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## Regulation of lipopolysaccharide-induced inflammatory response and endotoxemia by $\beta$ -arrestins

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### Abstract

$\beta$ -arrestins are scaffolding proteins implicated as negative regulators of TLR4 signaling in macrophages and fibroblasts. Unexpectedly, we found that  $\beta$ -arrestin-1 ( $\beta$ -arr-1) and -2 knockout (KO) mice are protected from TLR4-mediated endotoxic shock and lethality. To identify the potential mechanisms involved, we examined the plasma levels of inflammatory cytokines/chemokines in the wild type (WT) and  $\beta$ -arr-1 and -2 KO mice after lipopolysaccharide (LPS, a TLR4 ligand) injection. Consistent with lethality, LPS-induced inflammatory cytokine levels in the plasma were markedly decreased in both  $\beta$ -arr-1 and -2 KO, compared to WT mice. To further explore the cellular mechanisms, we obtained splenocytes (separated into CD11<sup>b+</sup> and CD11<sup>b-</sup> populations) from WT,  $\beta$ -arr-1 and -2 KO mice and examined the effect of LPS on cytokine production. Similar to the *in vivo* observations, LPS-induced inflammatory cytokines were significantly blocked in both splenocyte populations from the  $\beta$ -arr-2 KO compared to the WT mice. This effect in the  $\beta$ -arr-1 KO mice, however, was restricted to the CD11<sup>b-</sup> splenocytes. Our studies further indicate that regulation of cytokine production by  $\beta$ -arrestins is likely independent of MAPK and  $\text{I}\kappa\text{B}\alpha$ -NF $\kappa\text{B}$  pathways. Our results, however, suggest that LPS-induced chromatin modification is dependent on  $\beta$ -arrestin levels and may be the underlying mechanistic basis for regulation of cytokine levels by  $\beta$ -arrestins *in vivo*. Taken together, these results indicate that  $\beta$ -arr-1 and -2 mediate LPS-induced cytokine secretion in a cell-type specific manner and that both  $\beta$ -arrestins have overlapping but non-redundant roles in regulating inflammatory cytokine production and endotoxic shock in mice.

### Introduction

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) identified for their role in mediating signaling from the pathogen-associated molecular patterns (PAMPs) from microbial products (Beutler, 2004; Beutler, 2005; Beutler, 2009). TLRs are now implicated in a number of acute and chronic inflammatory diseases including endotoxemia and endotoxic shock (Sabroe et al., 2008; Salomao et al., 2008). Of particular importance, activation of Toll-like receptor-4 (TLR4) (stimulated by lipopolysaccharide (LPS) from gram-negative bacteria) is proposed as one of the primary mechanisms in the pathophysiology of endotoxemic shock and lethality in animals and humans (Armstrong et

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#Equal Contribution

al., 2004; Daubeuf et al., 2007; Leon et al., 2008; Lorenz et al., 2002; O'Brien et al., 2005; Salomao et al., 2008; Spiller et al., 2008). Endotoxemia and lethality in turn are mediated by production of pro- and anti-inflammatory cytokines/chemokines, the balance of which subsequently determines the disease outcome. LPS stimulation of TLR4 causes an increase in Inflammatory cytokines/chemokines production by mediating cell signaling pathways including that of MAPK and NF $\kappa$ B pathways (Miggin and O'Neill, 2006; O'Neill, 2008), (Symons et al., 2006), (Hayden and Ghosh, 2004; Sun and Ley, 2008).

MAPK signaling typically involves the ERK, JNK and p38 MAPK pathways. These kinases further phosphorylate a number of proteins including transcription factors leading to changes in gene expression (Symons et al., 2006). NF $\kappa$ B pathway is regulated by activation of I $\kappa$ B kinase (IKK) complex, which phosphorylates cytosolic I $\kappa$ B proteins (e.g. I $\kappa$ B $\alpha$ , a well-known member). Under unstimulated conditions I $\kappa$ B $\alpha$  binds to NF $\kappa$ B transcription factors (p50/p65) and retain them in the cytosol. Upon phosphorylation by IKK complex, I $\kappa$ B $\alpha$  proteins undergo ubiquitination and proteasome-dependent degradation resulting in the release of NF $\kappa$ B transcription factors (Hayden and Ghosh, 2004; Sun and Ley, 2008). These transcription factors then translocate into the nucleus and evoke expression of cytokines and chemokines. In addition to MAPK and NF $\kappa$ B signaling pathways, recent studies have clearly demonstrated an important role for chromatin modification, especially histone acetylation in the regulation of cytokine gene expression (Kuo and Allis, 1998). In this regard, histone acetyl transferase (HAT) and histone deacetylase (HDAC) activities have been shown to play a crucial role in cytokine expression and inhibition of these enzymes have profound effects on LPS-induced cytokine production ((Barnes et al., 2005; Choi et al., 2009a; Choi et al., 2009b; Choi et al., 2008; Leoni et al., 2005)).

Arrestins are scaffolding proteins originally discovered for their role in G-protein coupled receptor desensitization. There are four members of the arrestin family (Arrestin-1 to -4), of which arrestin-1 and -4 are restricted to the eyes and arrestin-2 and -3 are ubiquitously distributed. Arrestin-2 (also called  $\beta$ -arrestin-1) and Arrestin-3 (also called  $\beta$ -arrestin-2) are best known for their role in the desensitization and trafficking of  $\beta$ -adrenergic receptor (DeWire et al., 2007). Subsequent studies have demonstrated a much broader role for  $\beta$ -arrestins in cell signaling. Although individual knockout of  $\beta$ -arrestin-1 and -2 are viable with some unique phenotypes, homozygous double knockout of  $\beta$ -arrestin-1 and -2 is embryonically lethal, suggesting that at least one of the  $\beta$ -arrestins need to be present for proper embryonic development. Studies have also shown that depending on the cellular context and receptor examined,  $\beta$ -arrestins either mediate or inhibit receptor signaling. This is also the case for TLR4 signaling where  $\beta$ -arrestins' role is dependent on the cell type (Fan et al., 2007; Parameswaran et al., 2006; Wang et al., 2006). While these studies were primarily done in cell culture and *in vitro* models, the precise role of  $\beta$ -arrestins in TLR4 signaling *in vivo* is not clearly understood. Using both *in vivo* and *ex vivo* model systems, we demonstrate here that both  $\beta$ -arrestins are positive regulators of TLR4-induced inflammatory cytokine production and endotoxemia in mice and therefore suggest that  $\beta$ -arrestins may be potential drug targets in the treatment of TLR4-mediated inflammatory diseases.

## Materials and Methods

### Animals

$\beta$ -arrestin-1 and -2 KO mice have been backcrossed to C57BL6 background for more than 10 generations and have been described previously (Rajagopal et al., 2006). They were kindly provided by Dr. Robert Lefkowitz (Duke University). Wild type control C57BL6 mice were purchased from Jackson Labs. Animals were housed four to five mice per cage at 22–24°C in rooms with 50% humidity and a 12-h light–dark cycle. All animals were given mouse chow and water *ad libitum*. Purchased mice were housed for at least 1 week before

experimental use, and age- and sex-matched animals (~2–3 month old males) were employed as described. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and conformed to NIH guidelines.

### Survival study

Male mice (~2–3 month old) were injected with LPS (*E. Coli* 0111:B4 from Sigma) intraperitoneally (20 µg/g body weight) and survival of mice monitored for 48 hours (Greten et al., 2007).

### Cytokine assays

A mouse 23-plex multiplex-based assay was used to determine the indicated cytokine/chemokine plasma concentrations as per the manufacturer's instructions (Bio-Rad) via Luminex 100 technology as previously described (Appledorn et al., 2008). For ELISA assays, IL1β, IL-6, IL-10 and TNFα ELISA kits were obtained from eBiosciences and performed per manufacture's instructions. ELISA assays for IL12, IL5 and IFNγ were performed as described previously (Navuluri et al., 2006; Parvataneni et al., 2009).

### Quantitative RT-PCR

To determine relative levels of a specific RNA transcript, tissues were snap-frozen in liquid nitrogen and RNA was harvested using TRIzol reagent (Invitrogen) according to manufacturer's protocol and as described before (Appledorn et al., 2008; Loniewski et al., 2008). Synthesis of cDNA was performed with reverse transcriptase (RT) with the total RNA using the superscript II kit with Oligo-dt (12–18) primers as recommended by the manufacturer (Invitrogen). cDNA was amplified by PCR in a final reaction volume of 25 µl using SYBR Green Supermix (Invitrogen) with 10 pmol of each primer (Qiagen quantitect primers for COX2 and 18s rRNA primers from IDT). Real-time PCR was performed in MX3000P (Stratagene) thermocycler. RNA free samples (negative control) did not produce any amplicons. Melting curve and gel analysis were used to verify single products of the appropriate base-pair size. Data were analysed with MX3000P software using 18s rRNA for normalization.

### Splenocyte preparations

Mouse splenocytes were obtained and cultured as previously described (Parvataneni et al., 2009). Isolation of CD11<sup>b+</sup> and CD11<sup>b-</sup> populations was performed using CD11b antibody (Miltenyi Biotec) and by positive selection (for CD11<sup>b+</sup>) using MACS column (Miltenyi Biotec) according to manufacturer's instructions. The negatively selected splenocytes by this method were designated CD11<sup>b-</sup> cells.

### Flow cytometric analysis of Cell surface TLR4 expression

CD11<sup>b+</sup> splenocytes were obtained as described above. For bone marrow cells, bone marrows were flushed from tibiae and femurs of wild type, β-arrestin-1 and -2 KO mice and CD11<sup>b+</sup> cells were then separated as described for the splenocytes using the MACS column. Cells were then rinsed with staining buffer (1% FBS and 0.09% sodium azide in PBS) and stained with the following antibodies: anti-CD11b-FITC, anti-TLR4-PE, anti-CD3-PerCp-Cy5.5, anti-CD19-PE-Cy7. TLR4 expression was analyzed using LSRII (BD Biosciences) and Flowjo software (Tree Star).

### Western blotting

Splenocytes were lysed in lysis buffer (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 with protease inhibitors. Cell lysates were then centrifuged at maximum speed (13,000 × g) for 10 min at 4°C and the protein concentration

of the supernatant determined using Bradford method. Western blotting for p-ERK1/2, ERK2, I $\kappa$ B $\alpha$ , pJNK1/2, JNK1/2, pP38, and tubulin were performed as previously described (Loniewski et al., 2008; Loniewski et al., 2007; Patial et al., 2010). Briefly, equivalent concentrations of protein samples were run on polyacrylamide gels and transferred to nitrocellulose membranes. Blots were then probed with primary and fluorescent secondary antibodies as described (Patial et al., 2010). Blots were scanned and bands were quantified using Li-COR Odyssey scanner. For data analysis, p-ERK1/2 bands were normalized to ERK2, and I $\kappa$ B $\alpha$  levels to tubulin/actin for quantitation.

### HAT and HDAC activities

HAT and HDAC activities were assayed using nuclear extracts of the spleen obtained from mice injected with LPS as indicated. Nuclear extracts from the spleen were obtained as described before (Blackwell et al., 1997). HAT activity was performed using the HAT activity colorimetric assay kit from BioVision Research Product (California) following manufacturer's assay protocol. HDAC activity was performed using HDAC activity assay kit from Caymen, as per manufacturer's protocol.

### Statistics

All data in the figures and text are expressed as mean $\pm$ SE and data analyzed using GraphPad Prism Software. Each "N" represents individual mouse. The Student's t-test was used to compare mean values between two experimental groups and Analysis of Variance (with Bonferroni Post-test) for more than two groups. Differences in the survival were determined using the log-rank test. P Values <0.05 were considered significant.

## Results and Discussion

### LPS-induced mortality is inhibited in $\beta$ -arrestin-1 and -2 knockout male mice

To determine the role of  $\beta$ -arrestins in LPS-induced endotoxic shock, we injected wild type,  $\beta$ -arrestin-1 ( $\beta$ -arr-1) and -2 knockout mice with LPS (20  $\mu$ g/g body weight intraperitoneally) and monitored the survival of the three groups of mice for 48 hours. As shown in Fig 1, both  $\beta$ -arrestin knockout mice survived significantly better than the wild type mice. Only 10% of the wild type mice survived by 48 hours compared to 58% of  $\beta$ -arr-1 and 50% of  $\beta$ -arr-2 knockout mice. While the median survival of the wild type mice was 34 hours, median survival of the  $\beta$ -arr-2 KO mice was 47.5 hours and was longer than 48 hours for  $\beta$ -arr-1 KO mice. The surprisingly better survival of  $\beta$ -arr-2 KO male mice is in contrast to the findings of Wang *et al* who showed that mortality in female  $\beta$ -arr-2 KO mice is significantly quicker than the wild type mice in a Galactosamine-D (Gal-D) sensitized model of endotoxic shock (Wang et al., 2006). Our studies using the Gal-D-sensitized model of endotoxic lethality in males, exhibited the same phenotype as that of our LPS model, i.e. both  $\beta$ -arrestin-1 and -2 knockout had significantly lower mortality rates compared to wild type mice (data not shown). Thus, it is possible that the differences in the study results may be due to the different genders of mice used. Ample evidence indicates that male and female rodents respond quite differently with respect to inflammatory agents (Card et al., 2006; Marriott et al., 2006; Marriott and Huet-Hudson, 2006; Rettew et al., 2009; Rettew et al., 2008). Studies by Rettew *et al* (Rettew et al., 2009; Rettew et al., 2008) especially have shown that while testosterone reduces the cell surface expression of TLR4 in mouse macrophages, estrogen augments this effect. In addition, removal of endogenous estrogen decreases the plasma levels of inflammatory cytokines in LPS-injected mice. Whether the levels of estrogen are different between the female wild type and  $\beta$ -arrestin KO mice is not known and if the effects observed by Wang *et al* (Wang et al., 2006) may be attributed to the differences in the levels of TLR4 is also not clear. Interestingly, however, cell surface expression of TLR4 in male wild type and  $\beta$ -arrestin-1 and -2 knockout mice were similar

in our studies, as determined by flow cytometry (Fig 2). These results suggest that at least in the males,  $\beta$ -arrestins do not regulate basal cell surface expression of TLR4. Taken together, the difference between our studies and that of Wang *et al* suggest that the role of  $\beta$ -arrestins in modulating specific inflammatory responses may differ between males and females. Furthermore, our results clearly demonstrate that  $\beta$ -arrestin-1 and -2 are important modulators of endotoxic lethality in male mice.

### **$\beta$ -arrestins positively regulate LPS-induced inflammation *in vivo***

Endotoxic shock in mice is mediated by a complex array of pro- and anti-inflammatory cytokines and chemokines. Levels of these cytokines/chemokines increase in the plasma within hours after LPS injection and are strongly associated with mortality in mice. Based on the survival results, we hypothesized that  $\beta$ -arrestins either mediate pro-inflammatory cytokines/chemokines production or inhibit anti-inflammatory cytokines such as IL-10. To test this hypothesis, we injected wild type,  $\beta$ -arr-1 and -2 KO mice with LPS (20  $\mu$ g/g body weight, intraperitoneally) and determined the plasma levels of various cytokines and chemokines (using 23-plex multiplex cytokine assay) at 1, 3, 6 and 12 hours after injection. Levels of these cytokines/chemokines were also determined in uninjected as well as PBS injected mice to estimate basal/control levels. We did not find any difference in the basal levels of the cytokines between the genotypes (data not shown). Our results however, indicate distinct as well as overlapping roles for  $\beta$ -arrestins in LPS-induced cytokine regulation (Fig 3). Plasma levels of IL-1 $\beta$ , IL12p40, IFN $\gamma$ , IL-2, IL-3, IL-4, and IL-5 were significantly inhibited in both  $\beta$ -arrestin knockout mice compared to the wild types at the corresponding time points after LPS injection (Fig 3A). Some cytokines (IL1 $\alpha$ , IL-13, IL-17, Eotaxin and KC) however, were inhibited only in the  $\beta$ -arrestin-2 knockout mice (Fig 3B). Importantly, levels of some cytokines/chemokines (IL-9, IL-10, IL-6, IL-12p70, G-CSF, GM-CSF, MCP-1, MIP1 $\alpha$  and RANTES) were similar between wild types and  $\beta$ -arrestin-1 & -2 knockout mice suggesting selective regulation of inflammatory response by  $\beta$ -arrestins (Fig 3D). Interestingly, as shown by Wang *et al* (Wang et al., 2006) (for TNF $\alpha$ ), LPS-induced TNF $\alpha$  (at 1h) and MIP1 $\beta$  (at 3h) levels were significantly elevated in the  $\beta$ -arrestin-2 knockout mice compared to the wild types (Fig 3C). Given that many pro-inflammatory cytokines are inhibited in the  $\beta$ -arrestin-2 KO mice, the pathophysiological consequence of the negative regulation of TNF $\alpha$  and MIP1 $\beta$  in the current model is not clear. We initially reasoned that the enhanced TNF $\alpha$  in the  $\beta$ -arrestin-2 KO mice might render them more susceptible for LPS/D-Gal-mediated shock (Lehmann et al., 1987), as was shown by Wang *et al* (Wang et al., 2006). As indicated earlier, however, the survival of the male  $\beta$ -arrestin-2 KO mice was better in the LPS/D-Gal-mediated shock, compared to the wild type (data not shown). Thus the consequence(s) of the initial negative regulation of TNF $\alpha$  and MIP1 $\beta$  remains unclear in this model.

Previous studies have demonstrated a strong association between the plasma cytokine levels in sepsis and mortality in animals and humans [reviewed in (Rittirsch et al., 2008)]. In the present study, the better survival of the  $\beta$ -arr-1 and -2 KO mice is strongly associated with the diminished pro-inflammatory cytokine responses observed in the KO mice. In particular, many pro-inflammatory cytokines are significantly attenuated in the  $\beta$ -arrestin-1 and -2 knockout mice in response to LPS injection. Many of these cytokines have also been shown to have significant effects in the pathogenesis of endotoxemia (Greten et al., 2007; Kohler et al., 1993; Wsocka et al., 1995; Zisman et al., 1997). Thus, even though levels of anti-inflammatory cytokine such as IL-10 did not differ between the genotypes, the profound decrease in the pro-inflammatory cytokines (especially IL1 $\beta$ , IFN $\gamma$  and IL12) is probably sufficient to tip the “balance” towards the anti-inflammatory phenotype observed in the KO mice, and this may be associated with the better survival of these mice.



## Regulation of Cyclooxygenase-2 expression by $\beta$ -arrestins

In addition to the panel of inflammatory cytokines, LPS also induces the expression of cyclooxygenases (COX), enzymes that play a key role in the production of prostaglandins (PGs). Of the various PGs, PGE<sub>2</sub> has been shown to be critical in the pathogenesis of endotoxemia and sepsis (reviewed in (Cook, 2005; Funk, 2001)). Two isoforms of COX have been identified: COX1 and COX2. COX1 is constitutively expressed and is responsible for basal constitutive prostaglandin synthesis. COX2, however, is induced under various inflammatory states including endotoxemia and importantly, selective inhibition of COX2 has been shown to improve early survival in mouse model of endotoxemia (Reddy et al., 2001). Because deletion of  $\beta$ -arrestins in mice appears to be protective in terms of survival in endotoxemia, we hypothesized that in addition to the regulation of inflammatory cytokine production, prostaglandin production would also be attenuated in the  $\beta$ -arrestin-1 and -2 KO mice. To test this, we determined the levels of *ptgs2* (mRNA for COX2) in the spleen of mice injected with LPS for various time points. Similar to the cytokine response, our results demonstrate that the expression of COX2 is markedly inhibited in both  $\beta$ -arrestin-1 and -2 KO mice compared to the wild types (Fig 4), suggesting that this may also contribute to the better survival of the  $\beta$ -arrestin KO mice. Whether the decreased levels of COX2 in the  $\beta$ -arrestin-1 and -2 KO mice is a result of, or a consequence from, diminished cytokine responses is unclear and will be tested in future studies.

## Selective role of $\beta$ -arrestin-1 and -2 in CD11<sup>b+</sup> and CD11<sup>b-</sup> splenocytes

Our results so far suggest that both  $\beta$ -arrestins have overlapping as well as distinct functions in terms of cytokine production. To further elucidate the cellular and signaling mechanisms by which  $\beta$ -arrestins mediate the effects described above, we focused our studies on the splenocytes. We chose spleen because of its known role in the inflammatory responses observed in endotoxemia and sepsis models (Deriy et al., 2002; Moeniralam et al., 1997; Senaldi et al., 1999). We collected spleens from wild type,  $\beta$ -arrestin-1 and -2 knockout mice and separated the splenocytes into CD11<sup>b+</sup> and CD11<sup>b-</sup> populations (using a MACS column with CD11<sup>b</sup> antibodies). The negatively selected population in this manner was designated as CD11<sup>b-</sup> population. On an average the CD11<sup>b+</sup> population was about 5–10% of the total splenocyte population and using the MACS column we obtained about 85–90% pure CD11<sup>b+</sup> cells as assessed by flow cytometry (data not shown). Furthermore, the CD11<sup>b-</sup> population was ~95% pure (assessed by flow cytometry). Approximately 50% of the CD11<sup>b+</sup> cells were also positive for Gr-1 (marker for granulocytes, monocytes and plasmacytoid dendritic cells). Furthermore, macrophages (F4/80+) constituted ~30% of the CD11<sup>b+</sup> population whereas myeloid dendritic cells (CD11<sup>c+</sup>) constituted ~11% as assessed by flow cytometry (data not shown). Importantly, the CD11<sup>b-</sup> population contained mainly T- (CD3<sup>+</sup>, ~42%) and B-lymphocytes (CD19<sup>+</sup>, ~49%) and insignificant proportion of macrophages (F4/80<sup>+</sup>, 1%) and myeloid dendritic cells (CD11<sup>c+</sup>, 0.875%) as assessed by flow cytometry (data not shown). Both CD11<sup>b+</sup> and CD11<sup>b-</sup> populations were treated with LPS for various time points (0, 24, 48 and 72 hours) and the culture supernatants were collected and used for cytokine assay. We examined the levels of IL-1 $\beta$ , IL-12 (p40 and p70), IL-5, IFN- $\gamma$ , IL-6, IL-10 and TNF $\alpha$  using an ELISA assay. These cytokines were chosen based on the *in vivo* responses observed in the  $\beta$ -arrestin knockout mice after LPS injection as well as on the known role of these cytokines in endotoxic shock (reviewed in (Rittirsch et al., 2008)). In the wild type CD11<sup>b+</sup> splenocytes, LPS treatment caused a significant increase in IL-1 $\beta$ , IL-12 (p40 & 70), IFN $\gamma$ , IL-6, IL-10 and TNF $\alpha$  (Fig 5 and 6). There was, however, no detectable increase in IL-5 in the CD11<sup>b+</sup> splenocytes (data not shown). Although these responses were similar in the  $\beta$ -arr-1 KO cells, levels of IL-1 $\beta$ , IL-12, and IFN- $\gamma$  were significantly blocked in the  $\beta$ -arr-2 KO splenocytes, similar to *in vivo* observations (Fig 5). Interestingly, levels of IL-6 and TNF $\alpha$  (but not IL-10) were also modestly inhibited in the  $\beta$ -arrestin-2 knockout splenocytes suggesting that the local

production of these two pro-inflammatory cytokines may be potentially regulated by  $\beta$ -arrestin-2 similar to that of IL-1 $\beta$ , IL12 and IFN $\gamma$ . It should be noted, however, that even though TNF $\alpha$  levels were enhanced *in vivo* in the  $\beta$ -arrestin-2 KO mice after LPS injection, *in vitro* treatment of splenocytes resulted in a modest inhibition. Whether this can be attributed as an artifact of *in vitro* experiment or to the difference in the kinetics of *in vivo* *v* *s* *in vitro* production of TNF $\alpha$  is not known. Furthermore, IFN $\gamma$  secretion was enhanced in the  $\beta$ -arrestin-1 knockout cells (compared to wild types) but the significance of this to the *in vivo* findings is also not clear. Together, however, these results suggest that  $\beta$ -arrestin-2 but not  $\beta$ -arrestin-1 selectively mediates LPS-induced production of IL-1 $\beta$ , IL-12 and IFN $\gamma$  in CD11b<sup>+</sup> splenocytes. All three of these cytokines have been shown to play a major role in the development of endotoxic shock (Greten et al., 2007; Kohler et al., 1993; Wysocka et al., 1995; Zisman et al., 1997) and thus reduction in these cytokines observed in both *in vivo* and *in vitro* experiments in the  $\beta$ -arrestin-2 KO mice may in part explain the resistance of this genotype to endotoxic shock.

In CD11b<sup>-</sup> splenocytes, LPS treatment significantly increased IL1 $\beta$ , IL12p70, IL-5 and IFN $\gamma$ , (but not IL12p40) in the wild type mice (Fig 7 and data not shown for IL12p40). In contrast to the results in CD11b<sup>+</sup> splenocytes, LPS-induced IL-1 $\beta$ , IL12p70, IFN $\gamma$  and IL-5 were markedly inhibited in both  $\beta$ -arr-1 and -2 knockout splenocytes (CD11b<sup>-</sup> population) (Fig 7). Using this *in vitro* model system, our results reveal that the role of  $\beta$ -arrestin-1 and -2 are exquisitely cell type specific, in that, TLR4 signaling (with respect to cytokine production) is mediated only by  $\beta$ -arrestin-2 in the CD11b<sup>+</sup> splenocytes, whereas TLR4 signaling is mediated by both  $\beta$ -arrestins in the CD11b<sup>-</sup> splenocytes. Further *in vivo* studies are obviously necessary to distinguish the subpopulation of the splenocytes to determine which subtype of immune cell population is involved in this regulation. Based on our current analysis that, the proportion of macrophages and dendritic cells between the CD11b<sup>+</sup> and CD11b<sup>-</sup> population is markedly different, suggests that the role of  $\beta$ -arrestin-2 may be selective for macrophages/dendritic cells (predominant in the CD11b<sup>+</sup> population). On the contrary, both  $\beta$ -arrestins may be required for TLR4 signaling in lymphocytes (the predominant splenocyte population in the CD11b<sup>-</sup> cells, which constitute 90–95% of the total splenocyte population).

### Role of $\beta$ -arrestins in LPS-induced NF $\kappa$ B and MAPK pathways in the splenocyte populations

To understand the signaling mechanisms mediating the cytokine response in the  $\beta$ -arrestin-1 and -2 KO mice, we first examined the various components of the NF $\kappa$ B and MAPK signaling pathways in the CD11b<sup>+</sup> and CD11b<sup>-</sup> splenocyte populations after 30 and 60 minutes of LPS treatment. To assess the NF $\kappa$ B pathway, we determined the levels of I $\kappa$ B $\alpha$  in total cell lysates. Previous studies have shown that  $\beta$ -arrestins negatively regulate signal-induced I $\kappa$ B $\alpha$  degradation in cells in culture (Gao et al., 2004; Wang et al., 2006; Witherow et al., 2004). In this model, however, we did not observe any significant role for  $\beta$ -arr-1 or -2 in I $\kappa$ B $\alpha$  degradation in either CD11b<sup>+</sup> or CD11b<sup>-</sup> splenocytes (Fig 8). These results suggest that the cytokine effects observed in the  $\beta$ -arr-1 and -2 KO splenocytes are independent of I $\kappa$ B $\alpha$  and therefore likely to be independent of the canonical NF $\kappa$ B pathway.

$\beta$ -arrestins have also been shown to be important regulators of MAPK signaling pathways and depending on the cellular context,  $\beta$ -arrestins mediate or inhibit MAPKs. In particular, previous studies have shown that  $\beta$ -arrestins act as negative regulators as well as positive regulators of TLR4-induced ERK signaling pathway in different cell types (Fan et al., 2007; Parameswaran et al., 2006). Therefore, we examined the role of these MAPK pathways in the splenocyte population to determine if activation of these kinases is impaired in the  $\beta$ -arrestin1/2 KO mice cells. For this, we examined the levels of pERK, pJNK and pP38 in both CD11b<sup>+</sup> and CD11b<sup>-</sup> splenocyte populations. In the CD11b<sup>+</sup> cells, LPS treatment

increased pERK1/2 levels in the wild type mice and these effects were similar in the  $\beta$ -arrestin-1 and -2 knockout mice (Fig 9). In CD11<sup>b-</sup> splenocyte population however, there was no ERK activation (data not shown). Furthermore, we did not observe any consistent increase in either phospho-JNK or phospho-p38 after LPS treatment in the wild type splenocytes (both CD11<sup>b+</sup> and CD11<sup>b-</sup>) (data not shown). Taken together, these results indicate that neither  $\beta$ -arr-1 nor  $\beta$ -arr-2 KO mice splenocytes are defective in either I $\kappa$ B $\alpha$  or MAPK signaling. Therefore these results suggest that  $\beta$ -arrestins likely mediate TLR4-induced cytokine production independent of these pathways.

### **$\beta$ -arrestins regulate LPS-mediated histone deacetylase and histone acetyl transferase activities *in vivo***

Recent studies have suggested that  $\beta$ -arrestins not only play a role in cell signaling pathways, but also play an important role in gene expression by regulating histone acetyl transferases (HATs) (Kang et al., 2005). Other studies have shown that in addition to HATs, histone deacetylases also play a crucial role in chromatin modification and gene expression and that the balance of HAT and HDAC might eventually determine the outcome on gene expression. In addition, studies have shown that pharmacological modulation of HATs and HDACs lead to marked changes in inflammatory gene expression (Aung et al., 2006; Barnes et al., 2005; Choi et al., 2009a; Choi et al., 2009b; Choi et al., 2008; Kuo and Allis, 1998; Leoni et al., 2005; Sailhamer et al., 2008; Yu et al., 2002). Because neither I $\kappa$ B $\alpha$  nor MAPK signaling was consistently affected in  $\beta$ -arrestin-1 and -2 KO mice, we reasoned that  $\beta$ -arrestins might modulate chromatin modification and thus mediate production of several cytokines. To test this hypothesis, we obtained nuclear extracts from the spleen of wild type,  $\beta$ -arrestin-1 and -2 KO mice before and after LPS injection and performed HAT and HDAC activities. As shown in Fig 10, LPS injection in wild type mice led to a marked increase (2.5 fold) in the ratio of HAT:HDAC activity compared to the untreated wild type mice. Interestingly, compared to the wild type mice, HAT:HDAC ratio after LPS injection did not change in either  $\beta$ -arrestin-1 or -2 KO mice. These results suggest that LPS-induced increase in HAT:HDAC ratio *in vivo*, is mediated by  $\beta$ -arrestins.

Even though we observed specific differences in cytokine responses between  $\beta$ -arrestin-1 and -2 KO in the CD11<sup>b+</sup> cells, we did not see this difference *in vivo* using this whole spleen model likely because the CD11<sup>b+</sup> population constitutes only ~5% of the total cells. In the remaining CD11<sup>b-</sup> cells (~95%) role of  $\beta$ -arrestin-1 and -2 is similar with respect to TLR4-induced cytokine production. Further *in vitro* studies are necessary, however, to determine if  $\beta$ -arrestin-2 mediates TLR4 signaling in the CD11<sup>b+</sup> splenocytes through other mechanisms or if the mechanism of LPS-induced HAT/HDAC activities selectively utilizes only  $\beta$ -arrestin-2 and not  $\beta$ -arrestin-1 in this population. Based on the results presented here, we postulate that in the absence of  $\beta$ -arrestins, modulation of histone acetylation and chromatin modification by LPS is impaired. Because previous studies have demonstrated the importance of HAT/HDACs in the production of cytokines in response to LPS, we propose that  $\beta$ -arrestins modulate LPS-induced cytokine production and survival at least in part by regulating HAT:HDAC activity ratio.

It is important to note that in the present study we measured the total HAT and HDAC activities and therefore do not demonstrate which HDACs and HATs in particular are being regulated by  $\beta$ -arr-1 and -2. Also, whether  $\beta$ -arrestins directly regulate these enzymes or modulate them through other signaling pathways is not evident from our studies. In future work, we will determine members of HATs/HDACs that are modulated by LPS *in vivo* and, which HATs/HDACs are being regulated by  $\beta$ -arr-1 and -2, and whether there are cell-type specific differences. It is important to note that in addition to being critical for chromatin modification, HATs and HDACs also have cell signaling function i.e. acetylation/deacetylation of cell signaling proteins (Buchwald et al., 2009; Spange et al., 2009).



Therefore, it is also possible that the consequences of HAT/HDAC modulation by  $\beta$ -arrestins may in turn regulate other signaling molecules that might ultimately affect cytokine production. In addition, based on the role of chromatin modification in endotoxin tolerance (reviewed in (Biswas and Lopez-Collazo, 2009)), it is possible that the effect of  $\beta$ -arrestins on HAT/HDAC activity might reflect the ability of  $\beta$ -arrestin KO mice to modulate endotoxin tolerance. In this regard, Foster *et al* (Foster et al., 2007) showed that in LPS-tolerant macrophages, there are two subsets of genes being regulated: Inflammatory genes that are inhibited and, anti-microbial genes that are upregulated or are inducible. Based on its ability to regulate *in vivo* HAT/HDAC ratio, it would be interesting to test whether LPS-tolerant macrophages from  $\beta$ -arrestin KO mice are able to modify these subsets of genes during endotoxin tolerance. This will be examined in future studies.

Previous studies have shown that  $\beta$ -arrestin functions depend on the extracellular signal and the cellular context. Our studies suggest that *in vivo* it may depend on the pathogenic stimulus. In this regard, we reported that  $\beta$ -arr-1 and -2 have opposing functions in terms of innate immune response elicited by adenovirus vector (which signals via both TLR-dependent and -independent pathways) (Seregin et al.). We found adenovirus vector-induced cytokine response to be inhibited in the  $\beta$ -arr-1 knockout mice (similar to the present study). However, in contrast to the results presented here, we observed enhanced cytokine levels in response to adenovirus vector in the  $\beta$ -arr-2 knockout mice. Thus the role of  $\beta$ -arrestins, especially  $\beta$ -arrestin-2 in innate immune response may be dependent on the extracellular signal that induces the inflammatory response. This also translates into the possibility that  $\beta$ -arrestins' role in inflammatory disease may depend on the specific pathogenic aspect of the disease. It remains to be seen whether inhibition of  $\beta$ -arrestins' functions may be used as a strategy to treat inflammatory diseases.

In conclusion, our studies demonstrate that  $\beta$ -arrestin-1 and -2 have overlapping, non-redundant, previously undescribed positive regulatory role in TLR4 signaling *in vivo* and *in vitro*. Interestingly, majority of  $\beta$ -arrestins' functions are not compensated in one knockout by the presence of the other  $\beta$ -arrestin, in this LPS model. Furthermore,  $\beta$ -arrestin-1 and -2 have immune cell type-specific effects on TLR4 signaling, and their effects are largely independent of MAPK and  $\text{I}\kappa\text{B}\alpha$  pathways. Based on our results on HAT/HDAC activities in the spleen, we postulate that  $\beta$ -arrestins may mediate TLR4-induced cytokine production *in vivo* by modulating LPS-induced chromatin modification or by altering the acetylation status of some signaling proteins that may ultimately affect cytokine production and survival of mice.

## Acknowledgments

We wish to thank Dr. Robert J. Lefkowitz (Duke University) for his generosity in providing us with  $\beta$ -arrestin-1 and -2 knockout mice. We also appreciate the Michigan State University Laboratory Animal support facility for their assistance in the humane care and maintenance of the animals utilized in this work. We gratefully acknowledge the support of the National Institutes of Health grants (AR056680, HL095637 and AR055726) to N.P. We would also like to express our gratitude to Dr. Laura McCabe (Department of Physiology, MSU) for critical reading of this manuscript.

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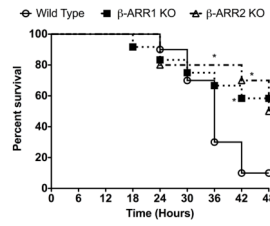
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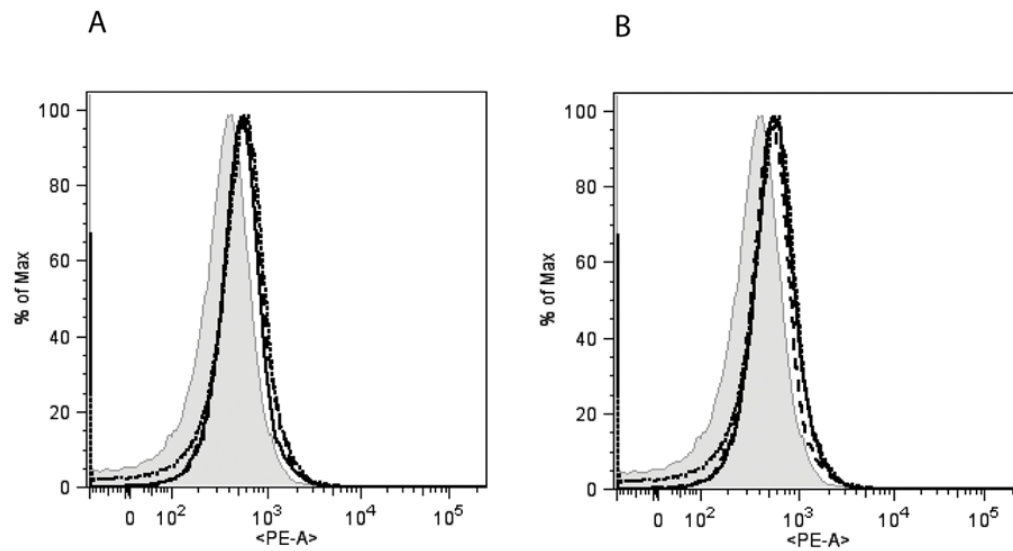
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**Figure 1. Endotoxin-induced mortality in wild type,  $\beta$ -arrestin-1 and -2 knockout male mice**  
 Wild type,  $\beta$ -arrestin-1 and -2 knockout mice were injected intraperitoneally with LPS (20  $\mu$ g/g body weight) and survival monitored for 48 hours. Survival curves were analyzed using Kaplan-Meier analysis with Graphpad PRISM software. P value = 0.05 for  $\beta$ -arrestin-1 KO mice compared to wild type; P value < 0.05 for  $\beta$ -arrestin-2 KO mice compared to wild type. N=10 mice for wild type and  $\beta$ -arrestin-2 KO each and N=12 mice for  $\beta$ -arrestin-1 KO mice. \*P < 0.05 compared to wild type by Fisher Exact Test.



**Figure 2. TLR4 expression in CD11<sup>b+</sup> splenocytes (A) and bone marrow cells (B) from wild type,  $\beta$ -arrestin-1 and -2 KO mice**

Cell surface expression of TLR4 in CD11<sup>b+</sup> splenocytes and bone marrow cells was determined by Flow-cytometry as described in the Methods. A representative experiment (showing the mean fluorescence intensity) from two such experiments is shown. Wild type=dotted line;  $\beta$ -arrestin-1 KO=dashed line and  $\beta$ -arrestin-2 KO=solid line; Isotype control (wild type)=shaded.

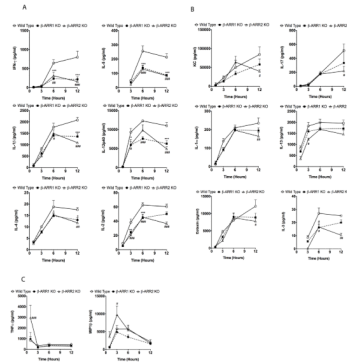
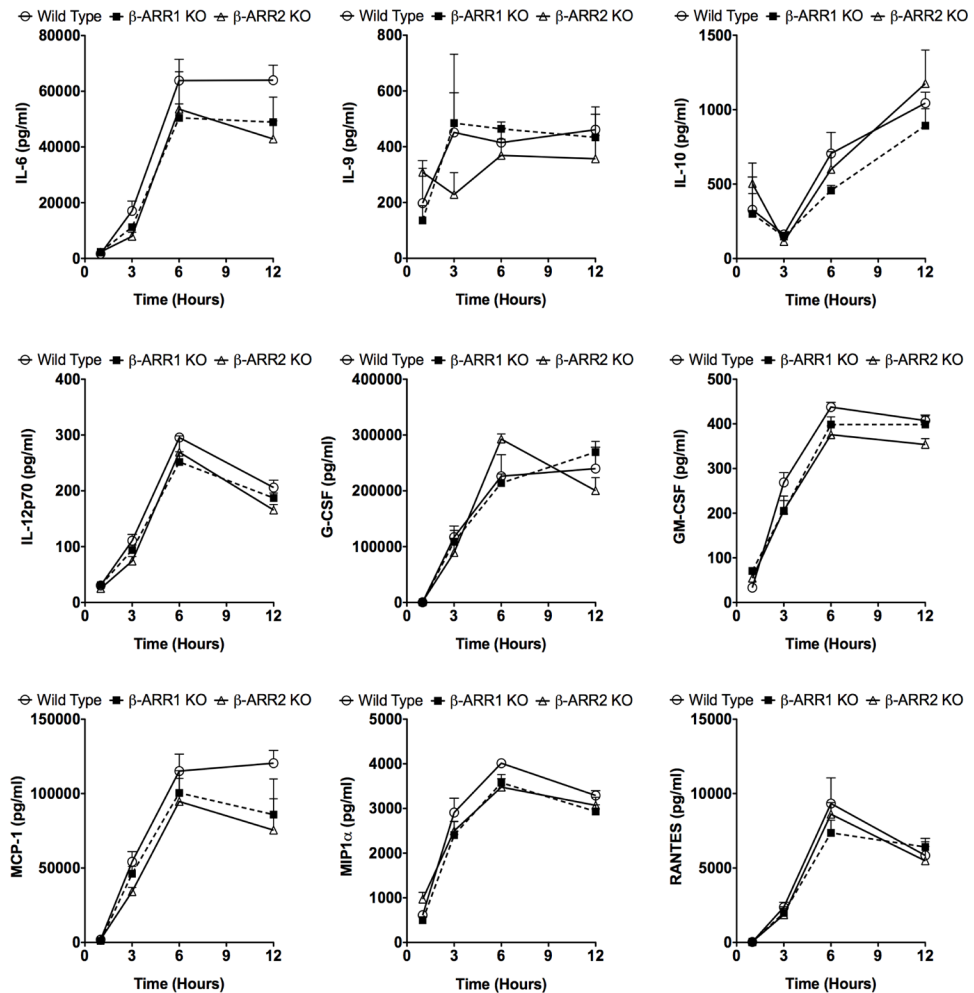


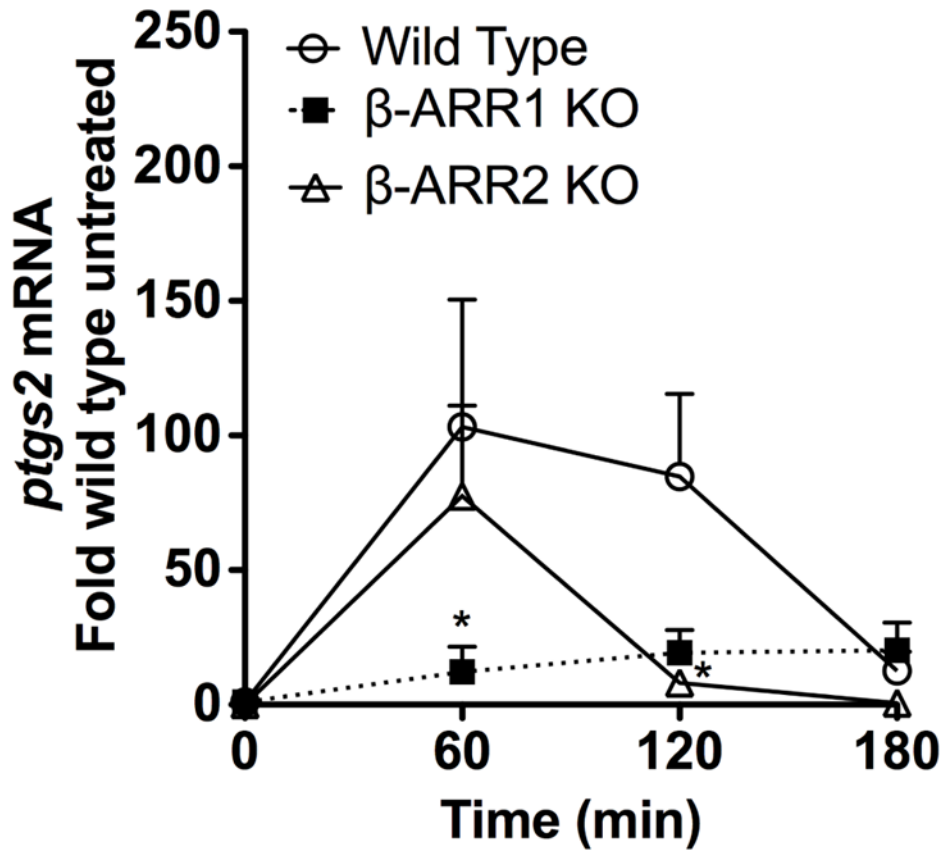
Figure 3D



**Figure 3. Inflammatory response in wild type,  $\beta$ -arrestin-1 and -2 knockout mice after lipopolysaccharide challenge**

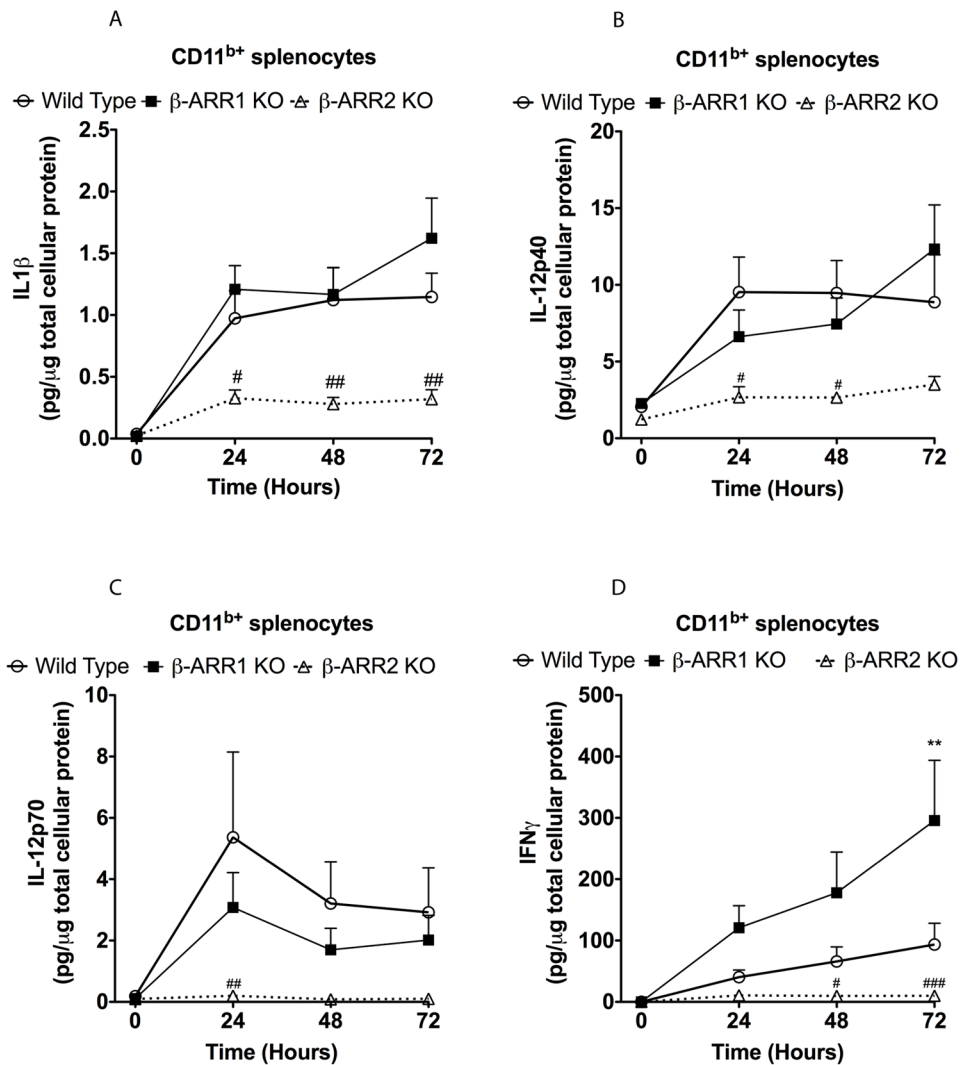
Wild type,  $\beta$ -arrestin-1 and -2 KO mice were injected intraperitoneally with LPS (20  $\mu$ g/g body weight) or vehicle and mice euthanized and plasma samples collected at the indicated time points. Cytokine/chemokine levels were measured using Biorad's 23-plex cytokine assay. Cytokine/chemokine levels were undetectable or were not different between uninjected WT,  $\beta$ -arr-1 and -2 KOs (data not shown). Cytokines/chemokines positively

regulated by both  $\beta$ -arrestins are shown in (A), cytokines/chemokines regulated only by  $\beta$ -arrestin-2 are shown in (B) and TNF $\alpha$  and MIP1 $\beta$  levels (which are negatively regulated by  $\beta$ -arrestin-2) are shown in (C). Cytokines/chemokines not regulated by  $\beta$ -arrestins *in vivo* are shown in (D). N=5 mice per genotype for each time point. Data were analyzed by two-way ANOVA with Bonferroni post-test. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared between  $\beta$ -arrestin-1 KO and wild type mice; #p<0.05, ##p<0.01, ###p<0.001 compared between  $\beta$ -arrestin-2 KO and wild type mice.



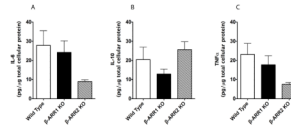
**Figure 4. Regulation of LPS-induced *ptgs2* mRNA by  $\beta$ -arrestins *in vivo***  
 Wild type,  $\beta$ -arrestin-1 and -2 KO mice were injected with LPS as described in Fig 2. Spleens were collected at the indicated time points after LPS-injection or from untreated mice (indicated as 0 time point). Messenger RNA levels for *ptgs2* was determined by real-time Q-RT-PCR. N=3 mice per genotype for each time point. Data were analyzed by two-way ANOVA with Bonferroni post-test. \* $p < 0.05$  compared between  $\beta$ -arrestin-1 or -2 and wild type mice at the respective time points.





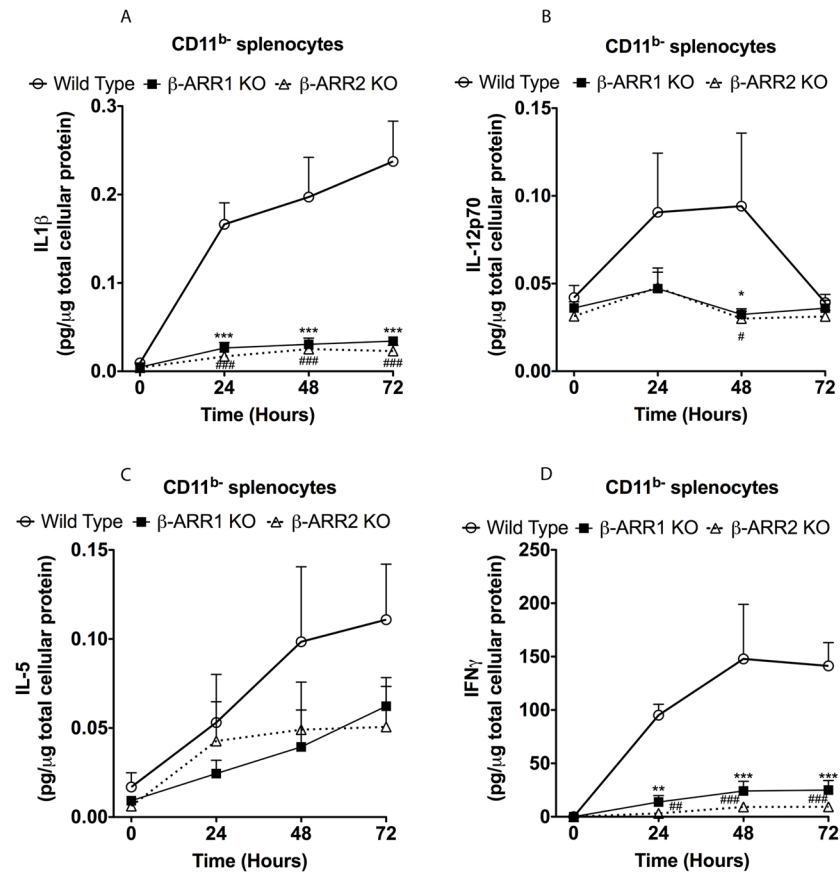
**Figure 5. Regulation of IL-1 $\beta$  (A), IL-12 (B&C) and IFN $\gamma$  (D) production by  $\beta$ -arrestins in CD11<sup>b+</sup> splenocytes**

CD11<sup>b+</sup> splenocytes were obtained as described in the methods. Cells were treated or not with LPS (5  $\mu$ g/ml) for the indicated time points, supernatants collected and cytokine levels determined by ELISA. The levels (in pg) were then normalized to  $\mu$ g of total cellular protein. N=5 mice per genotype. Data were analyzed by two-way ANOVA with Bonferroni post-test. #p<0.05, ##p<0.01, ###p<0.001 compared between  $\beta$ -arrestin-2 KO and wild type mice.



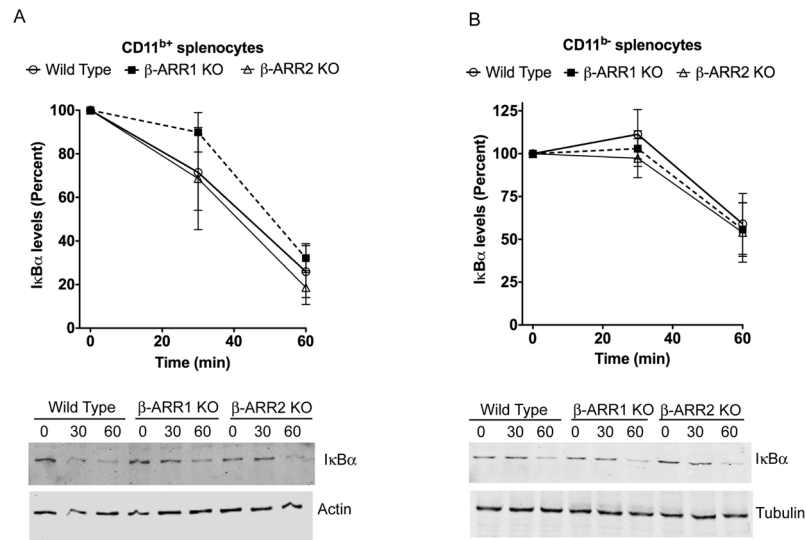
**Figure 6. Regulation of IL-6 (A), IL-10 (B), and TNF $\alpha$  (C) production by  $\beta$ -arrestins in CD11<sup>b+</sup> splenocytes**

Cell culture supernatants from LPS-treated (24 hour time-point) CD11<sup>b+</sup> splenocytes (experiment as described in Figure 4) were also tested for the levels of IL-6, IL-10 and TNF $\alpha$  by ELISA. N=5 mice per genotype.

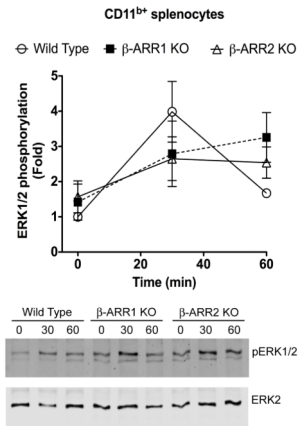


**Figure 7. Regulation of IL-1 $\beta$  (A), IL-12 (B), IL-5 (C) and IFN $\gamma$  (D) production by  $\beta$ -arrestins in CD11<sup>b+</sup> splenocytes**

Splenocytes were obtained as described in the methods. Cells were treated with LPS and ELISA and data analysis performed as described in Fig 3. N=5 mice per genotype. Data were analyzed by two-way ANOVA with Bonferroni post-test. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared between  $\beta$ -arrestin-1 KO and wild type mice; #p<0.05, ##p<0.01, ###p<0.001 compared between  $\beta$ -arrestin-2 KO and wild type mice.

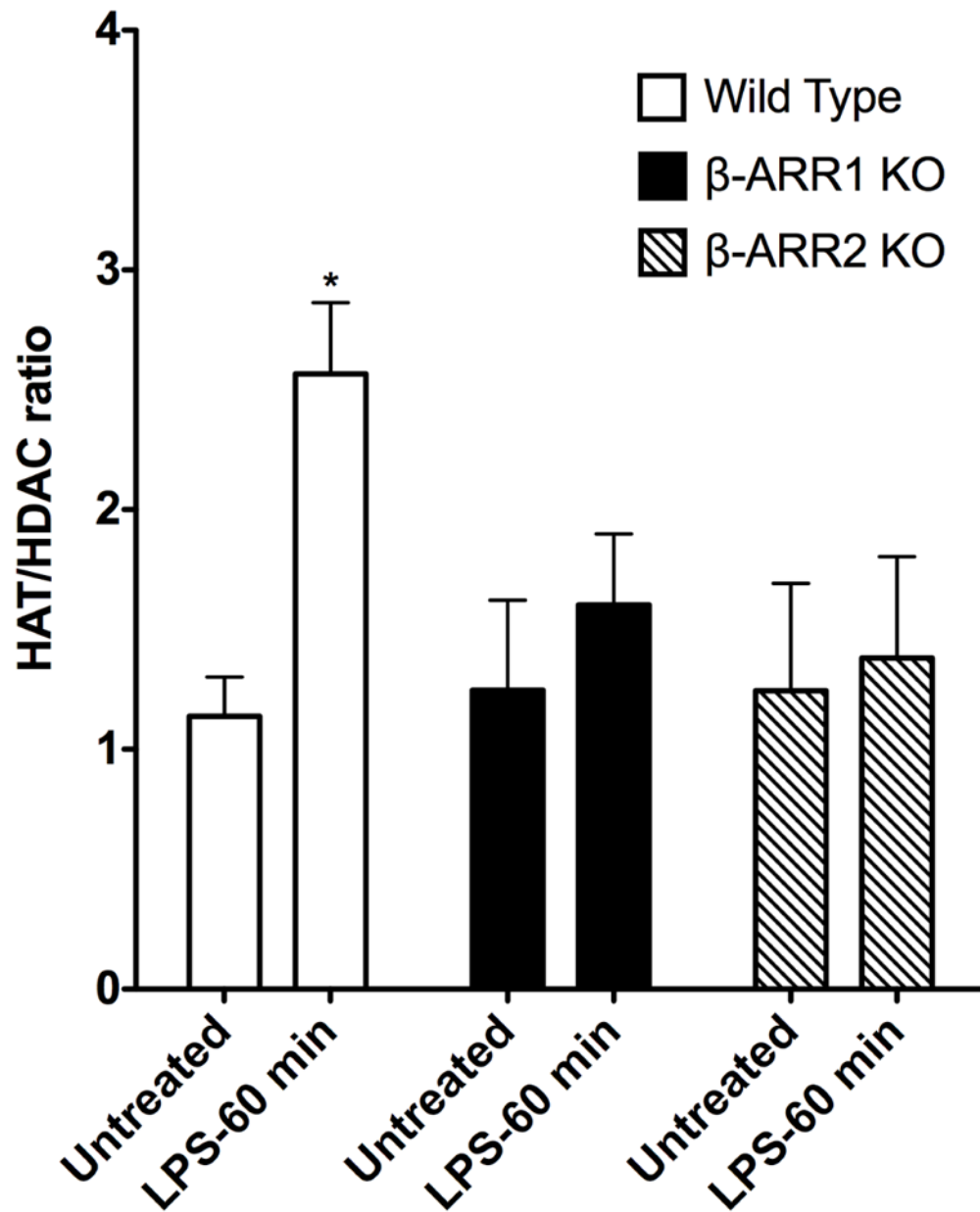


**Figure 8. Regulation of IκBα levels by β-arrestins in CD11<sup>b+</sup> (A) and CD11<sup>b-</sup> (B) splenocytes**  
 Splenocytes were obtained as described in the methods. Cells were then treated with LPS for the indicated time points and IκBα levels determined by Western blotting as described in the methods. IκBα levels were normalized to tubulin/actin for quantitation. N=4 mice per genotype. Quantitation is shown in the top and representative blots in the bottom.



**Figure 9. Regulation of ERK1/2 phosphorylation by  $\beta$ -arrestins in CD11<sup>b+</sup> splenocytes**  
 CD11<sup>b+</sup> splenocytes were treated with LPS for the indicated time points and lysates were Western blotted for pERK1/2 and ERK2 levels. pERK1/2 levels were normalized with ERK2 for quantitation. N=4 mice per genotype. Quantitation is shown in the top and representative blots in the bottom.





**Figure 10. Regulation of Histone deacetylase and histone acetyl transferase activities by  $\beta$ -arrestins *in vivo***

Wild type and  $\beta$ -arrestin KO mice were untreated or injected with LPS (for 60 min) and spleen collected and snap frozen. Nuclear lysates were extracted from the spleen and HAT/HDAC activities were determined as described in the methods. Results are expressed as ratio of HAT:HDAC activities in the untreated and LPS treated groups. N=3 mice each for  $\beta$ -arrestin-1 and -2 KO mice (per time point) and N=4 mice for wild type/time point. Data were analyzed by ANOVA with Bonferroni post-test. \*P<0.05 compared between wild type and  $\beta$ -arrestin-2 KO mice at the corresponding time point.