

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