MULTIFACETED ROLE OF β-ARRESTINS IN INFLAMMATION

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ABSTRACT

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The overall goal of the study is to understand the role of β-arrestins (intracellular scaffolding cell signaling proteins) in inflammation and pathogenesis of sepsis and colitis using mouse models. Using polymicrobial sepsis model, we have demonstrated that both β-arrestin (β-arr) 1 and 2 are critical negative regulators of sepsis-induced inflammation. Inspite of major emphasis on the role of β-arrestins in immune cells, we found that the negative regulatory role of β-arr1 in sepsis-induced inflammation is infact, mediated by its function in the non-hematopoietic compartment. Having demonstrated that β-arr1 aggravated colitis in response to chemically induced colitis models, we further examined the role of β-arrestin2 in gut inflammation. Absence of β-arr2 caused greater extent of intestinal inflammation even in the absence of any exogenous stimuli. Further, T cells from peripheral lymphoid organs in β-arr2 knockout mice had dysregulated activation potential. Consequently, in the dextran sodium sulfate (DSS) model of colitis, β-arr2 knockout mice exhibited significantly higher indices of colitis compared to wild type (WT) mice. Additionally, T cells deficient in β-arr2 displayed altered T cell differentiation pattern with higher Th1 and lower regulatory T cell (Treg) polarization potential. As a result, the colitogenic potential of T cells deficient in β-arr2 as assessed in RAG T cell transfer model of colitis was found to be higher. The systemic wasting disease response though was ameliorated in RAG mice reconstituted with T cells lacking β-arr2, suggesting distinct role for β-arr2 at the active site of microbial interaction (gut) and systemic sites where the response is perhaps initiated by different ligands. Nevertheless, these results demonstrate inhibitory role for β-arr2 in T cell activation,

providing protection against overt intestinal inflammation. Our studies therefore suggest cell type specific role for β-arrestins in regulating inflammation that is highly context dependent and further work on discerning the involved molecular mechanisms will likely lead to therapeutic strategies to target β-arrestins in inflammation.

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KEY TO ABBREVIATIONS

AP-2: clathrin associate protein

ATP: Adenosine triphosphate

BALF: Bronchoalveolar fluid

BMM: Bone marrow macrophages

CAIA: Collagen antibody induced arthritis

cAMP: cyclic adenosine monophosphate

CD: Cluster of differentiation

CF: Cystic fibrosis

CFTR: Cystic fibrosis transmembrane protein

CLP: Cecal ligation and puncture

CREB: cAMP response element binding

CSF-1: colony stimulation factor

C-terminus: carboxy terminus

DAG: Diacylglycerol

DSS: Dextran sodium sulfate

EAE: Experimental autoimmune encephalomyelitis

ERK: extracellular signal related kinase

FPR: formyl peptide receptor

GAS: gamma activation site

GPCR: G protein-coupled receptor

GRK: G protein coupled receptor kinase

GDP: Guanine diphosphate

GSK-3β: Glycogen synthase kinase

GTP: guanine triphosphate

HAT: Histone acetylase

HDAC: Histone deacetylase

HEK: Human embryonic kidney

IBD: Inflammatory bowel disease

IFN: Interferon

Ig: Immunoglobulin

IGF: Insulin Growth Factor

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IL: Interleukin

IPF: Idiopathic pulmonary fibrosis

JAK: Janus kinase

JNK: c-Jun N-terminal kinases

LIGHT: lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells

LPA: Lysophosphatidic acid

LPS: Lipopolysaccharide

MAPK: Mitogen activated protein kinase

MBP: myelin binding protein

MCMV: mouse cytomegalovirus

MDC: macrophage derived chemokine

MEF: Mouse embryonic fibroblast

MIP: monocyte

MMP: matrix metalloproteases

MS: multiple sclerosis

MyD88: Myeloid differentiation primary response gene 88

NES: Nuclear export signal

NFκB: Nuclear factor

NK: Natural Killer

NO: Nitric oxide

NOS: Nitric oxide synthase

NSF: N-ethyl-maleimide sensitive fusion

N-terminus: amino terminus

OVA: ovalbumin

PAR: Plasminogen activator receptor

PBC: primary biliary cirrhosis

PDE: Phosphodiesterase

PHA: Phytohaemaglutinin

PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PMA: phorbol myristate acetate

PRR: Pathogen recognition receptor

RANTES: regulated on activation, normal T cell expressed and secreted

ROS: reactive oxygen species

SDF: Stromal derived factor

SHP: Src-homology containing phosphatase

SIRS: Systemic inflammatory response syndrome

STAT: Signal Transducers and Activators of Transcription

TGF: Tumor growth factor

TCR: T cell receptor

TNBS: Trinitrobenzenesulfonic acid

TNF: Tumor necrosis factor

TRAF: TNF receptor associated factors

TRAIL: TNF-related apoptosis-inducing ligand

TRIF: TIR-domain-containing adapter-inducing interferon-β

Treg: regulatory T

TLR: Toll like receptor

UC: Ulcerative colitis

UV: Ultraviolet

VSV: Vesicular stomatitis virus

CHAPTER 1

LITERATURE REVIEW

β-ARRESTINS: INTRODUCTION

Arrestins are cytosolic proteins that include four distinct members divided into three subfamilies based on sequence homology and tissue distribution. The sub-families include (i) visual arrestin (S antigen), (ii) cone arrestin (C- or X- arrestin) and (iii) β Arrestins (β-arrestin1 and β-arrestin2). While the first two are restricted to rods and cones respectively in the visual system, β-arrestins (1 and 2) are ubiquitous proteins. Arrestins are highly conserved across the animal kingdom, with 39-50% sequence homology observed between vertebrates and invertebrates; and 44-84% homology within the vertebrate animals[1]. Sequence analysis for known structural motifs reveals consensus phosphorylation sequence and ATP-GTP binding motifs[1]. β-arrestin was first identified as a protein involved in β-adrenergic desensitization[2] and in addition to G protein coupled regulatory kinases (GRKs) was shown to be critical for canonical G-protein coupled receptor (GPCR) desensitization[3-6]. Canonical GPCR signaling includes agonist binding-induced conformational change in GPCR, allowing it to interact with and activate associated heterotrimeric G protein. G protein activation involves exchange of bound GDP for GTP, leading to its dissociation into Gα and Gβγ that induce downstream signaling through secondary messengers like cAMP, calcium and diacylglycerol (DAG). Signal termination and receptor desensitization is brought about by concerted action of GRKs and arrestins. Agonist binding also induces receptor cytoplasmic tail phosphorylation by GRKs that acts as a docking site for β-arrestin binding. A receptor thus bound is sterically unable to interact with G proteins, terminating the signal followed by receptor internalization through clathrin-coated pits. Over the course of time, this

simplified view was expanded to include contribution of β-arrestins to other aspects of GPCR signaling including receptor endocytosis and β-arrestin mediated downstream signaling.

β-arrestins association with GPCRs divides the latter into two distinct categories on basis of receptor trafficking. Class A receptors that include adrenergic (β2, α1B), μ opiod, endothelin A and dopamine D1A receptors have a higher affinity for β-arrestin 2 compared to β-arrestin 1 and interact transiently with β-arrestins. β-arrestin is recruited to the receptor following agonist binding and internalized through clathrin-coated pits. β-arrestins then dissociate and translocate back to the cytosol, while the receptor proceeds to endosomal compartment for dephosphorylation and rapidly recycling. Class B receptors on the other hand have equal affinities for both β-arrestins and their interaction is more stable. Receptor-β-arrestin complex is internalized and targeted to endosomal compartment as a single unit. These receptors recycle slowly and include angiotensin AT1a, vasopressin V2, neurotensin 1 and neurokinin NK1 receptors.

The two β-arrestins have 78% identical amino acid sequence, with most differences observed in the C-terminus[7] with some distinct and overlapping functions. Their contribution to GPCR signaling is not entirely redundant and even though individual knockouts develop normally, double knockouts are embryonic lethal[8]. β-arrestin 1 (53 kda, chromosome 7) knockout mice though impaired in adrenergic response, are fertile, produce normal litter size and do not exhibit any histopathological alterations in heart, kidney, brain, intestine, spleen and lung tissues. They also display normal blood chemistry in terms of hemoglobin, hematocrit, WBC and RBC count and splenic lymphocyte reconstitution[9]. β-arrestin2 (46 KDa, chromosome 11) knockout mice too are

viable with no gross abnormalities and no differences in basal body temperature[10]. The knockout were reported to have slightly lower body weight and fat proportion once they reached the age of 12 weeks[11]; although in our colony we have observed that young knockout mice have reduced body weight that recovers as the mice get older (unpublished data).

ROLE IN CANONICAL GPCR SIGNALING

The canonical role of β-arrestins involves receptor internalization and desensitization wherein it acts as a scaffold by interacting with proteins involved in receptor uptake and recycling as described below.

Endocytic adapter proteins

Receptor internalization is an important feature of GPCR desensitization, resensitization and signal transduction. It employs clathrin mediated or caveolae mediated endocytic vesicles. β-arrestin recruitment was found to be required for receptor internalization and it was eventually characterized as a scaffolding protein in formation and movement of clathrin coated pits. β-arrestin (1 and 2) through C terminus interact with clathrin protein heavy chain motif (LIEL/P) stoichiometrically following β-adrenergic agonist stimulation[12,13]. β-arrestin1 dephosphorylation at ser412 residue in response to agonist stimulation is required for its interaction with clathrin heavy chain, receptor internalization[14] and signaling through Src and ERK1/2[15]. On the other hand, β-arrestin2 dephosphorylated at Thr-383 residue in response to agonist stimulation is irrelevant to its interaction with clathrin molecules and downstream signaling[16, 17]. Further, while β-

arrestin1 phosphorylation is mediated by ERK1/2[15], β-arrestin2 phosphorylation is carried out by casein kinase II [16, 17]. β-arrestins also interact with other components of clathrin endocytic machinery including clathrin adapter protein AP-2[18,19] and NSF (N-ethyl-maleimide sensitive fusion) protein, an ATPase essential for intracellular transport[20]. AP-2 interaction with β-arrestin 2 was demonstrated through yeast two hybrid and receptor complex analysis and was dependent on arginine residues in C-termini of β-arrestin2[19]. NSF interaction with β-arrestins is dependent on ATP-bound state of NSF and independent of β-arrestin phosphorylation status[20]. Through it's interacting partners, β-arrestins initiate the process of receptor endocytosis, internalization and desensitization and therefore are critical regulators of these physiological processes.

ROLE IN NON-CANONICAL SIGNALING

Processes regulated by β-arrestins in response to GPCRs that form the paradigm of non-canonical signaling, including crosstalk between GPCR and non-GPCR with implications in inflammatory responses are discussed below:

Signal transduction

Mitogen Activated Protein Kinases (MAPK)

JNK3

c-Jun N-terminal kinases (JNK) are often activated following a stress response. The family includes three genes (JNK1, JNK2, JNK3) expressed in two isoforms p54 and p46. The cascade includes sequential phosphorylation by MAP3K 9ASK1, MEKK,

MLK) of MAP2K (MKK4, MKK7) that eventually activates JNK. Activated JNK can phosphorylate various cytosolic and nuclear targets. β-arrestin2 constitutively colocalizes with JNK3 in COS-7 cells even under basal conditions and is required for ASK-1 mediated JNK3 activation. This interaction was specific since neither β-arrestin1 nor JNK1 co-immunoprecipitated with a corresponding partner. β-arrestin2 interacts directly with ASK-1 and JNK3 through amino- and carboxy- termini respectively, although its interaction with upstream MKK4 is indirect, with the signalosome forming a complete MAPK module. In context of GPCR signaling, angiotensin induced JNK3 activation and endosomal translocation is β-arrestin2 mediated, demonstrating the ability of β-arrestin2 to regulate activation and subcellular localization of JNK MAPK module[21].

ERK1/2

Extracellular signal-related kinase (ERK) activation is associated with a proliferative response and similar to JNK activation involves a sequential activation of various kinases. Following activation, ERK1/2 translocate to nucleus and induce transcription of genes involved in mitogenesis and proliferation. The ability of β-arrestins to affect ERK1/2 activation was first shown in β2 adrenergic signaling; wherein its interaction with c-src and ability to induce receptor internalization were both required for optimal ERK activation[15]. In response to PAR2 agonist, β-arrestin forms a multimolecular complex with the receptor, Raf-1 and activated ERK, with p-ERK1/2 sequestered in cytosol, rendering its role of transcription activation and mitogenic potential ineffective[22]. Similar complex (Raf-1, receptor, β-arrestin and ERK)[23] and β-arrestin mediated sequestration of activated ERK1/2 in cytosol and decreased Elk driven transcription was also observed in response to angiotensin-II type 1a receptor (AT1aR) signaling[24].

Substance P mediated ERK1/2 signaling though occurs through a complex between NK1R, β-arrestin and Src, followed by nuclear translocation of pERk1/2 and downstream effects of proliferation and protection from apoptosis[25]. Therefore, similar to its regulation of JNK signaling, β-arrestins can scaffold both ERK and its upstream signaling mediators (Src, Raf-1) and can regulate both activation status and sub cellular localization of ERK MAPK. A model for the effect of activated ERK cellular location has been proposed, wherein nuclear ERK activates Elk driven transcription and cell proliferation; while cytosolic ERK phosphorylates other cellular targets, perhaps affecting crosstalk with other pathways[26,27].

p38 MAPK

p38 MAPK is another family of MAPK activated in response to a stress, including cytokines, LPS and growth factors. p38 activation in response to β2 adrenergic agonist is biphasic, with the first peak observed at 10 minutes, followed by another peak at 90 minutes, which is sustained for 6 hours. While the late activation is Gs/cAMP/PKA mediated, early activation is β-arrestin1 dependent. β-arrestin1 knockdown additionally inhibits Rac 1 membrane translocation, which too is required for early p38 activation, in addition to NADPH oxidase activity[28]. Early p38 activation therefore occurs via a β-arrestin1/Rac1/NADPH oxidase pathway while late activation occurs through canonical Gs signaling.

Nuclear Factor κB (NFκB) Pathway

NFκB includes a family of transcription factors that in basal state is kept inactivated by interaction with its inhibitory binding partner, IκBα. Activation of pathway includes

phosphorylation-induced degradation of IκBα, NFκB dimerization and translocation into the nucleus where it activates target genes. The role of β-arrestins in NFκB is highly controversial depending on the context. β2 adrenergic receptor induced NFκB activation is negatively regulated by β-arrestin2, which binds and stabilizes IκBα[29]. It also inhibits UV induced NFκB activation and anti-apoptotic signaling, that is further enhanced by β2 adrenergic stimulation[30]. In this case, the interaction with IκBα is regulated by the phosphorylation status of β-arrestin2 that can be modulated by UV and β2 adrenergic stimuli[29]. On the other hand, β-arrestin2 acts as a positive regulator of this pathway in response to lysophosphatidic acid (LPA)[31] and β-arrestin1 too acts as a positive regulator of endothelin induced NFκB activation, perhaps through its nuclear interaction[32].

Transcriptional regulation

Epigenetic modulation in form of histone acetylation that regulates chromatin structure and promoter accessibility is an important mode of transcriptional control mediated by a family of histone acetylases (HAT) and histone deacetylases (HDAC). In response to delta-opioid receptor signaling, β-arrestin1 through its interaction with HAT p300 mediates cAMP response element binding protein (CREB)-dependent promoter acetylation and transcription of proteins p27 and c-fos[33]. β-arrestin1 also regulates histone acetylation mediated gene transcription of anti apoptotic gene bcl-2 in CD4+ T cells[34]. In T cells isolated from primary biliary cirrhosis patients, β-arrestin1 can modulate expression of genes by virtue of affecting histone H4 acetylation. It positively mediates expression of CD40L, LIGHT, IL-17 and IFNγ while downregulating TRAIL,

Apo2 and HDAC7A expression[35]. These examples ascribe a critical scaffolding role to nuclear β-arrestin1 in regulating gene transcription.

Ubiquitination

Mdm2 is an E3 ubiquitin ligase protein that mediates p53 ubiquitination and proteosomal degradation. Its role in GPCR signaling was deciphered when it was found to interact with and ubiquitinate both β-arrestins and β2 adrenergic receptor. β-arrestin acts as a scaffolding protein to mediate that interaction; and ubiquitination of β-arrestin and receptor is essential for receptor internalization and degradation respectively[36]. β-arrestin2 by virtue of having a Nuclear Export signal (NES) also critically affects the subcellular localization of mdm2[37]. In response to agonist binding (dopamine or β2 adrenergic) receptor, β-arrestin and mdm2 form a ternary complex, reducing mdm2 self-ubiquitination that leads to lower p53 ubiquitination and subsequent degradation. β-arrestin2 expression level is thus able to affect p53-mediated apoptosis with higher level leading to mdm2 sequestration, higher p53 mediated apoptosis and vice versa[37]. β-arrestins therefore, provide an important link between GPCR and p53 signaling.

Apoptosis

The signal for apoptosis regulation by β-arrestins can originate from both GPCR and non-GPCR stimuli utilizing multiple pathways including MAPK and PI3K-AKT pathways. β-arrestin1 couples IGF-1 receptor to PI3K-AKT signaling in a G protein and ERK- independent mechanism to provide cytoprotective signal[38]. β-arrestins also protect against cell death caused by inadvertent ROS production in response to IL-8/CXCR2.

Double knockout (β-arrestin1/2) MEFs exhibit sustained MAPK activation, increased Rac-1 membrane translocation causing greater ROS production and consequent cell death[39]. Another study demonstrated a protective role of β-arrestins in cell death induced in response to multiple GPCRs- FPR, angiotensin II (type 1A) receptor, vasopressin and CXCR2 receptors[40]. In HEK293 cells, FPR in the absence of β -arrestins induces an apoptotic pathway involving PI3K, MAPK and Src activation leading to cytochrome c release and cleavage of caspases 3 and 9. Reconstitution with either -arrestins was able to inhibit this pathway, providing a survival benefit[40]. β-arrestins by virtue of interacting with ASK-1 protein, are capable of modulating its expression through ubiquitin mediated proteosomal degradation. This interaction is increased in response to hydrogen peroxide treatment, providing a cryoprotective signal[41]. β-arrestin2 also protect vascular smooth muscle cells and MEFs from hydrogen peroxide and ectoposide induced apoptosis. Further Angiotensin II survival signal that protects from above mentioned stimuli also requires β-arrestin2. The anti-apoptotic signal mediated through ERK and AKT leads to BAD phosphorylation changing its affinity for binding partners, bcl-xl and 14-3-3. In MEFs lacking β-arrestin2, AngII induced (i) ERK/AKT/BAD phosphorylation, (ii) reduction in BAD bound bcl-xl and (iii) increase in BAD bound 14-3-3 is reduced or abolished leading to loss of protective signaling[42]. This ability of β-arrestins to modulate ERK and AKT pathway for protection from apoptosis is also observed for β-arrestin1 in response to GLP1 signaling in pancreatic-beta cells[43] and β-arrestin2 in response to resveratrol[44] respectively. Staurosporine induced cell death is inhibited by β-arrestin1 in astrocytes[45] and by β adrenergic stimulation through a anti-apoptotic signalosome comprising of hsp27 and β-arrestins in human urothelial cells[46]. This cryoprotective

interaction with hsp27 is also observed for β-arrestin2 in response to apoptotic TRAIL stimulation[47]. Morphine induced cell death in breast cancer cell lines MCF-7 and MDA-MB231 is antagonized by β-arrestin2 through AKT and caspase 8 regulation[48]. β-arrestin2 is also protective in morphine induced lymphocyte apoptosis in conjugation with HIVgp120 priming[49] and TLR2 activation[50]. It also protects against serum starvation induced apoptosis that is increased further by TLR4 expression in HEK293 cells, via its ability to stabilize GSK-3β[51]. Further, both β-arrestins are protective in serum starvation induced apoptosis in MEFs through modulation of MAPK and AKT pathways[52]. Surprisingly though double knockouts exhibit apoptosis similar to wild type MEFs, suggesting overlapping and distinct roles for both β-arrestins in regulating apoptosis[52]. Contrary to these anti-apoptotic roles of β-arrestin2, colony stimulating factor (CSF-1) withdrawal induced cell death in bone marrow macrophages (BMM) is positively regulated by β-arrestin2 as demonstrated by higher viability in knockout BMM[53]. β-arrestin2 also positively affects p53 mediated apoptosis in response to GPCR stimulation by suppressing self ubiquitination of oncogene mdm2[37]. Additionally, in response to GABAB stimulation, β-arrestin stimulate JNK activation and consequent apoptosis in cancer cell lines, MCF-7 and T-47D[54]. β-arrestins can therefore regulate both pro- and anti- apoptotic signaling and its modulation by various GPCR agonists. The role of β-arrestins in apoptosis is further summarized in table 1.1.

Table 1.1: Role of β-arrestins in apoptosis

Signal Inducer	β-arrestin	Role	Mechanism	Reference
IGF-1	1	Protective	PI3K-AKT	[38]
IL-8/CXCR2	1/2	Protective	MAPK	[39]

Table 1.1 (Cont'd)

GPCR (fpR,CXCR2)	1/2	Protective	PI3K, MAPK	[40]
H_2O_2	1/2, 2	Protective	ASK-1, ERK/AKT/BAD	[41, 42]
H_2O_2 + Angiotensin	2	Protective	ERK/AKT/BAD	[42]
Morphine	2	Protective	AKT	[48]
Morphine + *HIVgp120*	2	Protective		[49]
TRAIL	2	Protective	Hsp27/Src	[47]
Resveratrol	2	Protective	AKT/GSK3β	[44]
Staurosporine	1	Protective	PI3K/AKT	[45]
Staurosporine + β2-adrenergic agonist	1/2	Protective	Hsp27	[46]
GLP1R	1	Protective	ERK/bad	[43]
GPCR	2	Susceptible	Mdm2/p53	[37]
GABAB	1/2	Susceptible	JNK	[54]
M-CSF withdrawal	2	Susceptible		[53]

The stimuli is mentioned in bold, secondary signaling that is cryoprotective is underlined while the one that further aggravates apoptosis is in italics.

β-ARRESTINS IN IMMUNE SYSTEM

Development of immune system is uncompromised by lack of β-arrestins. Equivalent proportion of neutrophils, lymphocytes, monocytes and eosinophils are observed in bone marrow[55]. Further splenic[9, 56, 57], thymic[57] and blood[56] composition of immune cells is unaffected by loss of β-arrestins. Equivalent CSF-1 and F4/80 expression is observed on BMM derived from WT or β-arrestin2 knockout mice[53]. Inflammatory response though in the absence of β-arrestins is altered by various stimuli via involvement of distinct and multiple pathways as described in following sections.

G-PROTEIN COUPLED RECEPTORS

Chemokine receptors

In the immune system, chemotaxis is an essential process for migration of immune cells to the site of inflammation, induced by cell polarization and motility. The role of β-arrestins in chemotaxis stems from their ability to regulate GPCR desensitization and signaling, interaction with clathrin adapters and signaling scaffolds. Most chemokine receptors are GPCRs associated with Gai or Gq subunits. β-arrestins can associate with various chemokine receptors, including CCR2 (mono mac-1 cells)[58], CXCR1[59], CXCR2[59,60], CXCR4[61,62,63], CXCR4:CXCR7 heterodimers[64], CCR5[63]. The role of β-arrestins in receptor desensitization and signaling following ligand binding is presented in table 1.2.

Table 1.2: Role of β-arrestins in chemokine receptor trafficking and signaling.

Receptor	Receptor trafficking	Signaling	β-arrestin	Reference
FPR	Effective recycling but not internalization	-	1/2	[65]
CXCR1	Internalization	-	1/2	[66]
CXCR2	Internalization	Higher calcium mobilization, GTPase activity and superoxide production	2	[67]
CXCR4	Internalization and desensitization	ERK and p38 activation	2	[61,62, 63]
CXCR4/7 (heterodimer)		ERK, p38 and JNK activation	2	[64]
CCR5	Desensitization	ERK and p38 activation; formation of multimeric complex containing Lyn, PI3K, Pyk2 and ERK	1/2	[63, 68,69]
CCR5/C5aR (heterodimer)	Internalization	?	1/2	[70]
PAR2	Internalization (1)	ERK activation	1/2	[71,22, 55, 72]

Chemokine signaling induced migration and other responses including degranulation are also regulated by β-arrestins and are summarized below.

Neutrophils

Even though CXCR2 mediated signaling is dependent on β-arrestins[67]; neutrophil infiltration in air pouch and cutaneous wound healing models was significantly elevated in β-arrestin2 knockout mice[67]. Neutrophil infiltration is also significantly elevated in β-arrestin2 KO mice in response to intraperitoneal oyster glycogen injection[73]. This highlights the differential regulation by β-arrestin2 of various aspects of CXCR2 signal transduction and directional migration. In response to PAR2 ligand, β-arrestins (1 and 2) are required for actin reorganization, pseudopodia polarization and subsequent directional migration. Therefore, leukocytes lacking β-arrestins (1 and 2) also display loss of chemotactic response to PAR2 ligands[55]. In neutrophils, stimulation with high concentration of IL-8 can also induce granule release via CXCR1- Src kinase (c-Hck and Fgr) mediated process. CXCR1 phosphorylation and association of β-arrestins (1 /2) with phosphorylated src kinase isoforms is essential for granule release in neutrophils[74]. β-arrestins therefore plays a diverse role in neutrophil migration as shown in response to various stimuli.

Lymphocytes

Defective chemotaxis was observed for lymphocytes lacking β-arrestin2 in response to CXCL12 (through CXCR4) in both transwell and trans endothelial assays, even though

GTPase activity in response the chemokine was higher[75]. Surprisingly though, β-arrestin2 deficient T and B cells also have an inconsistent increase in baseline chemokinesis[75] even though their numbers in spleen under homeostatic conditions are unaffected. CXCR7 though itself not coupled to a G protein is capable of forming heterodimers with CXCR4 and potentiate migration in response to SDF-1α. In U87-CD4, migration through the heterodimer in response to stromal derived factor-1α (SDF-1α) is β-arrestin2 dependent[64]. In allergic asthma model, lung lymphocyte infiltration is largely abrogated in mice lacking β-arrestin2[76]. As opposed to negative regulation in neutrophils, β-arrestin2 in lymphocytes is perhaps required for directional migration, atleast under conditions studied thus far.

Macrophages

In primary human macrophages, knockdown of β-arrestin 1 and 2 drastically decreases chemotactic response to MIP1β (CCR5)[69].

Other Cells

In non-immune cells such as HeLa and HEK293 cells, β-arrestin2 positively mediates SDF-1α induced chemotaxis and increases its efficiency[63]. In HEK293-CCR5, β-arrestin2 overexpression enhances CCR5 mediated chemotaxis in response to RANTES[63]. PAR2 mediated chemotaxis in breast cancer cell line MDA-MB 231 is impaired in the absence of both β-arrestins (1 and 2)[72]. β-arrestins are therefore required for chemotaxis of non-immune cells and this regulation can have implications in wound repair and cancer.

Complement receptors

C3aR

C3a is a GPCR that induces chemotaxis and degranulation in mast cells, basophils and eosinophils, playing important role in innate responses and asthma. In basophilic leukemia cell line RBL-2H3, receptor phosphorylation that causes β-arrestin recruitment to agonist bound C3aR was shown to induce CCL2 production but inhibit degranulation, suggesting distinct role for β-arrestin in mediating these processes[77]. In mast cells, C3aR induced signaling is differentially regulated by β-arrestin 1 and 2. While β-arrestin2 is required for receptor internalization and desensitization, β-arrestin1 is critical for degranulation. Further while both β-arrestins negatively regulate early ERK activation, only β-arrestin2 inhibits C3aR induced NFκB activation and consequent CCL4 (MIP1β) production[78]. β-arrestins thus have distinct and overlapping roles to play in C3aR mediated responses.

C5aR

C5aR signaling is mediated by two receptors, C5aR expressed at cellular membranes and intracellular C5L2. G protein coupling, however, is exclusively associated with C5aR. In response to agonist binding, C5aR is internalized and colocalizes with C5L2 and β-arrestin[79], suggesting an important role for β-arrestin in C5aR internalization.

In addition to complement receptor signaling, expression of complement genes can also be modulated by β-arrestins. C1q genes (a,b and c) is significantly lower in bone marrow macrophages lacking β-arrestin 2 (and not 1), both basally and in response to LPS stimulation[53].

Plasminogen activated receptor 2 (PAR2)

PAR2 is activated by protease trypsin leading to calcium mobilization and protein kinase C (PKC) activation. Its desensitization involves distinct mechanisms of irreversible receptor cleavage by trypsin, PKC mediated termination and endosomal receptor degradation[80]. Mast cell degranulation activates PAR2 leading to calcium mobilization and redistribution of tight junction proteins ZO-1 and occludin and perijunction protein F-actin. This process leads to increased transepithelial permeability and is affected by β-arrestin mediated ERK activation[81]. β-arrestins in response to PAR2 form a macromolecular complex with ERK, leading to p-ERK1/2 sequesteration in the cytosol; rendering its transcription and mitogenic activity ineffective[22]. PAR2 regulated increase in transepithelial permeability has huge implications in stress and inflammatory responses and could be modulated by β -arrestins.

NON G-PROTEIN COUPLED RECEPTORS

Toll Like Receptors (TLRs)

β-arrestins are widely expressed in macrophages and the expression β-arrestin1 is decreased in response to TLR2/4 (not TLR3/7) stimulation via a JNK-mediated mechanism involving both reduced transcriptional and increased degradation of the protein[82]. Even though the expression of β-arrestin2 is unaffected in primary macrophages (thioglycollate elicited) in response to LPS, it is reduced in RAW cells[83]. Further, in response to LPS β-arrestin2 stabilizes IκBα, thereby reducing NFκB

activation and NOSII expression[83]. This reduction is TRIF induced[84] and β2 adrenergic receptor mediated[83]. In another study using RAW cells, β-arrestin1 acts as a binding partner for NFκB1 p105 (p105), decreasing the downstream TPL2-MEK1/2 induced ERK1/2 activation[85]. In MEFs, both β-arrestin 1 and 2 negatively regulate NFκB activation while β-arrestin2 positively regulates ERK1/2 activation. Consequently, IL-6 production is lower following β-arrestin2 knockdown while both β-arrestin 1 and 2 were required for IL-8 production[86]. β-arrestin2 also negatively regulates cytokine production in response to LPS stimulation in BMMs[87]. In THP-1 cells, β adrenergic stimulation dampens IL-8 and TNFα production in response to TLR4 stimulation via a β-arrestin 2 mediated redistribution of surface TLR4/CD14 receptor[88]. Glycogen oyster elicited neutrophils from β-arrestin2 knockout mice produce greater extent of IL-6 and TNFα both basally and in response to LPS[73]. Consistent with this, thioglycollate elicited neutrophils from β-arrestin2 knockout mice respond to LPS stimulation with higher production of IL-6 and IL-10[56]. In addition to cytokine production, β-arrestin2 protects against serum starvation induced apoptosis aggravated by TLR4 signaling through its ability to stabilize GSK-3β[51]. β-arrestins can therefore differentially affect multiple facets of TLR4 signaling in different cell types.

Other studies have also shown in different cell types that in response to TLR (LPS, polyIC) and IL-1β ligands β-arrestins bind and inhibit TRAF6 (a E3 ubiquitin ligase protein) mediated NFκB activation. In this regard, the carboxy-terminus of β-arrestins interacts with TRAF6 through its TRAF domain, blocking the polyubiquitination site (K48) required for self-ubiquitination and IκB activation. Consequently, cytokine

production is higher in β-arrestin2 deficient BMM, in response to TLR4, TLR3 and TLR9 ligands[87].

Cytokine production to adenovirus infection is mediated through TLR, MyD88 and TRIF mediated signaling[89, 90]. β-arrestins differentially regulate chemokine and cytokine production following *in vitro* and *in vivo* adenovirus infection[91]. β-arrestin1 and β-arrestin2 act as positive and negative regulator respectively, suggesting that their modulation could alter adenoviral response and influence the use of adenovirus gene therapy.

Tumor Necrosis Factor Receptor (TNFR)

In HeLa cells, β-arrestin1 directly interacts with IκBα, as demonstrated by yeast two hybrid experiments and functions as a negative regulator of TNFα induced NFκB activation[92]. In MEFS though, β-arrestin (1/2) knockdown did not affect TNFα induced NFκB activation and IL-6 production [31]. On the other hand, in 3T3-L1 adipocytes, TNFα induces Src activation induced Gαq/11 phosphorylation that is β-arrestin 1 dependent. Further, downstream effects of ERK and JNK phosphorylation, MMP3 production and lipolysis are dependent on β-arrestin 1 and not the interacting G protein[93]. β-arrestin can thereby affect crosstalk between inflammatory and G protein signaling.

Transforming Growth Factor β Receptor (TGFβR)

β-arrestin2 mediates TGFβRIII internalization, thereby downregulating TGFβ signaling and its anti-proliferative role[94]. TGFβRIII signaling reduces migration via cdc42 and β-arrestin2 dependent actin remodeling process[95]. Further, TGFβ mediated Treg induction

is impaired in β-arrestin2 knockout mice in both, *in vitro* polarization assays and in secondary lymphoid organs in EAE model[96]. Thus, β-arrestin2 can modulate anti-proliferative and T cell differentiation potential downstream of TGFβ signaling, thereby affecting cancer progression, metastasis and T cell mediated immunopathologies.

Interferon receptors

IFNγ exhibits potent antiviral activity and signals through a STAT1 mediated pathway. IFNγ induces STAT1 tyrosine phosphorylation, dimerization and nuclear translocation where it binds target genes with IFNγ activation site (GAS) and induces transcription. It is antagonized by dephosphorylation by nuclear phosphatase TC45. β-arrestin1 interacts with both nuclear STAT1 (active) and TC45, as demonstrated through coimmunoprecipitation assays and affects STAT1 dephosphorylation, reducing the potency of IFNγ signaling[97]. MEFs and HeLa cells lacking β-arrestin1, have sustained STAT1 phosphorylation and greater induction of IFNγ inducible genes. Further, MEFs lacking β-arrestin1 pretreated with IFNγ before VSV infection have lower host cell death and viral loads; demonstrating a negative regulatory role for β-arrestin1 in IFNγ induced antiviral activity[97]. Viruses can modulate and interfere with STAT1 mediated IFN response[98]. β-arrestin1 expression increases for 8 days following Hepatitis B Virus infection before returning to baseline level in the liver (site of infection) but not the spleen[97]. This expression profile coincides with observed viral loads[99], suggesting that β-arrestin1 could be part of the machinery affecting IFN induced anti-viral response and modulation of its expression could be an effective therapeutic strategy.

IFNβ, an important type I IFN with immunomodulatory properties, used in MS therapy and is believed to reduce lymphocyte activation. In human mononuclear leukocytes, IFNβ rescues phytohaemagglutinin (PHA) induced decrease in β-arrestin1 protein expression. By itself, it increases β-arrestin1 mRNA expression but not protein levels[100], suggesting that β-arrestin1 could have a potential role in IFNβ mediated effects although it has not been demonstrated yet.

Natural Killer inhibitory receptors

NK cells have a repertoire of activating receptors, NKG2D and Natural cytotoxicity receptor (NCR) - NKp46/44/30 that induce signaling through phosphorylation of downstream signaling molecules to induce cytokine production or cytotoxicity against viral and tumor targets. They also have inhibitory molecules- killer immunoglobulin like receptors (KIRs) and NKG2A/C/E in humans and Ly49 in mice that by employing phosphatases counter the activation signal. β-arrestin2 is an important player in this KIR mediated cytotoxicity inhibition. It forms a complex with KIR2DL1 and phosphatases Src homology containing phosphatases-1 and 2 (SHP-1 and SHP-2) that counter and inhibit signal transduced through activating receptors. Similarly, mouse NK cells cytotoxicity is inversely proportional to β-arrestin2 expression as shown using NK cells from β-arrestin 2 transgenic (overexpression) and knockout mice. Further human KIR2DL allelic heterogeneity and inhibitory potential is associated with its ability to recruit β-arrestin2 and SHP-1 to receptor complex[101]. This ability of β-arrestin2 to affect NK cell mediated cytotoxicity has implications in mouse cytomegalovirus (MCMV) infection model that is largely dependent on NK cell activity. β-arrestin2 transgenic mice,

therefore, have higher viral loads, as there is greater inhibition to NK cell cytotoxicity[102]. T cell Immunoglobulin and ITIM domain receptor (TIGIT) is another inhibitory molecule expressed on NK cells, Treg, CD8+ and CD4+ T cells. In NK cell line YTS; it negatively regulates NFκB activation and IFNγ production in response to binding of its ligand poliovirus receptor (PVR) expressed on target cells. These inhibitory activities too are dependent on its ability to interact with β-arrestin2[103].

T Cell Receptor (TCR)

T cell receptor (TCR) ligation with cognate MHC: peptide complex activates cyclic AMP (cAMP) production that via PKA-Csk1 pathway inhibits optimal T cell stimulation. The inhibitory effect of cAMP is relieved by CD28 stimulation that recruits phosphodiesterase 4 (PDE4) in a PI3K and β-arrestin dependent manner. PKB-β-arrestin-PDE4 complex can be isolated from membrane fraction following CD3/28 stimulation and is inhibited by PI3K and Src inhibition[104]. Human primary T cells stimulated with CD3/28 for 20 hrs produce lower IL-2 and IFNγ following β-arrestin knockdown suggesting a positive role for β-arrestins in proximal T cell signaling[104]. Another study showed altered T cell differentiation in response to TCR stimulation in β-arrestin2 knockout cells. Even though the knockout T cells differentiate into Th1, Th2 and Th17 cells in proportion equivalent to the WT T cells, their polarization to regulatory T cells (Treg) is impaired. This impaired Treg differentiation has implications in EAE pathogenesis, with the knockouts suffering from increased susceptibility[105]. β-arrestin2 expression is higher in T cells isolated from asthma induced mice as compared to control[106, 107]. Modulating the expression of β-arrestin2 in CD4+ T cells isolated from

asthmatic animals affects Th2[107] and Th17[106] polarization potential and downstream production of IL-4 and IL-17, respectively. T cells lacking β-arrestin1, on the other hand, have equivalent Th1, Th2 and Treg differentiation while Th17 induction is negatively altered[108].

TLR2 pretreatment reduces stress-induced loss of splenocytes though a PI3K-Akt pathway that is β-arrestin2 dependent. Stress also alters T cell activation potential, decreasing IL-2 production while increasing IL-4; this effect too is abrogated by TLR2 agonist via a β-arrestin2 dependent mechanism[109]. β-arrestin2 knockout mice therefore, exhibit lymphocyte reduction and altered activation pattern that is not rescued by TLR2 agonists[109]. β-arrestins can therefore regulate different aspects of T cell response following an inflammatory stimulus.

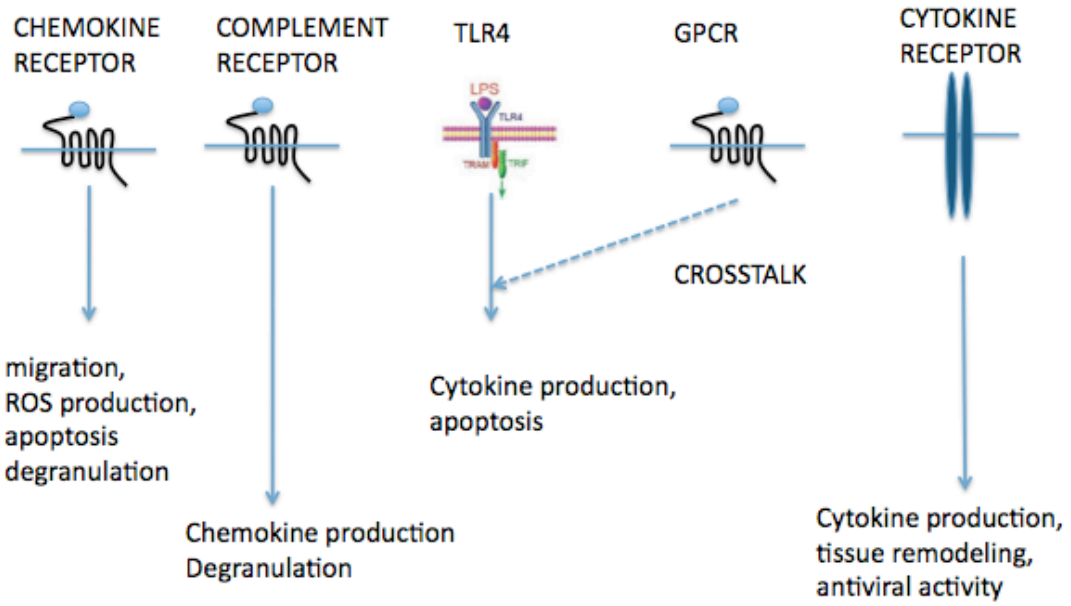

Figure 1.1: Schematic representation of signaling and inflammatory processes mediated by β-arrestins ROS-reactive oxygen species, GPCR- G protein coupled receptor

Table1.3: Summary of role of β-arrestins in immune cells

Assay	Stimuli	System	Outcome	Reference
Neutrophils				
Migration				
In vivo (air pouch)	CXCL1	β-arr2$^{-/-}$	Higher	[67]
In vivo (peritoneal cavity)	Oyster glycogen	β-arr2$^{-/-}$	Higher	[73]
Transwell	PAR2	β-arr1$^{-/-}$ β-arr2$^{-/-}$	Lower	[55]
Function				
Degranulation	IL-8 (CXCR1)	β-arr1 (mutant)	Lower	[74]
Superoxide production	CXCL1, CCL5	β-arr2$^{-/-}$	Higher	[67]
Cytokine production	LPS	β-arr2$^{-/-}$	Higher IL-6 and TNFα	[73]
		β-arr2$^{-/-}$	Higher IL-6 and IL-10	[56]
Macrophages				
Migration				
Transwell	MIP1β (CCR5)	β-arr1/2 KD	Lower	[69]
Function				
Survival	CSF withdrawal (BMM)	β-arr2$^{-/-}$	Higher	[53]
Complement gene expression	Basal and LPS (BMM)	β-arr1$^{-/-}$	Unaffected	[53]
		β-arr2$^{-/-}$	Lower	
Cytokine production	LPS (BMM) Conc.- 0.1ng/ml	β-arr2$^{-/-}$	Higher IL-6, TNFα, IL-12p40	[87]
	LPS (BMM) Conc.- 1-10ng/ml	β-arr2$^{-/-}$	Unaffected IL-6, TNFα	[110]
	LPS (BMM) Conc.- 10-500ng/ml	β-arr2$^{-/-}$	Lower IL-6 (100-500ng/ml) Higher TNFα (10ng/ml)	[56]

Table 1.3 (Cont'd)

	LPS (THP-1)	β-arr1 KD	Higher IL-6 and IL-8	[87]
	LPS (THP-1)	β-arr1 KD	Higher IL-6 and IL-8	[87]
	TLR3/TLR9/CD40L (BMM)	β-arr2$^{-/-}$	Higher IL-6, TNFα, IL-12p40	
	Adenovirus (peritoneal macrophages)	β-arr1$^{-/-}$	Lower RANTES, MCP-1	[91]
		β-arr2$^{-/-}$	Higher MCP-1, IL-12p40	
Basophils				
Functional				
Degranulation	IL-8 (CXCR1) RBL-2H3 cell line	β-arr1 mutant	Lower	[74]
Mast Cells				
Function				
Degranulation	C3a (HMC, LAD2 cell lines)	β-arr1 KD	Lower	[78]
Chemokine production		β-arr2 KD	Higher CCL4 (MIP1β)	
Mouse Embryonic fibroblast (MEF)				
Function				
Survival	IL-8 (CXCR1)	β-arr1/2$^{-/-}$	Lower	[39]
	Serum starvation	β-arr1$^{-/-}$, β-arr2$^{-/-}$	Lower	[52]
		β-arr1/2$^{-/-}$	Unaffected	
	GPCR- fPR, Angiotensin II (type 1A), V2 Vasopressin, CXCR2 ligands	β-arr1/2$^{-/-}$	Lower	[40]
Antiviral response	IFNγ primed VSV	β-arr1 KD	Higher	[97]
Cytokine production	LPS	β-arr1$^{-/-}$(β-arr1/2$^{-/-}$ with β-arr2)	Similar IL-6	[86]
		β-arr2$^{-/-}$	Lower IL-6	
		β-arr1/2$^{-/-}$	Higher CCL2, TNFα	[87]

Table 1.3 (Cont'd)

HEK293				
Migration				
	SDF1α(CXCR4), RANTES (CCR5)	β-arr2 KD	Lower	[63]
Function				
Survival	Hydrogen peroxide	β-arr2 over-expression	Higher	[41]
T Lymphocytes				
Migration				
Transwell	CXCL12 (CXCR4)	β-arr2$^{-/-}$	Lower	[75]
	SDF1α (CXCR4/7 heterodimer)	β-arr2$^{-/-}$	Lower	[64]
Allergic Asthma (Lung)	OVA	β-arr2$^{-/-}$	Lower	[76]
Function				
Gene expression through histone acetylation	naïve and activated T cells	β-arr1$^{-/-}$	Lower bcl-2 and apoptosis	[34]
	T cells from pulmonary biliary cirrhosis patients	β-arr1 KD	Altered gene expression, anti-inflammatory effect	[35]
Survival	Morphine	β-arr2$^{-/-}$	Lower	[50]
	Morphine with HIVgp120 (Jurkat T cells)	β-arr2 over-expression	Higher	[49]
		β-arr2 KD	Lower	
	Stress	β-arr2$^{-/-}$	Lower	[109]
T cell differentiation	CD3+CD28 under polarization conditions (Naïve T cells)	β-arr1$^{-/-}$	Lower Th17	[108]
		β-arr2$^{-/-}$	Lower Treg	[105]
Cytokine Production	CD3+CD28 (human primary T cells)	β-arr1 or 2 KD	Lower Il-2 and IFNγ in 20 hrs	[104]
	PMA+ConA (splenic CD4+ T cells from asthmatic mice)	β-arr2 KD	Lower IL-17A production	[106]

Table 1.3 (Cont'd)

	PMA+ConA with terbutaline (splenic CD4+ T cells from asthmatic mice)	β-arr2 KD	Lower Il-4 and GATA3 expression	[107]
	ConA (Splenic cells from stressed mice)	β-arr2$^{-/-}$	Lower IL-2 and higher IL-4 induction	[109]
	ConA (TLR2 mediated protection from stress)	β-arr2$^{-/-}$	TLR2 mediated increase in IL-2 and decrease in IL-4 abrogated	
Natural Killer Cells				
Function				
Cytokine production	PVR expressing target cells (TIGIT-YTS: NK cell line)	β-arr2 KD	Lower inhibition on IFNγ production	[103]
Cytotoxicity Inhibition	Tumor cell lines- CHO, YAC-1, RMA, RMA-S	β-arr2^{tg}	Higher	[102]
		β-arr2$^{-/-}$	Lower	
Antiviral activity	MCMV infection	β-arr2^{tg}	Lower	
		β-arr2$^{-/-}$	Higher	

KD- knockdown, Tg- transgenic (overexpression), OE- overexpression, PAR2- Plasminogen activated receptor 2, CSF- colony stimulating factor, BMM- bone marrow macrophages, LPS- Lipopolysaccharide, TLR- Toll like receptor, VSV- vesicular stomatitis virus, OVA-ovalbumin, TIGIT- T cell Immunoglobulin and ITIM domain receptor, Con A- concavalin A, PMA- phorbol 12 myristate 13 acetate.

β-ARRESTINS IN INFLAMMATORY DISEASE MODELS

Role of β-arrestins in various mouse models of inflammatory diseases are summarized below:

Experimental autoimmune encephalomyelitis (EAE)

Autoantibodies against β-arrestins are incident in human multiple sclerosis patients[111]. Further, T cell proliferative response to both retinal and β-arrestins is significantly higher in MS patients as compared to healthy controls and it further correlates positively with response to myelin binding protein (MBP), a dominant peptide in immunopathology of the disease[112]. These lines of evidence suggest incidence of epitope spreading in MS disease progression and involvement of β-arrestins in that process. β-arrestin1 knockout mice exhibit delayed onset, lower clinical score, reduced infiltration and demyelination in spinal cord sections in a mouse model of EAE. Conversely, transgenic mice with overexpression of β-arrestin1 have higher clinical score, increased infiltration in spinal cord section and greater demyelination. Mechanistically, β-arrestin1 promotes expression of anti-apoptotic gene bcl2 through its nuclear function of histone H4 acetylation; reducing apoptosis in both naïve and activated CD4+ T cells. CD4+ T cells from MS patients too have higher expression of β-arrestin1 and bcl-2 and knockdown of β-arrestin1 in these cells increases apoptosis. Therefore, higher survival of CD4+ T cells mediated by β-arrestin1 is posited to be a reason for its role as a positive mediator of the disease pathogenesis[34]. Another study reported similar upregulation of β-arrestin1 expression in the brains of MS patients and animal model of EAE as compared to respective control along with a concurrent decrease in A1 adenosine receptor expression. Glucocorticoid treatment that alleviates neuroinflammation and associated behavioral deficits causes an increase in A1AR expression concomitant with a reduction in β-arrestin1 expression, suggesting a reciprocal regulation between the two as an important determinant of MS pathogenesis[113].

While β-arrestin1 knockout mice are resistant to EAE pathogenesis, mice lacking β-arrestin2 are more susceptibility with disease symptoms being aggravated and sustained as compared to wild type mice. Mechanistically, the worsened phenotype is associated with lower peripheral Foxp3+ Treg (regulatory T cell) induction[105]. Infact, T cells lacking β-arrestin2 show poor conversion to iTregs *in vitro*; suggesting that lack of regulatory signaling is atleast partially responsible for overt activation of immune response. β-arrestins therefore have distinct roles to play in EAE inflammation and pathogenesis.

Meningitis

Meningitis is an acute inflammation of protective membrane in brain and spinal cord, also called meninges. It can be induced by viral, bacterial or fungal infections. *N. meningitides* is a gram negative bacterium that causes sepsis and meningitis. Once in the blood stream, it adheres to brain endothelium, multiplies at the cellular surface and crosses blood brain barrier to cause meningitis. It hijacks a β2 adenoreceptor/β-arrestin2-biased signaling pathway in endothelium cells to facilitate bacterial adhesion via src activation. Its penetration into tissue also requires β-arrestin mediated junctional proteins delocalization and gap formation[114]. β2-adrenergic agonists that induce receptor internalization are able to reduce bacterial adhesion pointing to their use as an effective strategy to combat infection. Further, in another study, β-arrestin2 expression is altered in peripheral blood monocyte cells (PBMCs) of patients suffering from meningitis caused by *Cryptococcus neoformans*, an opportunistic pathogen. Increased β-arrestin2 expression correlates positively with serum IL-10 and negatively with IFNγ levels. Further β-arrestin2 transfected PBMCs have lower cytotoxic activity while knockdown

leads to a non-significant increase in cytotoxic activity against *C. neoformans*[115]. This suggests a negative role for β-arrestin2 in inducing bacterial killing by perhaps inhibiting IFNγ production. β-arrestin2 expression therefore facilitates meningitis induction in response to these two microbes.

Allergic asthma

Mice lacking β-arrestin2 display drastically reduced physiological and inflammatory response in OVA sensitized allergy model. OVA induced T cell infiltration and Th2 response in the lung is markedly reduced in β-arrestin2 KO mice. IgG1 and IgE production and Th1 induction was unaffected indicating that peptide presentation and a skew towards Th1 respectively were not the reason for the observed phenotype. Alternately, T cell chemotaxis to macrophage derived chemokine (MDC) in vitro and its production *in vivo* is significantly lower in the KO mice, providing a mechanistic basis[76]. Further studies reveal a divergent role for both hematopoietic and non-hematopoietic β-arrestin2. While hematopoietic β-arrestin2 is required for eosinophil and lymphocyte infiltration; airway hyperresponsiveness is regulated by non-hematopoietic β-arrestin2[116]. Additionally, PAR2-induced modulation of inflammatory response in asthma is β-arrestin2 dependent[117]. Further, β-arrestin2 expression is significantly higher in T cells isolated from asthma-induced mice as compared to control[106, 107]. Modulating the expression of β-arrestin2 in CD4+ T cells isolated from asthmatic animals affects Th2[107] and Th17[106] polarization potential and downstream production of IL-4 and IL-17, respectively. β-arrestins can modulate asthma development by regulating various processes involved including T cell chemotaxis and differentiation.

Endotoxemia

The role of β-arrestins in endotoxemia model of sepsis is slightly controversial. In earlier studies, mice lacking β-arrestin2 were susceptible to D-galactosamine sensitized endotoxemia model with higher level of cytokines observed in plasma[87]. In contrast studies from our lab show lower production of plasma IFNγ and LPS induced mortality in mice lacking either β-arrestins[118]. Another study shows increased mortality in β-arrestin2 in response to LPS injection due to abrogation of anti-inflammatory IL-10 production[119]. The key difference in these studies with disparate results was use of galactosamine sensitization in Wang et al. and different doses of LPS in the other two; while Porter et al used 20 g/kg that induced 90% mortality in WT mice in 48 hours, Li et al used 10 g/kg LPS dose with less than 30% mortality in WT mice.

Sepsis

As opposed to their role in endotoxemia model of sepsis, both β-arrestins negatively regulate CLP induced inflammation and consequent mortality[57, 110]. In both knockout mice, increase cytokine levels are detected in plasma, peritoneal fluid (the site of infection) and lung tissue. Overt activation of NFκB pathway detected in lung tissues of septic mice for both knockouts, suggests inhibition of NFκB by β-arrestins as an important mechanism of controlling inflammation[56, 57]. Further mice heterozygous for both β-arrestins were protected from overt inflammation and enhanced mortality in CLP model of septic peritonitis[56, 57] suggesting one allele is sufficient for inhibiting sepsis induced inflammation. Chimeric mice generated with β-arrestin1 knockout mice

demonstrate that β-arrestin1 expression in non-hematopoietic cellular compartment is sufficient to inhibit the inflammatory response [57]. β-arrestin2 knockout mice subjected to polymicrobial injection to induce sepsis independent of the incidence of necrotic tissue, too exhibited increased inflammation and mortality[56]. Further, in both models of polymicrobial stimulation, β-arrestin2 KO mice have increased neutrophil sequesteration in the lung as ascertained by MPO activity[56, 73, 110]. This increased neutrophil sequesteration is independent of TLR4, since LPS injection does not have the same effect as microbial stimulation[56].

Inflammatory Bowel Disease (IBD)

IBD is a multifactorial disease perpetuated by a dysregulated immune response. Being the site of constant interaction between the immune system and foreign antigens, dietary or microbial makes the balance between inflammatory and regulatory responses particularly essential for homeostasis. β-arrestin1 knockout mice are protected from both dextran sodium sulfate (DSS) and trinitrobenzene sulfonic acid (TNBS) induced colitis based on clinical signs and histopathological scoring. The protective phenotype is associated with an altered inflammatory response. The knockout mice have markedly lower production of IL-6 and higher levels of IL-10 and IL-22 in colons of colitic mice, perhaps restricting inflammation and promoting epithelial cell repair[120].

Rheumatoid arthritis

Arthritis is an auto-inflammatory disorder, characterized by chronic inflammation in synovial joint causing cartilage and joint destruction. In a mouse model of collagen

antibody induced arthritis (CAIA), expression of both β-arrestins is significantly elevated in the joint tissue. Fibroblast like synoviocytes in response to hyaluron produce IL-6 and TNFα, the levels of which are increased by β-arrestin1 overexpression but decreased by overexpression of β-arrestin2[121]. β-arrestin2 knockout mice suffer from more severe arthritis in CAIA model with increased neutrophil and macrophage infiltration observed in the synovial tissue and cavity[121]. β-arrestin1 expression is upregulated in PBMCs from human patients suffering from RA and correlates positively with IL-17 expression. The role of β-arrestin1 further analyzed in collagen induced arthritis model in mice, demonstrates that the knockout mice are protected from disease incidence, ensuing joint swelling and destruction. β-arrestin1 expression increases in peripheral and synovial CD4+ T cells in response to arthritis induction. Further, lower IL-17A is detected in synovial joints of KO mice subjected to arthritis and β-arrestin1 knockdown in WT mice reduces production of IL-17 in the joints. Mechanistically, β-arrestin1 acts as a scaffold for JAK1-STAT3 mediated IL-6 signaling to positively regulate Th17 polarization[108]. β-arrestins thus have divergent roles to play in arthritis severity with β-arrestin 1 and 2 being pro- and anti-inflammatory respectively.

Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is an autoimmune disease associated with extensive humoral and cellular immune response. Autoreactive T cells specific for PDE-C2 antigen mediate destruction of biliary cells. In this model of T cell mediated pathogenicity, β-arrestin1 expression is significantly elevated predominantly in T cells isolated from blood of PBC patients as compared to healthy controls with the increase correlating with Mayo

risk score. Further autoreactive T cells with altered level of β-arrestin1 expression, by means of overexpression and knockdown demonstrate its positive role in regulating T cell proliferation and IFNγ production from them. Further, β-arrestin1 also modulates expression of genes involved in autoimmunity by virtue of affecting histone H4 acetylation; while expression of CD40L, LIGHT, IL-17 and IFNγ is upregulated, that of TRAIL, Apo2 and HDAC7A is found to be downregulated[122]. This warrants further investigation into the role of β-arrestins in initiation and development of this T cell mediated autoimmune disease.

Antiviral response

β-arrestin1 negatively impacts antiviral activity of IFNγ signaling as shown by VSV infection mediated cell death and viral loads being lower with β-arrestin1 knockdown in MEFs and HeLa cells[97]. Further, its own expression is modulated by and coincident with hepatitis B infection at the site infection (liver), whereas splenic β-arrestin1 expression is unaffected[97]. Since viruses are capable of affecting JAK-STAT pathway to evade the immune response and potentiate their infection[98], β-arrestin1 might be one way of doing so and perhaps regulating its expression might provide an effective antiviral therapy or potentiate existing IFN therapy. β-arrestin2 is an important player in clearance of MCMV infection that is largely dependent on NK cell mediated cytotoxic activity. β-arrestin2 in association with SHP-1 and SHP-2 is able to inhibit cytotoxicity in NK cells such that transgenic mice with higher β-arrestin2 expression have greater viral loads in organs following MCMV infection. β-arrestin2 knockout mice conversely and as expected have better clearance and lower viral titers[102]. β-arrestins therefore have distinct ways of

regulating anti-viral responses that requires further investigation for potential use in therapy development.

Pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a fatal disorder of unknown etiology that leads to loss of lung function. It is characterized by inappropriate fibrosis and involves excessive collagen deposition and distortion of lung architecture. Initiated by an airway injury, TGFβ and MMP are major players in pathophysiology affecting collagen synthesis, fibroblast proliferation, extracellular matrix remodeling and destruction of basement membrane. β-arrestins are important players in bleomycin induced mouse model of IPF with mice lacking either β-arrestin being protected from lung fibrosis and consequent mortality. Lung architecture distortion and collagen deposition is ameliorated in the absence of β-arrestins inspite of pulmonary inflammatory infiltration being unaffected. Even though TGFβ signaling and chemotaxis to bronchoalveolar fluid (BALF) is similar in primary lung fibroblasts; their invasiveness as assessed by matrigel invasion assay is severely impaired in the absence of β-arrestins (1 or 2). Further knockdown of β-arrestins in primary fibroblasts isolated from human IPF patients decreases their invasive potential; this effect being definite for β-arrestin2 but inconsistent for β-arrestin1, suggesting distinct roles for the two isoforms. Genes involved in extracellular matrix degradation and remodeling are further altered in lung tissue from β-arrestin knockout mice in response to bleomycin induced lung fibrosis[123]. Therefore, loss of β-arrestins is protective in IPF through regulation of fibroblast invasiveness and their localized inhibition could be a promising potential therapy.

Cystic fibrosis

Cystic fibrosis is a condition caused by loss of cystic fibrosis transmembrane conductance regulator (CFTR) protein, a cAMP regulated Ca2+ channel. CF cells exhibit higher cAMP signaling as measured by increased activity of cAMP response element binding protein (CREB) and altered cholesterol homeostasis. β-arrestin2 expression is elevated in CF cells in both mouse model and human patients[124]. CREB activity in nasal epithelium cells from CF model is enhanced via β-arrestin 2 mediated ERK activation[125]. Further β-arrestin 2 overexpression independent of CF is enough to induce cholesterol accumulation in epithelial cells in a cAMP dependent manner[126]. CF mice exhibit increased de novo cholesterol biosynthesis in the liver, which is also abrogated by loss of β-arrestin2[126]. Although, overall significance of these alterations is yet to be elucidated in a mouse model, these data suggest and important role for β-arrestin2 in CF complications.

Cutaneous flushing

Cutaneous flushing is a negative side effect of nicotinic acid treatment used to lower triglycerides and raise HDL, lowering the risk of cardiovascular diseases. Nicotinic acid binds to GPR109A, a 7-transmembrane receptor that activates pERK1/2 via involvement of both Gαi and β-arrestins. Nicotinic acid signaling induces interaction between β-arrestin1 and cytosolic phospholipase A2 (cPLA2), mediating latter's activation and release of arachidonate, precursor for vasodilator prostaglandin D2 (PGD2). Although, serum free fatty acid levels are similarly reduced in WT or β-arrestin (1 or 2) knockout

mice in response to nicotinic acid injection, ear perfusion is significantly reduced in β-arrestin1 knockout mice. Since PGD2 injection induces equivalent ear perfusion, the role of β-arrestin1 in niacin mediated flushing is most probably upstream of PGD2 production. Consequently, lower cPLA2 activity is observed in response to *ex vivo* nicotinic acid stimulation in β-arrestin1 knockout macrophages[127]. This data sheds light on the signaling induced by MK-0354, a GPR109A agonist that decreases FFA without inducing cutaneous flushing; suggesting that it functions as a biased GPCR ligand inciting therapeutic signaling without the side effects[127].

Table 1.4: Role of β-arrestins in inflammatory diseases.

Disease /Model	System	Outcome	Mechanism	Reference
MS/EAE	β-arr1$^{-/-}$	Protected	β-arr1 upregulates bcl-2; survival benefit to activated T cells	[34]
	β-arr1^{tg}	Susceptible		
	β-arr2$^{-/-}$	Susceptible	Lower Treg induction	[105]
Meningitis/ Cytotoxicity to *C. neoformans*	PBMC-β-arr2 OE	Lower	Negative regulation of IFNγ production	[115]
	PBMC-β-arr2 KD	Higher		
Asthma/ OVA sensitized model	β-arr2$^{-/-}$	Protective	Lower immune cell infiltration and airway hyperresponsiveness	[76, 116]
Endotoxemia/ LPS	β-arr1$^{-/-}$	Protective	Lower systemic response (IFNγ)	[118]
	β-arr2$^{-/-}$ (20mg/kg)	Protective	Lower systemic response (IFNγ)	
	β-arr2$^{-/-}$ (10mg/kg)	Susceptible	Lower IL-10 production	[119]
	β-arr2$^{-/-}$	Susceptible	Loss of anti-inflammatory regulation	[121]
Endotoxemia/ D-galactosamine + LPS	β-arr2$^{-/-}$	Susceptible	Higher systemic response (TNFα and Il-6)	[87]

Table 1.4 (Cont'd)

Sepsis/ cecal ligation and puncture or polymicrobial injection	β-arr1$^{-/-}$	Susceptible	Higher systemic response (IL-6); nonhematopoietic β-arr1 inhibits inflammation	[57]
	β-arr1$^{+/-}$	No effect	Similar systemic response (IL-6)	
	β-arr2$^{-/-}$	Susceptible	Higher systemic response (IL-6)	[110, 56]
	β-arr2$^{+/-}$	No effect	Similar systemic response (IL-6)	[56]
Colitis/ DSS and TNBS Arthritis/ CAIA	β-arr1$^{-/-}$	Protective	Lower IL-6 and higher IL-22 production Lower Th17 polarization	[120] [108]
	β-arr1$^{-/-}$	Protective		
Pulmonary Fibrosis/ Bleomycin induces lung fibrosis	β-arr1$^{-/-}$	Protective	Altered expression of genes involved in matrix production and degradation	[123]
	β-arr2$^{-/-}$	Protective		
Cystic Fibrosis/ CFTR knockout	β-arr2$^{-/-}$	?	Lower cholesterol synthesis and CREB activation	[125, 126]
Cutaneous Flushing/ Nicotinic acid injection	β-arr1$^{-/-}$	Reduced	Lowered prostaglandin D2 production	[127]

Tg- transgenic, OE- overexpression, KD-knockdown, LPS-lipopolysaccharide, OVA-ovalbumin, CAIA- collages antibody induced arthritis, DSS- dextran sodium sulfate, TNBS- 2,4,6-trinitro benzenesulfonic acid, PBMC- peripheral blood mononuclear cells,

Table 1.4 (Cont'd)

VSV- vesicular stomatitis virus, MCMV- murine cytomegalovirus, CFTR- cystic fibrosis transmembrane conductor regulator, CREB- cAMP response element-binding protein.

SEPSIS

Sepsis initiates as systemic inflammatory response syndrome (SIRS) in response to an infection; progressing through severe sepsis and septic shock characterized by organ failure and hypotension respectively. Inflammatory response is considered a prominent modulator of sepsis progression, affecting coagulation derangements[128], apoptosis of lymphoid and non-lymphoid tissues[129, 130] and organ dysfunction[131, 132], events that eventually cause mortality. Despite recent developments in early-goal directed therapies, sepsis remains a persistent clinical problem with reported mortality as high as 30-50%[133]. A review of discharge patients in USA reported the annual incidence of sepsis to be 751,000 cases, with 29% mortality[134]. Cardiac dysfunction is considered the last checkpoint for progression to mortality in septic patients[135, 136] and raises the mortality from 20% to 70-90%[137]. The economic burden of sepsis an be gauged from the fact that 1 in 5 patients admitted in ICU exhibit sepsis and treatment of each septic patient costs 6 times more than a non-septic patient[133].Therapies developed for specific treatment of sepsis include activated protein C[138], low dose steroids[139] and insulin therapy[140] that each decrease mortality only by 10% via unknown mechanisms. Current treatment for sepsis includes antibiotics for control of infectious agent and fluid resuscitation to maintain patient oxygen and blood pressure. According to recent findings of NIH GLUE grant (http://web.mgh.harvard.edu/TRT/new/cvs/MAX_MOF.html), β-arrestin1 expression in

immune cells correlates with the clinical attributes of sepsis in human patients. In animal models, it is an important regulator of inflammation in endotoxemia. Thus, understanding β-arrestin's role in the pathogenesis of sepsis and deciphering the involved mechanisms would provide clues for development of therapeutics for effective sepsis management.

Pathophysiology of sepsis

Inflammatory response

In sepsis, inflammatory response is initiated by recognition of pathogen recognition receptors (PRRs) to microbial components leading to production of inflammatory mediators and recruitment of additional effector cells. Cells of innate immune response, neutrophils, macrophages and dendritic cells form the first line of defense. In addition to production of immunomodulatory cytokines, macrophages and neutrophils are also involved in bacterial clearance via phagocytosis. Inflammatory mediators aid in defense against infection via upregulation of PRRs and potentiation of phagocytosis[141]. On the flip side, cytokines and secondary metabolites (NO, ROS) can lead to vasodilation, increased permeability and hypotension producing collateral damage to the host[142]. IL-6 signaling has been shown to be central to pathological sequelae of sepsis[143]. Therefore, regulated inflammatory response is an important criterion for resolution of infection with minimal damage to the host. Therefore, both exaggerated and ameliorated inflammation can be harmful to the host[132]. Often, the status of inflammatory response can be successfully used to predict mortality. IL-6 correlates well with organ dysfunction and mortality following sepsis in both humans[144] and animal models of sepsis[145, 146]. Ratio of TNFα and IL-10 has been used to successfully predict mortality in animal models [147].

Endothelial response

The vascular endothelium is capable of modulating systemic inflammation and coagulation derangements affecting cardiac dysfunction and multiple organ failure during sepsis[148]. Activated endothelium can recruit platelets, monocytes, and neutrophils that can initiate and amplify inflammatory and coagulation response[128]. The endothelium can undergo apoptosis and acquire a more pro-coagulant phenotype[149]. The activation status and loss of membrane integrity of endothelium is believed to induce organ dysfunction and global tissue hypoxia, an important feature of severe sepsis/shock[150].

Apoptosis

Apoptosis is extensively observed in lymphoid organs (spleen and thymus) and lymphoid tissues of large intestine, both in humans[151] and in animal models of sepsis[136]. In addition to lymphoid tissue, it affects gut epithelium, lung endothelium, kidney tubular cells and skeletal muscles[152]. While apoptosis of lymphoid cells can result in immunosuppression, apoptosis of non-lymphoid cells contributes to multi-organ dysfunction, leading to fatal consequences.

Cecal Ligation and Puncture as a model for polymicrobial sepsis

Cecal ligation and puncture is a clinically relevant model of sepsis analogous to sepsis as a result of perforated intestine (septic peritonitis) in humans. It closely mimics the clinical parameters associated with sepsis including inflammatory response, cardiovascular dysfunction, bacteremia, multiple organ dysfunction and death. It involves a procedure in which caecum is ligated below the ileocaecal valve and punctured. A small amount of fecal content is extruded to maintain the patency of the puncture for continuous bacterial

dissemination. The caecum is then returned to the peritoneal cavity, which is closed using a silk suture. This causes introduction of bacteria into otherwise sterile peritoneal cavity leading to inflammation and subsequent cascade of septic response. The control surgery wherein, caecum is exteriorized but not ligated or punctured is called sham. The intensity of sepsis can be varied depending on position of ligation, size of needle used for puncture and the number of punctures[153]. We will be using CLP as a model for polymicrobial sepsis to identify the role of β-arrestin1 in inflammation and progression of sepsis.

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is an inflammatory disorder affecting gastrointestinal tract that affects approximately 1.5 million people in the US with associated cost of approximately 4 billion dollars. Its etiology is largely unknown but involves a complex interplay between genetic and environmental factors, including microbiota, existing pathologies, dietary factors, etc. The initiation and perpetuation of disorder involves dysregulated inflammatory response to pathogenic or chemical insult leading to loss of epithelial architecture and oftentimes, extraintestinal pathologies. Certain genetic factors can additionally predispose an individual to breakdown of tolerance and overt response to enteric non-pathogenic bacteria. IBD is characterized by chronic inflammation of the gastrointestinal tract and can be classified into two types: ulcerative colitis (UC) and Crohn's disease (CD), based largely on the location of ongoing inflammation. While UC affects colon and rectum, is continuous and affects the mucosal layer; CD can affect any part of the GI tract, is patchy or discontinous and can involve all layers of the intestinal wall including mucosa, submucosa and muscularis.

Mouse models of IBD employed to study pathogenesis and development of therapeutic interventions can be broadly divided into four types- spontaneous, genetic, chemical and T cell transfer model of colitis. The two models used by the lab to determine the role of β-arrestin2 in mucosal inflammation are discussed below.

Dextran sodium sulfate induced colitis

DSS is a sulfated polysaccharide that induces colitis with inflammation restricted to colon and rectum, thereby making it a UC model of colitis[154]. Colonic erosions and ulcers are observed with increased weight loss, diarrhea, bloody stools and anemia observed in the mice. Even though the exact mechanism is involved, DSS is cytotoxicity to epithelial cells, can induce inflammatory response in macrophages[155] and affects epithelial proliferation and repair[156]. The inflammatory response includes a mixed Type1/2 and 17 type[157] and even though colitis can be induced in T cell deficient models[158, 159], a potent T cell response has been observed in this model of colitis[160]. Additionally, development of a competent innate immune system is critical for protection against colitis[161, 162]. The role of microbiota is controversial in this model although probiotics have been shown to improve the progression and outcome[163-165]. The colitis incidence is dependent on molecular weight, batch and lot of DSS[166]. The use of this model is very convenient because of its simplicity, low cost, rapid onset, reproducibility and the protocol can be modulated to study acute or chronic colitis.

T cell transfer model of colitis

This is an induced model of colitis, wherein introduction of $CD4^{+}CD45RB^{lo}$ T cells into mice lacking T cells leads to development of colitis. In the absence of regulatory control (only naïve T cells are introduced), T cells get activated by enteric microflora; undergo expansion and differentiation causing colitis progression and weight loss, loose stools starting approximately 4 weeks after transfer. Histopathological examination of tissue exhibits transmural inflammation, epithelial cell hyperplasia, with loss of goblet cells, lymphocytic and neutrophilic infiltration and crypt erosion[167]. The inflammation can be observed in both large and small intestine, can be discontinous and hence falls under CD-like features. Additionally, chronic hepatitis and bronchitis has been reported in this model of colitis[168]. Several lines of evidence indicate that the disease pathology is dependent on loss of regulatory controls. Firstly, introduction of CD4+ CD45RBhi T cells, that include Tregs, rescues development of colitis[168]. Secondly, unfractionated T cells from IL10 deficient mice fail to induce colitis[168]. Thirdly, onset is delayed in host containing functional B cells that have been ascribed regulatory role[169, 170]. Inflammation induced is of mixed Th1/Th17 type; with contradictory studies ascribing immuno-dominant and regulatory role to individual cytokine involved. Naive T cells isolated from both, IFNγ and Il-17A[171] knockout mice demonstrate increased colitogenic potential, suggesting that a complex interplay of inflammatory and regulatory network are responsible for immuno-pathology. Further microbiota has been ascribed important role in colitis progression with several studies indicating that enteric aerobes and anaerobes are responsible for induction and sustenance of colitis causing inflammatory response[172, 173].

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CHAPTER 2

This chapter represents a manuscript published as "**Gene dosage dependent negative regulatory role of β-arrestin2 in polymicrobial infection induced inflammation"** in Infection and Immunity, 2013 Aug;81(8):3035-44.

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ABSTRACT

β-arrestin2 (β-arr2) is a scaffolding protein of the arrestin family with a wide variety of cellular functions. Recent studies have demonstrated differential roles for β-arr2 in inflammation following endotoxemia and Cecal Ligation and Puncture (CLP) models of sepsis. Because CLP-induced inflammation involves response to fecal contents and necrotic cecum in addition to microbial challenge, in this study we examined the role of β-arr2 in an exclusively polymicrobial infection (PMI) model. In addition, we examined the role of gene dosage of β-arr2 in polymicrobial sepsis. Our studies demonstrate that β-arr2 is a negative regulator of systemic inflammation in response to polymicrobial infection and that one allele is sufficient for this process. Our results further reveal that loss of β-arr2 leads to increased neutrophil sequestration and overt inflammation specifically in the lungs following polymicrobial infection. Consistent with this, specific NFκB and MAPK signaling pathways were differentially activated in the β-arr2 knockout (KO) mice lungs compared to WT following PMI. Associated with enhanced inflammation in the KO mice, PMI-induced mortality was also significantly higher in KO compared to WT mice. To understand the differential role of β-arr2 in different sepsis models, we used cell culture systems to evaluate inflammatory cytokine production following endotoxin and polymicrobial stimulation. Our results demonstrate cell type as well as stimuli-specific roles for β-arr2 in inflammation. Taken together, our results reveal a negative regulatory role for β-arr2 in polymicrobial infection-induced inflammation and further demonstrate that one allele of β-arr2 is sufficient to mediate most of these effects.

INTRODUCTION

Arrestins are members of a family of scaffolding proteins that include α and β-arrestins. β-arrestins (1 and 2) were originally discovered for their role in G-protein coupled receptor (GPCR) desensitization[1]. However, recent studies have demonstrated that in addition to receptor desensitization, β-arrestins are also involved in receptor endocytosis and downstream signaling[2]. In fact the latter even has G-protein-independent and arrestin-dependent components[3]. Furthermore, β-arrestins can regulate signaling downstream of non-GPCRs by virtue of acting as scaffolds for major signaling molecules[4-6]. This places arrestins as critical regulators of various cellular and physiological processes important in maintenance of homeostasis. It is thus not surprising that β-arrestins have been implicated in the pathogenesis of many different diseases including arthritis[7], colorectal cancer[8], myeloid leukemia[9], multiple sclerosis[10], sepsis[11, 12], and colitis[13]. In addition to mammals, β-arrestins have been shown to control unique physiological processes in other species including *C. elegans*[14], drosophila[15] and zebra fish[16]. Furthermore, β-arrestins are critical for embryonic development in mammals as evidenced by the embryonically lethal phenotype of β-arrestin-1/2 double knockout mice[17].

The role of β-arrestins in regulating inflammation stems from their "traditional" role in modulating GPCRs such as C5aR[18], C3aR[19], PAR and chemokine receptors[20-22 23]. Furthermore, β-arrestins have been shown to act as scaffolding proteins for various signaling molecules important in mediating inflammatory responses including TRAF6[24], NFκB1p105[25], IκBα[21, 26, 27] and MAPKs[6, 20, 28, 29]. This role as a critical scaffolding molecule extends β-arrestins' capability in modulating inflammation beyond GPCRs to non-GPCRs such as Toll-like receptors[24, 25, 30]. Studies have shown that the role of β-arrestins in inflammation is highly context dependent and

that depending on the stimulus and disease model, β-arrestins can either mediate or inhibit inflammation[11, 12, 31, 32]. In this regard, we recently demonstrated that β-arr2 promotes increase in systemic levels of interferon-γ and other cytokines in the endotoxemia model[11] whereas it inhibits adenovirus-induced innate responses[33]. Additionally, recent studies have suggested that β-arr2 is a negative regulator of polymicrobial sepsis induced inflammation in the cecal ligation and puncture (CLP) model[12].

Sepsis is a complex pathophysiological disease process that involves an integrative response of the host to various pathogenic stimuli including surgery, necrosis, abscess and polymicrobial infections. While the CLP model of polymicrobial sepsis is a gold standard model, the pathogenesis of inflammation and mortality depends on multiple aspects including necrotic cecum and polymicrobial infection[24, 34]. In fact, studies have shown that removal of the necrotic cecum in animals subjected to CLP can significantly prevent mortality[24]. Given the differential roles for β-arr2 in endotoxemia and CLP models, we hypothesized that the difference is due to the latter causing a polymicrobial infection and not due to the effects of necrotic caecum and surgery. To test this hypothesis, we examined the role of β-arr2 in a polymicrobial infection model[35], without involving a necrotic tissue. Additionally, in this study we also determined the gene dosage effect of β-arr2 in mediating these events.

MATERIALS AND METHODS

Animals

β-arrestin2 knockout mice were kindly provided by Dr. Robert Lefkowitz and bred at Michigan State University[36]. Wild type C57BL/6 mice were purchased from NCI and bred in the same

facility. Animals were housed in rooms maintained at 22-24°C with 50% humidity and a 12 hr light-dark cycle. Mouse chow and water were provided *ad libitum* to all animals. All experiments were performed with age- and sex-matched mice between 8-12 weeks of age. Animal procedures were approved by Michigan state University institutional Animal Care and Use Committee (IACUC) and conformed to NIH guidelines.

Preparation of polymicrobial culture

Polymicrobial culture was obtained as described previously[35]. Briefly, cecal contents collected from WT mice were inoculated in sterile media (Brain Heart Infusion; BD Bacto) and cultured at 37°C, 220 rpm shaking for 18 hours. The contents were then centrifuged at 432 ×g for 10 min, the bacterial pellet was resuspended in 40% glycerol and stored at -80°C. For colony forming unit (CFU) measurements, 100 µl of the polymicrobial culture was inoculated in 100 ml media and grown for 14 hours, washed with PBS and plated on Muller-Hinton agar (BD Bacto) plates using serial dilution. Once the CFU/ml for the culture was determined, it was diluted to obtain the required CFU count for the experiments. The culture stock was determined to be consistent in terms of CFU count and was confirmed to be polymicrobial based on multiple colony morphologies as well as sequencing of the microbial community. To sequence the polymicrobial culture, genomic DNA was extracted from the culture and V3-V5 region of the 16S rRNA was amplified with barcoded primers. Amplicon sequencing was performed using the 454 GS Junior (Roche Diagnostics) platform according to the manufacturer's protocols. Sequences were analyzed using mothur (26) Version 1.29.1 (January 2013). Sequences were aligned to the SILVA reference alignment using the NAST-based aligner in mothur, trimmed to ensure that sequences overlapped, and pre-clustered, allowing a difference between sequences of

2 bp or less[37]. Chimeric sequences were removed using the mothur-implementation of UChime [38]; remaining sequences were classified using RDP training set version 9 (March 2012) and mothur's implementation of the kmer-based Bayesian classifier. Four genera of bacteria that dominated the polymicrobial culture were identified as *Bacillus*, *Enterococcus*, *Planococcus*, and *Streptococcus* (data not shown).

Polymicrobial sepsis

Age matched male mice were intraperitoneally injected with 10 x 10^6 CFU polymicrobial culture in 200 μl PBS. Six hours later, mice were euthanized, peritoneal fluid, spleen and plasma was collected, lung and liver tissue harvested and snap frozen in liquid nitrogen for further analysis as described previously[11]. Briefly, peritoneal cavity was lavaged with 4 ml 1640 RPMI (Gibco) media with 5% FBS (Gibco) and 55 μM β mercaptoethanol (Gibco), supernatant from this initial wash was stored at -80°C for cytokine analysis. The cavity was further washed with another 20 ml media and the cells from all washes pooled for cellular analysis. Blood was collected by cardiac puncture; 100 μl was subjected to RBC lysis and subsequent wash in media to get cells for flow cytometry analysis. The rest of it was centrifuged at 5000 rpm for 2 min to obtain plasma that was stored at -80°C for cytokine analysis. Spleen was crushed, subjected to RBC lysis and filtered through 40 μm nylon mesh. The cells were counted, resuspended in RPMI 1640 (with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM β mercaptoethanol) and plated at concentration of 10 x 10^6 cells/ml. Twenty four hours later the supernatant was collected for cytokine determination and stored at -80°C.

Survival study

Male mice were administered intra-peritoneal injection of polymicrobial culture (40 x 10^6 cfu in 200 µl PBS) and monitored for survival for 24 hours. The dose for survival studies was based on LD50 determined through a pilot experiment done on wild type mice.

Cytokine measurements

Cytokines were measured from plasma, splenic culture supernatant and peritoneal fluid using ELISA kits from eBiosciences, Inc. as per manufacturer's protocol and as described before[39]. In order to pool the data from different experiments, the raw values were converted to fold change over average WT concentrations for each experiment.

Flow cytometry

Peritoneal and blood cells collected from septic mice 6-hours post polymicrobial injection were processed as described above. They were then stained with antibody cocktail made in 2.4G2 supernatant (fcγR blocking antibody) to block non-specific binding and washed with staining buffer (PBS with sodium azide and BCS). The antibodies against cell surface markers CD11b, F4/80, Gr-1, CD3, CD19, CD11c were obtained from eBiosciences and used as per manufacturer's instructions.

MPO assay

Tissue myeloperoxidase (MPO) activity was performed as described before[39]. Briefly, snap-frozen lung and liver tissues were homogenized in 50 mM potassium phosphate (pH 6.0) buffer. After centrifugation, the pellets were resuspended and vortexed in 50 mM potassium phosphate (pH 6.0) buffer containing 0.5% hexadecyltrimethylammonium bromide to release MPO. An

aliquot of the supernatant was incubated at 25°C in 50 mM potassium phosphate (pH 6.0) buffer containing 0.0005% H_2O_2 and 167 μg/ml o-dianisidinehydrochloride. MPO activity was determined spectrophotometrically by measuring the change in absorbance at 450 nm over time using a 96-well plate reader. It was then normalized to total protein from the tissue initially homogenized, determined by Bradford method.

Quantitative RT-PCR

To determine the relative levels of a specific RNA transcript, RNA was isolated from snap frozen tissue using Qiagen RNeasy mini kit using manufacturers' protocol and as described earlier[40]. Reverse transcription was carried out with 1 μg of RNA using promega cDNA synthesis kit. Q-RT-PCR was performed with ABI fast 7500 (Applied biosystems) and all genes were normalized to HPRT as previously described[40]. Following primers were used for the respective genes: TNF-α- forward: TCTCATCAGTTCTATGGCCC-3, reverse GGGAGTAGACAAGCTACAAC; IκBα-forward: TGG CCA GTG TAG CAG TCT TG, reverse: GAC ACG TGT GGC CAT TGT AG; IL-6- forward: ACA AGT CGG AGG CTT AAT TAC ACA T, reverse: TTG CCA TTG CAC AAC TCT TTT C; KC- forward: CTTGAAGGTGTTGCCCTGAG, reverse: TGGGGACACCTTTTAGCATC; MIP2- forward: GGCAAGGCTAACTGACCTGGAAAGG, reverse: ACAGCGAGGCACATGAGGTACGA; HPRT- forward: AAG CCT AAG ATG AGC GCA AG; reverse: TTA CTA GGC AGA TGG CCA CA. Primers sets for β-Aarrestin1 and β-arrestin2 were obtained from Qiagen and were used as decribed earlier[41].

Western Blotting

Snap frozen liver tissue was homogenized in lysis buffer (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 and protease and phosphatase inhibitors. Homogenized tissue was spun at 13,000 rpm for 10 min at 4°C. Protein concentration of the supernatant was determined using bradford method. Western blot for pERK1/2, ERK2, IκBα, pJNK1/2, JNK1/2, pP38, pP105, and tubulin was performed as previously described[25]. 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 antibody as described. Blots were scanned and bands quantified using Li-COR Odyssey scanner. For data analysis pERK1/2 was normalized to ERK2; pI κ B α to I κ B α ; and pJNK, pP38 and pP105 to actin/ tubulin as loading controls.

Cell Culture

All cells were cultured in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM β mercaptoethanol and incubated at 37°C and 5% CO_2. In case of polymicrobial stimulation, initial stimuli was done in antibiotics-free media for an hour following which antibiotic mixture (100 U/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml gentamycin) was added for the rest of the duration.

Basal peritoneal cells were obtained by collecting peritoneal washes from naïve mice. Briefly, 30 ml media was used to lavage the peritoneal cavity. Cells were subjected to RBC lysis and plated at 0.4 million cells/well in 500 μl media.

Splenocytes were harvested and processed to obtain single cell suspension. Briefly, spleen was crushed, subjected to RBC lysis, filtered through a 40 μm filter and resuspended to give a final concentration of 5 million cells/well in 1ml media.

Bone marrow derived macrophages (BMDM) were obtained by culturing bone marrow cells in the presence of L929 cell conditioned media (LCCM) as described previously[42]. Briefly, tibia and femur was collected and flushed using a 27-gauge needle, cells were passed through the needle twice for single cell suspension. Following RBC lysis, cells were filtered and cultured in 30% LCCM for 7 days to generate BMDMs. The cells were finally plated at 0.5 million cells/ml in 1ml media for stimulation.

To obtain neutrophils, 1 ml thioglycollate was injected into the peritoneal cavity and cells harvested from the cavity 6 hours later as described above. We confirmed that the cells harvested from peritoneal cavity 6 hours after thioglycollate injection were predominantly neutrophils (~70%) with macrophages comprising only a minor population (~10%) (Flow cytometry data-not shown). Even though the cellular proportion was unaffected by loss of β-arr2, the total cell count was marginally (not statistically) decreased in the KO (KO $2.24 \pm 0.6 \times 10^6$) and unaffected in the HET ($5.34 \pm 0.4 \times 10^6$) compared to the WT mice ($5.15 \pm 0.9 \times 10^6$). The cells were eventually plated at 1 million cells/well in 1 ml media for stimulation.

For stimulation, ultrapure LPS (Invivogen) and polymicrobial culture (obtained as described above) were added at specified concentrations and multiplicity of infection (cells: bacteria) respectively. Supernatant was collected 18 hours later and stored at -80°C for further analysis.

Statistical Analysis

All experimental data in the figures is expressed as mean ± SEM and analyzed using GraphPad Prism Software. Each ‘‘N’’ represents individual mouse. Experiments were performed 3 times and total N represents the number of mice used in all 3 experiments combined. Student’s **t**-test (for comparing groups with equal variances) or Mann-Whitney (for comparing groups with

unequal variances) was used to compare 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

Gene-dosage-dependent effect of β-arrestin2 on PMI-induced cytokine production

To induce polymicrobial sepsis, we injected the polymicrobial culture intraperitoneally in the 3 groups of mice (wild type (WT), β-arr2$^{-/-}$ (KO), β-arr2$^{+/-}$ (HET)) and assessed cytokine production as a measure of inflammation at different sites (systemic and local), 6 hours following infection[35, 43, 44]. Uninfected mice from the three genotypes were used as controls. As shown in Fig 2.1A, plasma IL-6 and IL-10 levels were significantly enhanced in KO compared to the WT mice. Note that the Plasma IL-6 and IL-10 were below detection limits in uninfected mice and TNFα was not detectable at this time point in the infected or uninfected mice (data not shown). Similar to the plasma cytokine levels, splenic and peritoneal IL-6 and IL-10 were upregulated following microbial challenge and significantly enhanced in KO as compared to the WT mice, except peritoneal IL-6 ($p=0.09$) (Fig 2.1B and C). Interestingly, cytokine levels in these different sites were similar between the HET and WT mice suggesting that expression from one allele is sufficient to inhibit polymicrobial infection-induced cytokine production (Fig 2.1B). The difference in cytokine levels between the genotypes was not due to differential bacterial load since bacterial counts in blood and spleen was similar between the three groups (Fig 2.1D). Thus, βarr2 acts as a negative regulator of cytokine production following PMI even though there appears to be no difference in bacterial dissemination.

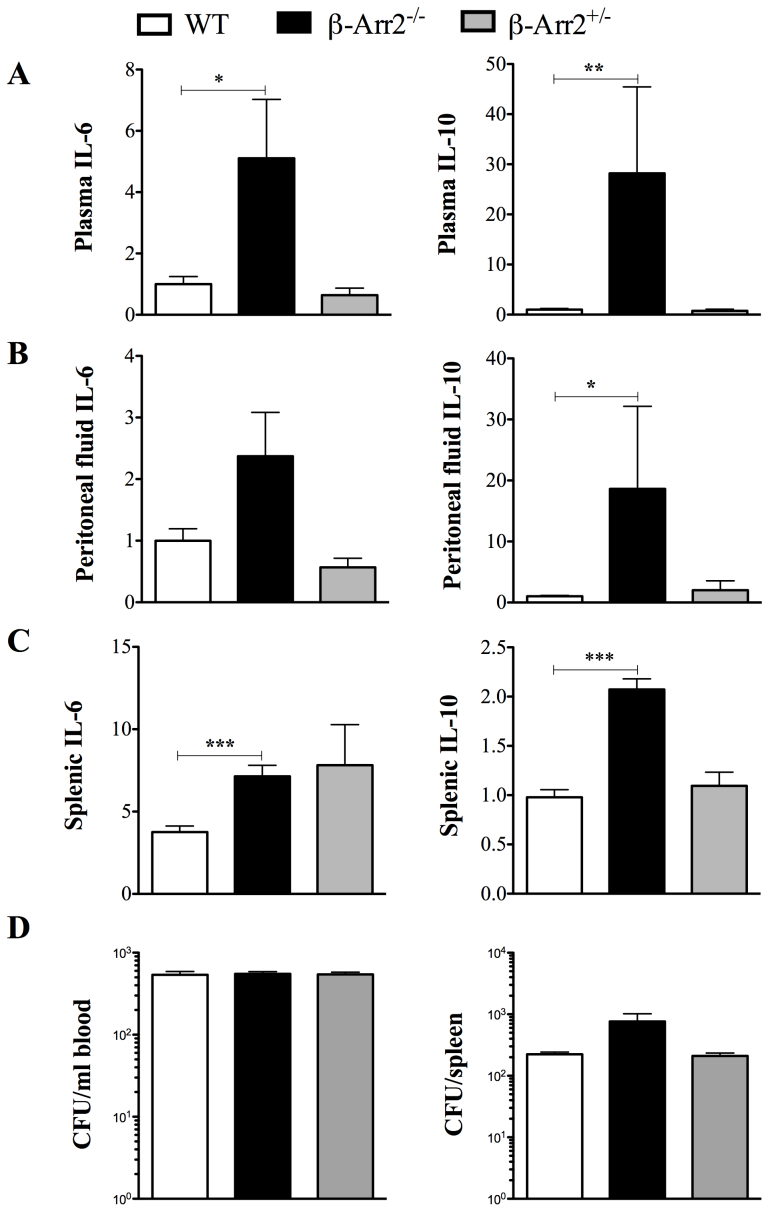


Figure 2.1: Cytokine production induced by polymicrobial injection is enhanced in β-arrestin2 knockout mice Wild type (WT), β-arrestin2 homozygous knockout (β-arr2$^{-/-}$) and β-arrestin2 heterozygous (β-arr2$^{+/-}$) mice were intraperitoneally injected with polymicrobial culture. Six hours later mice were euthanized and samples collected as described in the methods. A. Plasma cytokine B. Cytokines in splenic culture supernatant: Splenic cells were cultured at 5×10^6 cells/ml and 24 hours later the supernatant was collected and assayed for cytokine concentration.

Figure 2.1 (Cont'd)

C. Cytokines in peritoneal fluid. All concentrations were converted to fold change over septic WT for plasma and peritoneal fluid and over naïve WT for spleen. Uninfected naïve animals had undetectable levels of cytokines in plasma and peritoneum. D. Bacterial load in blood and spleen of infected mice represented on log scale. Uninfected naïve mice had no bacterial load. *$p<0.05$; **$p<0.01$; *** $p<0.001$ compared to WT using t-test or Mann-Whitney test. N=3 for naïve and 8-9 for septic mice for each genotype.

Differential regulation of immune cell infiltration by β-arrestin2

We also assessed local (peritoneal) and systemic (blood) infiltration of neutrophils and macrophages following PMI as another measure of inflammatory response following microbial challenge. The number of neutrophils and macrophages in blood and peritoneal cavity were comparable in naive mice from the three genotypes (data not shown) and increased following polymicrobial injection. At the local site of infection (i.e. peritoneal cavity) the number of macrophages but not the neutrophils was significantly lower in KO compared to the WT mice (Fig 2.2A). Interestingly, infiltration of these cells was similar between the peritoneal cavities of HET and WT mice. In contrast to the peritoneal cavity, blood neutrophil and macrophage numbers were significantly increased in KO (but not HET) mice as compared to the WT (Fig. 2.2B). These results suggest that in the complete absence of β-arr2 protein there is an increased influx of innate immune cells into blood but not to the site of infection.

To assess the fate of cells entering the bloodstream, MPO content, an indicator of neutrophil sequestration was determined in lung and liver tissue from septic mice. Basal MPO levels were equivalent in lungs from WT, KO and Het mice (data not shown) and microbial

challenge did not cause an increase in MPO content in WT mice (Fig. 2.2C). However, lungs from septic KO mice had significantly enhanced MPO activity compared to the WT (Fig. 2.2C). Interestingly, MPO activity in liver was unaffected by loss of β-arr2 or microbial stimulation (Fig 2.2C). The role of β-arr2 thus appears to be organ-specific in terms of neutrophil sequestration following polymicrobial sepsis. More importantly, increased MPO content was not observed in lungs from septic HET mice, suggesting gene dosage effect of β-arr2 in regulating neutrophil migration.

To determine if lipopolysaccharide (LPS, a major component of gram negative bacteria) following systemic infection is the likely mediator of this differential neutrophil migration in the KO, MPO content was determined in lung tissue following intraperitoneal LPS injection. In contrast to PMI stimulation, MPO content of lung tissue from KO mice was comparable to the WT following LPS administration (Fig 2.2D) suggesting that the effect on MPO activity in the lung is specific to PMI model.

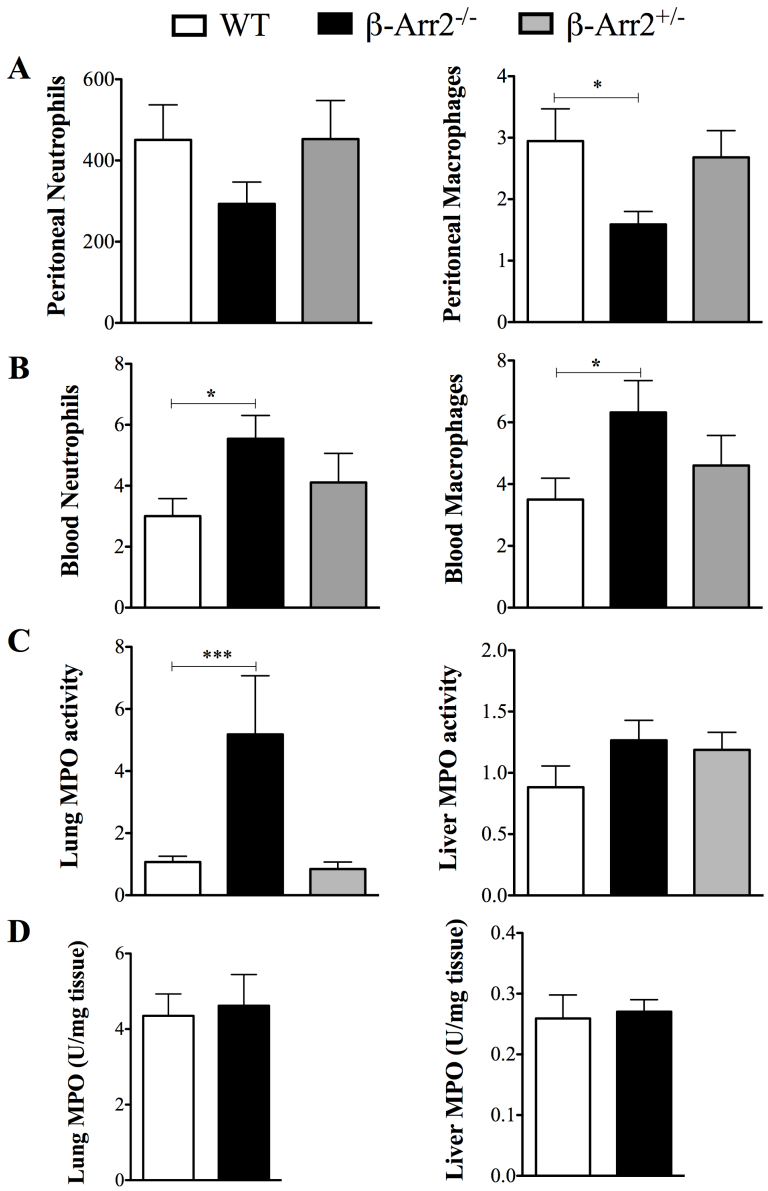


Figure 2.2: β-arrestin2 differentially regulates immune cell infiltration following polymicrobial infection Wild type (WT), β-arrestin2 homozygous knockout (β-arr2$^{-/-}$) and β-arrestin2 heterozygous (β-arr2$^{+/-}$) mice were intraperitoneally injected with polymicrobial culture. Six hours later mice were euthanized and samples collected as described in the methods. Cells were subjected to flow cytometry and identified on the basis of cell surface markers CD11b^{+}Gr-1^{+} (Polymorphonuclear cells (PMN)) and CD11b^{+}F4/80^{+} (macrophages). A. Total number of

Figure 2.2 (Cont'd)

cells harvested from peritoneal cavity of each mouse. B. Total number of cells/ml of blood. C. MPO activity as a marker for neutrophil sequestration was determined in lung and liver tissue following PMI. Data normalized as fold naïve WT; basal values were similar in all three genotypes. D. Lung and Liver MPO activity following LPS injection. Wild type and β-arrestin2 knockout mice were intraperitoneally injected with LPS (5 μg/g body weight) and lung and liver tissue was collected for MPO activity 6 hours later. *$p<0.05$; **$p<0.01$; *** $p<0.001$ compared to septic WT using t-test or Mann-Whitney test. N=3 for naïve and 8-9 for septic mice of each genotype.

β-arrestin2 regulates inflammatory gene expression in lungs following PMI

Impaired neutrophil chemotaxis in β-arr2$^{-/-}$ mice following polymicrobial sepsis was specific for lung and not observed in the liver. Hence, we further examined the role of β-arr2 on cytokine and chemokine genes in these organs following septic peritonitis. As is evident from figure 3a, cytokines and chemokine expression was induced in WT lung following PMI. Interestingly, uninfected KO lung had higher expression of *Tnfα* and *Il10* compared to uninfected WT lung. However, septic KO lung tissue demonstrated significantly enhanced mRNA *for Il6*, *Tnfα, Il10, Kc* and *Mip2* compared to septic WT mice (figure 2.3A). In addition, mRNA expression of *Iκbα* (a gene tightly regulated and induced by NFκB pathway[45]) was significantly elevated in KO lung tissue compared to the WT mice. Contrary to the distinctly enhanced "inflammatory signature" in KO, the HET mice had reduced expression levels of *Kc*, *Mip2* and *Iκbα* expression but similar levels of *Il6*, *Tnfα, and Il10* compared to the WT lung.

Basal expression of tested inflammatory mediators were unaffected by loss of β-arr2 in liver tissue. Unlike lung, *IL-6*, *Tnfα* and *Iκbα* expression was not even induced in the liver tissue in response to bacterial challenge. Additionally, except for *Mip2* expression, which was higher in the KO liver tissue, all other tested inflammatory markers were comparable between WT and KO liver (fig 2.3B). Further, *Il6*, *Tnfα* and *Il10* expression was lower in septic HET liver tissue as compared to the WT. Thus, both enhanced neutrophil sequestration and increased expression of inflammatory genes appears to be specific to lung tissue in KO mice following polymicrobial sepsis. Furthermore, both these observations are dependent on loss of both β-arr2 alleles since neither was enhanced in the HET mice.

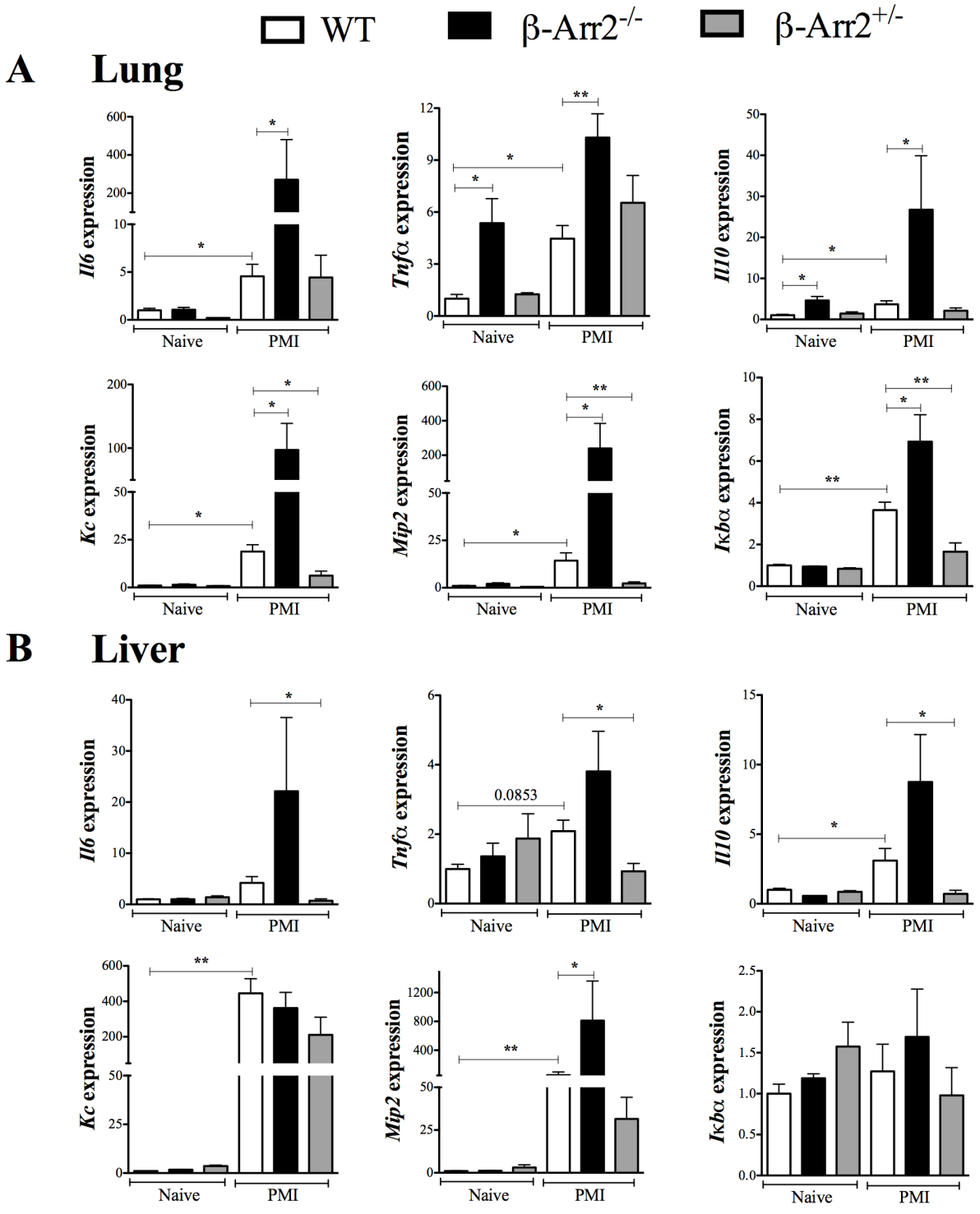


Figure 2.3: Gene dosage-dependent regulation of inflammatory genes in lung by β-arrestin2 following polymicrobial infection RNA was obtained from lung (A) and liver (B)

Figure 2.3 (Cont'd)

tissue samples from wild type (WT), β-arrestin-2 homozygous knockout (β-arr2$^{-/-}$) and β-arrestin-2 heterozygous (β-arr2$^{+/-}$) mice, 6 hours post-polymicrobial infection. Messenger RNA expression of the indicated inflammatory genes was performed using real time Q-RT-PCR as described in the methods. The values were converted to fold naïve WT for each experiment. *$p<0.05$, **$p<0.01$ using t-test or Mann-Whitney test. N=3 for naïve and 7-9 for septic mice of each genotype.

Differential regulation of signaling in the lungs by β-arrestin2 following PMI

β-arr2 has been shown to act as a scaffolding protein for NFκB and MAPK signaling molecules [6, 21, 24, 26-28, 46]. To examine the signaling mechanisms associated with hyper-inflammation observed in the KO lungs, we determined the phosphorylation status of major NFκB (IκBα, P105) and MAPK (ERK, JNK, P38) signaling molecules. Interestingly, compared to the uninfected controls, MAPK signaling was significantly down regulated in infected WT mice at this time point. In contrast, pP105 levels were significantly elevated post-infection, while pIκBα showed no difference. In addition, when compared to infected WT mice, KO infected mice had significantly elevated pJNK and pIκBα levels, while pP38 showed a similar trend ($p=0.06$ two tailed t-test) (Fig 2.4C). Lung tissue from septic HET mice had MAPK and NFκB activation similar to the WT. Together, these data suggest that β-arr2 is likely important for down-regulating some of these specific NFκB and MAPK pathways in the lungs following polymicrobial infection.

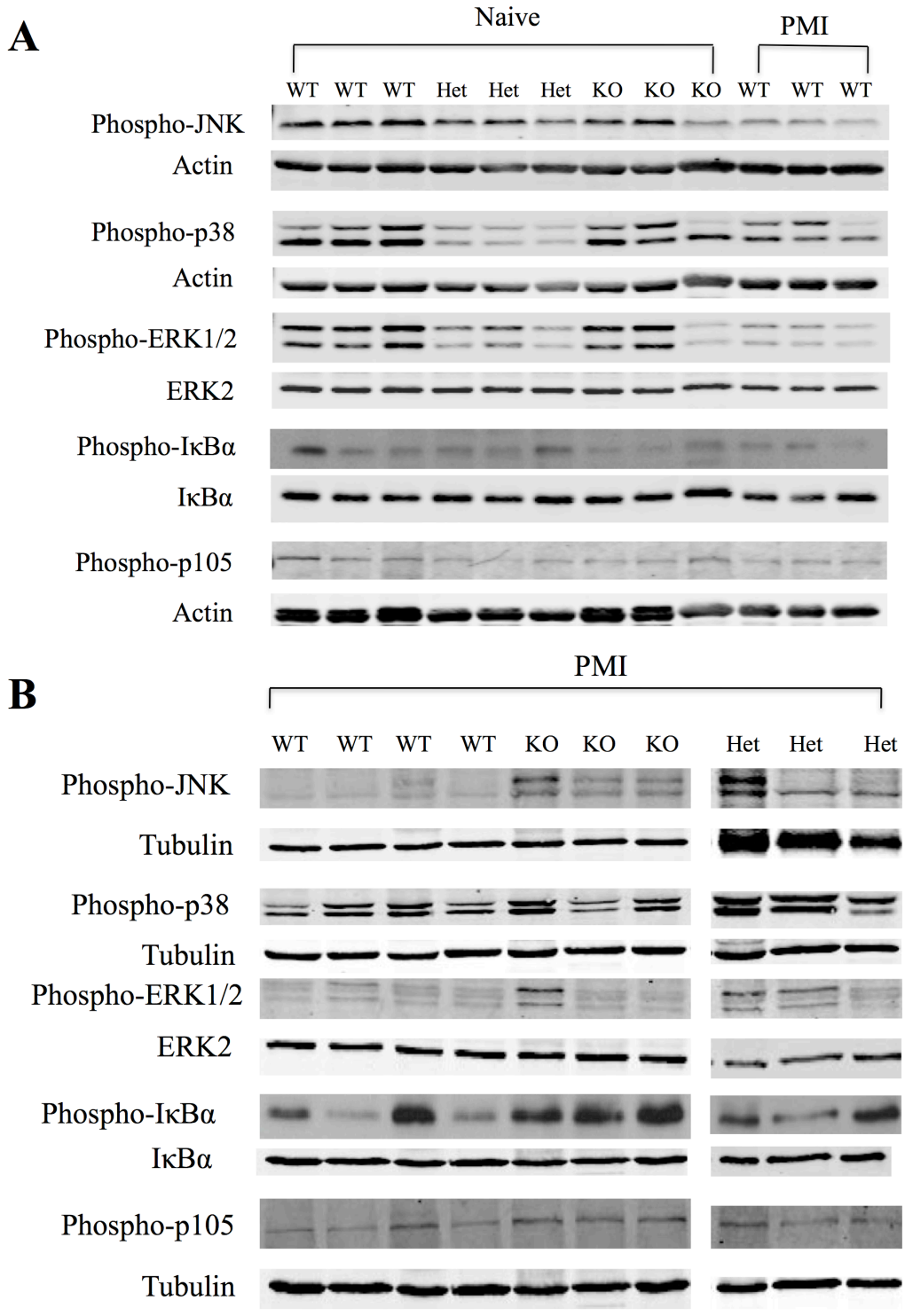
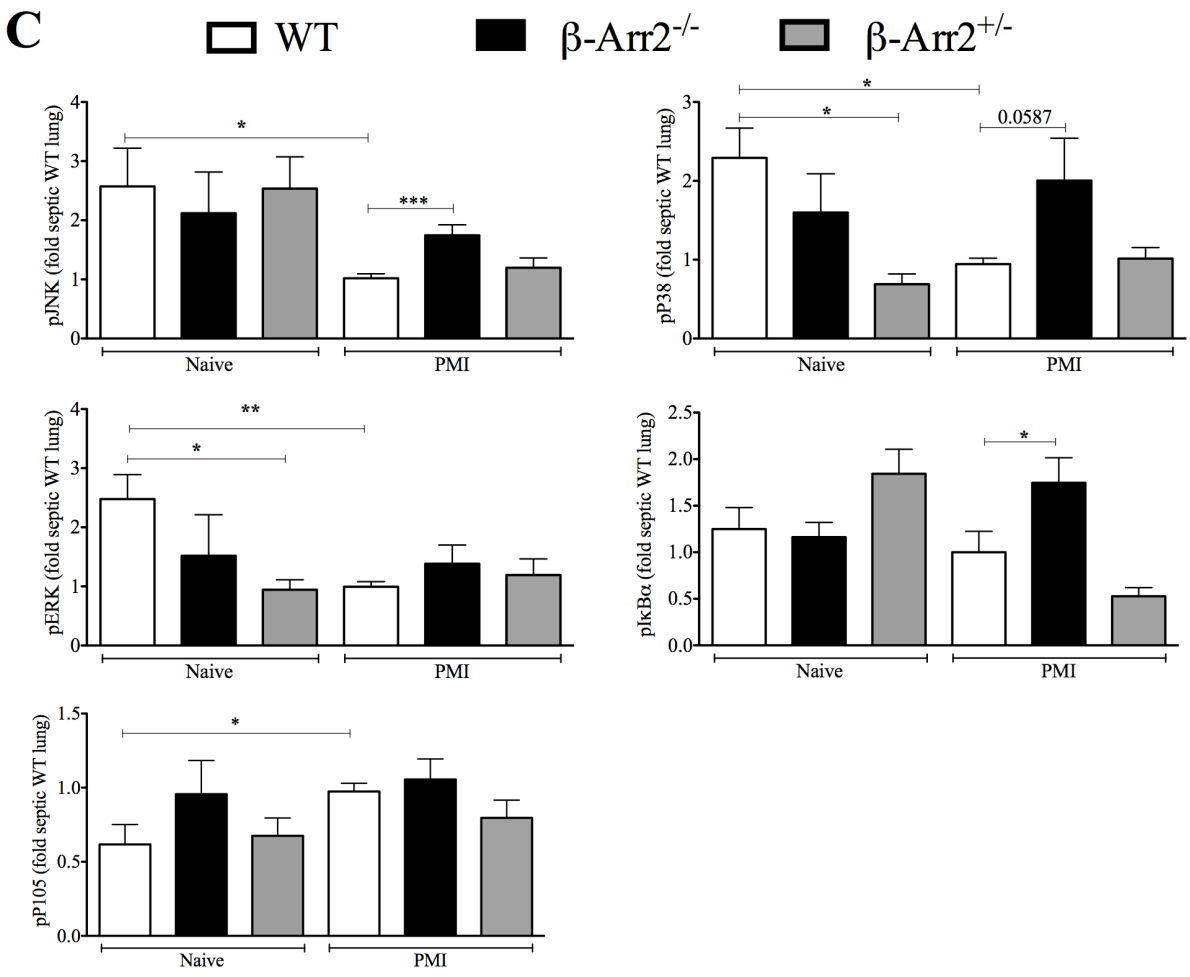


Figure 2.4: Differential regulation of MAPK and NFκB kinase pathways by β-arrestin2 in the lung following polymicrobial infection

Figure 2.4 (Cont'd)



Lung protein lysates from wild type (WT), β-arrestin2 homozygous knockout (β-arr2$^{-/-}$) and β-arrestin2 heterozygous (β-arr2$^{+/-}$) mice, 6 hours post-polymicrobial infection were assessed for phosphorylation status of major signaling molecules as described in methods section. Note that the blots were probed with multiple primary antibodies and later analyzed by Licor Odyssey (except for pIκBα) as stated in the methods. Representative blots from (A) control and (B) septic mice. C) Quantitative analysis of signaling molecules. pERK was normalized to ERK; pIκBα to IκBα; pJNK to JNK/actin; pP38 and pP105 were normalized to actin/tubulin as loading controls.

Figure 2.4 (Cont'd)

Raw values were converted to fold septic WT from each blot. *p<0.05, ** p<0.01 using t-test or Mann-Whitney test. N= 3-6 for naïve and 8-9 for septic mice for all genotypes.

β-arrestin2 mRNA expression is upregulated in lungs from WT mice following PMI

β-arrestin1 and β-arrestin2 have distinct as well as overlapping functions [19, 31, 33, 47]. Keeping that in mind, we examined the possibility that the level of β-arr1 might be differentially regulated in KO mice to carry out compensatory functions. To test this, we determined the expression levels of both arrestins in the lung tissue of naive and septic mice. *β-arr1* mRNA expression was unaffected by loss of β-arr2 under basal conditions and furthermore was unaltered following induction of polymicrobial sepsis in all three genotypes. *β-arr2* mRNA expression on the other hand was significantly upregulated in the WT septic lungs following PMI (Fig 2.5). Both alleles of β-arr2 however, were necessary for this upregulation since there was no increase in *β-arr2* levels in the HET mice following PMI. β-arr2 thus acts as an important negative regulator of pulmonary inflammation and its expression is upregulated in the lungs following microbial challenge.

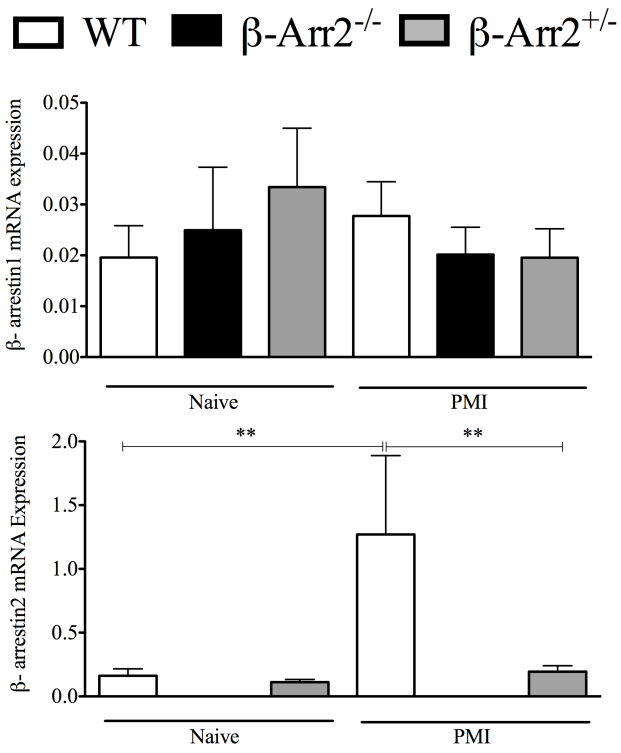


Figure 2.5: Differential regulation of β-arrestin expression in the lung following polymicrobial infection Lung RNA samples described in Fig 3 were subjected to real time Q-RT-PCR for determining the expression levels of (A) β-arrestin1 and (B) β-arrestin2 in mice injected with polymicrobial culture. Untreated control mice were used as basal controls. **p<0.01 using Mann-Whitney test. N= 7-9 for each treatment group and genotype.

β-arrestin2 modulates sepsis-induced mortality

IL-6, IL-10 and other cytokines have often been used to predict early deaths in sepsis, hinting at presence of cause and effect or correlative mechanisms at play for the two events[7, 43, 44]. In the present study, consistent with the systemic cytokines profile, polymicrobial injection resulted in higher mortality in the β-arr2 knockout mice compared to WT mice (Fig. 2.6). HET mice however, had mortality comparable to WT following lethal PMI. Together, our results

demonstrate a close association between inflammatory response and mortality with both being higher in KO compared to WT mice following polymicrobial infection (Fig. 2.6).

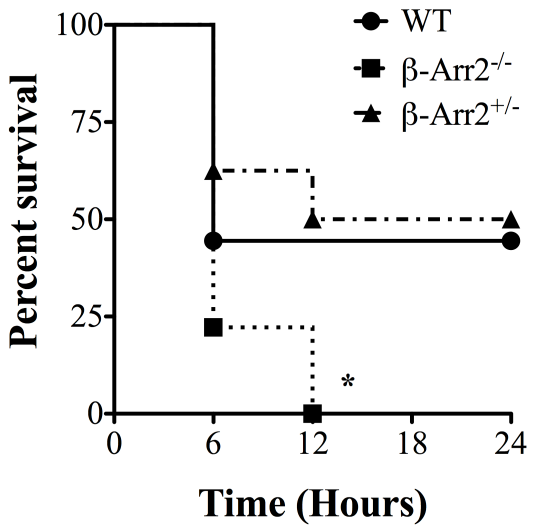


Figure 2.6: Gene dosage-dependent role for β-arrestin2 in preventing mortality following polymicrobial infection Wild type (WT), β-arrestin2 homozygous knockout (β-arr2$^{-/-}$) and β-arrestin2 heterozygous (β-arr2$^{+/-}$) mice were intraperitoneally injected with polymicrobial culture (40 x 10^6 CFU) in 200 µl volume. Mice were then monitored for survival for 24 hours. *$p<0.05$ compared to WT by log rank (Mantel Cox) test. N=7 mice for each genotype.

Role of β-arrestin2 in regulating cytokine production *in vitro*

β-arr2 has been ascribed contrasting roles in regulating inflammation following different stimuli[11, 12, 24, 33]. Additionally, following PMI we observed a site-specific regulation of cytokine production by β-arr2. We hypothesized that β-arr2 has a cell type-specific and stimuli-dependent role in regulating inflammation. To test this we used peritoneal cells from naïve mice, as well as

splenocytes, bone marrow-derived macrophages (BMDMs) and thioglycollate-induced neutrophils as cellular models to examine the effect of LPS and polymicrobial challenge on IL-6, TNFα and IL-10 production. Peritoneal cells and splenocytes from KO mice demonstrated enhanced cytokine production compared to the WT (Fig. 2.7-2.8). Compared to these two populations, IL-6 and TNFα production was lower but IL-10 was higher from KO BMDMs compared to the WT cells in a stimulus-specific manner (Fig 2.9). HET BMDMs produced lower IL-6 in response to LPS but higher IL-10 following polymicrobial stimulation. Thus, in BMDMs in contrast to peritoneal cells and splenocytes, β-arr2 has a diverse role in cytokine production, acting as a positive regulator of pro-inflammatory and negative regulator of anti-inflammatory cytokines. In contrast to BMDMs, thioglycollate-elicited neutrophils from KO mice produced significantly enhanced IL-6 and IL-10 compared to WT mice following LPS but not microbial stimulation (Fig 2.10). Neutrophils from HET mice had lower IL-10 compared to WT following microbial stimulation. These results suggest that β-arr2 is a negative regulator of IL-6 and IL-10 production in these neutrophils in a stimulus-specific manner. Overall, these results suggest a stimuli and cell type specific role for β-arr2 in mediating cytokine production.

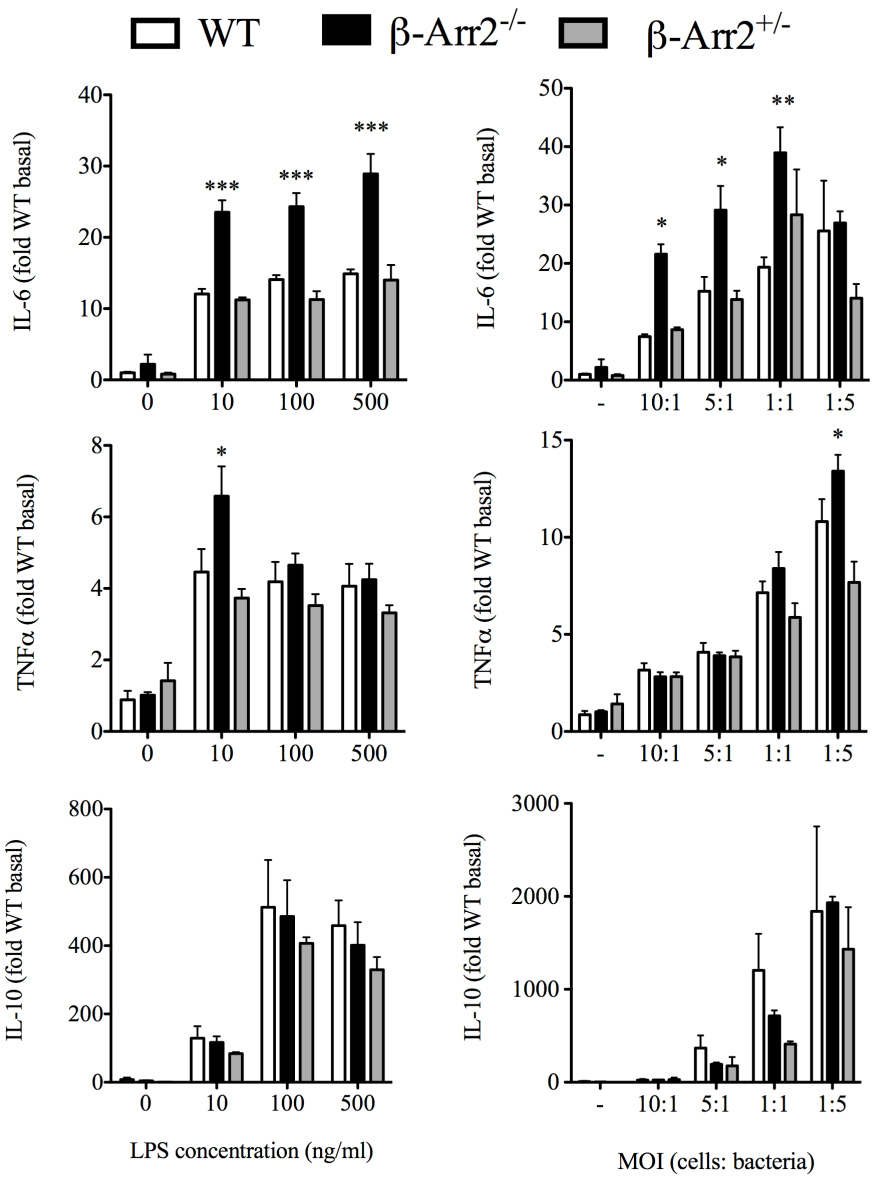


Figure 2.7: β-arrestin2 negatively regulates cytokine production in resident peritoneal cell population Resident peritoneal from naïve wild WT, β-arr2$^{-/-}$ and β-arr2$^{+/-}$ mice were obtained, processed and plated as described in the methods. The composition was similar in all three genotypes as determined by flow cytometric analysis. Cells were then stimulated with LPS and polymicrobial culture at different concentrations and multiplicity of infection (MOI) respectively. Cells were stimulated for 18 hours and supernatants assayed for IL-6, IL-10 and TNFα

Figure 2.7 (Cont'd)

concentrations. Cytokine levels were transformed as fold over WT basal. *p<0.05; **p<0.01; ***p<0.001 compared to WT as determined by 2-way ANOVA followed by Bonferroni post test. N=4-5 mice for each genotype.

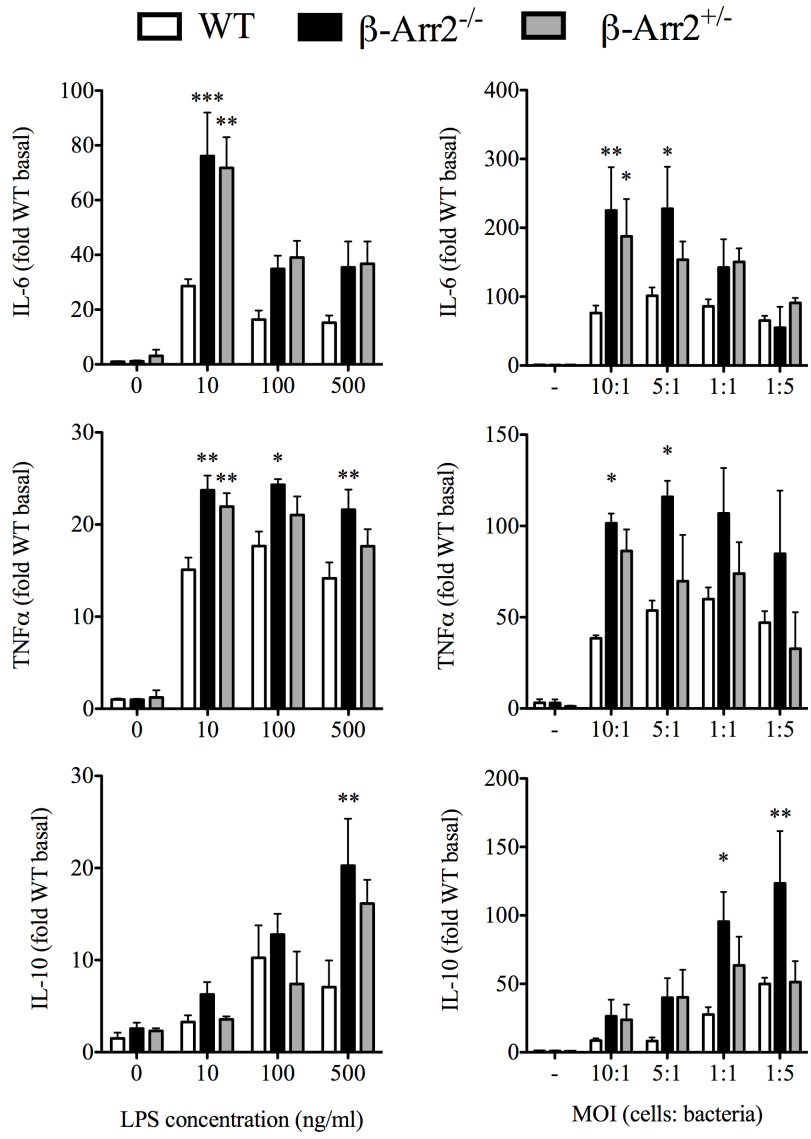


Figure 2.8: β-arrestin2 negatively regulates cytokine production in splenocytes Splenocytes from WT, β-arr2$^{-/-}$ and β-arr2$^{+/-}$ mice were processed and plated as described in the methods. Cells were then stimulated and samples processed as in Fig 2.7. Cytokine levels were

Figure 2.8 (Cont'd)

transformed as fold over WT basal. *p<0.05; **p<0.01; ***p<0.001 compared to WT as determined by 2 -way ANOVA followed by Bonferroni post test. N=4-5 mice for each genotype.

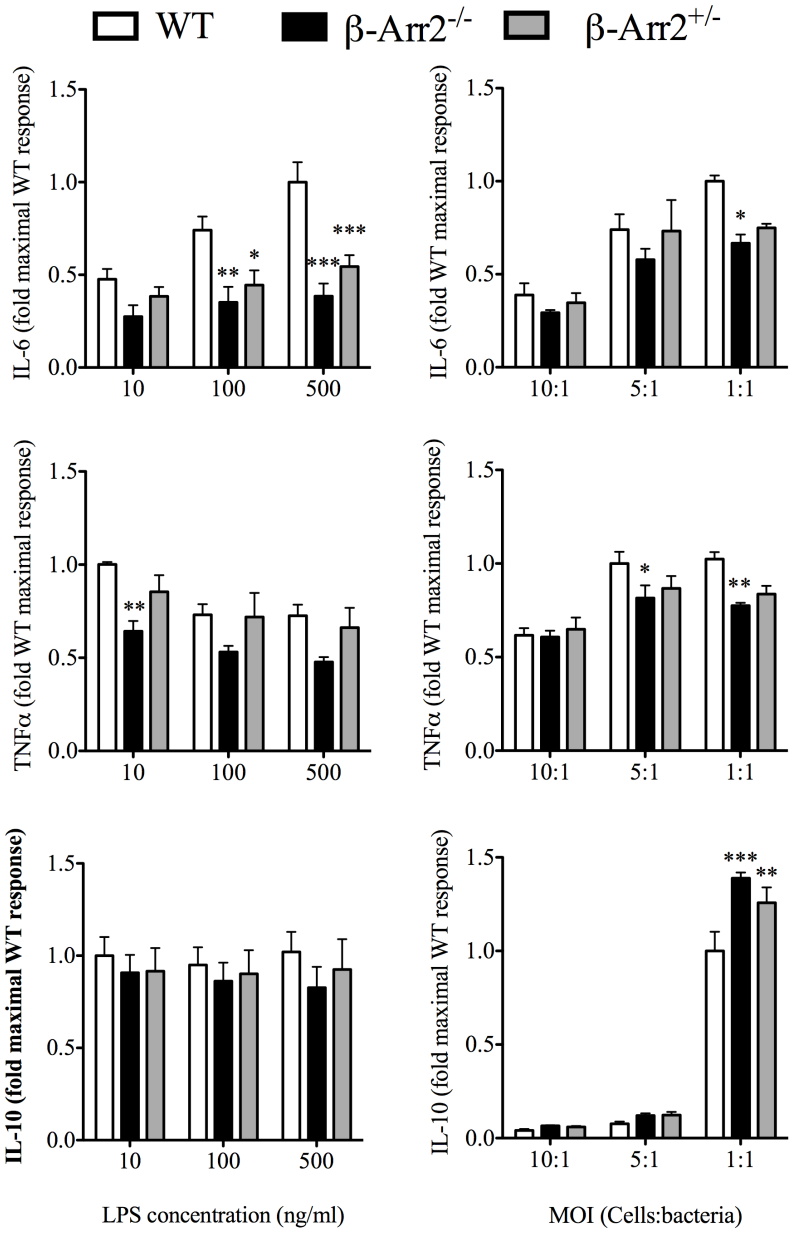


Figure 2.9: Differential regulation of IL-6, TNFα and IL-10 by β-arrestin2 in Bone marrow derived macrophage Bone marrow derived macrophages were obtained WT, β-arr2$^{-/-}$

Figure 2.9 (Cont'd)

and β-arr2$^{+/-}$ mice and plated as described in methods. Cells were then stimulated and samples processed as in Fig 2.7. The data was transformed to fold WT maximal response. *p<0.05; **p<0.01; ***p<0.001 compared to WT as determined by 2-way ANOVA followed by Bonferroni post test. N=4-5 mice for each genotype.

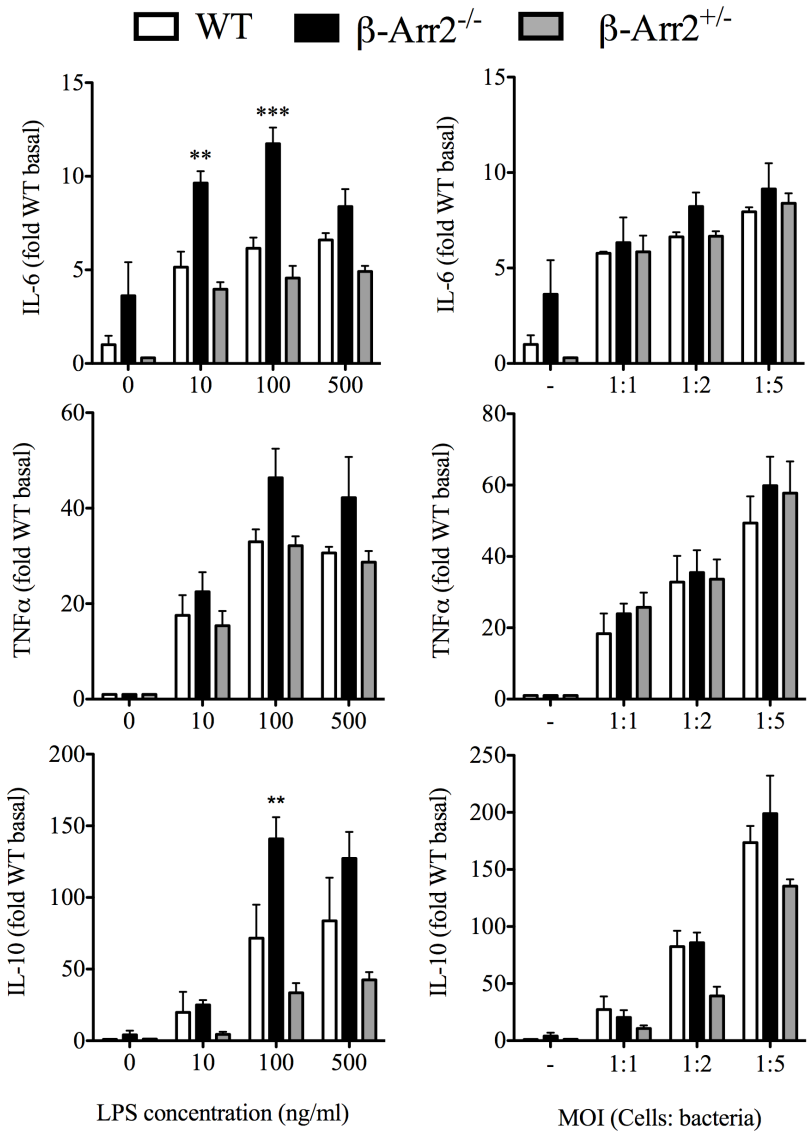


Figure 2.10: Stimulus-specific role of β-arrestin2 in regulating cytokine production from neutrophils Neutrophils were obtained from thioglycollate-injected WT, β-arr2$^{-/-}$ and β-arr2$^{+/-}$

Figure 2.10 (Cont'd)

mice as described in methods. Cells were then stimulated and samples processed as in Fig 2.7. Cytokine levels were transformed as fold over WT basal. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to WT as determined by 2-way ANOVA followed by Bonferroni post test. N=3 for each genotype.

DISCUSSION

The role of β-arrestin2 (β-arr2) in modulating inflammatory changes in different sepsis models have yielded contradictory results with β-arr2 KO having higher mortality in cecal ligation and puncture (CLP) model[12] but lower mortality in endotoxemia model[11]. Unlike the endotoxemia model, in CLP the inflammatory stimuli include microbes, fecal material and necrotic tissue[48], and β-arr2 was shown to be a negative regulator of both inflammation and mortality in this model. We therefore used polymicrobial infection (PMI) model to determine whether the β-arr2 has similar function following microbial challenge independent of the effects of necrotic tissue and surgery. Similar to the CLP-induced sepsis, but in contrast to the endotoxemia, β-arr2 KO exhibited exacerbated systemic inflammation and poor survival following PMI, indicating that microbial challenge is the dominant stimuli in response to which β-arr2 exerts its influence in polymicrobial sepsis models. Additionally, even though TLR4 signaling has been ascribed a critical role in pathogenesis of PMI model[35], the role of β-arr2 in microbial infection as a negative regulator appears to be dominant over and distinct from its role as a positive regulator of inflammation in response to LPS. Distinct roles for β-arr2 in regulating cytokine production in response to LPS and microbial challenge were observed *in vitro* as well.

In addition to being model-specific, β-arr2 appears to regulate inflammatory genes in a tissue-specific manner in the PMI model. Expression of certain inflammatory mediators (*IL-10*, *Kc*) was enhanced in the lung but not liver of septic KO mice, suggesting that β-arr2's negative regulatory role in this model is tissue specific. While it is possible that there could be differences in kinetics of cytokine production in different tissues, the site-specific regulation could also be due to cell type-specific role of β-arr2. The latter scenario is supported by our in vitro experiments wherein the role of β-arr2 in mediating cytokine production was found to be responder-specific. This was particularly interesting given the contrasting roles ascribed to β-arr2 when using different cell models[12, 24, 31]. Using the *in vitro* systems led us to further postulate that given the integrative nature of systemic inflammatory responses, functions observed *in vitro* might not be reflective/indicative of this protein's *in vivo* role. Even though the biochemical basis for these differences are not clear, our results underscore the importance of β-arr2 in inflammation, as well as suggest that the role of β-arr2 in inflammation may be disease specific depending on the stimulus and the dominant cell type involved in disease pathogenesis.

β-arr2 has been shown to be an important regulator of chemotaxis in different models[21-23, 32]. Consistent with that our studies also reveal that β-arr-2 is an important regulator of directional migration of neutrophils. Although polymicrobial infection did not cause any differential infiltration of immune cells to the site of infection (peritoneum), MPO activity in lung but not liver was significantly elevated in the β-arr2 KO mice. This neutrophil sequestration in KO lungs following microbial challenge was also observed in the surgical CLP model of polymicrobial sepsis[12, 32]. This site-specific regulation in the KO could be due to specific "neutrophil-favoring" chemokine gradients regulating infiltration into the lungs (since *Kc* and *Mip2* mRNA expression were higher in KO lung as compared to the WT) or due to an inherent defect in the ability of β-

arr2 KO neutrophils to carry out "directional migration" towards the site of infection. It is however clear that this directional migration is specific for PMI model since LPS-induced neutrophil sequestration in the lungs was unaffected by the loss of β-arr2.

In addition to enhanced sequestration of neutrophils in the KO lungs, inflammatory gene expression was also enhanced in the lungs from KO mice. β-arr2 has been shown to act as a scaffolding protein for NFκB and MAPK signaling molecules[6, 21, 24, 26-28, 46]. Altered activation of some of these pathways in the lung and enhanced gene expression suggests that β-arr2 likely regulates these pathways negatively, thereby affecting pulmonary inflammation. Interestingly, at the 6-hour time point that we studied, MAPK activation (pERK, pJNK and p-p38) appears to be decreased following infection. It is possible that these pathways are activated at early time points and regulatory mechanisms come in to play to negatively regulate these pathways and suppress consequent exacerbated inflammation. This mechanism appears to be largely lost in the β-arr2 KO, suggesting that β-arr2 is important for downregulation of these specific pathways at the time point we tested. Enhanced NFκB activation as evidenced by higher Iκbα phosphorylation could in part explain exacerbated inflammatory genes in the KO mice infected lungs. Consistent with that, mRNA expression of *Iκbα* (NFκB-regulated gene) was significantly higher in the KO lung compared to the WT. Interestingly pulmonary mRNA expression of *β-arr2* itself was upregulated following infection. This regulation of β-arr2 expression under inflammatory conditions has been previously observed in arthritis model[49] and was found to have an important role to play in its pathogenesis. A similar regulation of β-arr2 expression in sepsis model suggests an important role for β arr2 in regulating pulmonary inflammation following sepsis.

Consistent with previous studies on CLP-induced mortality as well as the "hyper-inflammatory" phenotype observed following PMI, β-arr2 KO had higher lethality than the WT

mice. Again, as was observed for most other parameters, one allele of β-arr2 was sufficient in preventing PMI-induced mortality. Enhanced lethality of the β-arr2 KO mice is likely because of higher systemic and lung inflammatory cytokines and likely pulmonary damage in the KO mice. Interestingly, enhanced pulmonary neutrophil sequestration in KO mice was observed only in microbial models of sepsis and not in endotoxemia. These data taken in conjunction with our previous observations on the decreased mortality of β-arr2 KO in endotoxemia model, suggest that the enhanced lethality in the β-arr2 KO is specific for models of microbial challenge.

Even though β-arr2 knockout mice were shown to be susceptible to CLP-induced septic mortality[12], our studies demonstrate that the susceptibility of the KO mice is dependent solely on microbial challenge and independent of other affects of surgery or necrotic caecum. In future studies we will determine the molecular mechanisms by which β-arrestin2 modulates bacterial infection which would likely result in identification of new therapeutic targets to treat bacterial sepsis.

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CHAPTER 3

This chapter represents a manuscript titled "**Non-hematopoietic β-arrestin1 inhibits inflammation in a murine model of polymicrobial sepsis"** published in American journal of Pathology (2014). June 16

Authors who contributed to the work included Deepika Sharma, Nandakumar Packiriswamy, Ankit Malik, Peter C. Lucas and Narayanan Parameswaran.

ABSTRACT

β-arrestin1 (β-arr1), a scaffolding protein critical in GPCR desensitization has more recently been found to be important in the pathogenesis of various inflammatory diseases. The goal of this study was to understand the role of β-arr1 in sepsis pathogenesis using a mouse model of polymicrobial sepsis. Whereas in previous studies we established that β-arr1 deficiency protected mice from endotoxemia, here we demonstrate that the absence of β-arr1 remarkably renders mice more susceptible to mortality in polymicrobial sepsis. In accordance with the mortality pattern, early production of inflammatory mediators was markedly enhanced in β-arr1 KO mice systemically and locally in various organs. In addition, enhanced inflammation in the heart was associated with increased NFκB activation. Compared to these effects, immune cell infiltration, thymic apoptosis and immune suppression during polymicrobial sepsis were unaffected by deficiency of β-arr1. Additionally, enhanced inflammation and consequent higher mortality were not observed in heterozygous mice suggesting that one allele of β-arr1 was sufficient for this protective negative regulatory role. We further demonstrate that, unexpectedly β-arr1 in non-hematopoietic cells is critical and sufficient for inhibiting sepsis-induced inflammation while hematopoietic β-arr1 is likely redundant. Taken together, our results reveal novel and previously unrecognized negative regulatory role of the non-hematopoietic β-arr1 in sepsis-induced inflammation.

INTRODUCTION

Sepsis is a serious medical condition that to this date incurs high mortality (~30-50%) in spite of high expenditure in terms of patient care. Early detection, antibiotics and life support to maintain organ homeostasis remain the only line of defense with no specific treatments available. Therefore understanding the mechanistic basis of sepsis progression is critical in identifying potential therapeutic targets for future drug development. Among the various pathophysiological events that occur through sepsis progression, inflammation remains a double-edged sword for the host, with dysregulated pro-inflammatory phase causing tissue destruction[1] and a prolonged immunosuppressed phase causing excessive microbial burden. A balanced inflammatory response protects patients from sepsis-induced morbidity and mortality. Keeping that in mind, elucidating mechanisms regulating inflammation is critical to our understanding of sepsis pathogenesis.

β-arrestins (1 and 2), initially identified as being involved in GPCR desensitization are now known to have diverse array of roles in GPCR-dependent and -independent signaling[2]. This gives them an opportune foothold in various physiological functions and places them as important regulators of homeostasis. Their role in mediating inflammation stems from their ability to modulate chemotaxis[3], cytokine production and signaling via non-canonical regulators of inflammation, such as β-adrenergic[4], angiotensin[5], lipids and other receptors. Additionally, they can act as scaffolding proteins for major signaling complexes, including MAPK[6,7] and NFκB pathways[8,9]. *In vitro* studies have demonstrated a negative regulatory role for β-arr1 in TLR-4 and TNFR signaling[8,10,11]. However, we demonstrated in previous studies that β-arr1 is a critical

mediator of inflammation and mortality in endotoxemia model of sepsis[12]. Consistent with that, other studies have also shown that β-arr1 mediates the pathogenesis of various inflammatory diseases, such as rheumatoid arthritis[13], colitis[14], cancer[15] and multiple sclerosis[16].

In spite of the limitations of mouse models in replicating human sepsis, cecal ligation and puncture (CLP)-induced polymicrobial sepsis has been shown to encompass several immunopathological features of human sepsis[17]. Based on previously reported role of β-arr1 in endotoxemia and other inflammatory models, we hypothesized that β-arr1 deficient mice will be protected from polymicrobial sepsis-induced inflammation and mortality. Our studies however, reveal a previously unappreciated negative regulatory role of β-arr1 in polymicrobial sepsis and consequent mortality. We further demonstrate that β-arr1 in non-hematopoietic compartment is required and sufficient, in regulating polymicrobial sepsis-induced inflammation.

MATERIALS AND METHODS

Animals

β-arrestin1 knockout mice on C57BL/6 background (kindly provided by Dr. Robert Lefkowitz, Duke University) have been described earlier[12]. Wild type C57BL/6 mice were purchased from NCI and all mice were bred or housed at Michigan State University in rooms maintained at 22-24°C with 50% humidity and a 12-hr light-dark cycle. Mouse chow and water were provided *ad libitum* to all animals. All experiments were performed with age- and sex-matched mice between 8-12 weeks of age. Animal procedures were approved by Michigan state University institutional Animal Care and Use Committee (IACUC) and conformed to NIH guidelines[18].

CLP surgery

Animals were subjected to CLP as described earlier[19]. Briefly, mice were anaesthetized using intraperitoneal injection of xylazine (5 mg/kg) and ketamine (80 mg/kg). The cecum was exteriorized, ligated and punctured either once (single puncture, SP) with a 16G needle (16G-SP) or twice (double puncture, DP) with a 20G needle (20G-DP). The cecum was then inserted back and peritoneal cavity sutured with 5.0 silk. Sham surgery wherein cecum was exteriorized but neither ligated nor punctured was used as control. All animals were given a subcutaneous injection of 1 ml saline (pre-warmed to 37°C) after the surgery. For mortality studies, mice were observed for 7 days after surgery.

Generation of chimeric mice

Chimeric mice were generated using lethal irradiation and bone marrow reconstitution. Briefly, mice were irradiated with a total dose of 11 gy (5.5 gy X 2, 3 hours apart) and 12 hours later injected with $5X10^6$ bone marrow cells from donor. Immediately after irradiation and reconstitution, mice were put on water with antibiotics (sulfamethoxazole and trimethoprim, hi-tech pharmacal) for a period of 4 weeks and mice were used for experiments 8 weeks after reconstitution.

Sample Processing

At pre-determined time of harvesting, mice were euthanized using CO_2 asphyxiation. Peritoneal fluid, plasma and organs were harvested and processed as previously stated[20]. Briefly, the peritoneal cavity was flushed with R10 media (RPMI 1640 with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM β mercaptoethanol), the cells collected and processed for

FACS analysis. The first peritoneal wash was done in 4 ml media and supernatant saved for further analysis. The cavity was then lavaged twice with 10ml media and cells from all washes collected and counted for further analysis. Blood was centrifuged at 300 rcf for 5 min and supernatant stored at -80°C for ELISAs. The organs were harvested, flash frozen and stored at -80°C. Spleen was crushed, subjected to RBC lysis and filtered through 40 μm nylon mesh. For further stimulations, cells were counted, resuspended in R10 at concentration of 10 X 10^6 cells/ml and incubated at 37°C for 18 hours with or without LPS (100ng/ml). For flow cytometric analysis, $2X10^6$ cells were used and processed. Thymus was processed similar to the splenocytes and sample prepared for cytometric analysis. For BAL (bronchoalveolar lavage) collection, the thoracic cavity was opened, the trachea cannulated and secured with ligation. Bronchoalveolar space was lavaged thrice using R10 media and cells pooled from the three washes for cytometric analysis.

Flow cytometry

Peritoneal, BAL, spleen and thymus cells collected from septic mice were processed as described above. They were then stained with antibody cocktail made in 2.4G2 supernatant (fcγR blocking antibody) to block non-specific binding and washed with staining buffer (PBS with sodium azide and BCS). The antibodies against cell surface markers CD11b, F4/80, Gr-1, CD3, CD19, CD4 and CD8 were obtained from ebioscience and used as per manufacturer's instructions. Cells were run on LSR II and data analyzed using Flowjo software. Neutrophils were gated as CD3-CD19-CD11b+Gr-1+ cells, macrophages as CD3-CD19-CD11b+F4/80+ cells and T cells as CD19-CD3+ cells. T cells were further marked as CD4+ (Th), CD8+ (Tc) and CD4+CD8+ (DP) T cells based on CD4 and CD8 expression.

Cytokine/chemokine measurements

Cytokines were measured from plasma, splenic culture supernatant and peritoneal fluid using ELISA kits from ebioscience as per manufacturer's protocol. In order to pool the data from multiple bone-marrow transfer experiments, the raw values were converted to fold change over average WT concentrations for each experiment.

Bacterial Counts

Bacterial load was determined in peritoneal fluid and blood. Briefly, sample was serially diluted and plated on Mueller-Hinton Agar plates (Difco). The plates were the incubated at 37°C for 24 hours and the number of colony forming units (CFU) counted and recorded.

Preparation of polymicrobial culture

Polymicrobial culture was obtained as described previously[20]. Briefly, cecal contents collected from WT mice were inoculated in sterile media (Brain Heart Infusion; BD Bacto) and cultured at 37°C, 220 rpm shaking for 18 hours. The contents were then centrifuged at 432 ×g for 10 min, the bacterial pellet was resuspended in 40% glycerol and stored at -80°C. For colony forming unit (CFU) measurements, 100 µl of the polymicrobial culture was inoculated in 100 ml media and grown for 14 hours, washed with PBS and plated on Muller-Hinton agar (BD Bacto) plates using serial dilution. Once the CFU/ml for the culture was determined, it was diluted to obtain the required CFU count for the experiments.

Bacterial killing assay

Intracellular and total killing assay was done using thioglycollate-elicited neutrophils. To obtain neutrophils, mice were injected with 1 ml thioglycollate intraperitoneally and four hours later, cells collected using peritoneal lavage. *E. coli* (ATCC 25922*)* was cultured in tryptic soy broth overnight and a secondary culture started from it for the assay. Four hours later, the culture was spun at 5000 rpm for 15 minutes; washed twice with sterile PBS and expected CFU calculated based on OD value and previously determined growth curve. It was then opsonized with heat-inactivated serum (55°C for 1 hour) for 1 hour at 37°C with mild shaking (100 rpm). Neutrophils and bacteria were mixed at MOI of 1:5 and incubated at 37°C with mild shaking (100 rpm). For total killing assay, bacteria alone control was setup as well and at indicated times both control and experimental groups were serially diluted and plated to obtain CFU counts. For intracellular killing assay, 20 minutes later, gentamycin (10 μg/ml) was added to kill extracellular bacteria. Twenty minutes later the cells were washed in PBS to remove gentamycin and incubated at optimum conditions for indicated time periods. At the end of each time point, the cells were lysed in 0.1% triton X and serially diluted and plated to obtain CFU counts.

Quantitative RT-PCR

To determine the relative levels of a specific RNA transcript, RNA was isolated from snap frozen tissue using Qiagen RNeasy mini kit using manufacturers' protocol and as described earlier. Reverse transcription was carried out with 1 μg of RNA using promega cDNA synthesis kit. Q-RT-PCR was performed with ABI fast 7500 (Applied biosystems) and all genes were normalized to HPRT as previously described[20]. Primer sequences are provided in table 3.1.

Table 3.1: Primer Sequences used for QPCR.

Gene name	Forward Primer	Reverse Primer
TNFA	5' TCT CAT CAG TTC TAT GGC CC 3'	5' GGG AGT AGA CAA GCT ACA AC 3'
IL6	5' ACA AGT CGG AGG CTT AAT TAC ACA T 3'	5' TTG CCA TTG CAC AAC TCT TTT C 3'
NFKBIA	5' TGG CCA GTG TAG CAG TCT TG 3'	5' GAC ACG TGT GGC CAT TGT AG 3'
NOS2	5' TCT TTG ACG CTC GGA ACT GTA GCA 3'	5' ACC TGA TGT TGC CAT TGT TGG TGG 3'
NOS3	5' CTG CTG CCC GAG AAT ATC TTC 3'	5' CTG GTA CTG CAG TCC CTC CT 3'
ICAM	5' GGC ACC CAG CAG AAG TTG TT 3'	5' GCC TCC CAG CTC CAG GTA TAT 3'
VCAM	5' GGA GAG ACA AAG CAG AAG TGG AA 3'	5' ACA ACC GAA TCC CCA ACT TG 3'
SERPINE1	5' GGC ACA GTG GCG TCT TCC T 3'	5' TGC CGA ACC ACA AAG AGA AAG 3'
F3 coagulation factor III	5' CAT GGA GAC GGA GAC CAA CT 3'	5' CCA TCT TGT TCA AAC TGC TGA 3'
PROCR	5' AGC GCA AGG AGA ACG TGT 3'	5' GGG TTC AGA GCC CTC CTC 3'

Western Blotting

Snap frozen heart tissue was homogenized in lysis buffer (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 and protease and phosphatase inhibitors. Homogenized tissue was spun at 15,000 rcf for 10 min at 4°C. Protein concentration of the supernatant was determined using Bradford method. Western blots were performed as previously described[20]. Briefly, equivalent concentrations of protein samples were run on polyacrylamide gels and transferred to nitrocellulose membranes. Primary antibodies used were p-ERK (Cell signaling, 9101L), ERK2 (Santa Cruz, Sc-1647), pIκBα (Cell signaling, 9246S), and IκBα (Cell signaling, 9242S) and secondary (anti-rabbit or anti-mouse) were bought from invitrogen or licor. Blots were probed with primary followed by IR-dye/HRP conjugated secondary antibody

and then scanned. Bands were quantified using Li-COR Odyssey scanner or ImageJ software. For data analysis, pERK1/2 was normalized to ERK2 and pIκBα to IκBα as loading controls.

Caspase activity

Caspase 3 activity was assessed in thymocytes using the fluorescence assay described earlier[21]. Briefly, thymocytes were lysed in CHAPS buffer (50 mM HEPES, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF and 10 μg/ml Leupeptin). The cell lysate was quantified and 10 μg protein was incubated with fluorescent substrate (Ac-DEVD-AFC) in assay buffer (100 mM HEPES, 10% sucrose and 0.1% CHAPS and 10 mM EDTA, pH7.4). Fluorescence of cleaved product was measured (Excitation at 400 nm and emission at 505 nm) using Tecan Spectra FlourPlus fluorescence plate reader. Data was analyzed and presented as picograms of cleaved AFC per milligram protein per minute calculated using standard curve of free AFC.

Phagocytosis and ROS potential

Cells harvested from septic mice, 12 hours post-surgery were used as source of neutrophils for phagocytic potential and ROS generation. Briefly, peritoneal cavity was lavaged with R2 media (RPMI 1640 with 2% FBS), and cells counted for the assay. For phagocytosis, pHrodo *E. coli* bioparticle conjugate (invitrogen) was used as described in manufacturer's manual. Briefly, 1 x 10^5 cells were incubated with bio-particles for 30 min at either 37°C (experimental) or 4°C (control) and reaction stopped by washing with cold FACS wash buffer. For ROS detection, 1 x 10^5 cells were preloaded with 5 mM DHR-123 dye (invitrogen) and stimulated with PMA (1 ng/ml). The cells were stained with Gr-1 antibodies to detect neutrophils and increase in MFI over control recorded as phagocytic potential and ROS generation, respectively.

Histopathology

Liver, kidney, spleen and lung tissues were collected from mice subjected to CLP and fixed in 10% formalin overnight. They were then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The hallmarks of injury and inflammation (infiltration) were assessed by a board certified pathologist (P. C. L) in a blinded manner.

Statistical Analysis

All experimental data in the figures is expressed as mean±SEM and analyzed using GraphPad Prism Software. Each ‘‘N’’ represents individual mouse. Student’s t-test (for comparing groups with equal variances) or Mann-Whitney (for comparing groups with unequal variances) was used to compare two experimental groups. Differences in the survival were determined using the log-rank test. P-values <0.05 were considered statistically significant.

RESULTS

β-arrestin1 inhibits sepsis-induced mortality and inflammation

To assess the importance of β-arrestin1 in modulating progression of sepsis, we subjected wild type (WT), and β-arrestin1 KO (KO) mice to CLP surgery (16G-SP) and followed their survival over the course of 7 days. We also included the β-arrestin1 heterozygous (HET) mice to assess the gene dosage effect of β-arr1 in sepsis-induced mortality. In contrast to the role of β-arr1 in endotoxemia-induced mortality, we observed that all the KO mice succumbed to sepsis with in 3 days of surgery, while only ~45% of the WT mice died (P=0.0032) (Fig 3.1A). Interestingly,

HET mice had mortality similar to WT mice suggesting that one allele of β-arr1 is sufficient to inhibit sepsis-induced mortality (Fig. 3.1A). Since all the knockout mice died within 3 days of surgery, we theorized that the deaths likely were consequent to an early dysregulated inflammatory response[22,23,24]. Given that plasma cytokine levels, especially IL-6 at an early time point is predictive of mortality in this model we assessed its production in response to the septic insult. Associated with accelerated mortality, plasma IL-6 levels were significantly elevated in septic KO mice at both 6 and 12 hours post sepsis compared to the WT (Fig 3.1B). At 24 hours post-sepsis, IL6 levels decreased significantly and were similar between the WT and KO mice. Similarly, TNFα, IL-12p40 and IL-10 were also significantly elevated in septic KO mice as compared to the WT, 12 hours post-CLP and decreased to WT levels by 24 hours post sepsis (Fig 3.1C). This exaggerated early cytokine response was not observed in septic HET mice, consistent with their mortality being similar to that of WT.

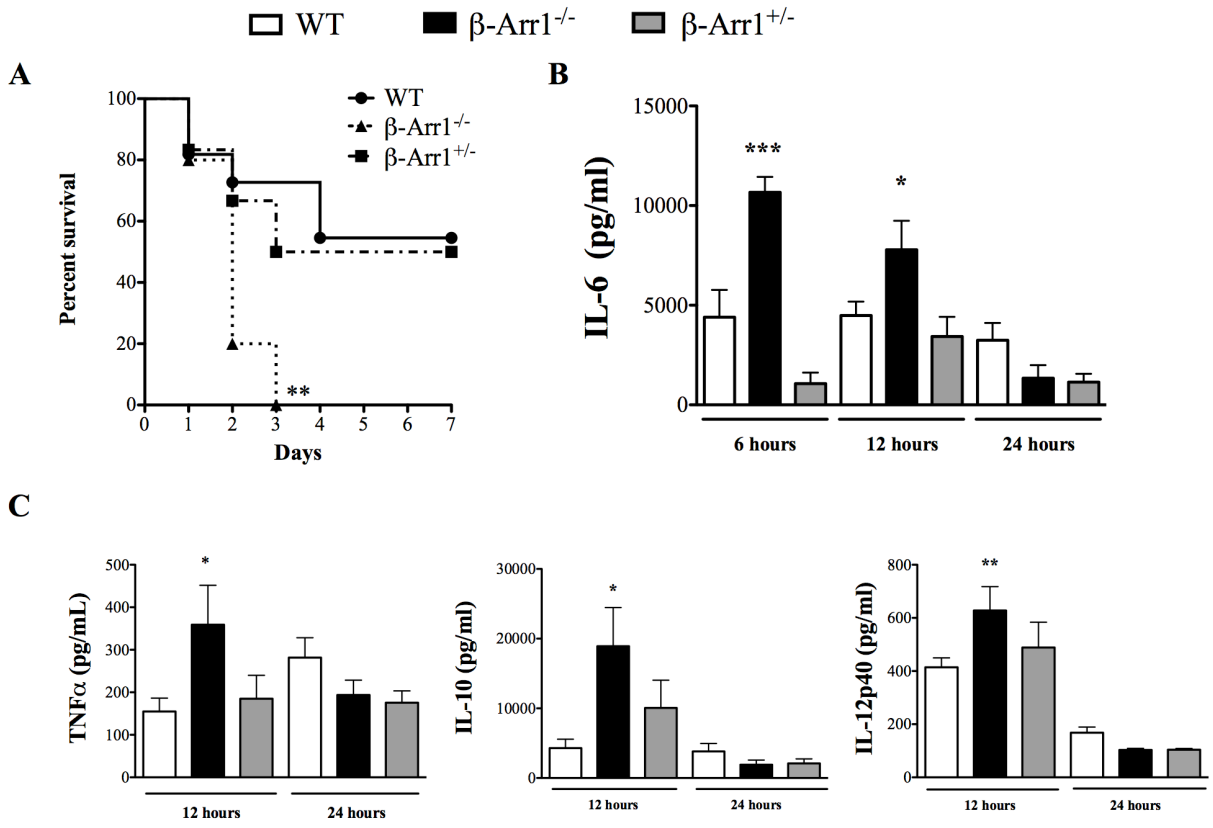

Figure 3.1: Role of β-arrestin1 (β-arr1) in sepsis-induced mortality and inflammation A) Wild type (WT), β-arr1 knockout (β-arr1$^{-/-}$) and βarr1 heterozygous (β-arr1$^{+/-}$) mice were subjected to 16G-single puncture (16G-SP) surgery and observed for mortality over 7 days. **p<0.01 compared to WT by log rank (Mantel Cox) test. N= 10-12 mice for each genotype. B-C) Mice from the different genotypes were subjected to CLP as indicated in (A) and plasma cytokine concentrations in septic mice determined at indicated time points post-surgery. *p<0.05, **p<0.01 and ***p<0.001 compared to WT using t-test . N= 8-14, data pooled from atleast two independent experiments.

Regulation of bacterial clearance and cellular infiltration by β-arrestin1

Cellular infiltration into the site of injury and bacterial clearance are critical factors impacting both inflammation and mortality[25,26] and could potentially contribute to hyper-inflammatory phenotype in septic KO mice. Examination of cellular infiltrate into the peritoneal cavity revealed marked neutrophil infiltration in response to septic insult but no significant difference between the three genotypes at both 12 and 24 hours post surgery (Fig 3.2A). In this grade of sepsis, peritonitis did not induce an increase in macrophage numbers at the site of infection even by 24 hours, which was nonetheless similar between the genotypes (Fig 3.2A). This suggests that cellular infiltration to the site of infection is likely not regulated by β-arrestin1.

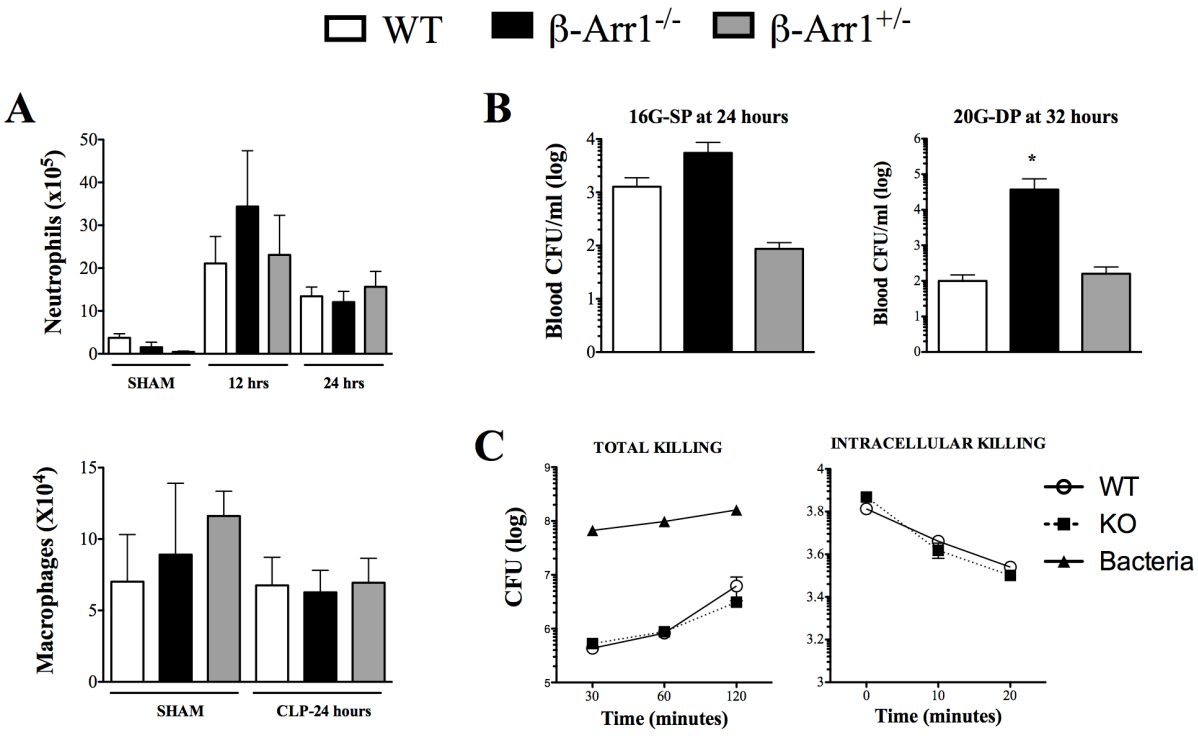

Figure 3.2: Role of β-arr1 in cellular infiltration and bacterial killing Wild type (WT), β-arr1 knockout (β-arr1$^{-/-}$) and β-arr1 heterozygous (β-arr1$^{+/-}$) mice underwent sham or CLP surgeries and were euthanized at defined time points. A) Neutrophil and macrophage

Figure 3.2 (cont'd)

infiltration in peritoneal cavity of sham mice at twenty-four and septic mice at indicated time points post surgery, determined using flow cytometry. B) Bacterial load represented as CFU/ml in blood of septic mice at indicated time points and grades of sepsis. N=9-14 for septic mice and N=3 for sham. Data for septic mice pooled from three independent experiments. C) Bacterial killing capacity of thioglycollate elicited neutrophils from WT and KO mice depicted as surviving bacteria (CFU) recovered from extracellular media at indicated time points for total bacterial killing and from cellular lysate at various time points post 20 minutes uptake for intracellular killing assay. N=3-5, *P<0.05 using student's t-test.

Interestingly, bacterial load in blood too was similar between WT and KO septic animals subjected to 16G-SP (Fig 3.2B). Bacterial load however, was higher in the septic KO mice subjected to 20G double puncture (20G-DP) suggesting that the role of β-arr1 in bacterial clearance *in vivo* is perhaps dependent on the severity of sepsis induction [27](Fig 3.2B). HET mice however, had bacterial loads similar to septic WT mice in both CLP models. To probe the potential role of β-arr1 in bactericidal activity independent of the confounding effect of ensuing inflammation, we examined the ability of thioglycollate-elicited neutrophils from WT and KO mice to kill bacteria *in vitro*. As shown, we did not observe any role for β-arr1 in intracellular or total *in vitro* bacterial killing assays (Fig 3.2C). It is possible that even though β-arr1 KO neutrophils do not seem to have any apparent defect in their ability to carry out efficient bacterial killing, β-arr1 might differentially regulate the effect of inflammation on bactericidal activity. It must be noted that in both models (16G-SP and 20G-DP), early levels of plasma IL-6 and IL-10

were significantly higher in septic KO mice as compared to the WT (Fig 3.1 and Fig 3.3A) even though bacterial clearance was differentially affected (Fig 3.2B). Additionally, peritoneal infiltration of neutrophils and macrophages was similar between the septic WT and β-arr1 KO animals in both grades of sepsis (Fig 3.2 and Fig 3.3B). Thus the exacerbated systemic cytokine levels in the septic KO mice are likely independent of systemic bacterial load or cellular infiltration to the site of infection.

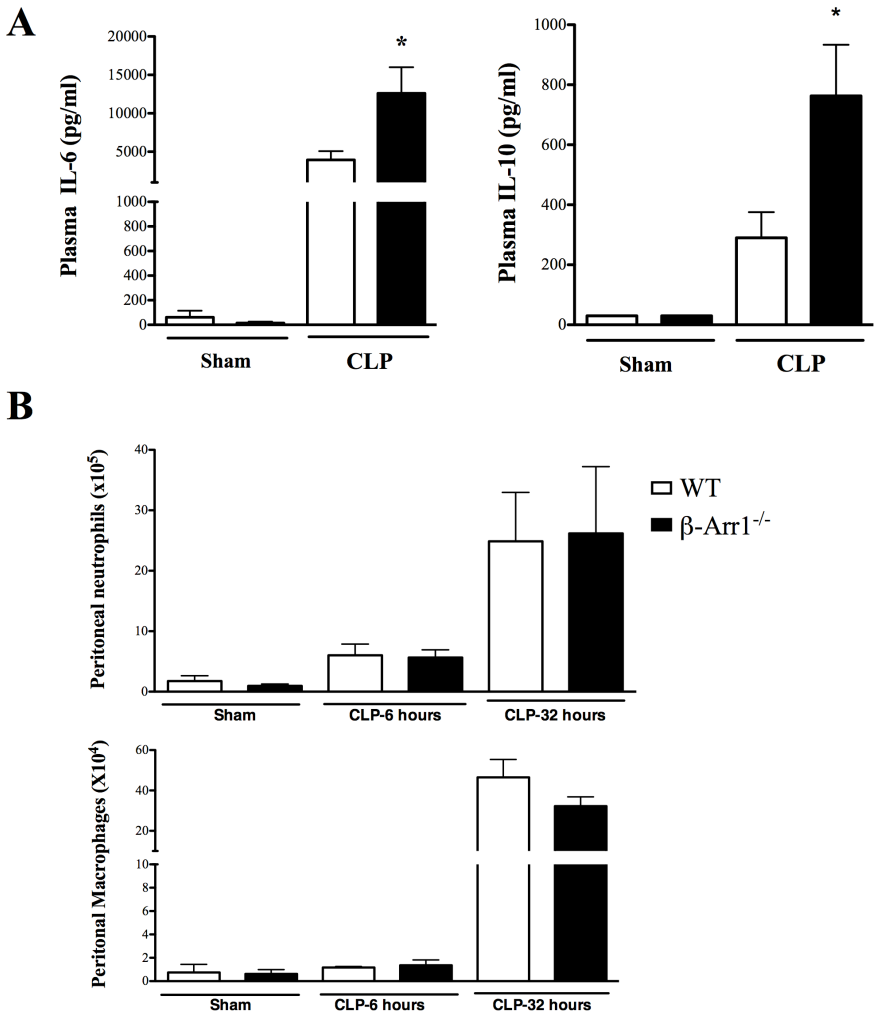

Figure 3.3: Role of β-arr1 in systemic cytokine production and cellular infiltration in sepsis

(A) Plasma cytokine concentrations in septic (20G-DP) and sham mice 6 hours post surgery. (B) Neutrophil and macrophage infiltration in peritoneal cavity of septic mice shown for the

Figure 3.3 (Cont'd)

indicated time points (6 hours for Sham). N=9-14 for septic mice and N=3 for sham. Data for septic mice pooled from three independent experiments. *P<0.05 using student's t-test.

β-arrestin1 inhibits tissue inflammation

Inflammatory mediators induced by septic insult are capable of inflicting host tissue damage due to excessive/injudicious production[28,1]. Even though histopathological lesions of tissue damage are not evident in early sepsis in major tissues including lung and liver[29,30], mortality is consequent to multiple organ dysfunction in sepsis. Given the mortality pattern of septic KO mice, we hypothesized that expression of inflammatory mediators (associated with organ dysfunction) would be higher in septic KO mice. Similar to plasma, IL-6 levels were significantly higher in heart, liver and lung of septic KO as compared to WT mice 12 hours post CLP (Fig 3.4A). In addition to assessing IL-6 protein levels, we also examined the mRNA expression of several inflammatory mediators in various tissues as follows:

Cardiac mRNA expression of IL6 and TNFα was significantly higher in the septic KO, as compared to septic WT mice (Figure 3.4B). Associated with these two pro-inflammatory cytokines, expression of NOS2/NOS3 expression was also markedly enhanced in KO hearts, suggesting increased NO production from iNOS allele and possible loss of protective effects of eNOS[31]. Together in the presence of higher levels of cytokines such as IL-6, this could lead to exacerbated cardiac dysfunction in the KO mice.

In contrast to the cardiac tissue, liver mRNA expression of IL6 and TNFα was similar in septic mice of both genotypes (data not shown), even though IL6 protein level was higher in tissue from septic KO mice (Fig 3.4A). However mRNA expression of coagulation factors, F3

"coagulation factor III" (TF) and PROCR "protein C receptor, endothelial" (EPCR) as well as NOS2/NOS3 were markedly elevated in septic KO livers compared to the WT mice (Figure 3.4C). Increase in PROCR expression in endotoxemia model has been shown to occur via PAR-1 dependent thrombin signaling, linking it to increased thrombin production and impaired anti-inflammatory and anti-coagulation pathways[32]. This pro-coagulant and increased *NO* production is potentially detrimental for liver tissue and could lead to excessive liver dysfunction in the KO mice [33].

Similar to the cardiac tissue, lungs from septic KO mice also exhibited significantly higher mRNA expression of IL6 and TNFα, as compared to the septic WT mice (Figure 3D). In addition, we also observed enhanced mRNA expression of pro-coagulant factor SERPINE1, "serpin peptidase inhibitor, clade E, member 1" (PAI, Plasminogen Activator Inhibitor) (Figure 3.4D) as well as that of adhesion molecules, ICAM and VCAM in the septic KO compared to the WT mice lungs (Figure 3.4D). Consistent with enhanced adhesion molecule expression, the number of BAL neutrophils was significantly higher in KO compared to the WT septic mice, at a later time point (24 hours) (Figure 3.4E). Since lung neutrophil sequestration has been shown to correlate with lung tissue damage in sepsis model[34] enhanced inflammation in the septic KO lung could lead to higher pulmonary dysfunction in septic KO animals.

Overall, the tissues examined showed an exacerbated inflammatory gene expression profile in knockout mice compared to the wild types, which together with the higher mortality suggest a greater extent of organ dysfunction in response to polymicrobial sepsis. In contrast to the KO and as expected, tissues for septic HET mice had similar levels of inflammatory mediators to that of septic WT mice (Fig 3.4A-E), consistent with comparable mortality between WT and HET mice. Histological examination of major organs (including lung, liver, kidney and heart) from septic

animals however, displayed minimal differences between WT and KO animals at both 24 and 48 hours after surgery (data not shown). Histopathological assessment of organ injury in the CLP model has been a bit controversial and recent studies have demonstrated that there are minimal histopathological differences in major organs of septic mice predicted to live versus those predicted to die even up to 48 hours after surgery[30,29].

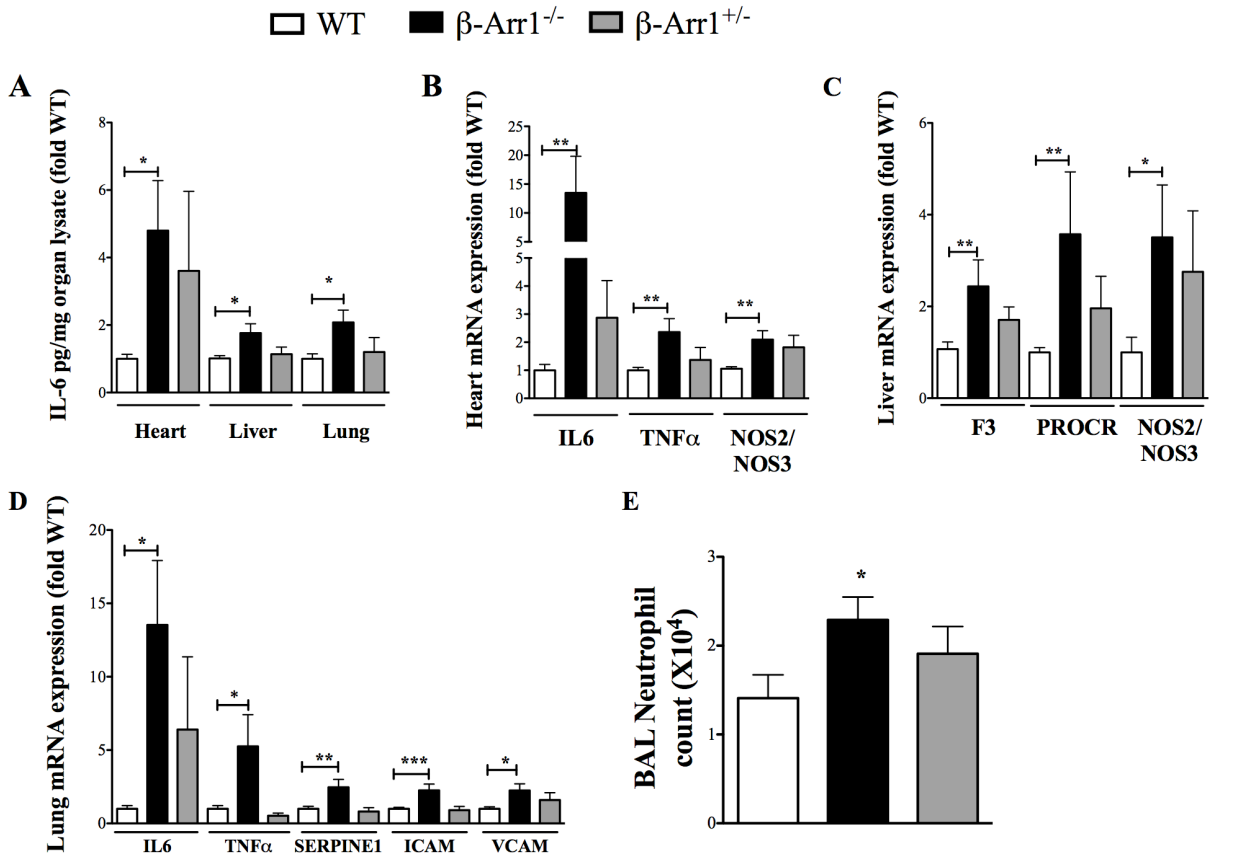

Figure 3.4: Role of β-arr1 in sepsis-induced organ inflammation Wild type (WT), β-arr1 knockout (β-arr1$^{-/-}$) and β-arr1 heterozygous (β-arr1$^{+/-}$) mice were subjected to 16G-single puncture (16G-SP) surgery and indicated organs collected at defined time points for analysis. A) IL-6 levels in organ lysates (determined by ELISA) from septic mice 12 hours post surgery. Quantitative real-time PCR analysis of inflammatory mediators in (B) heart (C) liver and (D) lung tissue from septic mice 12 hours post surgery. E) Total number of

Figure 3.4 (Cont'd)

neutrophils as determined by flow cytometry isolated from BAL of septic mice 24 hours post surgery. Protein and RNA data (A to D) is represented as fold WT and is pooled from atleast two independent experiments. Messenger RNA expression was normalized to HPRT prior to converting to fold WT. N=8-17 for each genotype. *P<0.05, **p<0.01 and ***p<0.001 using student's t-test.

β-arrestin1 inhibits cardiac IκBα phosphorylation

The enhanced pro-inflammatory signature observed in tissues from β-arr1 KO mice suggested enhanced activation of signaling pathways. β-arr1 has been shown to be an important regulator of NFκB[8] and MAPK pathways involved in production of inflammatory mediators[35,7,36,9]. To ascertain whether β-arr1 modulates signaling via any of these pathways and to correlate the gene expression to signaling, we determined activation status of these pathways in the heart lysates from septic mice. Consistent with the expected pattern, phospho-IκBα levels (Figure 3.5A-B) were enhanced in the heart lysate from septic KO mice as compared to WT. This was specific for IκBα since phosphorylation status of other pathways including ERK (Figure 3.5A,C) was comparable in heart lysates from septic WT and KO mice. Note that phosphorylation of other NFκB and MAPK molecules including P65, P105, JNK and p38 in septic mice of either genotype was either undetectable or very low in either genotype (data not shown). However, since this was a single time point analysis, differential activation of these signaling players during the course of the infection cannot be ruled out. Nonetheless, because there was enhanced activation of IκBα phosphorylation in the KO mice, we examined the mRNA levels of NFKBIA (IκBα) whose promoter is activated by NFκB[37]. Consistent with enhanced IκBα phosphorylation

in the KO hearts, mRNA expression of Iκbα was significantly enhanced in the KO compared to the WT hearts (Fig 3.5D). Together, these results suggest negative regulatory role for β-arr1 in NFκB activation in cardiac tissue in response to a septic insult and potentially links increased inflammatory mediator production to higher NFκB activation in the β-arr1 knockout mice.

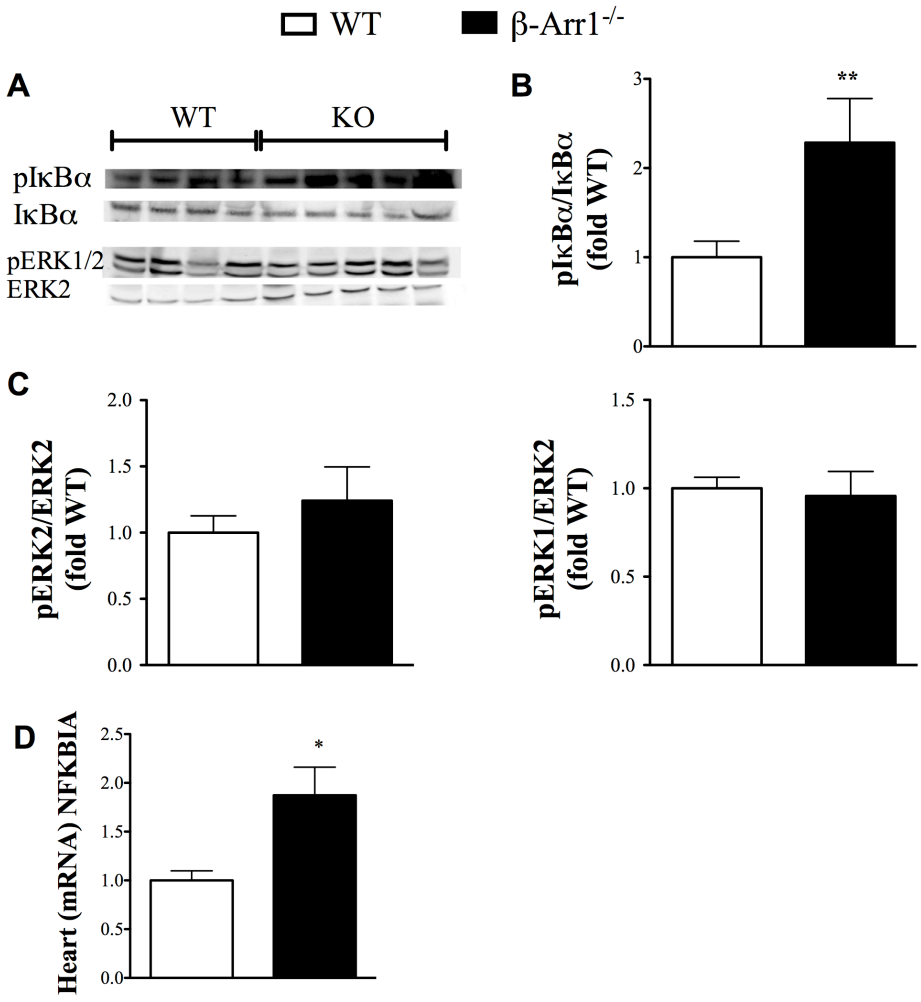

Figure 3.5: β-arrestin1 inhibits cardiac NFκB signaling Wild type (WT), and β-arr1 knockout (β-arr1$^{-/-}$) mice were subjected to 16G-single puncture (16G-SP) surgery and heart tissue collected 12 hours post-surgery. A) Representative blots and quantitative presentation of phosphorylation status of (B) IκBα and (C) ERK1/2 in heart lysates from

Figure 3.5 (Cont'd)

septic mice. Note that pIκBα was normalized to IκBα and pERK1/2 to ERK2 for loading control. D) Real time Q-RTPCR for NFKBIA (IκBα) mRNA expression in heart tissue from septic mice. N=8-10 for each genotype. Data is pooled from two independent experiments. *p<0.05, using student's t test.

Thymus Apoptosis and immune-suppression were unaffected by loss of β-arrestin1

Lymphocyte apoptosis and innate immune-suppression have drastic consequences in progression of sepsis[38] and have been correlated with dysregulated inflammatory cascade associated with mortality[39,40]. Given that sepsis progression was worse in the KO mice, we wanted to examine if the detrimental effects of lymphocyte apoptosis and immune-suppression played any role in the final outcome. To evaluate the extent of apoptosis induced in response to sepsis, lymphocyte cellular profile was evaluated in thymus and spleens of sham and septic mice. Note that the cellular profile in thymus and spleen of sham mice was unaffected by zygosity of β-arr1 (Table 3.2). Sepsis induced a drastic reduction in number (and proportion) of thymic CD4+CD8+ (DP) T cells, that was comparable between WT, KO and HET septic mice (Figure 3.6A). Additionally, caspase-3 activity that was significantly induced in response to sepsis was similar between thymic tissues from septic mice of all genotypes (Figure 3.6B). While septic WT spleen did not exhibit significant loss in $CD4^+$ (p=0.0563) or $CD8^+$ T-cells compared to sham mice, numbers of both T cell types were significantly lower in septic KO mice as compared to septic WT (Figure 3.6C). Splenic $CD4^+$ T cells were also lower in septic HET mice similar to that of the KO. Additionally, caspase-3 activity in spleen, even though slightly induced at this time point, was similar between the three genotypes in response to sepsis (Figure 3.6D). The discrepancy in the

spleen and thymus of KO septic mice with regard to the effect on T-cells might be due to lower expression of β-arr1 in thymus as compared to the spleen[41]. Additionally, $CD4^+$ T cells have greater amount of nuclear β-arr1, hence might be affected to a greater extent by its zygosity as compared to $CD8^+$ T cells.

Table 3.2: T cell distribution in lymphoid organs

THYMUS	WT	B-arr1$^{-/-}$	B-arr1$^{+/-}$
CD4+CD8+	$5.0\pm0.6\times10^7$	$5.7\pm1.6\times10^7$	$3.8\pm1.2\times10^7$
SPLEEN			
CD4+	$6.6\pm0.3\times10^6$	$6.3\pm0.6\times10^6$	$7.3\pm0.9\times10^6$
CD8+	$4.8\pm0.2\times10^6$	$4.5\pm0.5\times10^6$	$5.3\pm0.8\times10^6$

Thymus and spleen cells were processed, stained and run on LSRII, to identify T cell types. Total T cell counts were observed and recorded.

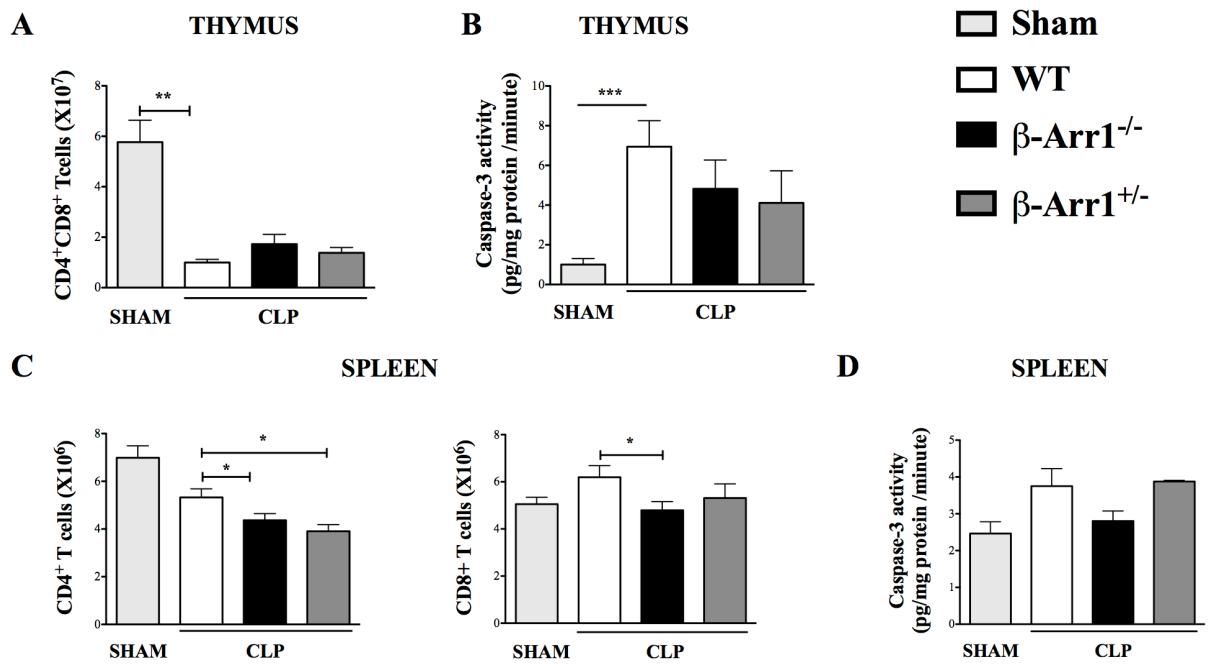

Figure 3.6: Role of β-arr1 in sepsis-induced lymphocyte apoptosis Wild type (WT), β-

Figure 3.6 (Cont'd)

arr1 knockout (β-arr1$^{-/-}$) and β-arr1 heterozygous (β-arr1$^{+/-}$) mice were subjected to 16G-single puncture (16G-SP) surgery and thymus and spleen collected 24 hours post-surgery for the indicated parameters/analysis. A) $CD4^+CD8^+$ T cells in thymus as determined by flow cytometry; and (B) caspase-3 activity in thymic lysates of septic mice as compared to WT-sham. C) $CD4^+$ and $CD8^+$ T cells in spleen as determined by flow cytometry and (D) caspase-3 activity in splenic lysates from septic mice as compared to WT-sham. N=4-6 for sham and N=10-19 for septic mice for each genotype and data is pooled from atleast three independent experiments for septic mice, except for E that has N=4-5. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ using student's t-test.

To assess immune-suppression, peritoneal and splenic cells from septic mice were stimulated with LPS as secondary stimuli and extent of cytokine production determined. Both cell populations responded to further stimuli even though splenic response (IL-6 $p<0.01$; TNFα $p<0.005$; IFNγ $p<0.01$) was more pronounced as compared to peritoneal cells (IL-6 $p=0.07$; TNFα $p<0.05$; IFNγ $p<0.05$, using one-tailed t test) (Fig 3.7A-B). In the absence of secondary stimuli (control), KO splenocytes exhibited significantly higher TNFα and IFNγ production (Fig 3.7A), while peritoneal cells produced higher IFNγ (Fig 3.7B; $p= 0.05$). Given that the cells had undergone exposure to LPS *in vivo,* to assess immune-suppression in response to secondary LPS stimulation, data was converted to fold change over control for each individual animal. As shown in Table 3.3, there was no statistically significant difference between the three genotypes in regard to the ability of splenic or peritoneal cell populations to respond to the secondary stimuli. However, spleen TNFα production was significantly higher in the KO compared to the

WT, both without and with secondary stimulation (Fig 3.7A). To further assess the role of β-arr1 in innate cell dysfunction, we determined the phagocytic potential and ROS generation capacity of peritoneal neutrophils from septic mice[27] and found neither to be affected by loss of β-arr1 in response to a septic insult (Fig 3.7C). Taken together, these results suggest that innate immune suppression following septic insult was unaffected by the loss of β-arr1.

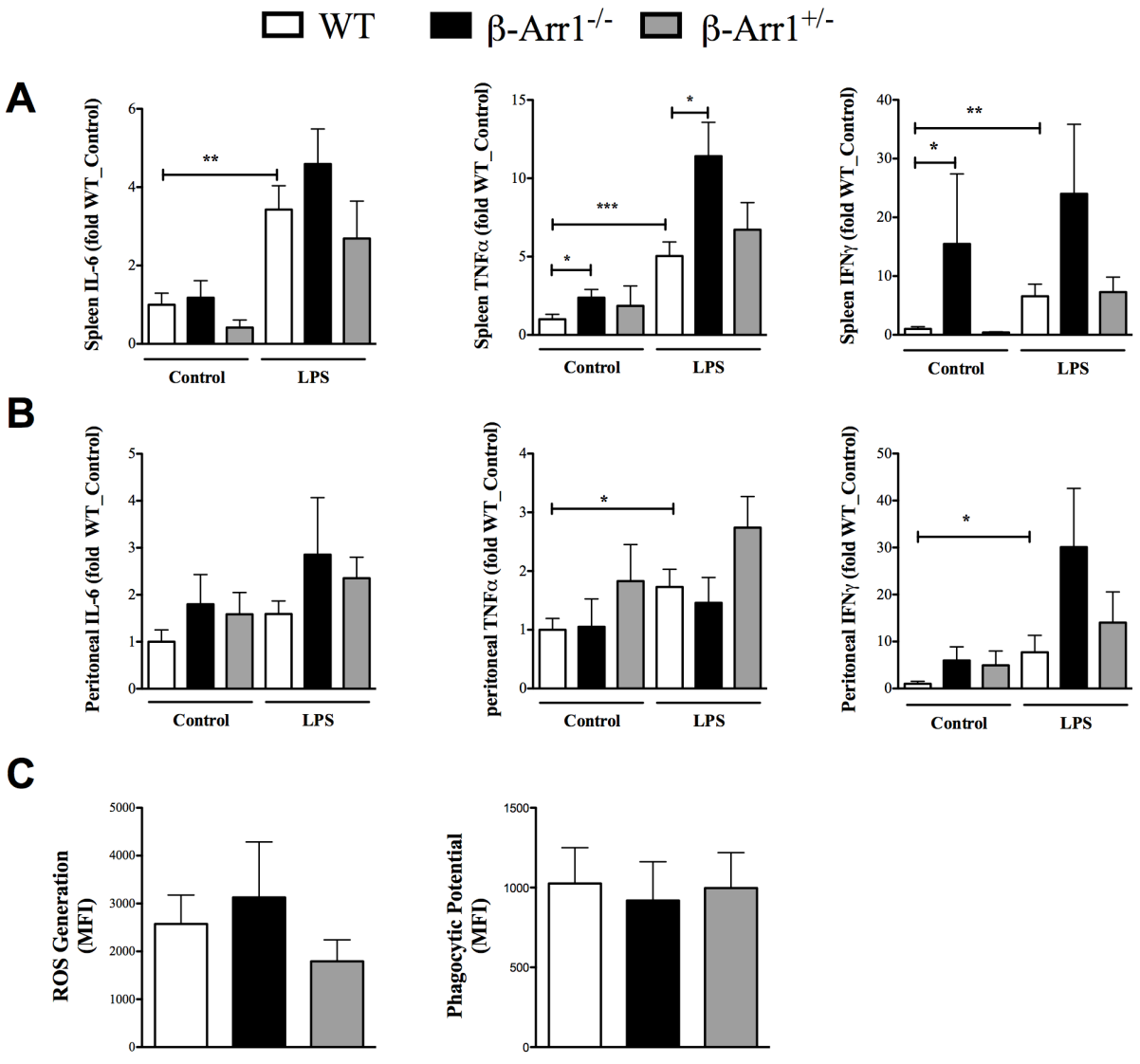

Figure 3.7: Role of β-arr1 in sepsis-induced immune-suppression Wild type (WT), β-arr1 knockout (β-arr1$^{-/-}$) and β-arr1 heterozygous (β-arr1$^{+/-}$) mice were subjected to 16G-

Figure 3.7 (Cont'd)

single puncture (16G-SP) surgery, and spleen and peritoneal cells collected 24 hours post-surgery and processed as described in the methods. Cells were then plated and left untreated (control) or stimulated for 18 hours with LPS (100 ng/ml). Cytokine levels in the supernatants in (A) splenocytes and (B) peritoneal cells in control and LPS stimuli as determined by ELISA. Data is presented as fold change over WT-control. C) Phagocytic potential and ROS generation in peritoneal cells from septic mice presented as MFI increase over controls. N=8-10 with data pooled from three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 using student's t-test.

Table 3.3: Immune suppression in septic mice

Splenocytes	**WT**	**β-arr1$^{-/-}$**	**β-arr1$^{+/-}$**
IL6	9.7±3.3	6.5±2.6	51.9±42.6
TNFα	36.8±19.2	15.2±7.6	45.9±30.1
IFNγ	4.6±2.2	1.1±0.2	8.9±2.3
Peritoneal Cells	**WT**	**β-arr1$^{-/-}$**	**β-arr1$^{+/-}$**
IL6	7.6±5.1	10.7±4.8	3.8±0.9
TNFα	5.3±2.1	25.2±12.3	3.6±0.7
IFNγ	37.2±27.3	36.3±29.5	5.2±2.2

Cytokine production by splenocytes and peritoneal cells following *ex vivo* LPS stimulation shown as fold change over unstimulated cells for each mouse. N= 6-8 mice for each genotype with data pooled from two independent experiments.

Non-hematopoietic β-arrestin1 negatively regulates inflammation following sepsis

Based on the mortality pattern, we predicted that exacerbated-inflammation was the likely cause of poor outcome in KO animals. In accordance, both systemic and tissue inflammation from septic animals were significantly higher in mice lacking β-arr1. To further determine the

biochemical mechanisms, we used splenocytes and peritoneal cells from naive mice and stimulated them with LPS and polymicrobial culture. Interestingly, LPS-induced IL-6 and TNFα from the KO splenic cells were significantly higher compared to the WT cells (Fig 3.8A). This response was quite opposite in the peritoneal cells from the KO mice with respect to IL-6 production (Fig 3.8B). There was no difference however, in cytokine production following polymicrobial stimulation of either population. This suggests that β-arr1 plays a cell and stimuli specific role in mediating cytokine production *in vitro*. Given the range of receptors and signalosomes employed in polymicrobial sepsis[42] and the potential for β-arr1 to intercept and regulate downstream effects[43,10,9], we first wanted to test whether the hyper-inflammatory phenotype observed in septic KO mice stems from β-arr1's role in the immune cells.

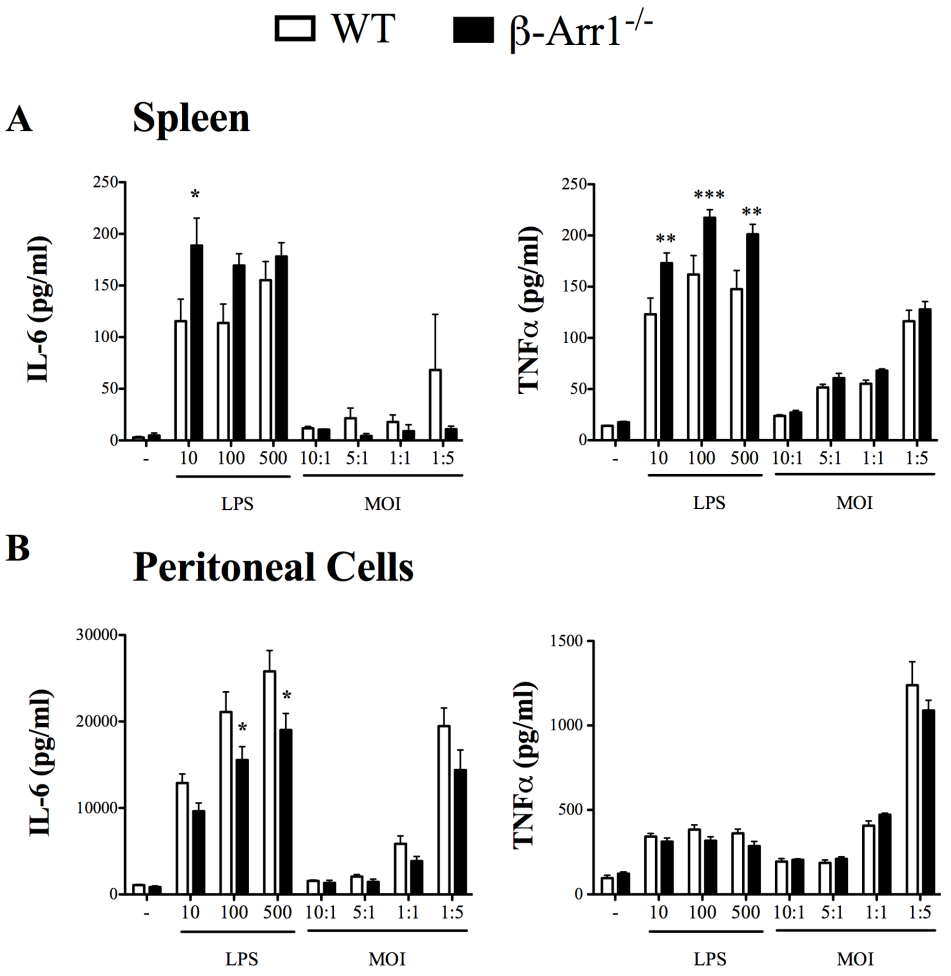

Figure 3.8: Role of β-arr1 in cytokine production using *in vitro* cell culture models

Spleen and resident peritoneal cells from the three genotypes were collected and processed as described in the methods. Equivalent number of cells were plated and treated with LPS and polymicrobial culture at different concentrations and multiplicity of infection (MOI) respectively for 18 hours. Supernatants were then assayed for IL-6 and TNFα concentrations using ELISA. Data from splenocytes shown in (A) and from peritoneal cells in (B). N=4-5 mice for each genotype. *p<0.05; **p<0.01; ***p<0.001 compared to WT as determined by 2-way ANOVA followed by Bonferroni post test.

To assess this, we generated bone marrow chimeras of WT and β-arr1 KO with donor and recipient genotypes comprising the hematopoietic or the non-hematopoietic compartments, respectively. The chimeric mice were found to have >92% donor derived leukocytes in the blood, using flow cytometry to distinguish between 45.1 and 45.2 alleles, except in the case of KO>KO transfers. The four groups of chimeric mice were subjected to CLP and cytokine production, immune cell infiltration (to site of infection) and bacterial clearance determined. Contrary to our expectations, we found that following 12 hours post-CLP; the non-hematopoietic β-arr1 knockout mice had significantly elevated levels of IL-6, IL-10, TNFα and MCP-1 in plasma, peritoneal fluid and spleen compared to the WT septic group (Figure 3.9A-C). Importantly levels of these cytokines were similar between the hematopoietic β-arr1 KO and the WT septic mice demonstrating that immune cell-specific β-arr1 is not the likely regulator of sepsis-induced inflammation. Consistent with these systemic effects, lung and liver IL-6 levels were significantly elevated in non-hematopoietic β-arr1 KO mice compared to the other groups (Figure 3.9D). Neither neutrophil infiltration to the initial site of infection (Figure 3.9E) nor systemic bacterial load (Figure 3.9F) was affected by loss of β-arr1 in either cellular compartment. Together these data demonstrate that the non-hematopoietic β-arr1 exerts negative regulatory role in sepsis-induced inflammation in mice.

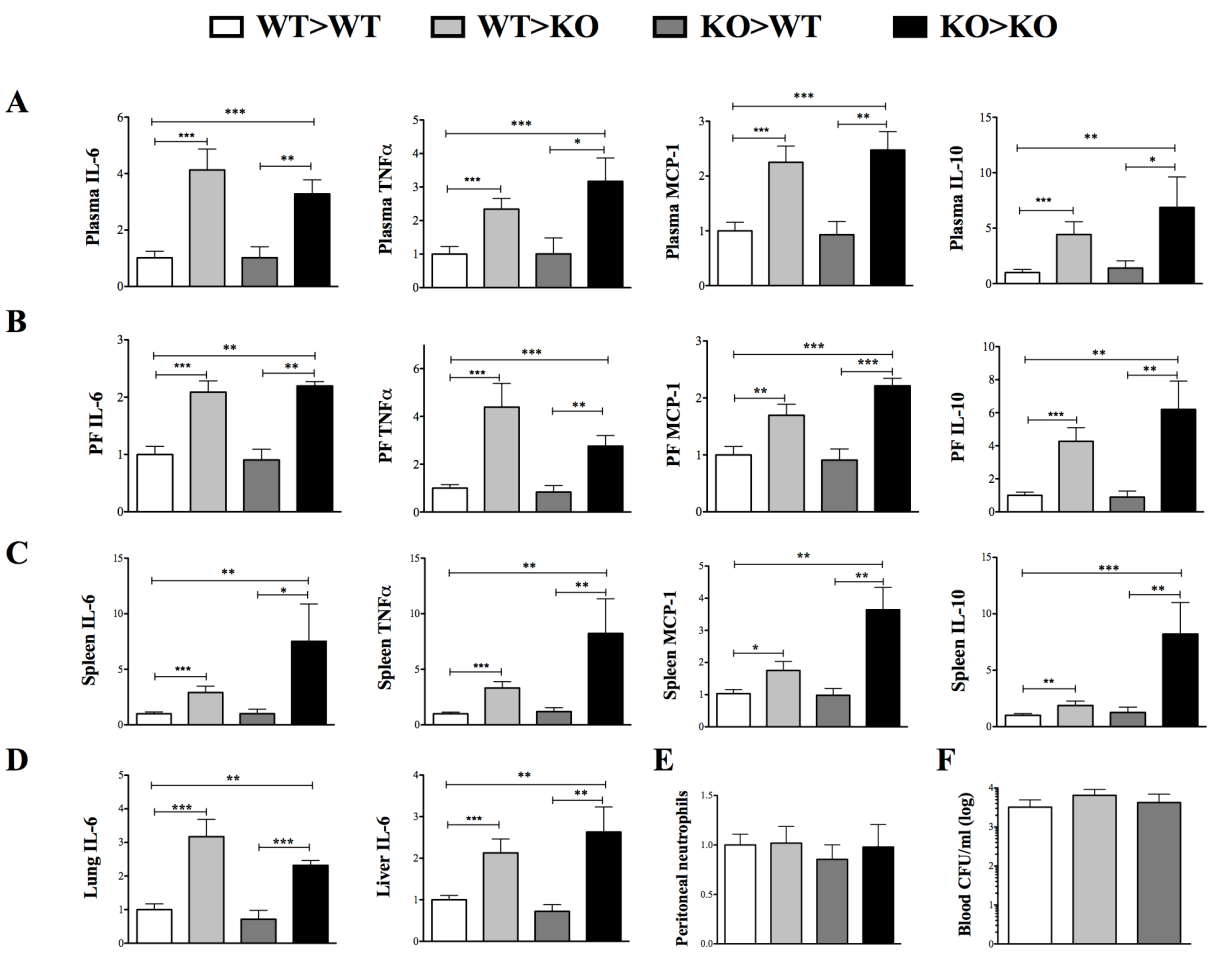

Figure 3.9: Non-hematopoietic-β-arr1 in sepsis-induced inflammation Bone marrow Chimeras were generated as described in methods. The four groups of mice were subjected to CLP (16G-SP) and 12 hours later euthanized for sample collection. Cytokine levels as determined by ELISA in (A) plasma, (B) peritoneal fluid, (C) spleen and (D) lung and liver lysates. (E) Peritoneal neutrophil infiltration and (F) blood bacterial load in septic mice shown as total count and CFU/ml respectively. Data in A-D is presented relative to WT for each group. The chimeric nomenclature used is donor>recipient such that the chimeric mouse has donor's hematopoietic cells and recipient's non-hematopoietic cells. N= 8-21 for each chimeric group except KO>KO that has N=5. Data is pooled from atleast 2 independent

Figure 3.9 (Cont'd)

experiments, except for KO>KO group. *p<0.05, **p<0.01 and ***p<0.001 using student's t-test.

DISCUSSION

Sepsis is a highly integrative pathophysiological disorder that if left untreated can result in multiple organ damage and mortality. In animal models of sepsis, host inflammatory sequelae including humoral response, cellular infiltration, lymphoid apoptosis and consequent immune-suppression have been identified as important factors in determining host susceptibility. The data presented here demonstrates a critical role for β-arr1 specifically in sepsis-induced inflammation and mortality, while ruling out the likely role for β-arr1 in chemotaxis (to the site of infection), bacterial killing, thymic apoptosis and immune suppression. Interestingly, the role of β-arr1 in polymicrobial sepsis-induced mortality is strikingly opposite to our previous findings on β-arr1 in the endotoxemia model of sepsis. It must be noted, however, that in both models, inflammation correlated and could be predictive of susceptibility to disease progression. This difference in outcome in endotoxemia and polymicrobial sepsis has also been observed for β-arrestin2 (β-arr2)[44,12] and IFN alpha-β receptor (IFNAR) knockout mice, which were similarly protected from former but susceptible to the latter model of sepsis[45]. This highlights the difference in pathophysiology of endotoxemia and polymicrobial sepsis with the instigating stimuli being endotoxin in the former versus gut microbes and necrotizing tissue in the latter.

β-arr1 is also a potential modulator of other inflammatory diseases, including colitis[14], arthritis[46] and EAE[41], although its loss is, surprisingly, protective in these models. This suggests

that the stimulus and ensuing inflammatory sequelae dictate the role β-arr1 plays in modulating the disease and therefore, understanding its mode of action in these different diseases in the context of instigating stimuli is important. Similar to the role of β-arr1, β-arr2 (aka arrestin-3) has also been shown to negatively regulate polymicrobial sepsis, both in surgical CLP as well as a non-surgical sepsis model[44,20]. Whether β-arr1 and 2 regulate similar or distinct pathways in polymicrobial sepsis will be pursued in future studies.

In various disease models and *in vitro* studies, β-arr1 has been ascribed diverse regulatory roles affecting immune cells. We therefore examined the differences in response to polymicrobial and LPS stimulation, using splenocytes and basal peritoneal cells as *in vitro* models. Curiously, the role of β-arr1 in cytokine production (specifically, IL-6) in response to LPS was found to be different based on cellular model used, similar to what we had observed earlier with respect to IFNγ production from $CD11b^-$ and $CD11b^+$ splenocytes[12]. Because of this perplexity, we further tested whether β-arr1 in immune cells is responsible for the observed results in sepsis and surprisingly uncovered the dominant negative regulatory role for β-arr1 in the non-hematopoietic cells in sepsis-induced inflammation. Although the identity of these cells remains the subject of future research, our results demonstrate that the role of β-arr1 is highly context dependent and therefore highlights major drawbacks in concluding the role of β-arrestins in inflammation based solely on *in vitro* cell culture studies.

Given the importance of non-immune cells such as endothelial and neuronal cells in sepsis progression, the role of non-hematopoietic compartment in sepsis-induced inflammation in general is not surprising. However, since previous studies on the role of β-arr1 in modulating inflammation has been extensively studied in, and attributed to, cells of hematopoietic origin (immune cells)[12,43, 9, 41] our results on the role of non-hematopoietic β-arr1 in septic inflammation

is unexpected and deserves further attention. It should be noted that β-arr1 was originally discovered for its role in desensitization of GPCRs[3] and more recently has been implicated in biased signaling from GPCRs[47]. Many GPCRs including C5aR[48,49], adenosine receptors[50,51], adrenergic receptors[52] have all been shown to play a critical role in sepsis pathogenesis. Interestingly, similar to our results, adenosine receptor (A2B) in the non-hematopoietic cells was also found to have a critical role in negatively regulating inflammation in response to sepsis [51,50]. Whether β-arr1 is involved in regulating these or other GPCRs in the non-hematopoietic cells in context of sepsis progression will be examined in future studies.

Taken together in this study we provide evidence that β-arr1 is a negative regulator of sepsis-induced inflammation and mortality. Even though previous studies have focused on the role of immune cell-specific β-arr1 in inflammation, our results demonstrate that β-arr1 in the non-hematopoietic cells functions as a negative regulator of sepsis-induced inflammation. Future studies will focus on identifying the appropriate physiological model to understand the biochemical basis by which β-arr1 suppresses sepsis-mediated inflammation. These studies will likely open up new avenues for development of therapeutic strategies against this devastating disorder.

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CHAPTER 4

This chapter represents work done towards a manuscript titled "**β-arrestin2 protects against colitis: Inhibitory role in T cell activation"** that will be submitted for publication.

Contributing authors include Deepika Sharma, Ankit Malik, Michael Steury and Narayanan Parameswaran.

ABSTRACT

β-arrestin2 (β-arr2), identified as a scaffold in GPCR desensitization and signaling have lately been shown to be important regulators of inflammation. Considering its role as a negative regulator of inflammation in polymicrobial stimulation, we wanted to investigate the role of β-arr2 in intestinal inflammation, a site of persistent microbial stimulation. In the absence of β-arr2, mice exhibited greater extent of mucosal inflammation determined by cellular infiltration and expression of inflammatory mediators even in the absence of exogenous stimuli. This increased their susceptibility to DSS induced colitic insult. β-arr2 knockout mice (KO) exhibit greater body weight loss; higher disease activity index and shortened colon as compared to wild type (WT) mice in response to DSS induced colitis. Higher susceptibility to colitis was independent of microbiota diversity since the distinct phenotype is maintained in co-housed mice. Further, one allele of β-arr2 sufficiently curbs the colitic response. T cells under both basal and colitic conditions displayed an altered activation status, implicating their involvement in disease induction. Further assessment of the role of β-arr2 in intrinsic T cell differentiation confirmed its importance in T cell polarization. T cells lacking β-arr2 exhibited a higher colitogenic potential, although the concurrent systemic wasting disease manifestation was lower. This study highlights the T cell specific role for β-arr2 in affecting colitis progression and a distinct role in regulation of systemic response.

INTRODUCTION

IBD is a multifactorial disease perpetuated by a dysregulated immune response. Being the site of constant interaction between the immune system and foreign antigens, diet and/or microbiota renders the balance between inflammatory and regulatory responses particularly essential for homeostasis. An imbalance by virtue of aberrant T cell activation or macrophage response contributes to pathogenesis of IBD[1]. Genetic susceptibility and environmental triggers can disrupt this balance and lead to inflammation against microbial or "self" antigens[2]. Current therapies against IBD include anti-inflammatory and immunosuppressive agents, but given the diverse etiology of the disease a significant proportion of patients is unresponsive to these. Further understanding of progression and factors affecting pathogenesis of IBD are therefore required to identify inflammatory nodes or pathways that could be targeted for novel therapies.

β-arrestins identified as scaffolding proteins required for GPCR desensitization have lately been found to be important in receptor endocytosis and GPCR-dependent and independent signaling. The family of GPCRs includes various receptors involved in immune responses including chemotaxis, proliferation and differentiation of leukocytes. It is an important player in inflammation and consequent pathogenesis of sepsis[3,4], allergic asthma[5], EAE[6] and rheumatoid arthritis[7]. Its involvement in innate responses to TLRs[8], adenovirus[9] and microbial stimulation[4] has been demonstrated. Further in T cells, CD28 mediated PDE4 recruitment[10] and T cell differentiation into Tregs was found to be dependent on β-arr2 expression[6]. Involvement of G proteins in T cell activation[11] and colitis[12] has also been previously demonstrated. Importantly, β-arrestin1 deficiency was found to be protective in DSS and TNBS induced colitis[13]. Together, these studies demonstrate the potential of G proteins and arrestins to mediate immune responses

via GPCR dependent or independent signaling, especially relevant to mucosal inflammation. Given the ability of β-arr2 to mediate innate an adaptive immune responses, we hypothesized that it would play a role in mucosal inflammation.

MATERIALS AND METHODS

Animals

β-arrestin2 knockout mice on C57BL/6 background (kindly provided by Dr. Robert Lefkowitz, Duke University) have been described earlier[14]. Wild type C57BL/6 mice were purchased from NCI and all mice were bred or housed at Michigan State University in rooms maintained at 22-24°C with 50% humidity and a 12-hr light-dark cycle. Mouse chow and water were provided *ad libitum* to all animals. All experiments were performed with age- and sex-matched mice between 8-12 weeks of age. Animal procedures were approved by Michigan state University institutional Animal Care and Use Committee (IACUC) and conformed to NIH guidelines. For co-housing experiments, Wild type and knockout animals were co-housed at weaning stage (approx 4 weeks) and used 8 weeks later. Alternatively, mice were housed separately with cages interchanged twice each week for 8 weeks.

DSS induced model of colitis

Mice were provided 3.5% or 5% DSS (w/v) in drinking water for 6 days and water for an additional day and sacrificed at this point or earlier if the body weight loss was over 25% initial weight. Over this period, mice were weighed everyday and observed for disease activity index indicated through stool consistency (1-loose); blood in stool (1-mild, 2-gross) ruffled hair coat (0

or 1); crusty eyes (0 or 1) and hunched posture (0 or 1). At the time of harvesting, splenic weight, colon length and thymic weight were noted as measures of inflammation.

RAG T cell transfer model of colitis

RAG mice, obtained from NCI were injected with 0.5 million cells, intraperitoneally. As follow up of colitis development, they were weighed once a week for first 3 weeks and thrice a week after that. Signs of disease development were observed and recorded in form of ruffled hair coat and hunched posture. At the time of harvesting colon length, weight and splenic weight were noted as measures of inflammation.

Sample Processing

At pre-determined time of harvesting, mice were euthanized using CO_2 asphyxiation. Plasma, spleen and MLN was harvested and processed as previously stated[4]. Briefly, spleen and MLN was crushed, subjected to RBC lysis, filtered through 40 μm nylon mesh and counted for stimulation. Colon length was noted and 5mm segments from distal end flash frozen for mRNA isolation; rest or part of the colon was processed as previously described[15]. Briefly, colon was cut into 5mm segments and incubated in epithelial dissociation buffer at 25°C with gentle shaking for 30 minutes. The segments were further cut into 1mm segments and incubated for an hour in 0.5mg/ml collagenase D. It was then strained through 100μm filter and loaded onto 80:40 percoll gradients. Cells were collected form the interface and used as leukocyte fraction.

T cell sorting

Spleen was processed as described above and subjected to CD4+ T cell enrichment by negative selection using miltenyi beads as per manufacturer's instructions. The enriched population was stained with CD4, CD25, CD44 and CD62L in RPMI media and washed with the same. Cells were sorted using Influx as naïve (CD4+CD25-CD44-CD62Lhi) and activated (CD4+CD25+CD44+CD62L-) under sterile conditions and stimulated as follows. For RAG T cell transfers, the CD4+ T cell enriched population was stained with CD4 and CD45RB antibodies and CD4+CD45RB hi cells were sorted under sterile conditions for transfer.

TCR Stimulation

Single cell suspensions from spleen or MLN were counted and stimulated with plate bound CD3 (5μg/ml) and CD28 (4μg/ml) for 48 hours and supernatant collected for cytokine analysis. Naïve and activated T cells isolated from spleen as described above were stimulated with plate bound CD3 (5μg/ml) and soluble CD28 in the presence of differentiating factors: IL-12 (10ng/ml) for Th1; IL-4 (40ng/ml) and anti-IFNγ (5μg/ml) for Th2; IL-6 (10ng/ml), TGFβ (1ng/ml), anti-IL2 (10μg/ml) and anti IFNγ (5μg/ml) for Th17 and TGFβ (1ng/ml) and anti IFNγ (5μg/ml) for Treg. The stimulation condition without these factors was termed Th0. Following 5 days of stimulation, the supernatant was collected for ELISA analysis while cells were further stimulated with phorbol ester and ionomycin in the presence of golgi stop brefeldinA for four hours and processed for flow cytometry.

Flow cytometry

Processed cells were surface stained with antibody cocktail made in 2.4G2 supernatant (fcγR blocking antibody) to block non-specific binding and washed with staining buffer (PBS with

sodium azide and BCS). When intracellular staining was required, cells were fixed using fixation buffer (ebioscience) and permeabilised and washed with perm buffer (PBS with sodium azide and saponin). The antibodies against cell surface markers CD11b, F4/80, Gr-1, CD3, CD19, CD4 and CD8; intracellular cytokines, IFNγ, IL-17A and IL-4; and transcription factors RORγT and Foxp3 were obtained from ebioscience and used as per manufacturer's instructions. Cells were run on LSR II and data analyzed using Flowjo software.

Cytokine/chemokine measurements

Cytokines were measured from plasma, splenic culture supernatant and peritoneal fluid using ELISA kits from ebioscience Inc. as per manufacturer's protocol.

Quantitative RT-PCR

To determine the relative levels of a specific RNA transcript, RNA was isolated from snap frozen tissue using Qiagen RNeasy mini kit using manufacturers' protocol. Reverse transcription was carried out with 1 μg of RNA using promega cDNA synthesis kit. Q-RT-PCR was performed with ABI fast 7500 (Applied biosystems) and all genes were normalized to HPRT as previously described[4]. Primer sequences are provided in Table 4.1.

Table 4.1: Primer sequences used for QPCR

Gene	Forward	Reverse
IL-6	ACAAGTCGGAGGCTTAAT TACACAT	TTGCCATTGCACAACTCTTTTC
TNFα	TCTCATCAGTTCTATGGCCC	GGGAGTAGACAAGCTACAAC

Table 4.1 (Cont'd)

IL-1β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
GmCSF	ATGCCTGTCACGTTGAATGAAG	GCGGGTCTGCACACATGTTA
IL-18	ACTGTACAACCGCAGTAATACGG	GGGTATTCTGTTATGGAAATACAGG
TGFβ	AGGGCTACCATGCCAACTTC	CCACGTAGTAGACGATGGGC
IL-12p40	GACCCTGCCCATTGAACTGGC	CAACGTTGCATCCTAGGATCG
IL-15	GCAGAGTTGGACGAAGAC	
IP-10	GATGACGGGCCAGTGAGAATGAG	CTGGGTAAAGGGGAGTGATGGAGA
CXCL9	TCCTTTTGGGCATCATCTTC	TTCCCCCTCTTTTGCTTTTT
CXCL11	GCATGTTCCAAGACAGCAGA	AGTAACGGCTGCGACAAAGT
CCL20	TTGCTTTGGCATGGGTACTG	TCGTAGTTGCTTGCTGCTTCTG
CX3CL1	GCCGCGTTCTTCCCATTTG	TGGGATTCGTGAGGTCATCTT
KC	CTTGAAGGTGTTGCCCTGAG	TGGGGACACCTTTTAGCATC
MIP2	GGCAAGGCTAACTGACCTGGAAAGG	ACAGCGAGGCACATGAGGTACGA
IFNγ	TGACCTCAAAGCCTGTGTGAT	AAGTATTTCCTCACAGCCAGCAG
IL-17A	CAGCAGCGATCATCCCTCAAA	CAGGACCAGGATCTCTTGCTG
IL-22	ATACATCGTCAACCGCACCTT	AGCCGGACATCTGTGTTGTTAT

Table 4.1 (Cont'd)

Tbet	CATGCCAGGGAACCGCTTA	GACGATCATCTGGGTCACATT
RORγT	ACCTCCACTGCCAGCTGTGTGCTG TC	TCATTTCTGCACTTCTGCATGTA GACTGTCCC
GATA3	TTATCAAGCCCAAGCGAAG	CCATTAGCGTTCCTCCTCCA
Foxp3	CACCCAGGAAAGACAGCAACC	GCAAGAGCTCTTGTCCATTGA

Histopathology

Colon was harvested and a section swiss rolled and fixed overnight in 10% formalin followed by 70% ethanol. It was then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Histopathology score was calculated by a certified pathologist.

Statistical Analysis

All experimental data in the figures is expressed as mean±SEM and analyzed using GraphPad Prism Software. Each ''N'' represents individual mouse. Student's t-test (for comparing groups with equal variances) or Mann-Whitney (for comparing groups with unequal variances) was used to compare two experimental groups. P-values <0.05 were considered statistically significant.

RESULTS

β-arrestin2 inhibits gut mucosal inflammation under homeostatic conditions

Given the negative regulatory role of β-arr2 in polymicrobial sepsis, we hypothesized that it might be an important regulator of intestinal inflammation. To ascertain its role in mucosal

homeostasis, we determined cellularity in colonic lamina propria (cLP) and expression of inflammatory and regulatory mediators in colon tissue of wild type (WT), β-arrestin2 knockout (KO**)** and β-arrestin2 heterozygous (HET) mice. Interestingly, cLP in KO mice exhibited a higher number of cells of both innate (macrophages, dendritic cells and neutrophils) and adaptive (CD4+ and CD8+ T cells) immune system (Fig 4.1). Production of inflammatory mediators including a vast array of innate cytokines, chemokines and T cell effectors were significantly higher in KO colon tissue even in absence of any exogenous stimuli (Table 4.2). Particularly pronounced was the increase in type I T cell cytokine IFNγ, transcription factor T-bet, modulating genes (TNFα, GmCSF, IL-1β) and downstream genes IP-10, CXCL9, CXCL11. Similar increase was observed in type 17 responses with higher expression of IL-17A and transcription factor RORγT. IL-22 expression, on the other hand was lower in KO colon tissue suggesting likely decreased anti-microbial defenses. Unlike KO, HET colons exhibited inflammatory mediator expression similar or lower to WT colon (Table 4.2) even though T cell infiltration into cLP was higher (Fig 4.1). This distinction in two parameters of inflammation of gene dosage could be explained by two possibilities: 1. β-arr2 inhibits inflammatory genes and cellular infiltration by distinct mechanisms and expression of one allele of β-arr2 is sufficient to inhibit gene expression but not T-cell infiltration. 2. Even though T cell numbers are higher, lack of equivalent increase in antigen presenting cells (APC) in the heterozygous mice could halt their activation and therefore consequent gene expression.

Inspite of the altered activation status no overt signs of clinical disease or histopathological colitic manifestations were observed in the mice lacking β-arr2 (data not shown).

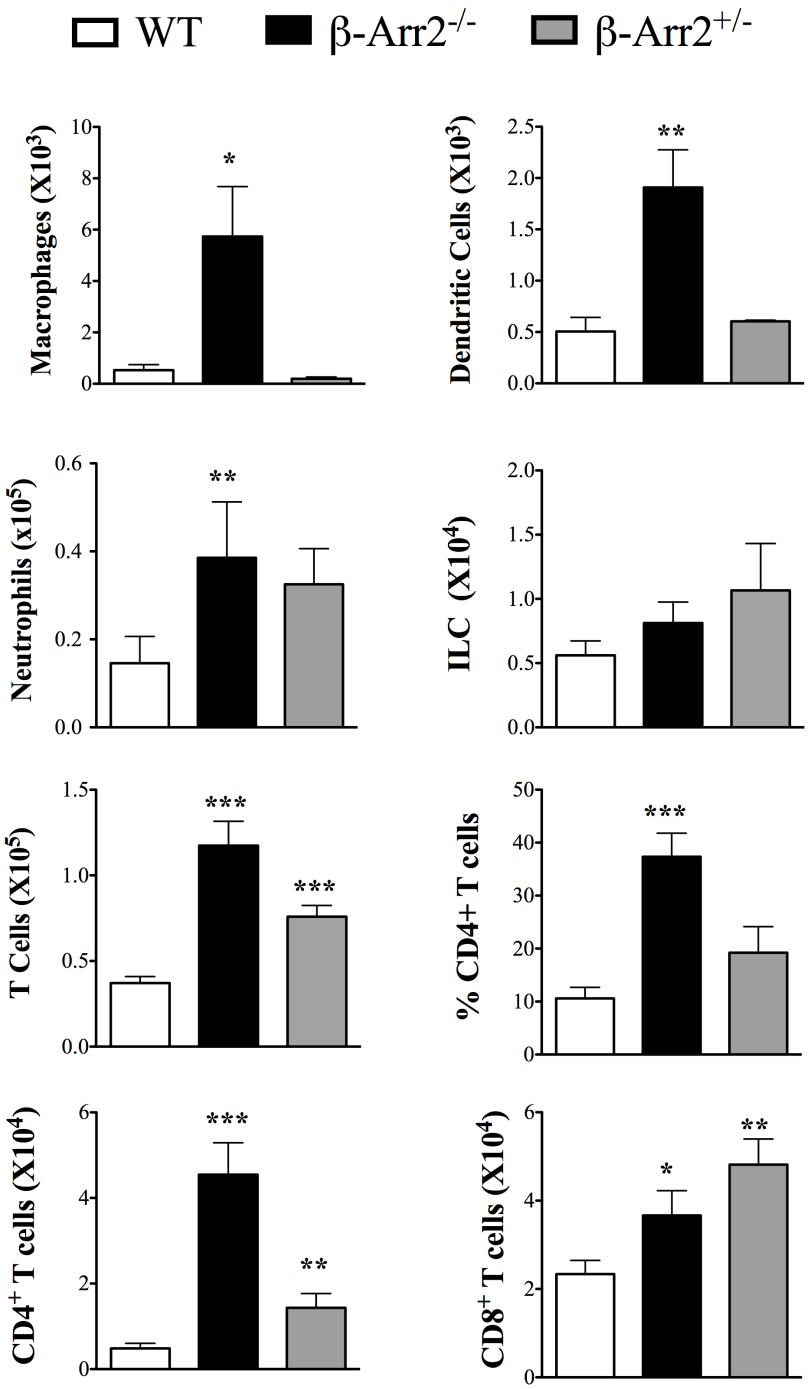

Figure 4.1: β-arrestin2 negatively regulates mucosal inflammation under homeostatic conditions Total number of cells obtained from colonic lamina propria (cLP) of Wild type (WT), β-arrestin2 knockout (β-arr2$^{-/-}$) and β-arrestin2 heterozygous (β-arr2$^{+/-}$) mice kept under basal

Figure 4.1 (Cont'd)

conditions. N=5-13 mice for each genotype. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as compared to WT using student's t-test.

Table 4.2: Inflammatory mediator expressions in basal colon

	WT	β-arr2$^{-/-}$	β-arr2$^{+/-}$
Innate cytokines			
IL-6	0.9±0.2	**22.5±15.0**	0.5±0.2
TNFα	1±0.2	**4.6±1.6**	1±0.1
IL-1β	1±0.2	**11.8±6.7**	0.6±0.2
GmCSF	1±0.2	**7.3±2.4**	0.8±0.3
IL-18	1.1±0.3	0.9±1.3	
TGFβ	1±0.2	**1.4±0.1**	
IL-12p40	1±0.3	2.4±1.1	**0.4±0.2**
IL-15	1±0.1	**1.9±0.4**	2.4±0.7
Chemokines			
IP-10	1±0.4	**3.4±0.9**	0.7±0.3
CXCL9	1.±0.3	**26.8±13.0**	1.8±0.9
CXCL11	1±0.2	**5.6±1.3**	1.9±0.4
CCL20	1±0.2	0.8±0.2	
CX3CL1	1±0.2	1.1±0.3	0.9±0.3
KC	1±0.4	0.7±0.2	**0.2±0.1**
MIP2	1±0.42	2.3±0.7	**0.3±0.1**
T-Cell cytokines/Differentiation markers			
IFNγ	1±0.2	**10.8±4.2**	2.2±0.8
IL-17A	1±0.1	**6.6±3.4**	0.3±0.2
IL-22	1±0.4	**0.6±0.2**	0.5±0.5
Tbet	1±0.1	**3.0±0.6**	0.7±0.2
RORγT	1±0.2	**1.5±0.1**	**0.3±0.1**
GATA3	1±0.1	2.5±0.8	3.1±2.4
Foxp3	1±0.3	1.4±0.5	**0.5±0.1**

Table 4.2 (Cont'd)

RNA was extracted from distal colon segments of WT, β-arr2$^{-/-}$ and β-arr2$^{+/-}$ mice, subjected to cDNA synthesis and QPCR to quantify the expression of mentioned genes using primers noted in table 4.1. Data is presented as fold change over WT. Genes that are differentially regulated and are statistically significant in β-arr2$^{-/-}$ and β-arr2$^{+/-}$ colon are compared to the WT are marked in bold.

Loss of β-arrestin2 alters T cell activation status under homeostatic conditions

T cells can be identified as effector, central memory or naïve based on surface expression of activation marker CD44 and homing integrin CD62L. Effector, central memory and naïve T cells are CD44^{+}CD62L^{lo}, CD44^{+}CD62L^{hi} and CD44^{-}CD62L^{hi} respectively. MLN and spleen are critical lymphoid organs associated with T cells activation and homing to the gut. While the gut has largely central memory T cells, lymphoid organs act as reservoirs of naïve T cells. Colonic lamina propria (cLP) from KO mice had significantly higher proportion of effector T cells (CD4+ and CD8+); while the spleen and MLN from the same mice had higher proportion of naïve CD4+ T cells (Fig 4.2). The colon is therefore, disposed to not only higher numbers but also greater proportion of activated T cells.

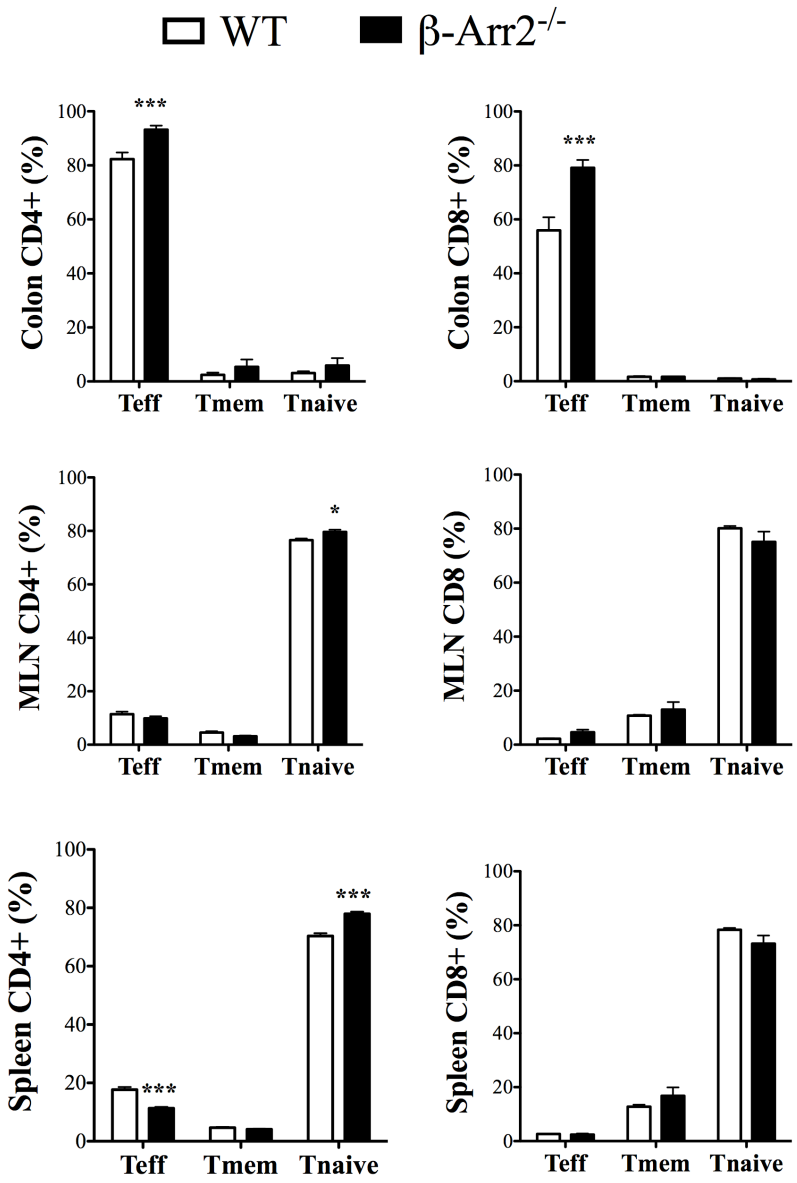

Figure 4.2: Loss of β-arrestin2 affects T cell activation status and distribution in gut and associated lymphoid tissues Percentage of effector memory (Teff), central memory (Tmem) and naïve (Tnaive) CD4 and CD8 T cells in colon (cLP), mesenteric lymph node (MLN) and spleen; identified as $CD44^{+}CD62L^{l0}$, $CD44^{+}CD62L^{hi}$ and $CD44^{-}CD62L^{hi}$ respectively in WT and β-arr2^{-}

Figure 4.2 (Cont'd)

$^{/-}$ mice. N=4-5 mice for each genotype. * $p<0.05$ and ***$p<0.001$ as compared to WT using student's t-test.

To determine the functional effect of β-arr2 in T-cells from spleen and MLN in response to T-cell receptor activation, we stimulated spleen and MLN cells with CD3 and CD28 ligation and examined IFNγ, IL17A and IL10 production. Interestingly, T-cell stimulation led to greater extent of IFNγ (Th1 cytokine) and IL-17A (Th17 cytokine) in cells from KO mice (Fig 4.3). Splenic KO cells also produced higher IL-10 as compared to the WT. Cells from Het mice produced either equivalent or as in case of spleen even lower IFNγ following T cell stimulation (Fig 4.3), reinstating the ability of β-arrestin2 expression from one allele to inhibit overt activation.

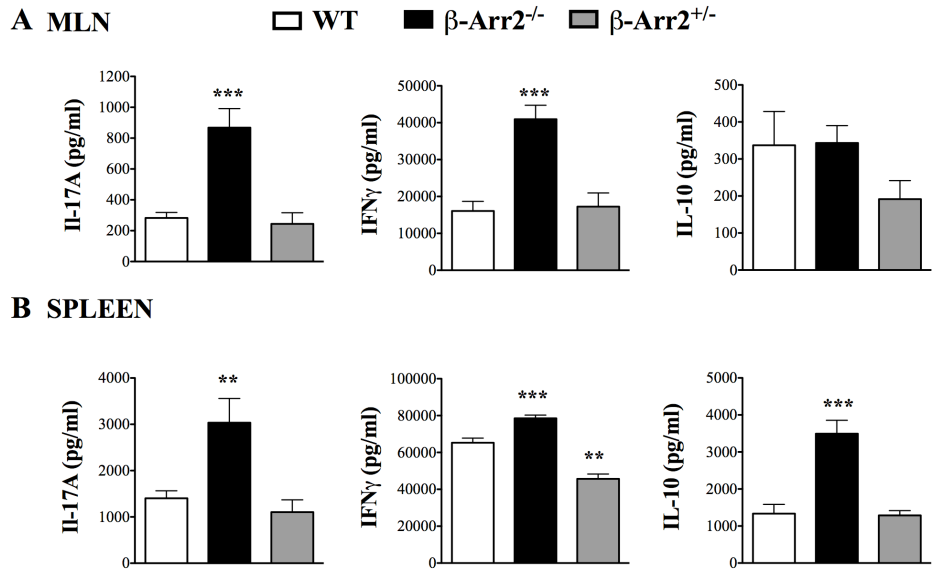

Figure 4.3: Loss of β-arrestin2 affects T cell differentiation potential in MLN and spleen

IL-17A, IFNγ and IL-10 production in response to CD3/28 stimulation of MLN and spleen T

Figure 4.3 (Cont'd)

cells for 72 hours in WT, β-arr2$^{-/-}$ and β-arr2$^{+/-}$ mice. N=8-16 for WT, β-arr2$^{-/-}$ mice and 4 for β-arr2$^{+/-}$ mice. **p<0.01, ***p<0.001 as compared to WT using student's t-test.

β-arrestin2 is protective in DSS induced colitis model

Since the KO mice had higher extent of intestinal inflammation we hypothesized that they would therefore be predisposed to colitis. To test this, mice were subjected to colitis through ingestion of 3.5% DSS in drinking water. All mice lost body weight starting day 5; exhibited signs of disease measured as disease activity score (DAI; indicated by loose and bloody stools, hunched posture, crusty eyes and ruffled hair coat) and had markedly shortened colons at the end of the period. All these indices of colitis and associated wasting disease were markedly higher in mice lacking β-arr2; KO mice lost significantly more weight as early as day 4; had higher DAI at days 6 and 7; and reduced colon length in response to DSS induced colitis (Figure 4.4A-C). HET mice, on the other hand, had colitis induction as measured by these parameters to the extent similar to WT (Figure 4A-C), suggesting that one allele is able to compensate and inhibit disease progression. Additionally, there was an inflammation induced thymic weight loss that was significantly higher in the KO colitic mice (Figure 4D). β-arr2 has been shown to be important in preventing stress induced lymphocyte reduction in a PI3K-Akt dependent manner [16], On the other hand, splenic weight increased in response to colitis and this increase exhibited a trend towards being higher in the colitic KO animals (Figure 4E). In the basal mice, splenic weight was significantly higher for Het mice as compared to WT even though it increased further in response to colitis. These results demonstrate that β-arr2 is protective against DSS induced colitis and associated wasting disease.

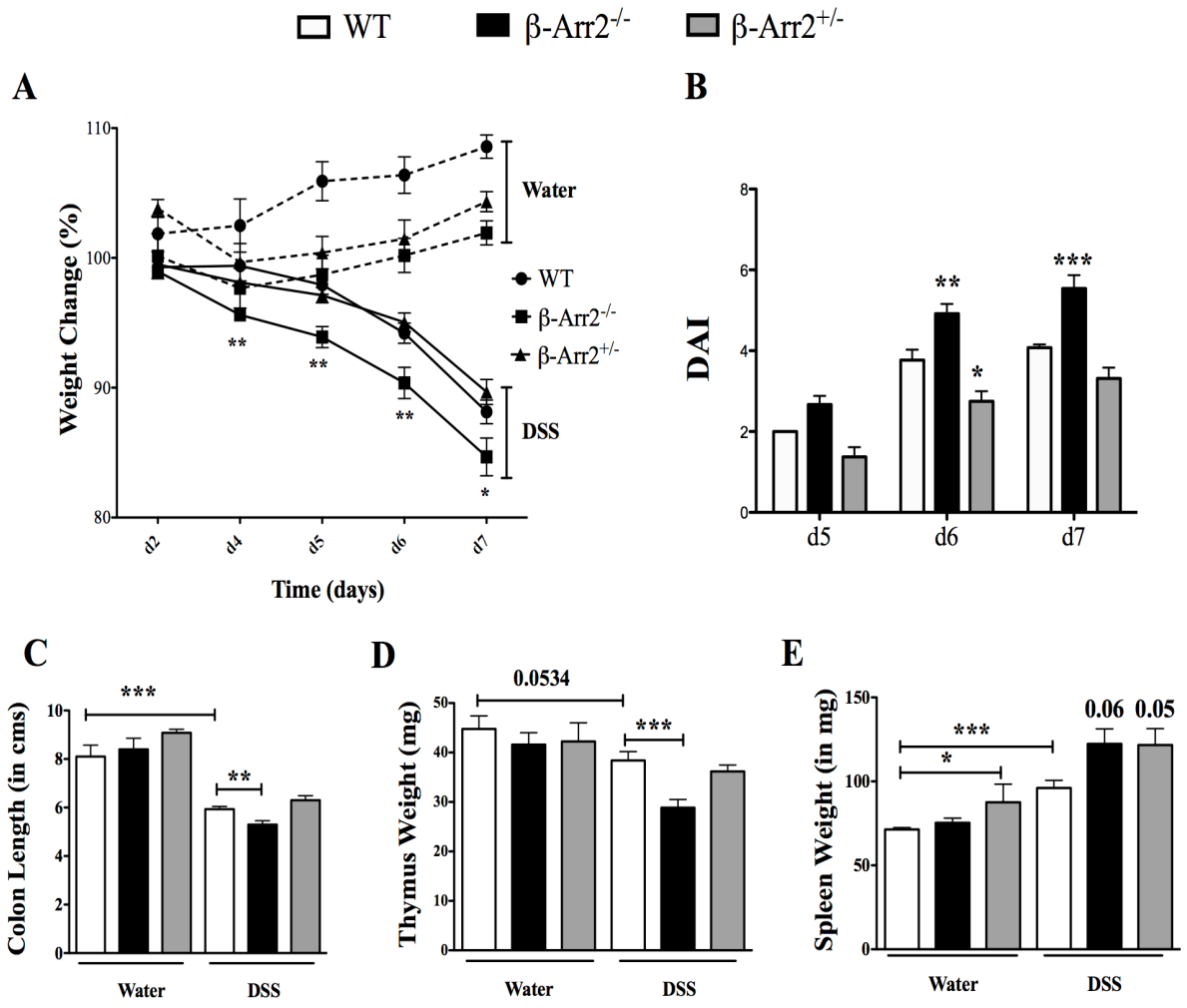

Figure 4.4: β-arrestin2 is protective in DSS induced colitis Wild type (WT), β-arrestin2 knockout (β-arr2$^{-/-}$) and β-arrestin2 heterozygous (β-arr2$^{+/-}$) mice were fed 3.5% DSS in their drinking water for 6 days to induce colitis and euthanized on day7. (a) Percentage body weight loss and (b) disease activity index observed over the course of the experiment. (c) Colon length, (d) thymus and (e) spleen weight recorded for control and colitic mice at day7. N=14-28 mice per genotype. Data pooled from atleast three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as compared to WT using 2-way ANOVA for weight loss and DAI and student's t-test for colon length, spleen and thymus weight.

β-arrestin2 inhibits DSS induced inflammation

Analysis of plasma cytokine revealed that even though IL-6, and IL-1β were produced to similar levels in colitic mice of all genotypes, IFNγ production was significantly higher in β-arr2 KO colitic mice (Figure 4.5). Plasma TNFα was not detectable in any of the genotypes (data not shown).

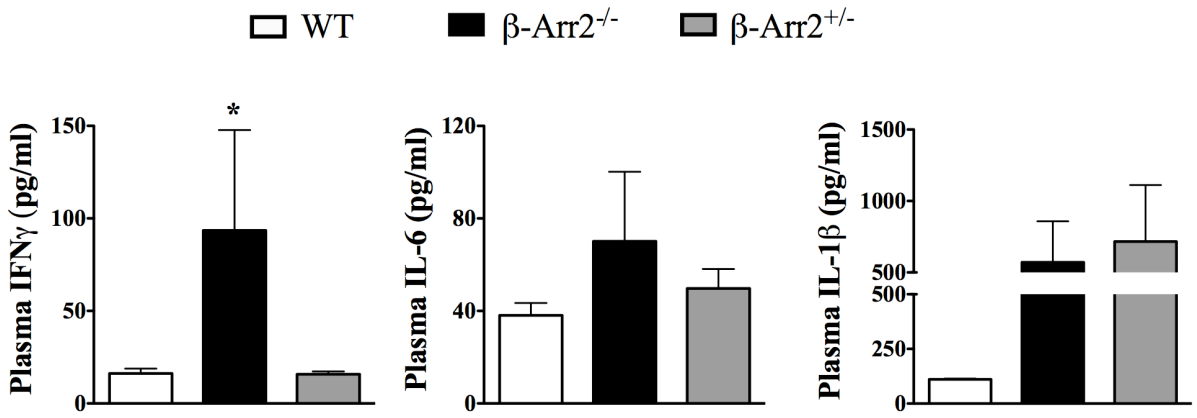

Figure 4.5: β-arrestin2 inhibits systemic cytokine response in colitic mice Observed cytokine concentration in plasma from colitic mice at day 7. N=13-15 mice for each genotype. * $p<0.05$ as compared to colitic WT using student's t-test.

In the colon, inflammatory gene expression as determined by QPCR analysis revealed increased expression of IL-1β and Th2 transcription factor GATA3; while RORγT expression was significantly lower in the colon from colitic KO mice (Table 4.3). Further analysis of cLP cellular infiltrates demonstrates increased infiltration of all analyzed cell types (innate and adaptive) in WT colitic mice as compared to the control mice (Fig 4.6). Colon from KO colitic mice showed increased infiltration of dendritic cell, innate lymphoid cells and neutrophils over control mice, although T cell numbers were unaffected by induction of colitis (Table 4.4). Het

mice exhibited increased infiltration of all innate cells (DC, macrophages, neutrophils, ILCs) but not T cells over Het controls (Table 4.4). Comparison of cellular infiltration in colitic mice showed increased numbers of CD4+ T cells but lower neutrophil numbers in KO colons as compared to the WT (Fig 4.6). Thus, even though colitis induced a potent inflammatory response in WT mice that in some respect was similar to the KO mice; T cell infiltration remained high in the KO perhaps thereby, mediating expedited and greater incidence of colitic and systemic response.

Table 4.3: Inflammatory mediator expression in response to DSS-induced colitis

	WT	β-arr2$^{-/-}$	β-arr2$^{+/-}$
Innate cytokines			
IL-1β	1±0.2	**4.0±1.8**	2.6±1.6
GmCSF	1±0.2	2.6±1.1(p=0.07)	1.4±0.4
T-Cell cytokines/Differentiation markers			
RORγT	1±0.2	**0.4±0.1**	0.6±0.3
GATA3	1±0.2	**5.3±3.1**	3.4±2.4

RNA was extracted from distal colon segments of WT, β-arr2$^{-/-}$ and β-arr2$^{+/-}$ colitic mice from experiment in fig 4.4 was used to quantify the expression of mentioned genes using primers noted in table 4.1. Data is presented as fold change over WT. Genes that are statistically significant in β-arr2$^{-/-}$ and β-arr2$^{+/-}$ colon are compared to the WT are marked in bold.

Table 4.4: Cellular infiltration in colonic lamina propria

	WT	**β-arr2$^{-/-}$**	**β-arr2^{+-}**
Macrophages	**3507±1180**	4692±1109	**3041±408**
Neutrophils	**140858±19340**	**73389±7598**	**110991±14907**
DCs	**8582±1672**	**6549±1267**	**5237±1422**

Table 4.4 (Cont'd)

ILCs	**22028±3301**	20209±3079	23033±3518
T cells	**105728±13127**	119670±19146	115321±13613
CD4+ T cells	**19562±2870**	51174±8669	18247±4112
CD8+ T cells	**56585±14162**	76354±19590	74338±12115

Total number of cells isolated from colonic lamina propria of control and DSS treated wild type (WT), β-arrestin2 knockout (β-arr2$^{-/-}$**)** and β-arrestin2 heterozygous (β-arr2$^{+/-}$) mice obtained from experiment in figure 4.4. DCs and ILCs are dendritic and innate lymphoid Cells, respectively. Genes that are statistically upregulated in response to DSS in each respective genotype are marked in bold.

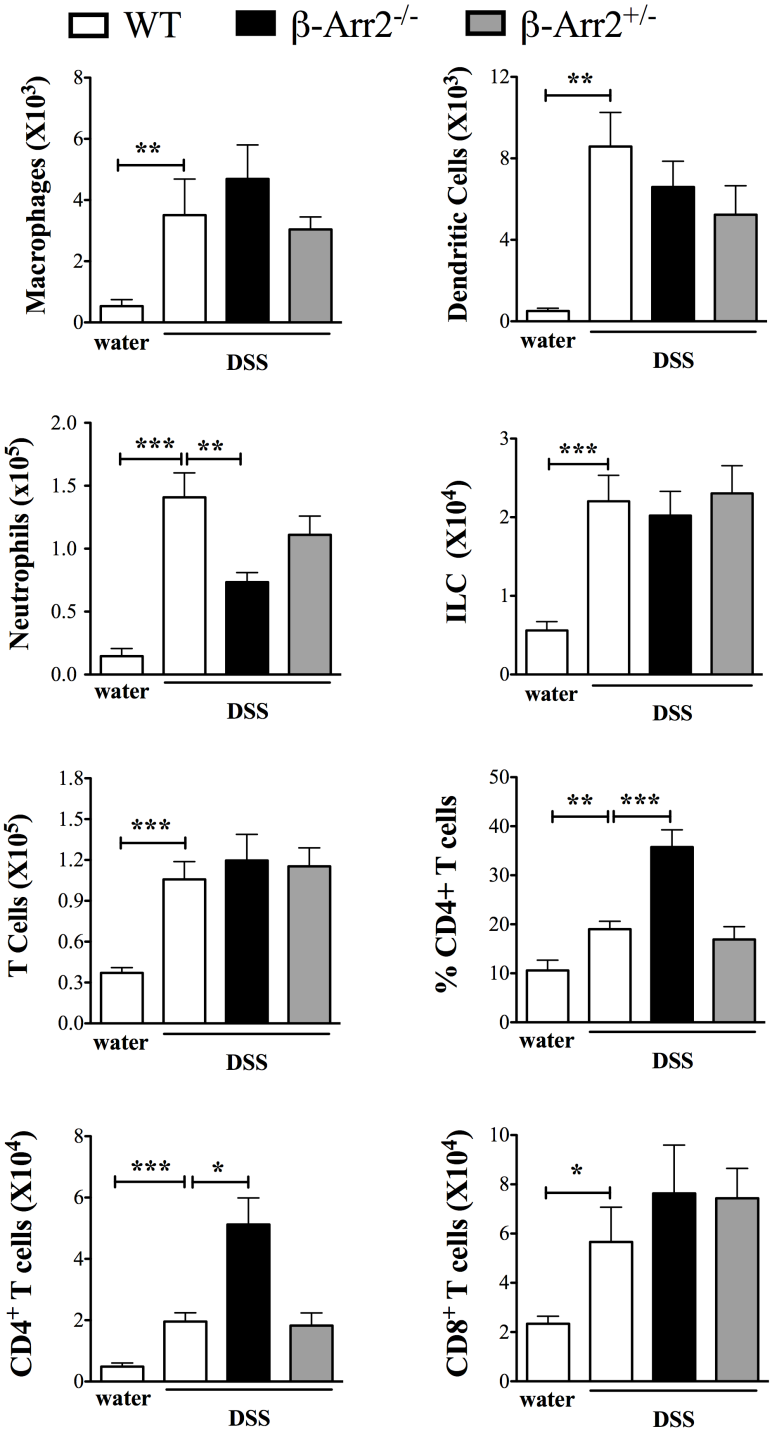

Figure 4.6: Cellular infiltration in colonic lamina propria of colitic mice Total number (and proportion) of inflammatory cells isolated from colonic lamina propria of colitic mice shown along with cell numbers in control WT mice (from figure 1). N=6-14 mice for each genotype.

Figure 4.6 (cont'd)

Data pooled form atleast 2 independent experiments. *$p<0.05$, **$p<0.01$, $p<0.001$ as compared to indicated group using student's t test.

Ex vivo T cell stimulation of cells isolated from colitic mice was further analyzed for their activation potential. Compared to WT, T cells from KO colitic tissue had markedly increased induction of Th1 and Th17 response, as suggested by increase in number of IL-17A+, IFNγ+ and double positive (DP, IL-17A+IFNγ+) CD4+ T cells (Figure 4.7B). Additionally, KO colon also exhibited higher proportion of IL-17A+ (Fig 4.7A) and RORγT+ CD4+ T cells (Fig 4.7C). This enhanced T cell differentiation was not observed in HET colitic mice, perhaps explaining why in spite of starting with higher T cell numbers in basal state, colitic progression was comparable to the WT mice. β-arrestin2 knockout mice therefore, exhibit increased T cell activation that is likely responsible for worsening of colitis.

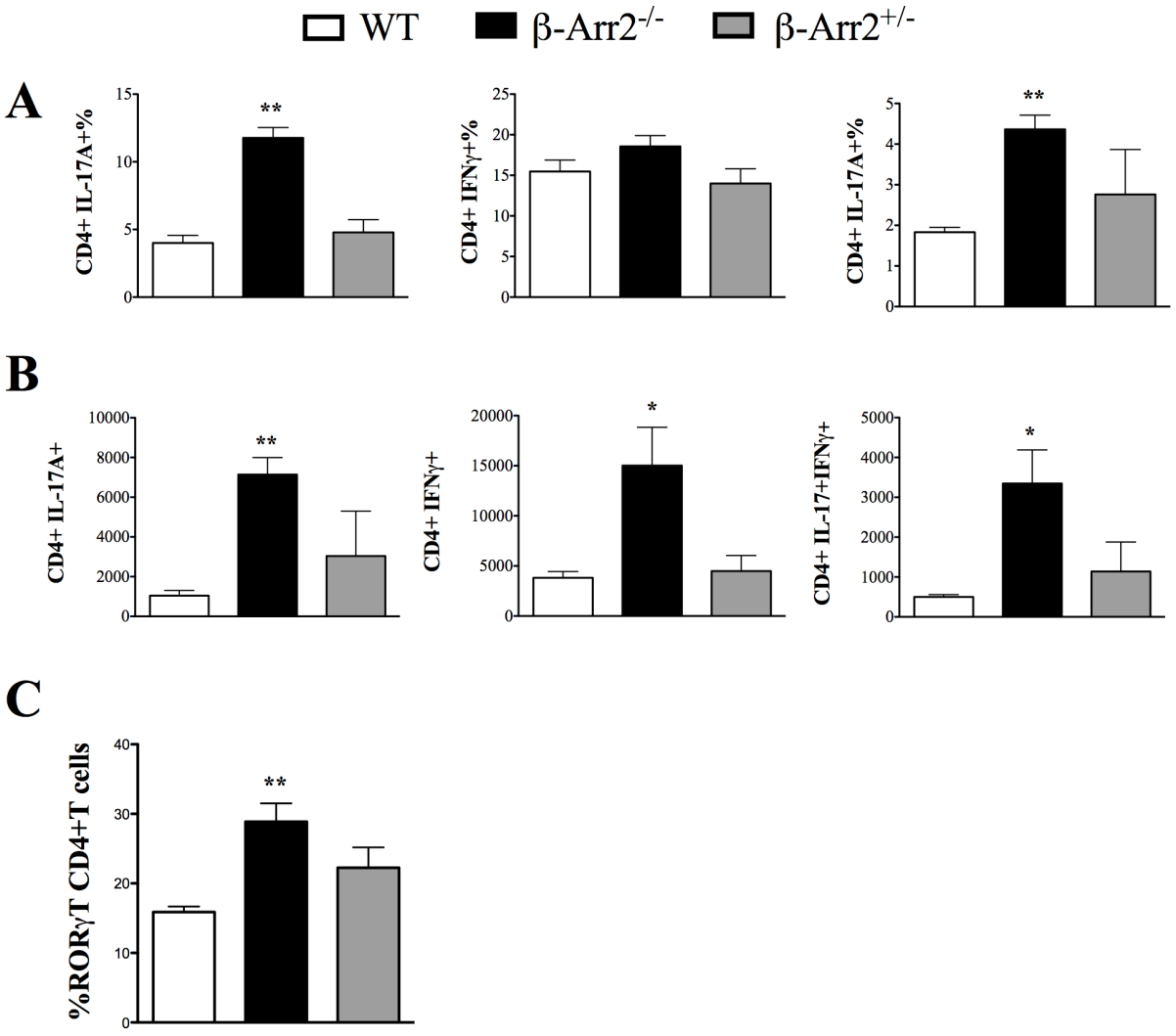

Figure 4.7 T cell differentiation in colitic mice cLP cells processed from colitic colons were processed and stimulated ex vivo to determine a) proportion and b) total number of CD4+ T cells producing IL-17A, IFNγ or both cytokines. Cells were also stained to determine proportion of RORγT+ CD4+ T cells in the colons of colitic mice. N=4-7 mice for each genotype. *p<0.05, **p<0.01, p<0.001 as compared to WT using student's t test.

β-arrestin2 protects against colitis independent of differences in microbial composition

To demonstrate that the role of β-arr2 in mediating DSS induced colitis was independent of microbiota, WT and KO mice were co-housed and then subjected to colitis. Even under these conditions, KO mice had significantly higher weight loss and greater shortening of colon in response to DSS treatment (Figure 4.8). Further, the trend for higher splenic weight and statistical significance for lower thymic weight in response to colitis was observed even in the co-housed KO animals (Figure 4.8). Together, these data suggest that, mice lacking β-arr2 suffer from exacerbated colitis and associated wasting disease and this phenotype is likely independent of microbial diversity.

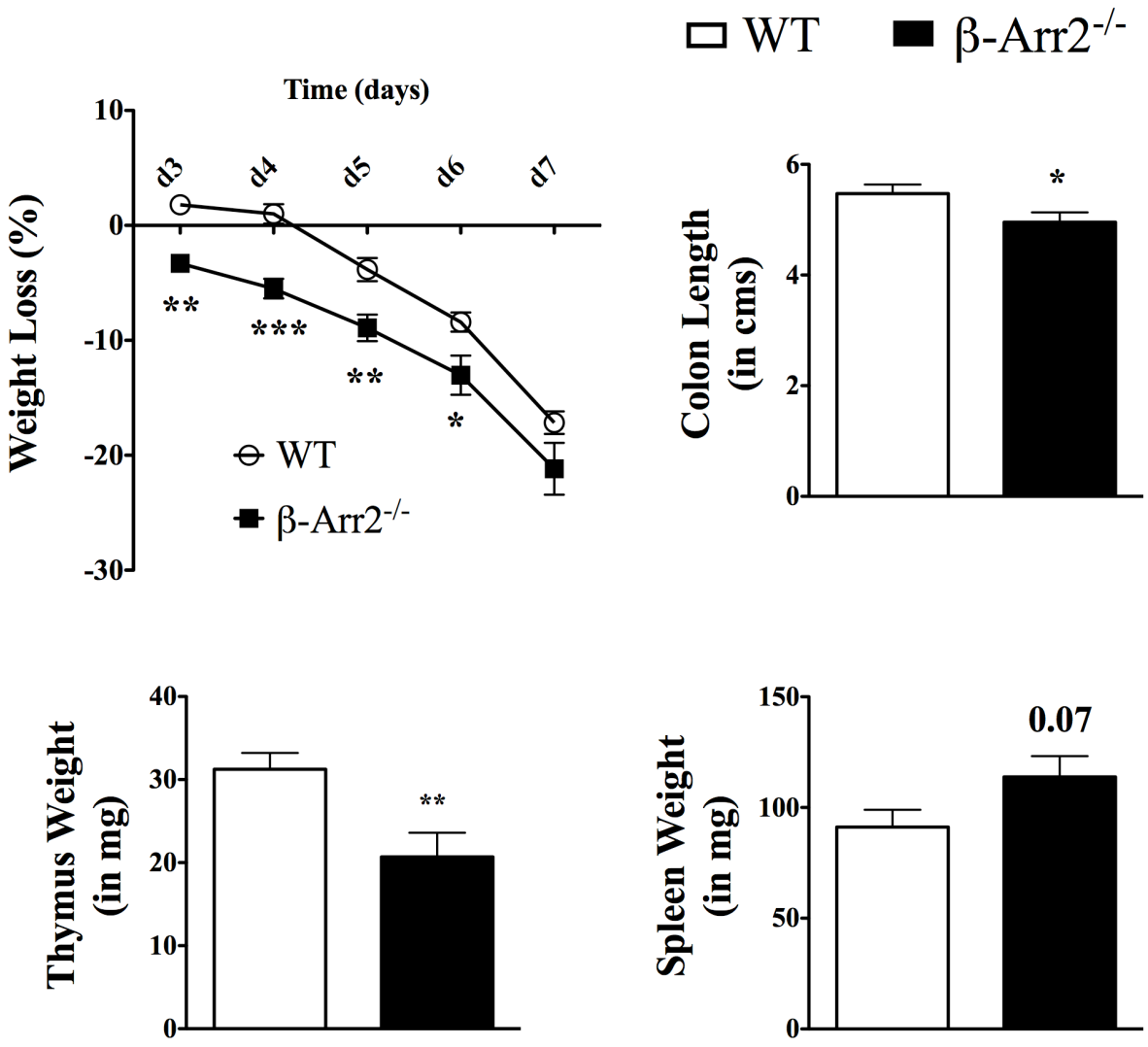

Figure 4.8: β-arrestin2 protects against colitis independent of microbial composition Wild type (WT) and β-arrestin2 knockout (β-arr2$^{-/-}$) were co-housed at the time of weaning (4 weeks) for eight weeks and subjected to colitis. Colitic mice were observed for percentage body weight loss through course of the experiment and colon length, spleen and thymus weight at the end (day7). N=9-11 mice per genotype. Data pooled from two independent experiments. * $p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to WT using 2-way ANOVA for weight loss and student's t-test for colon length, spleen and thymus weight.

β-arrestin2 inhibits T cell differentiation/response to TCR stimulation

Based on the role of β-arr2 in regulating homeostatic and colitic inflammation through T cell activation, we surmised that β-arr2 could have a direct role to play in T cell differentiation. A similar role for β-arr2 has been previously demonstrated in induction of regulatory T cell (Treg) [6] and our own data suggests its existence and implications (Fig 4.3). Activation potential of T cells in lymphoid organs depends on homeostatic and inflammatory regulation over the course of time and therefore could be suggestive of an extrinsic or intrinsic role for β-arr2 in T cell differentiation. To address this question further, naïve T cells ($CD4^+CD44^-CD25^-CD62L^{lo}$) were sorted from the spleen and activated in vitro under differentiation conditions favoring Th1, Th2, Th17 or Treg induction. T cells lacking β-arr2 had a skewed differential potential which was higher for Th1 (%IFNγ+) but lower for Treg (Foxp3+) and similar to WT for Th2 (IL-4+) and IL-17 (Il-17A+) (Fig 4.9A). Cytokine production though did not entirely mirror differentiation potential as ascertained by intracellular staining. IFNγ production under Th1 and IL-4 production under Th2 conditions was higher while IL-17A under Th17 conditions was slightly albeit significantly lower from T cells lacking β-arr2 (Fig 4.9B). Strikingly, even under Th0 (neutral) conditions, IFNγ production was significantly higher from T cells lacking β-arr2 (Fig 4.9B). Total number of CD4+ T cells at the end of the differentiation potential too were significantly different and lower for KO T cells under all differentiation conditions (Fig 4.9C), that could be a product of altered proliferation or apoptosis. Nevertheless, when cytokine production was normalized for total cell number (Fig 9d) or cells positive for a particular cytokine as determined in 4.9A (Fig 4.9E), T cells lacking β-arr2 have a strikingly higher differentiation potential towards Th1 and Th2. In case of Th17, lower cell numbers might explain lower cytokine release with no effect on differentiation potential as observed in KO T cells. β-arr2 therefore is

important for Treg induction and its absence greatly enhances IFNγ and IL-4 production from Th1 and Th2 cells, respectively.

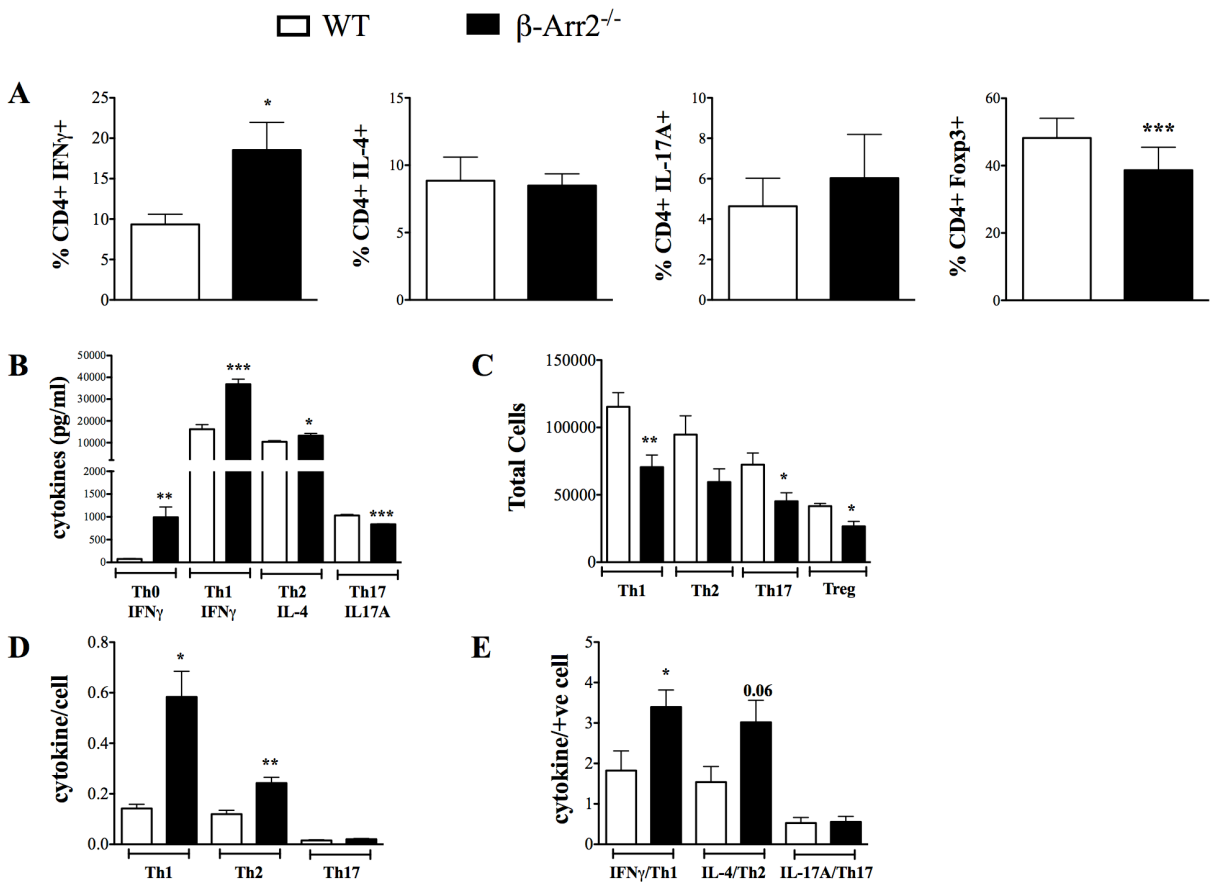

Figure 4.9: Loss of β-arrestin2 alters T cell differentiation potential Sorted naïve (CD4+CD44-CD25-CD62Lhi) CD4+ T cells isolated from WT and β-arr2$^{-/-}$ mice were stimulated with CD3/28 under different conditions to induce Th0, Th1 (IL-12), Th2 (anti-IFNγ, IL-4), Th17 (anti-IFNγ, anti-IL-2, IL-6, TGFβ) and Treg (anti-IFNγ, TGFβ) differentiation for 5 days. a) Proportion of differentiated Th1, Th2, Th17 and Treg cells. b) Levels of indicated cytokine released in response to different differentiation protocol. c) Total number of cells recovered at the end of in vitro differentiation and ex vivo stimulation. Cytokine production normalized to (d) total number of cells and (e) total number of cells positive for the cytokine as

Figure 4.9 (Cont'd)

determined in (a). N=6, with data pooled form 3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as compared to WT paired t-test.

β-arrestin2 deficient T cells have a higher colitogenic potential in T cell transfer model of colitis

Even though in vitro stimulation provides a snapshot at T cell differentiation potential it does not recapitulate the pattern under complex and antagonistic conditions observed in vivo. To determine T cell functionality and consequent colitogenic potential, CD4+CD45RBhi T cells were sorted, injected into $RAG2^{-/-}$ mice and the recipient mice followed over course of time for signs of colitis and associated wasting disease. Contrary to our expectations based on DSS colitis studies, weight loss and disease activity index were higher in $RAG2^{-/-}$ mice that received WT T cells compared to the $RAG2^{-/-}$ that received KO T cells as early as 4 weeks post transfer (Fig 4.10). While the weight loss became similar between the two sets of mice at later time points, disease activity index continued to be higher in mice injected with WT cells until the end of the experiment (7-8 weeks) (Fig 4.10). However, when we examined the mice at necropsy for various parameters of colitis, surprisingly, we observed that the shortening of colon length and increase in colon weight due inflammation were significantly higher for $RAG2^{-/-}$ injected with KO T cells as compared to mice that received WT T cells. Plasma IFNγ too was significantly higher in mice colitic in response to injection of KO T cells.

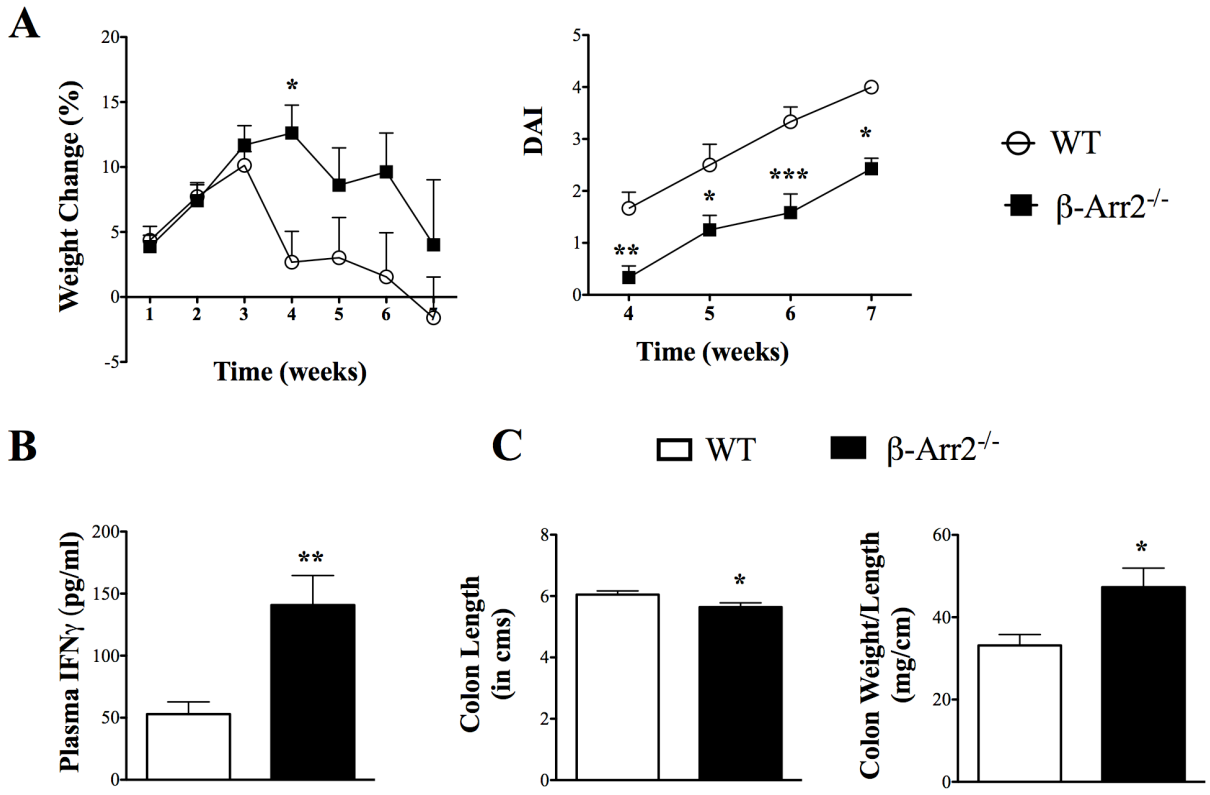

Figure 4.10: β-arrestin2 inhibits T cell colitogenic potential Sorted CD4+CD45RBlo isolated from WT and β-arr2$^{-/-}$ mice were injected into RAG2-/- mice to induce T cell mediated colitis. a) Weight loss and disease activity index of T cell injected mice over the period of 7 weeks. b) Plasma levels of IFNγ (c) Colon length and (d) colon weight normalized to colon length as a marker of inflammation at the end of the experiment. N=12 with data pooled from two independent experiments. * $p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to WT using 2-way ANOVA for weight loss and disease activity index (a) and student's t-test for (b-d).

To determine the homing potential and activation status of injected T cells, cells from colonic lamina propria and lymphoid organs were harvested and assessed for T cell differentiation. Although, there was no difference observed in homing of WT or KO T cells to cLP or spleen (Fig 4.11A), their differentiation pattern was drastically different. Treg induction (Foxp3+CD4+)

in T cells isolated from cLP, spleen and MLN of RAG2$^{-/-}$ transferred with KO T cells was significantly lower in comparison to their WT controls (Fig 4.11B). Ex vivo stimulation of T cells showed no difference in the proportion of Th1 and Th17 cells, while double positive T cells were significantly higher in the colon and MLN of RAG2$^{-/-}$ injected with β-arr2 KO T cells. Overall, ratio of IFNγ+ and IL-17A+ T cells to Foxp3+ that would be reflective of overall T cell activation status was significantly higher in T cells originating from RAG2$^{-/-}$ injected with KO T cells in all three lymphoid organs. T cells lacking β-arr2 therefore, are deficient in their ability to differentiate into regulatory T cells while Th1 and Th17 differentiation is largely unaffected *in vivo*, thereby leading to enhanced colitogenic potential.

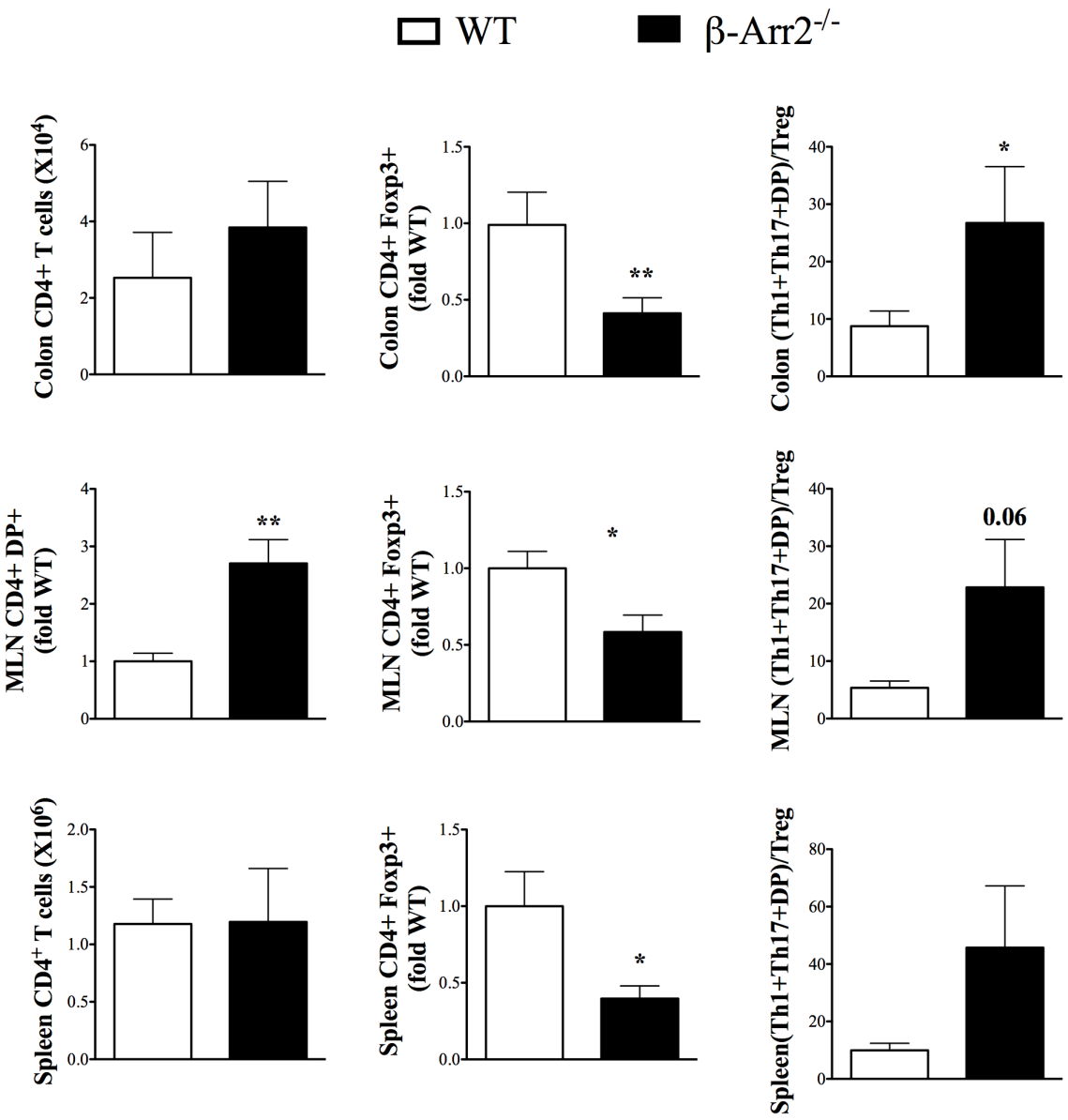

Figure 4.11: β-arrestin2 inhibits Treg induction *in vivo* and alters T cell activation balance

a) Total number of CD4+ T cells isolated from colon and spleen of colitic mice following T cell transfer. b) Treg induction and (c) ratio of activated T cells -Th1, Th17 and double positive (DP) to Foxp3+ T cells in colon and associated lymphoid organs. N=12 with data pooled from two independent experiments. * $p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to WT using student's t test.

DISCUSSION

In this study, we show that mice lacking β-arr2 exhibit enhanced intestinal inflammation even under basal conditions and disruption of intestinal barrier by DSS leads to exacerbated colitis in these mice. Further in the absence of β-arr2, T cells were skewed in their differentiation and had increased colitogenic potential. The role of β-arr2 in mucosal inflammation could have its basis in its ability to inhibit innate response to LPS (microbial component) and polymicrobial stimulation as demonstrated in previous studies[4,17,18]. Further increased T cell numbers could be due infiltration in response to higher T cell chemokine production or *in situ* T cell proliferation instigated by higher number of APCs. The latter could also provide stimulation for increased differentiation and activation as observed by proportion of effector T cells in cLP of KO animals. This T cell activation-mediated negative regulatory role of β-arr2 in intestinal inflammation is in contrast to its role in allergic asthma model of lung inflammation[5], where T cell infiltration and consequent disease induction was abrogated in the absence of β-arr2. In the same study, LPS induced T cell infiltration was unaffected by loss of β-arr2, implicating that the role of β-arr2 at distinct organ sites is likely stimuli dependent. Further, the role of β-arr2 in gut inflammation has a greater involvement of microbial stimulation perhaps making it more sensitive to β-arr2 mediated negative regulation, even under basal conditions.

Further, even though DSS induced colitis in co-housed mice argues against the role of microbial diversity in colitis progression, development of mucosal immunity is microbiota driven. The effect of microbiota and its modulation on development of gut immunity in β-arr2 knockout mice will be examined in future studies.

Even though there were some discrepancies in T cell differentiation observed in the two different colitis models versus in vitro polarization- they do not undermine the role β-arr2 plays in regulating T cell differentiation. It only underscores the involvement of different co-stimulatory molecules (in addition to CD28 used in vitro) and cytokine milieu in context of ongoing inflammation, guiding and dictating the final differentiation potential. Other studies have demonstrated contrasting role for β-arr2 in T cell differentiation in allergic asthma model[5,19,20] and in human primary T cells[10]. Although the use of siRNA to modulate β-arr2 expression and lack of data on T cell activation status in the human T-cell model makes them difficult to compare to our current study. Another study using magnetically enriched naïve T cell population and a different CD3/28 concentration observed no difference in Th1/2/17 polarization but observed a similar decrease in Treg induction[6]. Similar to colitis model, β-arr2 mediated Treg induction was also shown to be important in pathogenesis of EAE and the KO consequently exhibited the disease with greater severity[6]. Further, it is possible that this deficiency in Treg induction in the absence of β-arr2 has a role to play in both basal and DSS induced inflammation. Even though β-arr2 has been shown to be important for TGFβRIII receptor endocytosis and downstream anti-proliferative potential[21]; further work needs to be done to determine the role of altered TGFβ signaling in context of T cell differentiation. Similar to β-arr2, G proteins have been shown to have an important role in T cell activation[22,23, 24] implicating a potential mode of mechanism for β-arr2 in T cell function.

Even though colonic inflammation in both models of colitis was higher in the absence of β-arr2; weight loss and disease activity index in the two models showed completely different modulation

by β-arr2. It is quite possible that distinction stems from difference in pathogenesis of acute and chronic models of colitis. It must also be noted that while DSS is considered an equivalent of ulcerative colitis; T cell transfer is a model for crohn's disease. Nevertheless, the role of β-arr2 in differentially affecting weight loss and colitis induction in T cell transfer model of colitis was quite surprising. Similar distinction was also observed in animals treated with an anti-TLR4 antibody[25] and TLR4 signaling in T cells can directly regulate TCR activation and colitis progression[26]. Given the positive regulatory role for β-arr2 in LPS mediated inflammation[8], it is possible that these effects are TLR4 mediated; with TLR4 having a direct or indirect role on T cell mediated disease pathogenesis. The role and differential production of other inflammatory mediators at systemic or local site cannot be ruled out. In T cell transfer in SCID mice, antibiotics and TNFR-Fc preventive treatment were shown to affect only colitis or weight change respectively[27], suggesting differential involvement of microbial components and TNF signaling in these parameters.

The role of β-arrestin2 was distinct from that of β-arrestin1 in colitis induction, with the latter being a positive regulator of gut inflammation[13]. This is similar to distinct role of these two proteins in pathogenesis of other autoimmune diseases, EAE[6,28] and arthritis[7,29]. It is interesting that even though both proteins have similar roles in endotoxemia and polymicrobial sepsis, their roles in auto-immune diseases, some of which are modulated by TLR signaling are quite distinct. One possibility is tissue-specific role for role of β-arrestins as observed in sepsis[30], EAE[6,28] and allergic asthma model[31]. One potential cause of divergent roles in gut inflammation could be production of IL-22, which was higher (protein expression) in β-arrestin1 knockout following

DSS induced colitis[13] but was lower (mRNA expression) in basal β-arrestin2 colon tissue. The source and effect of IL-22 in context of β-arrestins though requires further investigation.

In summary, this study demonstrates an important role for β-arr2 in regulating mucosal inflammation under both homeostatic and colitic conditions. Its mode of action involves negative regulation of T cell activation and its requirement for induction of regulatory T cells. Further work would determine if either TLR4 or other G proteins have a role to play in this and provide insight into crosstalk between TCR, TLR and G protein pathways in regulation of inflammatory diseases.

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CHAPTER 5

This chapter summarizes the major findings from previous chapters (2-4) and concludes the multifaceted role of β-arrestins in inflammatory disorders

SUMMARY AND CONCLUSIONS

The overall goal of this thesis was to understand the role of β-arrestins (intracellular scaffolding cell signaling proteins) in regulating inflammation and in the pathogenesis of sepsis and colitis using mouse models. β-arrestins, given their critical role in intracellular signaling, chemotaxis, apoptosis and cytokine production are poised to act as important mediators of inflammatory response. Their role in endotoxemia model had been previously discerned, where both β-arrestins acted as positive mediators of cytokine response and consequent mortality. We further wanted to study their role in a more relevant clinical setting of sepsis in response to polymicrobial stimuli. Additionally, gut being a site of constant interaction between microbes and the immune component is particularly sensitive to a dysregulated immune response and hence serves as an appropriate model to determine the role of β-arrestins in inflammation. The individual aims and main findings from each project are summarized below.

Role of β-arrestin 2 in polymicrobial sepsis

β-arrestin2 knockout (β-arr2 KO) mice had shown to be resistant to LPS mediated mortality but sensitive to the cecal ligation and puncture model of sepsis. The aim was to assess inflammation and associated mortality in response to a polymicrobial infection independent of the effects of surgery, including response to fecal contents and necrotic tissue.

Results

1. β-arrestin 2 is a negative regulator of polymicrobial-induced inflammation.

2. Pulmonary tissue is particularly affected in β-arr2 KO mouse in response to polymicrobial sepsis with increased neutrophil sequesteration and inflammatory mediator production observed in β-arr2 KO lung.
3. MAPK and NFκB activation was increased in lung tissue from septic β-arr2 KO mice and β-arrestin 2 expression was upregulated in response to sepsis.
4. β-arr2 KO mice were more sensitive to polymicrobial sepsis induced mortality
5. One allele of β-arrestin 2 was sufficient to provide protection against overt inflammation and mortality.
6. β-arrestin 2 mediates cytokine production in a cell and stimuli specific manner.

Conclusion

β-arrestin 2 negatively regulates inflammation in response to polymicrobial stimuli, perhaps by virtue of modulating MAPK and NFκB activation. The difference in endotoxemia and polymicrobial model is likely due to β-arr2 KO cells responding differentially to the two stimuli.

Limitations and future directions

While we demonstrate a cell type and stimuli specific role for β-arr2 in regulating inflammation, the cause of this discrepancy is not known. Use of cell type specific knockout mice would be better at teasing out the mode of regulation.

Role of β-arrestin 1 in polymicrobial sepsis

The aim of this project was to determine the role of β-arrestin1 (β-arr1) in inflammation in response to septic peritonitis.

Results

1. β-arr1 KO mice are more susceptible to mortality following septic peritonitis.
2. β-arr1 KO mice exhibit overt systemic inflammatory response to sepsis; observed both in plasma and various organs.
3. Other critical parameters affecting mortality like immune cell infiltration, thymic apoptosis and immune-suppression were largely unaffected.
4. Cardiac NFκB activation was higher in septic β-arr1 KO mice.
5. One allele was sufficient to protect against overt inflammation and associated mortality.
6. β-arrestin1 expression in non-hematopoietic cells was required and sufficient in inhibiting the overt inflammation observed in whole body β-arr1 KO mice.

Conclusion

β-arr1 similar to β-arr 2 acts as a negative regulator of polymicrobial stimuli induced inflammation, through a mechanism involving but not limited to NFκB activation. This negative regulatory role in inflammation is dependent on non-hematopoietic β-arr1 expression, demonstrating an important role for β-arr1 in inflammation in non-immune cells.

Limitations and future directions

Even though we know the importance of non-hematopoietic β-arr1, the particular cell type or mode of mechanism is still largely unknown. In vitro models do not mimic or translate into an *in vivo* response, making further deductions even more difficult. RNA sequence analysis of RNA isolated from liver of septic chimeric mice is being used to get an idea of wider range of mediators regulated differentially in chimeric mice lacking β-arr1 in hematopoietic or non-hematopoietic cellular compartments; which could potentially lead to an upstream regulator/stimuli causing this distinct response.

Role of β-arrestin 2 in gut inflammation

The aim of this study was to discern the role of β-arr2 in gut inflammation. We hypothesized that since β-arr2 is important for negative regulation of inflammatory response to microbes, in its absence gut would exhibit dysregulated inflammation.

Results

1. β-arr2 KO mice exhibited increased inflammation even in absence of an exogenous stimulus. Additionally, T cells in peripheral lymphoid organs exhibited altered T cell differentiation potential.
2. In response to dextran sodium sulfate (DSS) ingestion, β-arr2 KO displayed higher indices of colitis induction associated with higher Th1 and Th17 differentiation.
3. Higher colitic response in β-arr2 KO mice was independent of microbiota, as increased colitic response was observed in β-arr2 KO mice co-housed with WT mice.

4. Mice heterozygous for β-arr 2 were protected from overt inflammation under basal and DSS-induced colitic conditions.
5. β-arr2 intrinsically altered T cell polarization potential, with higher Th1 and lower Treg induction observed in *ex vivo* differentiation assay.
6. T cells deficient in β-arr2 had greater colitogenic potential in T cell transfer model of colitis.

Conclusion

We demonstrate an important role for β-arr2 in inhibiting intestinal inflammation via regulating T cell activation. Basally, in response to DSS and in T cell transfer model of colitis, T cells lacking β-arr2 demonstrate dysregulated inflammatory response perhaps leading to greater extent of colitis observed in both models of colitis. This regulation was further shown to be intrinsic to T cells and while β-arr2 expression in other cell types too is perhaps important as demonstrated in previous studies, its role in T cells particularly raises their colitogenic potential.

Limitations and future studies

While we know that β-arr2 in T cells is definitely involved in inhibiting gut inflammation, its contribution in other cells cannot be ruled out. Further, the mechanism for dysregulated T cell activation is not known. Particularly perplexing is the distinct regulation by β-arr2 of systemic and colitic response observed in T cell transfer model of colitis, it could involve differential TLR signaling but that has not been followed in this study. Most hints towards mode of regulation are deductions from other studies and

would need validation as mechanism employed by β-arr2. Future studies would concentrate in dissecting the role of β-arr2 in intrinsic T cells activation and TGFβ signaling, required for optimal Treg induction. Another important question is the incidence of a "leaky gut" in β-arr2 knockout and determine the role of β-arr2 in epithelial cell proliferation and repair.

Multifaceted roles of β-arrestins in inflammation

Overall, the thesis has demonstrated distinct and overlapping roles for β-arr1 and β-arr2 in regulating inflammation in response to various stimuli. The distinct features of the role of β-arrestins in modulating inflammation are listed below:

1. Their roles are not redundant. Loss of one β-arrestin is unable to compensate for other as observed in various stimuli and inflammatory disease models.
2. Their roles in cytokine production are cell-type and stimuli specific. This also highlights discrepancies observed in literature with use of different cell lines. Use of cell specific knockout would shed further light on the source and implications of this observation.
3. Their role is also dependent on the dose of stimuli as can be gauged by various our in vitro studies and in vivo studies using different doses of LPS in endotoxemia model. This suggests that β-arrestins could potentially act as rheostats of inflammation instead of having a definite role as positive or negative regulator.
4. Their roles could be similar or distinct. While the responses of both knockouts to microbial stimuli (LPS, polymicrobial infection) were similar, the two β-arrestin

knockouts exhibited distinct roles in auto-inflammatory diseases, including arthritis, colitis and EAE. While β-arr1 KO mice are protected, β-arr2 mice suffer from exacerbated phenotype through diverse mechanisms, with regulation of T cell responses as a potential common theme. Further experiments would be done to tease out how T cell activation and differentiation is altered in the absence if β-arrestins with particular focus on Treg and Th17 induction. It would be worthwhile to further delve into the overall "immune status", T cell repertoire and activation to "self-antigens" in these mice.

Table 5.1: Summary of role of β-arrestins in inflammatory disorders.

Model	β-arrestin 1	β-arrestin 2
Endotoxemia	**Protective**	**Protective**
Polymicrobial sepsis	**Susceptible**	**Susceptible**
Arthritis	Protective	Susceptible
EAE (MS)	Protective	Susceptible
Colitis	**Protective**	**Susceptible**

Those in bold are studies done in Parameswaran Lab.

5. Their roles in regulating inflammation could stem from non-hematopoietic regulation. While this was shown for β-arrestin1 in sepsis, it needs to be further explored in context of β-arrestin2 and other disorders. In allergic asthma, β-arrestin2 regulated distinct aspects of disease induction through hematopoietic

and non-hematopoietic compartments. Ascertaining the level at which control is exerted by β-arrestin could further aid in identifying the mode of regulation. Neuro-modulation of inflammation is another avenue that needs to be studied with respect to the role of β-arrestins.

In conclusion, β-arrestins act as critical regulators of inflammation in various disease models and further work on discerning the involved molecular mechanisms will likely lead to therapeutic strategies to target β-arrestins in inflammation. Translational implications of this work stem from identifying the receptor/signaling platform β-arrestin utilize to modulate inflammation and use of biased ligands to specifically modulate certain aspects of that signaling.