ARRESTIN'S FUNCTION ON TLR4 SIGNALING IN HUMAN MACROPHAGE

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ABSTRACT

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Inflammation is a host immune response against infectious, traumatic or autoimmune injury, normally leads to recovery. However if not properly controlled, inflammation can cause persistent tissue damage. Macrophage is a leukocyte that plays an important role in inflammation by detecting inducers and producing mediators. TLRs are a group of receptors that macrophage harbor to recognize PAMPs. TLR4 can be activated by LPS and embarks on a complex signaling cascade that leads to the transactivation of many inflammation and autoimmunity related genes. Arrestins are a group of scaffolding proteins that can desensitize 7TMR G protein signaling and also initiate signaling. Arrestin has been indicated to regulate non-7TMR receptor signaling including TLR4. Arrestin's function in TLR4 signaling in human macrophage was studied in this research using THP-1 cell model. THP-1 was induced to differentiate into macrophage by PMA. siRNA was applied to knock down arrestin 2 level. LPS was used to stimulate TLR4 signaling and relevant genes were measured by PCR array. The results showed that arrestin 2 level was increased during THP-1 differentiation and arrestin 2's regulation effects are different according to different gene groups. This research may provide novel insights on the mechanisms of inflammatory gene expression.

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ABBREVIATIONS

AP-1	Activator Protein 1
CCL	chemokine (C-C motif) Ligand
CD14	Cluster of Differentiation 14
CXCL	chemokine (C-X-C motif) Ligand
FADD	Fas-Associated protein with Death Domain
GM-CSF	Granulocyte/Macrophage Colony Stimulation Factor
GPCR	G Protein Coupled Receptor
GRK	G protein coupled Receptor Kinase
hr	hour
IFN	Interferon
IκB	NF-κB Inhibitor
IKK	Inhibitor of nuclear factor Kappa-B Kinase
IL	Interleukin
IRAK	Interleukin-1 Receptor-Associated Kinase)
IRF3	Interferon Regulatory Factor 3
JNK	c-Jun N-terminal Kinase
K63	lysine 63
KD	Knock Down
KO	Knock Out
LBP	Lipid Binding Protein
LPS	Lipopolysaccharide

LRR	Leucine Rich Repeats
M-CSF	Macrophage Colony Stimulation Factor
MAPK	Mitogen Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MD-2	Lymphocyte antigen 96
MyD88	Myeloid Differentiation primary response gene 88
NEMO	NFκB Essential Modulator
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
qPCR	quantitative Polymer Chain Reaction
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RIP1	Receptor-Interacting Protein 1
TAK1	Transforming growth factor β Activated Kinase1
TBK	TANK-Binding Kinase 1
TIR	Toll IL1 Receptor
TIRAP	TIR domain-containing Adaptor Protein
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
TRADD	Tumor necrosis factor Receptor type 1-Associated Death Domain
TRAF	TNF Receptor Associated Factor

TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR domain-containing adaptor Inducing IFN-beta
VD3	Vitamin D3

Chapter One: Introduction

Inflammation: balance is the key

Inflammation is a host response of organism to get rid of damaged tissue or foreign invaders (bacteria, virus, fungus, parasite, toxin and irritant etc.). The classical signs of inflammation are redness, swelling, heat, pain and loss of function, which is due to increased blood flow and infiltration to the inflamed area. These responses are essential for the recruitment of immune cells and subsequent recovery [1, 2]. Therefore, inflammation is fundamentally a protective response at a cost of transient decline of function. Too little inflammation can often leads to susceptibility to various infections and poor wound healing [3].

However, sometimes inflammation can be inappropriately triggered, poorly controlled and contributes to the pathogenesis of many diseases characterized by altered homeostasis. These diseases include sepsis, asthma, atherosclerosis, neurodegenerative diseases, type 2 diabetes, obesity, aging and some autoimmune diseases [4]. Therefore, it is very important to understand inflammation in human health and disease in order to avoid its unpleasant aspect and boost its beneficial effect.

A typical inflammatory response consists of 4 components: 1, inducer, 2, sensor that detects inducer 3, mediators produced by the sensor cells 4, target tissue that are affected by those mediators. Each component comes in multiple forms. For example,

the inducer could be bacteria components, virus RNA or DNA, host heat shock protein etc., sensor could be toll-like receptor expressed on tissue-resident macrophages or dendritic cells. Mediators could be inflammatory cytokines e.g. TNFα, IL1β, IL6 or chemokines e.g. IL8, CCL4, MCP-1, RANTES as well as prostaglandins produced by sensor cells and the target tissues could be local blood vessels, immune cells. Some mediators can also have systemic effect when secreted in large amount into the circulation system. Therefore, study of inflammation can be performed at multiple levels [3, 5, 6].

The big picture of my research is to understand more about inflammation. Especially I will be focus on one sensor cell type “macrophage” at cell and molecular level.

Microphage biology

Macrophage is a phagocytotic leukocyte. It originates from bone marrow hematopoietic stem cells, and its direct precursor cells are generally considered monocyte from peripheral blood, which migrate into different tissue loci to replenish residential macrophage pool when needed. Macrophages are highly heterogeneous and can be divided into various types or subpopulations according to location or activation state [7, 8]. Macrophage can be spotted by several makers including CD14, CD11b, F4/80 (mice)/EMR1 (human), lysozyme M, MAC-1/MAC-3 and CD68 by flow cytometry or immunohistochemical staining [9].

Macrophage generally performs 2 functions. First, homeostatic function: in normal physiological condition, macrophages phagocytose senescent/apoptotic cells and maintain tissue homeostasis. Second, innate immune function: during pathogen invasion situation, macrophages launch the first wave immune response, they recognize, engulf and destroy pathogens, secrete cytokines to recruit more immune cells and present antigens to adaptive immune cells such as T or B lymphocytes and eventually lead to adaptive immunity and immunological memory [7]. In the context of inflammation, macrophage is a major contributor to the production of inflammatory cytokines and chemokines [6].

Toll like receptor

For macrophage to recognize pathogens, a group of receptors called Toll like receptors (TLR) plays an essential role. TLR is one sort of pattern recognition receptors, which recognize molecular pattern that is exclusively associated with pathogens (PAMP) e.g. components of bacteria cell wall, bacteria genome DNA, virus, fungal products etc [10]. Also, TLRs interact with endogenous molecules released from damaged tissues or dead cells, the so-called stress or damage associated molecular patterns and regulate many sterile inflammation processes [11].

The nomenclature of “toll like receptor” is derived from Toll protein discovered in drosophila (Toll means wild in German), mutations in the Toll signaling pathway in drosophila, dramatically reduce survival of drosophila after fungal infection [12]. Pathways similar to Toll signaling in Drosophila have been indentified in a variety of other organisms ranging from plants to human [12, 13]. So far, more than 10 TLRs has been identified in mammals, TLR1 to TLR10 are found in human, which are also conserved in mouse, while TLR10 is not functional in mouse, TLR11 to TLR13 are only found in mouse. Therefore, there are certain species specific TLRs, most members are conserved [14].

TLRs are type I trans-membrane glycol-protein receptor (please refer to figure 3 in reference [15] for illustration), their extracellular portion contains 16-28 leucine rich repeats (LRR) and exhibit a horse-shoe like structure, these LRRs deviate a lot in terms of number and sequence between different TLRs, contributing to their distinct ligand

recognition function. The extracellular portion of TLR is linked to the intracellular portion by a trans-membrane alpha-helix portion, the intracellular portion is homologous to interleukin-1 receptor, therefore it was named TIR (Toll IL1 receptor) domain [16].

TIR domain is also shared by the adapter proteins downstream of TLR including MyD88, TIRAP, TRIF, TRAM. Upon receptor activation, it is believed that a TIR-TIR interaction complex is formed between the receptor and the adapter TIR domains. TIR domains are structurally conserved. However, the surface electrostatic properties of TIR domains are distinctive between TIR domain containing receptors or adaptor proteins, which might explain specific interaction between adaptors and distinct TLRs [17].

TLR ligand activate TLR signaling by bridging together 2 TLRs, form a “m” shaped extracellular structure (please refer to figure 4 in reference [18] for illustration), most TLR can form homodimers, some can also form heterodimers e.g. TLR1/2, TLR2/6 dimers, TLR also needs several coreceptors e.g. CD36, CD14, MD-2 etc. for proper signaling function, Dimerization of TLRs triggers the recruitment of specific adaptor proteins to the intracellular TIR domains and subsequent downstream signaling which eventually leads to transcription factor activation and gene expression [10, 18, 19].

TLRs can be divided into 2 groups by their cellular location. In human, TLR1, 2, 4, 5, 6 are exposed on cell surface responsible for detecting pathogen outside host cells, such as lipid or protein structures that are expressed on the surface of pathogens. TLR3, 7, 8, 9 are on the surface of endosomes responsible for intracellular microbes recognition

such as nucleic acids which is usually confined inside a pathogen but can be exposed after engulfed by immune cells. Further, TLR7, 9 need a proteolytic modification to function, their endo-lysosomal location renders this operation [20, 21]. Table 1 from reference [22] provides a thorough description of all TLRs in terms of locations, ligands, co-receptors, signaling adaptors, transcription factors and effector cytokines induced.

TLR4 signaling

My research is focused on TLR4. TLR4 signaling is the most extensively studied TLR signaling pathway, because of its peculiar features in ligand recognition, adaptor recruitment and patho-physiological significance. TLR4 was first demonstrated to be the receptor for lipopolysaccharide (LPS). LPS is a component of gram-negative bacteria outer cell wall. LPS consists of a hydrophilic polysaccharide chain, known as O-antigen, a core and a hydrophobic lipid moiety, known as lipid A (please refer to figure 1 in reference [23] for illustration), which is responsible for the toxic effects. The polysaccharide chain is highly variable within the same bacterium and amongst different bacteria species [24]. LPS is an endotoxin, and induces a strong response from normal animal immune systems [25].

Several synthetics mimics of lipid A such as Eritoran, Lipid IVa act as TLR4 antagonist, they compete with LPS for binding site however failed to elicit receptor dimerization and signaling, showing potential therapeutic effect towards overactive immune response e.g. sepsis [26, 27].

According to the current model, LPS is carried by lipid binding protein (LBP) in the serum which delivers it to CD14 on the cell membrane, which in turn transfers it to another non-anchored protein MD2 to form a monomeric LPS:MD-2 complex that binds to TLR4 and trigger TLR4 dimerization and signaling [28].

LBP is a 58 to 60 kDa glycoprotein that is secreted in the serum mainly by hepatocytes

as an acute phase protein, The LPS binding site of LBP contains a cluster of positively changed residues that interact with phosphorylated head of lipid A moiety. LPS binding site of LBP is located in the N terminus whereas; the C terminus is responsible for interaction with membrane and CD14 [29].

CD 14 is a 55 kDa glycoprotein expressed on the surface of myelomonocytic cells including macrophages as a glycosylphosphatidylinositol anchored receptor or secreted in a soluble form. The extracellular portion of CD14 has 11 LRR and form a horse-shoe shaped three-dimensional structure similar to TLR. CD14 shows ligand promiscuity, it can binds different forms of LPS with high affinity as well as a number of other TLR ligands, acting as a co-receptor for TLR1, TLR2, TLR6, TLR4, and TLR3. CD14 is crucial for low dose LPS recognition [30], but largely dispensable for the response of high concentrations of LPS, which occur almost normally in CD14 deficient macrophages suggesting a CD14 independent LPS sensing [31, 32].

MD-2 is a 25-30 kDa soluble protein and physically associates with TLR4 through hydrogen and electrostatic bonds between two complementary charged patches located on each molecule [33], MD-2 can bind LPS directly [30, 34, 35] and has been demonstrated to be the LPS binding protein in TLR4/MD-2 complex, it is not clear whether TLR4 can bind LPS directly without MD2 [28, 36]. MD-2 is also essential for ligand induced TLR4 dimerization, upon LPS binding, a symmetric ‘‘m’’-shaped multimer composed of two TLR4:MD-2 heterodimers is formed. 5 out of 6 acyl chains of LPS are resided in MD-2 hydrophobic pocket and the remaining chain is interacting with the

second TLR4 by means of hydrophobic interaction. In this way, two TLR4/MD-2 were brought together [28] (please refer to figure 1 in reference [28] for illustration). Among all of the TLR4 accessory molecules, MD-2 is the only one that is absolutely required for the response to LPS [35].

LPS stimulation of TLR4 includes the participation of several molecules, and the currently favored LBP/CD14/MD-2/TLR4 model is overly simplified, How a system will react to LPS varies significantly according to a number of parameters, including host species, cell type or cell differentiation/activation state and the nature, concentration, or duration of the stimulus. A growing list of accessory molecules involved in LPS recognition has been indentified. Different usage of co-receptors may results in different LPS responses [32, 37].

After ligand recognition and TLR4 dimerization, the downstream signaling pathways are tremendously complicated. It is a sophisticated network starting with several TIR domain containing adaptor proteins: MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein, also known as Mal, MyD88-adapter-like) TRIF (TIR domain-containing adaptor inducing IFN-beta), and TRAM (TRIF-related adaptor molecule), then ending with several transcription activators including NF-κB, AP1 (c-jun/c-fos), IRF3 (Interferon regulatory factor 3) etc. In between, there is a vast network of cross interacting signal transducers e.g. IRAK, TRAF6, TAK1, MAPKKK, MAPK (P38/ERK/JNK), TRAF3, TBK, IKK, IκB etc. to name a few.

To simplify this signaling network, traditionally, TLR4 signaling are divided into MyD88 dependent and MyD88 independent/TRIF dependent signaling pathways. Most TLRs except for TLR3 signal via adaptor MyD88 and activate transcription activator AP-1 and NF-κB, TLR3 signals via adaptor TRIF and produces similar biological outcome. Intracellular, nucleic acid sensing TLRs (TLR3, 7, 8, 9) can also activate transcription factors IRF3, 7, which largely regulates the expression of type 1 interferon (IFN). However, TLR4 is a unique exception in that TLR4 recruits both MyD88 and TRIF to induce the activation of NF-κB and AP-1. And, in a manner similar to TLR3, it uses TRIF to stimulate the production of type 1 IFNs, although in response to non-nucleic acid ligands [22, 32]. Figure 1 from reference [22] is a simplified illustration of TLR signaling network, which highlighted most if not all key joints.

MyD88 dependent TLR4 signaling pathway

MyD88 contains a C terminal TIR domain that is responsible for interaction with TLR4, and an N terminal death domain that is the effector part of MyD88, interacting with downstream IRAK (Interleukin-1 receptor-associated kinase) protein family that contains both a death domain and a kinase domain. MyD88 can directly interact with some TLRs (TLR5, TLR7, TLR8, TLR9), however, in TLR4 signaling, an intermediate bridging adaptor protein TIRAP, is needed for MyD88 recruitment. TIRAP contains a PtdIns(4,5)P2 binding domain and localizes to PtdIns(4,5)P2 rich lipid rafts in resting cells [38]. The phenotypes of MyD88 and TIRAP deficient mice upon TLR4 stimulation are largely overlapping, with a completely abolished pro-inflammatory cytokine production. However, a delayed activation of NF-κB and AP-1 is still detectable. This

late wave of NF-κB activation, as well as the expression of type 1 IFN genes is a hallmark of the MyD88-independent signaling pathway that is triggered by adaptor TRIF. This fine orchestrated cytokine production event is partly controlled by TLR4 and its adaptors' cellular localization and trafficking [39, 40].

Recruitment of IRAK protein family results in the activation of IRAK4 by auto-phosphorylation, which is responsible for the subsequent phosphorylation of IRAK1. Phosphorylated IRAK1 shows an increased binding affinity for TRAF6 and recruits TRAF6 to the receptor complex. TRAF6 belongs to TRAF (TNF receptor associated factor) family, which plays important roles in the signaling transduction to NF-κB triggered by a number of receptors including TLRs [41]. The distinctive feature of TRAF proteins is a C-terminal TRAF domain, which is composed by TRAF-N (N-terminal coiled-coil region) and TRAF-C (C-terminal beta-sandwich region). TRAF domain mediates protein-protein interactions. TRAF-N regulates self-oligomerization and TRAF-C regulates binding to upstream molecules. TRAF6 is the crucial TRAF protein in MyD88-dependent NF-κB activation [42]. TRAF6 is an E3 ubiquitin ligase that can promote the attachment of lysine-63 (K63) linked polyubiquitin chains to substrate molecules including TRAF6 itself [43]. K63 polyubiquitination is different from the classical K48-linked polyubiquitination. Instead of signaling for proteasomal degradation, K63-linked polyubiquitination serves as a signaling moiety that recruits specific ubiquitin-binding domain containing proteins [44].

Recruitment and clustering of TRAF6 at receptor complex, stimulates its auto-

ubiquitination. Ubiquitinated TRAF6 in turn interacts with TAK1 (Transforming growth factor β activated kinase1, a member of the MAPKKK family) and also promotes K63 linked polyubiquitination of TAK1 and IRAK1, which in turn recruit NEMO (NFκB essential modulator) to the receptor complex. NEMO also named IKKγ (inhibitor of nuclear factor kappa-B kinase subunit gamma) is the regulatory subunit of IKK complex, which also contains 2 other kinase subunits (IKKα and IKKβ). TAK1 recruits IKK complex to the receptor complex at the plasma membrane and promotes IKK activation, which ultimately results in activation of NF-κB [32].

In addition to TRAF6, IRAK1 also mediates the recruitment of TRAF3 and cIAP1/2 to the receptor complex where TRAF6 catalyzes K63-linked polyubiquitination of cIAP1/2. K63-linked polyubiquitinated cIAP1/2 is enzymatically active as an E3 ligase that promotes degradative K48-linked poly-ubiquitination of TRAF3 and possibly IRAK1. Upon subsequent proteasomal degradation of TRAF3 and IRAK1, the TRAF6-nucleated complex containing TAK1 dissociates from the receptor and is released into the cytosol. Once in the cytoplasm TAK1 acts as MAP3K (Mitogen activated protein kinase kinase kinase) and initiate MAPK signaling cascade and AP-1 activation [32].

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP-1 are the major transcription activators activated in TLR4 MyD88-dependent signaling pathway. NF-κB function as dimers. NF-κB family contains 5 members. p50 (NF-κB1) and its precursor p105, p52 (NF-κB2) and its precursor p100, RelA (p65), RelB, and c-Rel; all of these share an N-terminal Rel-homology domain (RHD) that mediates homo- and

hetero-dimerization as well as sequence-specific DNA binding. NF-κB transcriptional activity is sequestered by IκB (NF-κB inhibitor) proteins, including IκBα, β and ε. IκB protein binds NF-κB dimmers, blocks its DNA binding site and keeps NF-κB in the cytosol. Upon NF-κB signaling activation, IκB is rapidly K48-linked ubiquitinated and subsequently degraded by proteasome, resulting in the release of NF-κB dimer that moves into nucleus, binds DNA and promotes gene expression. IκB degradative ubiquitination is dependent on a previous site-specific phosphorylation event that is operated by an activated IKK complex. Although how IKK is activated remains unclear, it might result from IKK trans-autophosporylation as a consequence of NEMO mediated oligomerizaiton of IKK complexes, or the phosphorylation maybe operated by an IKK kinase perhaps TAK1, whose activation is linked to receptor stimulation. Regardless, recruiting of NEMO to the activated receptor is very important [32, 45].

AP-1 is also a dimeric transcription factor. It is composed of members of the Jun, Fos, Maf and ATF subfamilies of basic leucine zipper proteins [46]. One important way of AP-1 activity regulation is via phosphorylation by MAPKs. AP-1 activation by inflammatory stimuli is mostly mediated by JNK, p38, and ERK groups of MAPKs, which are phosphorylated by the upstream MAPK-kinase: MKK4/7, MKK3/6, and MKK1/2, respectively. MAPKK is phosphorylated and activated by MAPKKK (MAP3K), TAK1 is the MAP3K that is involved in TLR4 signaling, which links AP-1 activation to receptor stimulation [32]. To get a more straightforward idea of MyD88 dependent TLR4 signaling please refer to figure 2 in reference [32].

TRIF dependent TLR4 signaling pathway

Activation of TLR4 by LPS induces endocytosis of TLR4 from plasma membrane to the endosome [47]. During endocytosis it is known that PtdIns(4,5)P2 concentrations drop [48]. This results in the dissociation of TIRAP from TLR4 allowing for the recruitment of TRIF through bridging adaptor protein TRAM via TIR-TIR domain interaction, which leads to the induction of type 1 IFN around endosome [49] as well as the second/late wave of NF-κB activation and pro-inflammatory cytokine production [39, 40].

TRIF leads to 2 signaling pathway branches: RIP1-dependent NF-κB activation and TRAF3-dependent IRF3 activation.

TRIF does not have a death domain as MyD88, but a RIP homotypic interaction motif (RHIM) at its C-terminal region, which interacts with RIP1 (receptor-interacting protein 1). RIP1 is a serine/threonine kinase and acts as a scaffold protein that recruits another 2 scaffold proteins FADD and TRADD. TRADD and FADD can leads to subsequent recruitment and activation of TAK1 and IKKs. TAK1 and IKK activation further leads to downstream NF-κB and MAPK activation [32, 50].

Upon LPS stimulation, TRAF3 is also recruited to the endosomal TLR4 via TRIF. This results in K63 polyubiquitination of TRAF3. Consequently, TBK1 and IKKε are recruited to the ubiquitinated TRAF3. TBK1/IKKε then is activated via trans- or auto-phosphorylation. Activated TBK/IKKε then phosphorylate IRF3 monomers [51], which in turn dimerize and translocate into the nucleus to promote type 1 IFN gene expression

[32, 52].

Interferons named after their ability to "interfere" with viral replication within host cells are a group of glycoprotein secreted by host cells when exposed to pathogens. Their main function is to boost innate immune activity via activating innate immune cells such as macrophages and natural killer cells. Interferon has 3 major types: Type 1 interferon, all type 1 interferon binds to a cell surface receptor known as IFN-α receptor (IFNAR), The type 1 interferons in human are IFN-α, IFN-β and IFN-ω. Type 2 interferon contains only one member IFN-γ, which binds to IFN-γ receptor (IFNGR). Type 3 interferon was recently classified, consists of three IFN-λ molecules [53]. Type 1 IFN production is controlled at the transcriptional level by the IRF family transcription factors, with IRF3 and IRF7 being the key regulators. Both IRF3 and IRF7 are required for TLR3 mediated type 1 IFN production, however, IRF3 is the only one that activated downstream of TLR4 [32]. To get a more straightforward idea of MyD88 independent/TRIF dependent TLR4 signaling please refer to figure 3 and figure 4 in reference [32].

Arrestin

Arrestins are a group of cytosolic scaffold protein. In the late 1980s, It was first discovered as a desensitizing regulator of G protein coupled receptor (GPCR) signaling in the visual rhodopsin system [54] and in the beta adrenergic receptor signaling system [55]. Now, based on phylogenetic analysis, Arrestin family is divided into 2 subfamilies: alpha arrestin and beta arrestin. The well-studied beta arrestin subfamily (also known as beta/visual arrestin) are members of a small branch of the protein family that emerged relatively recently [56]. There are 4 subtypes of beta arrestin subfamily in mammals: arrestin 1 and arrestin 4 are the visual arrestins, whose expression is restricted in photoreceptors cells. Arrestin 1 (also named S-arrestin or visual arrestin) is mainly found in the rod cells and arrestin 4 (also named X-arrestin or cone arrestin) is mainly found in the cone cells. Arrestin 2 and 3 are the non-visual arrestins. Arrestin 2 was first cloned and showed higher binding preference to beta-adrenergic receptor than rhodopsin. Rhodopsin and beta-adrenergic receptors are the 2 only purified GPCRs at the time. Therefore, arrestin 2 is also commonly named β-arresitn 1, hence, the later discovered arrestin 3 is sometimes called β-arrestin 2 [57]. Non-visual Arrestin 2 and 3 are expressed ubiquitously in all cells and tissues. It is now generally established that arrestin serves as a general regulator of GPCRs, also known as the conventional seven-transmembrane domain receptors (7TMR) [58, 59].

Arrestin structure

The four arrestin subtypes are structurally similar. Each has an N-terminal domain and a C-terminal domain composed almost entirely of anti-parallel β-sheets and is connected

by a 12-residue hinge region. A hydrogen bonded network of polar amino acid residues are embedded between these 2 domains. Which is disrupted and disposed while binding to activated GPCRs, and releasing of C tail which contains binding sites for adaptor protein-2 and clathrin [60] (please refer to figure 1 in reference [60] for illustration). The amino acid sequences of the two non-visual arrestins are 78% identical, with most of the coding difference located in the C termini [61].

Knockout studies showed that mice lacking either arrestin 2 or 3 are viable [62, 63], whereas the double-knockout phenotype is embryonic lethal [64], implying that each β-arrestin functionally substitutes for the other isoform to some degree. However, molecular study showed that the two arrestin subtypes are not redundant [61].

Arrestin desensitize 7TMR G protein signaling

Illustration of how arrestin desensitize 7TMRs can be found in figure 1 in reference [65]. Simply, upon agonist stimulation, 7TMRs undergo conformational change that exposes the binding sites to heterotrimeric G protein. This leads to change of GDP for GTP on the Gα subunit initiating the dissociation of Gα and βγ dimer that acts as signaling units and activate various downstream effectors. Meanwhile, agonist-occupied 7TMRs become immediate substrate for a group of protein kinase named GRK (G protein coupled receptor kinase) and get phosphorylated. The phosphorylated 7TMRs recruit arrestin, which blocks further G protein activation by sterically hindering accession to the receptor causing desensitizing of G protein signaling. In addition, arrestin also scaffolds enzymes that degrade second messengers generated by G protein coupling,

which provide another layer for dampening of G protein signaling. Arrestin also plays a role in 7TMR endocytosis. Agonist stimulation promotes rapid internalization of cell-surface 7TMRs into clathrin-coated vesicles. This internalization is facilitated by arrestin binding, which has specific binding domains for clathrin and AP2 interactions [59, 65].

7TMR arrestin dependent signaling

Besides terminate G protein signaling, arrestin has been shown to initiate signaling. Receptor internalization is originally considered as a way to diminish signaling, since the absence of receptor on the cell surface will decrease agonist binding. However, it is evident that, signaling persists after internalization, especially, in arrestin dependent signaling. Arrestin functions as receptor activated scaffold that holds together up- and down-stream components of particular signaling pathways, form the so-called signalsome (signaling receptor complex or scaffold associated with endosome). For example, arrestin can binds cRAF-1 (MAPKKK), MEK1 (MAPKK) and ERK2 (MAPK) thus activates ERK2 signaling. Figure 4 from reference [66] illustrated different kinds of GPCR-arrestin signalsomes and their diverse functions. Therefore, Arrestin dependent signaling is temporally and spatially distinct from the initial second messenger dependent G protein signaling [66, 67]. Certain biased ligands can preferentially activate β-arrestin signaling while blocking or minimally activating G protein signaling or vice versa [68].

Arrestin regulate non-7TMR cell surface receptors and ion channels

Arrestins are discovered in the context of 7TMRs, however increasing evidence suggests that arrestin functions as adaptors for a diverse range of cell surface receptors. A recent proteomic screen study have revealed that arrestins can bind a broad range of catalytically active proteins and recruit them into receptor-based signalsome complexes [69]

β-arrestins have been shown to regulate signaling and/or endocytosis of IGF1R (insulin-like growth factor 1 receptor) [70], Frizzled [71, 72], smoothened [73], TGFβRIII (Transforming growth factor, beta receptor III) [74], LDLR (Low-Density Lipoprotein Receptor) [75], nephrin [76], NHE1,5 (Na+/H+ exchanger isoform 1,5) [77, 78], and Drosophila Notch [79]. β-arrestins are also key regulators of several ion channels including ligand-gated ion channel nicotinic cholinergic receptor [80], cardiac Ca(v)1.2 voltage-gated channels [81] and the transient receptor potential (TRP) ion channel, TRPV4 [82]. Therefore, the biological roles of β-arrestin in signal transduction are likely much broader than we currently know. Arrestin may have a general function as adaptor for phosphorylated forms of receptors or non-receptor proteins.

Arrestin nuclear function

Arrestin 2 and 3 differ in their partitioning between the cytosol and nucleus. Arrestin 3, but not arrestin 2, has a discrete nuclear export signal (NES) [83, 84]. As a result, arrestin 3 exists exclusively in the cytoplasm, and arrestin 2 has been shown to reside both in the cytoplasm and nucleus, which suggests that at least one member of the

family has nuclear function. Kang et al. provide evidence that arrestin 2 moves to the nucleus in response to GPCR stimulation, where it regulates gene expression by facilitating histone acetylation at specific gene promoters [85]. Another proteomics study indicates that more than 68 nuclear proteins, including a number of nucleic acid binding proteins, nuclear kinases, and nuclear signaling proteins, were found to interact with β-arrestins, suggesting previously unsuspected nuclear functions of β-arrestins [69].

Arrestin in TLR4 signaling

As the research continues, the number of cell surface receptors and intra-cellular signaling protein that arrestin scaffolds and regulates is increasing. Arrestin has been shown to play a crucial role in regulating different aspects of inflammation, such as chemokine-induced migration and mobilization of inflammatory cells, mainly through regulation of chemokine receptor signaling, which belongs to GPCR families. Arrestin's expression level in immune cells are dynamically regulated in response to inflammation as well [86]. Arrestin has also been shown to inhibit NF-κB signaling (which is essential in inflammation) via binding to IkBα and protecting it from degradation in the context of TNFα signaling [87]. Whether arrestin is also involved in TLR4 signaling (which is an important player in inflammation) is obviously a new research direction for arrestin function research. So far, arrestin's function in TLR4 signaling has been investigated by a couple of groups and only a handful of literature has been published.

Wang et al. showed that arrestin 2 or 3 interact directly with TRAF6 after TLR4 activation, which prevented auto-ubiquitination of TRAF6 and subsequent activation of NF-κB and AP-1. LPS treated arrestin 3 deficient mice had higher expression of pro-inflammatory cytokines and were more susceptible to endotoxic shock. Thus, they concluded that arrestins are negative regulators of TLR4 signaling [88].

Fan et al. used a HEK cell model and demonstrated that arrestin 2 and 3 differentially regulate LPS-induced signaling and pro-inflammatory gene expression: arrestin 3 positively regulates LPS-induced ERK1/2 activation and both arrestin 2 and 3 negatively

regulate LPS-induced NFκB activation, suggesting that arrestins exert opposing effects on the TL4 induced ERK1/2 and NFκB pathways [89].

Parameswaran et al. found that arrestin 2 play a negative role in TLR4 signaling in Raw264.7 macrophage. They demonstrated that knockdown of arrestin 2 enhance LPS-induced phosphorylation and degradation of p105 (one kind of IκB) and TPL2 (MAP3K) release, and subsequent MEK1/2 phosphorylation. They also showed evidence that arrestin 2 directly binds to the C terminus of p105. They suggested arrestin 2 is a negative regulator of TLR4 signaling potentially through masking p105 from degradation [90].

Porter et al. found that arrestin 2 and 3 knock out (KO) mice are protected from TLR4 mediated endotoxic shock and lethality and LPS induced inflammatory cytokine levels in the plasma were also decreased in both knock outs. However, Arrestin 2 and 3 KO mice exhibit difference in LPS induced inflammatory cytokine from splenocyte. In arrestin 3 KO mice, significantly reduction of cytokine was found in both CD11b+ and CD11b- splenocyte population. But, in arrestin 2 KO mice, this effect is restricted to CD11b- splenocyte. Their studies also suggested that chromatin modification is the underling mechanism for regulation of cytokine levels by arrestins in vivo [91].

Lattin et al. shows that arrestin 3 but not arrestin2 is required for constitutive and LPS induced C1q expression in mouse macrophages, probably through arrestin 3 restricted activation of JNK, arrestin 3 contains a C-terminal JNK binding motif that is not in

arrestin 2 [92].

Seregin et al. showed that adenovirus vector induced innate immune responses are differentially regulated both in vivo and in vitro by arrestin 2 (positive) and arrestin 3 (negative), and adenovirus can trigger several signaling pathways including TLR signaling pathway [93].

Arrestin 3 has been indicated to potentially regulate TLR4 apoptotic signaling via regulating PI3K/AKT/GSK-3b/PPA2 pathway [94, 95]. Arrestin 3 has also been shown to regulate cross-talk between TLR4 and beta-adrenergic receptor [96-98].

In summary, these discoveries are inconclusive and somehow contradictory and lacking interpretation from a molecular perspective. No descriptive signaling regulation model has been proposed either. The general consensus are: 1, arrestins can regulate TLR4 signaling, so far, no evidence regarding arrestin regulates TLR4 endocytosis or trafficking. 2, arrestin 2 and 3 seems to have different functions in terms of TLR4 signaling regulation. 3, arrestins exhibit regulation of TLR4 induced inflammatory response both in vitro and in vivo.

Till now, the discoveries about how arrestin regulate TLR4 signaling are all at the intermediate steps, e.g. TRAF6, ERK/JNK, IκB, PI3K/Akt etc, These adapters may also be involved in other signaling pathways. Is arrestin a common regulator, which functions at certain cross-talking hub of TLR4 signaling pathway and other signaling pathway, or a specific key mediator of TLR4 signaling? Will arrestins directly interact with TLR4 as it

does with GPCRs or only indirectly regulate TLR4 signaling by forming signaling congregate through other adaptor proteins? All these questions need to be further investigated.

My research focus

My research described in this thesis focused on how arrestin regulate TLR4 signaling in human macrophage context. So far, most studies about arrestin's function in TLR4 signaling are based on mouse models. The genetic, biochemical and physiological difference of human and mice are obvious. Therefore, my study will not only provide more supporting evidence in arrestin's function in TLR4 signaling generally, but also provide data in human context. Hopefully, my data may contribute to the discovery of new therapeutic target for damaging inflammation and immune dysfunction.

In my research, I used THP-1 cell line, which is a human acute monocytic leukemia cell line. THP-1 is derived from the peripheral blood of a 1-year-old human male with acute monocytic leukemia [99]. THP-1 cell represent a model for precursor cells of mature macrophage, and can be induced to differentiate into mature macrophage-like cells using various protocols. In my research, in order to get an easily available cell model of human macrophage, I used THP-1 cells. THP-1 cell can be induced to differentiate into macrophage-like phenotype by phorbol-12-myristate-13-acetate (PMA). PMA is an analog of diacylglycerol and can activate PKC signaling pathway, which leads to THP1 differentiation. PMA differentiated macrophage-like THP-1 cells resembles primary macrophage in terms of morphology and macrophage specific markers, as well as other functions of macrophage, e.g. cytokine secretion when activated by pathogens [100].

I used siRNA to knock down arrestins in THP-1 derived macrophage cells and then stimulated the cell with TLR4 agonist LPS and harvested cells lysate for subsequent human inflammatory response & autoimmunity pathway focused PCR-array

experiments. By comparing array results of control and arrestin knock down cells, conclusions can be made regarding how arrestin regulation TLR4 signaling in human macrophage context.

I hypothesized that, compared to control, knock down of arrestin will cause a different expression profile of inflammatory and autoimmunity pathway involved genes in macrophage-like THP-1 cells.

Chapter Two: Material and Methods

Material:

THP1 cells (ATCC), RPMI1640 (GIBCO Cat# 11875), HI FBS (GIBCO Cat#10082), 2-mercaptoethanol (GIBCO Cat# 21985-023), PenStrep (GIBCO # 15140), Phosphate buffered saline (SIGMA D8537) Phorbol 12-myristate 13-acetate (SIGMA Cat# P8139-1MG), TranIT-TKO Transfection Reagent (Mirus Cat #MIR 2150), Human ARRB1 siRNA pool (Thermo Scientific Dharmacon ON-TARGETplus SMART pool Cat# L-011971-00), Control siRNA pool (Thermo Scientific Dharmacon ON-TARGETplus Non-targeting Pool, Cat# D-001810-10-20), Lipopolysaccharides (Invivogen Cat# LPS-SM Ultrapure), Arrestin2 antibody is a gift from Dr. Jeffrey L. Benovic from Dept. of Biochemistry and Molecular Biology, Thomas Jefferson University, 233 South 10th St., Philadelphia, PA. RNeasy Mini Kit (50) (QIAGEN Cat# 74104), SuperScript III reverse transcriptase (invitrogen Cat# 18080-044), Random primers (Promega Cat# C118A 29819605), dNTP Mix (invitrogen Cat#18427-013), Ribonuclease H (invitrogen Cat# 18021-014), RNase inhibitor (Applied Biosystems Cat# N808-0119), Platinum SYBR Green qPCR SuperMix-UDG kit (invitrogen Cat# 11733-046), RT² Profiler™ PCR Array Human Inflammatory Response and Autoimmunity (SABiosciences Cat# PAHS-077E-4), RT² SYBR Green/ROX PCR Master mix (SABiosciences Cat# PA-012-8), RT² First Strand Kit (SABiosciences Cat# C-03), RT² PCR Array Loading Reservoir (SABiosciences Cat# PA-027), Human IL-6 ELISA Ready-set GO! (ebioscience Cat# 88-7066-22)

Primers for qPCR were designed and ordered from IDT (http://www.idtdna.com)

Human ACTB primers: Forward 5’-CATCGAGCACGGCATCGTCA-3’; Reverse 5’-TAGCACAGCCTGGATAGCAAC-3’

Human HPRT1 primers: Forward 5’-GACCAGTCAACAGGGGACAT-3’; Reverse 5’-AACACTTCGTGGGGTCCTTTTC -3’

Human IL6 primers: Forward 5’-CCACTCACCTCTTCAGAACG-3’; Reverse 5’-CATCTTTGGAAGGTTCAGGTTG-3’

Human Arrestin 2 primers: Forward 5’-ATCCCTCCAAACCTTCCATG-3’; Reverse 5‘-TGACCAGACGCACAGAATTC-3’

Human Arrestin 3 primers: Forward 5’-AAGCACGAGGACACCAAC-3’; Reverse 5’-AAAAGGCAGCTCCACAGAG-3’

Methods

THP1 cell maintenance and differentiation

THP1 cells are grown in complete growth media (RPMI1640+10%FBS+ 0.1% 2-mercaptoethanol + 1% PenStrep) in 37C and 5% CO2 incubator. Cell density should be kept in-between 0.2 million/ml to 0.8 million/ml.

To differentiation THP1 cells, THP1 cells was seeded at 0.75 million/ml in complete growth media in appropriate culture dishes, for example 15ml cell suspension were added to 10cm culture dish), PMA (50ng/ml) were added to induce differentiation, THP1 cells will attach after 48 hour PMA induction, normally, after 72hours, THP1 cells will exhibit elongated pseudopodia and granules, resembling mature macrophage.

siRNA transfection and LPS induction

Warm up transfection reagent into room temperature, To transfection attached cells in 10cm culture dish, gently mix 1500ul serum free media (RPMI1640 + 0.1% 2-mercaptoethanol + 1% PenStrep) with 30ul Mirus transfection reagent, keep in room temperature for 15min then add 7.5 ul siRNA (100uM) gently mix well, then keep in room temperature for 15min till usage. Aspirate complete culture media after 48hr PMA induction, change media into fresh complete culture media with PMA (50ng/ml) , half the previous volume, for example, in 10 cm dish, the previous cell suspension volume is 15ml after 48hr differentiation and attachment, change media into 7.5ml. Dropwise add siRNA, transfection reagent and serum free media mixture onto the entire surface of cell culture, shake culture dish to distribute transfection mixture evenly. After 3 days of transfection, aspirate thoroughly culture media and wash it 3 times with PBS (this step it

to completely wash away PMA and serum), then add serum free media (7.5ml in 10cm dish). Serum starve for 3 hours, stimulate with LPS (1ug/ml) for appropriate time course. Harvest cells by thoroughly remove media and snap frozen in -80C freezer.

Western blot

For western blot 250~500 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, Roche protease inhibitor mixture and phosphatase inhibitor) were added to 10cm culture dish, lysates were clarified, and then protein concentration was determined, and equivalent amounts of protein were loaded on the gels for Western blot analysis. Immuno-blotting was performed as described previously [101].

RNA purification and RT-real-time PCR

RNA was harvested and purified using RNeasy Mini Kit strictly according to manufacturer's handbook. RNA concentration and quality were determined by NanoDrop Spectrophotometer. First-Strand cDNA synthesis was performed using SuperScript III Reverse transcriptase kit strictly according to the recommended protocol for random primers. Real-time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG kit, according to manufacturer's protocol. Quantitative PCR was performed on ABI Prism 7900HT Sequence Detection System at MSU RTSF Genomics core.

PCR array

After RNA purification and quantification, Pathway-Focused Gene Expression Profiling using Real-Time PCR array was performed strictly according to SABioscineces RT 2 Profiler PCR Array System User Manual / Handbook on ABI Prism 7900HT Sequence Detection System at MSU RTSF Genomics core.

ELISA

Supernatant from cell culture were collected and clarified. Supernatant were stored in -80C freezer and IL6 level in the supernatant were measured by ELISA using ebioscience ELISA Ready-set GO! Kit, according to manufacturer's protocol.

Phase contract microscopy

Phase contract microscope digital photos of THP1 cells and differentiated THP1 cells were taken using Nikon microscope system. Digital images were adjusted using MetaMorth software.

Chapter Three: Results and Discussion

Arrestin 2 expression level was increased during THP1 PMA induced macrophage-like differentiation

Cell morphology change and arrestin expression levels were monitored during PMA macrophage-like differentiation of THP-1 cells, in order to be certain first this differentiation regime is applicable and second arrestin levels are expressed highly enough in THP-1 macrophage model for practical research.

Before induction, THP-1 were round, suspended cells with few cellular granules (Figure 1 left), 48hours after PMA induction, THP-1 cells were almost 100% attached to culture dish, and at 72 hours, THP-1 cells exhibited elongated pseudopodia and contained more granules and cytoplasm compare to undifferentiated cells, resembling mature activated macrophage (Figure 1 right). During 3 days of siRNA knockdown time window, macrophage like THP-1 morphology did not change and only very few cells were detached and dying. This morphology change is similar to previous publication [100].

Arrestin 2 mRNA levels are increased by 1.5 times as measured by qPCR at the end of 2-day PMA induction plus 3day siRNA knockdown regime (Figure 2a). Also, arrestin 2 protein level was increased by 2.5 times as measured by western blot (Figure 2b). Arrestin 3 mRNA was also raised about 6 time (data not shown). Due to the lack of good human arrestin 3 antibody, how arrestin 3 protein level changes during the differentiation process are unknown.

This discovery is serendipitous. Few people had ever studied arrestin expression level change during a differentiation process. Arrestin 2 mRNA levels and protein level have been shown to increase in rat brain during postnatal development [102]. Arrestin 3 expression has been reported to be enriched in mouse macrophages compare to other mouse cell type [92]. Arrestin is also involved in many discrete developmental pathways e.g. Hedgehog, Wnt, Notch, and TGF pathways. These previous findings indicate a potential role of arrestin in macrophage differentiation and mature macrophage function.

Therefore, my discovery that arrestin level is up-regulated during THP1 PMA induced macrophage-like differentiation raised many interesting questions:
1, is this phenomenon artificial to THP1 cell PMA induced differentiation model or have a general physiological role?

2, is arrestin level increase specific to macrophage differentiation, or it is a common phenomena that happens in other differentiation process e.g. Dendritic cell differentiation, Adipogenesis, fibroblast genesis, neurogenesis etc.

3, is arrestin a prerequisite for monocyte to differentiate into macrophage or it is an outcome of differentiation.

For the first question, I performed experiment using different differentiation regimes to inducing THP-1 macrophage-like differentiation, and measured arrestin 2 protein level. I used VD3 [103], IL4+GM-CSF, IFNγ+LPS [104], and M-CSF [105, 106] regimes.

IL4+GM-CSF is a dendritic cell like monocyte differentiation regime [107], which was included to test whether this phenomena is specific to macrophage-like differentiation branch of monocyte. The other regimes are all known macrophage-like monocyte differentiation regimes. My results demonstrated that only IFNγ+LPS regime caused about 0.5 times arrestin 2 protein level increase, others showed no significant increase (data not shown). Consistent with this is that only IFNγ+LPS regime cause significant attachment of THP-1 cells to the culture dish and morphology change, although not as dramatic as PMA induced morphology change (data not shown). Another serendipity is that, during my experiment one maintenance culture flask caught bacteria contamination. And all the THP-1 are differentiated and attached to the culture surface, It is known that, pathogens can stimulate monocyte differentiate to mature active macrophage [7, 8]. Therefore, I postulate that the attached THP-1 cell caused by bacterial contamination is actually mature active macrophage, and I harvested these cells and measured arrestin 2 protein level and there was a huge increase about 3.5 times of undifferentiated control THP-1 cells (data not shown). My new hypothesis for this question is that arrestin 2 level increase in PMA induced THP-1 cell differentiation actually is not artificial but it happens in physiological condition as well. To further investigate this question, I could harvest primary monocyte as well as primary mature macrophage from animal or human and compare their arrestin levels. Also, I may compare arrestin 2 level in different macrophage populations from different inflammatory disease scenarios.

For the second question, my hypothesis is that arrestin expression increase is not restricted to macrophage differentiation, my rationale for this is that, arrestin has a broad role in many pathways and functions e.g. vehicle trafficking. As cells differentiate from precursor cells to functional cells, it is possible that more arrestin is needed for sufficient function. I hypothesis that arrestin level will also increase in neurogenesis, since one important function of neurons is neurotransmitter release and receipt which is regulated by arrestin. To test this question, I can monitor arrestin level change in differentiation processes of various cell lineages, such as adipogenesis, neurogenesis etc. or different differentiation branches of certain lineage, such as macrophage differentiation branch of monocyte versus dendritic cell differentiation branch of monocyte. One possible interesting discovery could be that: arrestin level increase is restricted to macrophage differentiation but not dendritic cell differentiation of monocyte. If this were true, it will be a very interesting future investigation subject.

For question three, I can simply knock down arrestin, then examine whether macrophage-like differentiation is impaired or not. If indeed differentiation is hindered, this indicates that arrestin is a prerequisite for macrophage differentiation. If cells can still be differentiated into macrophages, this indicates that arrestin level increase is just an outcome. However, I should always consider the functional redundancy of arrestin 2 and arrestin 3 while interpreting my data.

In THP1 PMA derived macrophage, siRNA can significantly knock down arrestin 2 in both mRNA and protein levels

After 48 hours PMA induction, arrestin 2 siRNA pool was applied on attached THP-1 cells for 72 hours, no detectable damage of cells was observed, meaning minimal toxicity of the knock down system. Cells were harvested and arrestin 2 protein level was measured by western blot. Arrestin 2 protein level was successfully knocked down by 50% (Figure 3). Arrestin's mRNA level was also measured via qPCR on cell lysate samples used for PCR array experiments (for PCR array sample treatment details please refer to following result content). The results also showed that arrestin 2 mRNA level were knocked down by 70% (data not shown), meanwhile, arrestin 3 mRNA level was increased to about 50% in arrestin 2 siRNA knock down samples for PCR array (data not shown). This result indicates possible compensation of arrestin 2 by up-regulating arrestin 3. Arrestin 2 and arrestin 3 are known to have redundancy functions. Therefore, when interpret data generated from arrestin 2 or arrestin 3 single deficiency experiments, I should always consider the influence of the other arrestin subtype's compensation.

Genes involved in inflammation and autoimmunity were differently affected by arrestin 2 siRNA knock down in LPS stimulated macrophage-like THP-1 cells

After siRNA knockdown, THP-1 cells are washed with PBS 3 times and then serum starved for 3 hours, 1μg/ml LPS was then added onto serum starved THP-1 cells for TLR4 stimulation. After 6hr stimulation, cell lysate were harvested and cDNA are synthesized by reverse transcription. Then PCR array or regular qPCR were performed in order to determine the mRNA expression level of genes of interest. We used a 384 well PCR array plate that contains predesigned primers for 96 genes in every 4 wells in order to detect expression level of 96 different genes in 4 samples via real time PCR simultaneously. For details of this technique please refer to material and method part of this thesis and SAbiosciences company website.

Besides 5 housekeeping gene and 7 controls for this technique, 84 inflammation and autoimmunity pathway focused gene are included in this PCR array plate, for detailed array layout and gene table please refer to Table 1. These 84 genes can be grouped in to several categories: chemokines (CCL11, CCL13, CCL16, CCL17, CCL19, CCL2, CCL21, CCL22, CCL23, CCL24, CCL3, CCL4, CCL5, CCL7, CCL8, CXCL1, CXCL10, CXCL2, CXCL3, CXCL5, CXCL6, CXCL9, IL8); cytokines (CD40LG, CSF1, FASLG, FLT3LG, IFNG, IL10, IL18, IL1A, IL1B, IL1F10, IL1RN, IL22, IL23A, IL6, LTA, LTB, TNF, TNFSF14); cytokine receptors (IL10RB, IL1R1, IL1RAP, IL22RA2, IL6R, CXCR1, CXCR2); chemokine receptors (CCR1, CCR2, CCR3, CCR4, CCR7, CXCR4, CXCR1, CXCR2); TLR receptors (TLR1, TLR3, TLR4, TLR6); genes involved in cytokine-cytokine receptor interaction (CCR1, CD40, IL18RAP, IL23R); gene involved in acute-

phase response (CEBPB, CRP, IL22, IL6); gene involved in inflammatory response (BCL6, C3, C3AR1, C4A, CCL11, CCL13, CCL16, CCL17, CCL19, CCL2, CCL21, CCL22, CCL23, CCL24, CCL3, CCL4, CCL5, CCL7, CCL8, CCR1, CCR2, CCR3, CCR4, CCR7, CD40, CD40LG, CEBPB, CRP, CXCL1, CXCL10, CXCL2, CXCL3, CXCL5, CXCL6, CXCL9, FOS, HDAC4, IL10, IL10RB, IL18RAP, IL1A, IL1B, IL1F10, IL1R1, IL1RAP, IL1RN, IL22, IL8, CXCR1, CXCR2, IL9, ITGB2, KNG1, LY96, MYD88, NFATC3, NFKB1, NOS2, NR3C1, RIPK2, TIRAP, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TNF, TOLLIP); and Gene involved in humoral immune response (C3, C4A, CCL16, CCL2, CCL22, CCL3, CCL7, CCR2, CCR7, CD40, IL10, IL18, IL1B, IL6, ITGB2, LY96, NFKB1).

In one of this 384-well PCR array plate, 4 samples can be arrayed simultaneously. The samples that were tested together are 1: cells treated with control siRNA and no LPS stimulus, 2: cells treated with arrestin2 siRNA and no LPS stimulus, 3: cells treated with control siRNA and 6hr LPS stimulation, 4: cells treated with arrestin2 siRNA and 6hr LPS stimulation. This experiment was repeated for 4 times for biological replicates. Each biological replicate start from an independent flask of THP-1 culture originated from the same frozen stock cells. Since THP-1 cells are floating cells, to grow THP-1 cells, I just need to dilute too condensed cell culture with new media in order to keep an appropriate cell growth density. The concept of cell passages is not applicable here. The difference between the 4 biological replicates are: cells used in replicate 2 is about 4 days older than cells used in replicate 1 and cells used in replicate 3, 4 are about 2

weeks older than cell used in replicate 2, and cells used in replicate 3, 4 are taken at the same time but from different culture flasks.

All 4 biological replicates of PCR array experiments are very successful as indicated by perfect quality control data from control wells on the PCR array plates (data not shown) and successful LPS stimulation as indicated by IL6 induction measured by regular qPCR in all 4 biological replicates used for array (Figure 4b). Arrestin 2 was also successfully knocked down in all 4 biological replicates as indicated by regular arrestin 2 gene qPCR (Figure 4a). Genes such as inflammatory cytokine IL-1β, IL6, TNFα and chemokine CCL3 (MIP1a), CCL4 (MIP1b) which are known to be induced upon TLR4 stimulation in monocyte/macrophage were also up-regulated in LPS stimulated control samples compared to resting control sample as shown in array data (Figure 8a, Figure 8b).

I used SABiosciences provided web based PCR array analysis software to analyze all 4 biological replicates together. A cluster image was generated (Figure 5). Genes with similar regulation profile were clustered together. As indicated by the cluster image, all genes tested can be roughly grouped into 5 clusters representing different regulation patterns (Figure 5): 1, genes that were not influenced by either arrestin 2 knock down or LPS stimulation; 2, genes that were up-regulated by arrestin2 knock down but not influenced by LPS stimulation; 3, genes that were induced by LPS stimulation and further enhanced by arrestin 2 knockdown; 4, genes that were highly induced by LPS stimulation but down regulated by arrestin 2 knockdown; 5, genes that were down

regulated by LPS and basal level expression were reduced by arrestin 2 knockdown, in the LPS stimulated state, arrestin 2 knockdown does not influence much. This classification of genes are just a description of expression regulation pattern trend in general, may not apply to individual gene.

The cluster of genes that were highly induced by LPS but was down regulated by arrestin 2 knock down caught my interest specially. If these data were true, indicates that arrestin 2 plays a positive regulation role in LPS induced TLR4 signaling pathway leading to the induction of these genes. This cluster including IL8, NFkB1, CXCL3, CXCL1, CXCL2, CCL3, CCL4, CXCL6 and IL1B. I further took a close look at gene array data of all 4 biological replicates of this cluster of gene specifically (Figure 6).

A discrepancy in arrestin's effect between replicates were noticed. Take IL8 as an example (Figure 6), in replicate 1 IL8 was highly induced by LPS stimulation, arrestin 2 knockdown further enhanced IL8 level a bit, in replicate 2 the effect of arrestin 2 knockdown is similar to replicate 1, that is arrestin 2 knockdown enhance LPS stimulated IL8 expression but to a larger extent. However, data from replicates 3 and 4 show opposite effect of arrestin 2 knockdown, being that arrestin 2 knock down negatively regulates LPS induced IL8 expression. The magnitudes of down-regulation are similar between replicates 3 and 4. Arrestin 2 knockdown and LPS induction had been confirmed by regular qPCR of arrestin 2 and IL6 on the same RNA sample used for array. Another supporting evidence of successful LPS induction is that: The supernatants of array samples were also collected and IL6 concentrations in

supernatants were tested using ELISA. Significant increases of IL6 concentration were observed in supernatants corresponding to LPS stimulated samples (data not shown). IL6 also exhibit similar regulation pattern as IL8 in PCR array, being opposite between replicates 1, 2 and replicates 3, 4 (Figure 4b). This ruled out possible sample switching, labeling or manipulation errors and indicated that what has been observed is indeed what happened. As reviewed in introduction, arrestin's function in TLR4 signaling has not been investigated extensively. Some of the discoveries are contradictory. Regarding IL8, Fan et al, has shown that arrestin 2 positively regulate IL8 secretion in HEK cells over-expressing TLR4, possibly through ERK signaling [89], whilst, Wang et al, suggest that arrestin 2 negatively regulate IL8 secretion by blocking TRAF6 in THP1 cells [88]. A possible explanation for this kind of discrepancy is that, the model cell systems used between different researchers are different. Therefore, different cell physiology will cause different regulation dynamics. Of my 4 biological replicates, replicate 1 is about 4 days older than replicate 2, which is then about 2 weeks older than replicate 3, 4. Replicates 3 and 4 are taken at the same time. This is consistent with gene regulation pattern: replicates 3 and 4 share similar trend, while replicates 1 and 2 share similar trend although slightly different in magnitude and opposite to replicates 3 and 4. It might be possible that because the 4 biological replicates are in different aging states, which causes different cell physiology thus leads to different gene expression dynamic profiles. One possible scenario/hypothesis of how arrestin 2 functions has been proposed here: arrestin 2 functions differently at the early gene induction stage from the late gene diminishing stage, as exemplified by Figure 7. Arrestin 2 knock down elongates gene induction, plateau, and diminishing time span. Assuming the cell

populations represented by replicate 1, 2 and replicate 3, 4 are at two different physiological conditions, with replicate 3, 4 represented cells exhibit longer gene induction/plateau/diminishing time span than replicate 1, 2. Therefore, 6 hr after LPS induction, gene expression in replicate 3, 4 may still be at induction stage in both wild type (control) and arrestin 2 knock down samples. The conclusion is arrestin 2 knock down negatively effects gene expression level. Whilst in replicate 1 and 2, the wild type (control) cells have already gone through gene induction/plateau stage and are at gene level diminishing stage. But, the arrestin 2 knock down cells are still at gene induction stage as shown in Figure 7. Hence, gene expression level is lower in wild type (control) cells than arrestin 2 knock down cells. This result resembles arrestin 2 knock down positively affects gene expression.

To test this hypothesis, I just need to sample at very early and late stages of LPS induction then measure gene levels, conclusion can be made.

Due to the discrepancy of the 4 biological replicates regarding arrestin 2's function, further analysis is difficult if including all data. Therefore, I used biological replicates 3 and 4 (taken at same time, representing same cell physiological condition) to do further analysis. 3 groups of genes whose expression were significantly (more than 2-fold change of gene expression magnitude) affected by arrestin 2 knock down in LPS stimulated THP-1 cells were selected (Figure 8). These 3 groups represent 3 different regulation effects of arrestin 2 on TLR4 signaling. Interestingly, if genes of one group are negatively affected by arrestin 2 knock down in replicates 3 and 4, this group of

gene tends to be all positively affected by arrestin 2 knock down in replicate 1 and 2 (data not shown) and vice versa. Therefore, no matter in what hypothetical cell physiological/metabolic state, they are regulated similarly by arrestin 2 and clustered together, possibly through shared signaling branch of TLR4 signaling network. Further bioinformatics study of the promoter region of these genes in one group, may provide shared binding motif for certain transcription activator e.g. AP-1, NF-κB, IRF3 etc. which may generate targets for further molecular mechanism study of how arrestin specifically affect TLR4 signaling. My new hypothesis is that: arrestin 2's function on gene group including CD40LG, IL8RB, NOS2, TNFSF14 (Figure 8c) is regulated at TLR4 signaling pathway leading to IRF3 activation. The rationale is that all these genes are somehow related to interferon. While arrestin 2's function on gene group including IL8, IL6, NFKB1, TNF, IL1B, CCL4, CCL3 etc (Figure 8a) is regulated at TLR4 signaling pathway leading to MAPK or NF-κB activation. Because these groups of genes are pro-inflammatory cytokine/chemokine genes which are known to be activated by MAPK or NF-κB under TLR4 signaling. To test this, on top of arrestin manipulation, I can activate or inhibit certain steps in individual signaling pathway branch and test associated gene expression levels. When choose gene, I should focus on genes with most dramatic regulation fold changes, for example, CCL3, CCL4, IL1B and IL6 from gene group illustrated in Figure 8a.

Chapter 4: Conclusion

Based on my research summarized in this thesis, I conclude that arrestin 2 expression level is increased during PMA induced macrophage-like differentiation of THP-1 cells, and arrestin 2 does regulate TLR4 signaling in human cell model context. Further, Arrestin 2 exhibits different regulation effects on inflammatory and autoimmunity involved genes expression under TLR4 stimulation. Probably because these genes are induced by different branches of TLR4 signaling and arrestin 2 regulates these branches differentially. My discovery is significant because it provided a relatively large pool of information about how arrestin 2 regulate TLR4 signaling in term of inflammation related gene expression in human cell context for the first time. Similar large-scale study has never been proposed in this field. However, there are also some drawbacks of my research.

1, the macrophage differentiation is induced by PMA, which remotely resembles macrophage differentiation/activation in real human physiology. For the future investigation, I could use controlled bacteria induced differentiation (mimic certain disease model) of THP-1 cells or other macrophage precursor cells as a model system or use primary monocyte isolated directly from human being. Considering the heterogeneity of macrophages, I should always think about what is the macrophage subpopulation in real organism closest relevant to the cell model system being used.

2, LPS was used at 1 μg/ml, which is a rather high dose. TLR4 can be activated without the help of co-receptor CD14 at this dose of LPS [31]. However, TLR4 co-receptor's

function is essential for TLR4 proper function in reality. High dose of LPS may mask certain delicate regulation of TLR4 signaling by its co-receptors. For the future, low dose stimulation or biased agonist stimulation may reveal more information about how TLR4 and its co-receptors are regulated by arrestin 2.

3, siRNA knockdown can not be achieved 100%, when interpreting data, I should always take into the account of arrestin 2's residual function and arrestin 3's compensation, since it is impossible to get knock out cells in human study, double knockdown and over-expression system could always be included as added evidences.

4, It is not a wise choice to collect/analyze samples at 6hr after LPS stimulation. Now I have learned that, in TLR4 signaling, genes induction is regulated in different waves. There are so-called primary, secondary response genes, that are regulated by different categories of transcription factors [6] (please refer to figure 1 in reference [6] for illustration). Further, for a single transcription factor e.g. NF-κB, there are early and late phase activation [97]. Gene expression kinetics varies with cell types and cell physiological conditions as well. 6hr time point may rest just in the middle of this gene induction orchestra. Therefore, discrepancy from different experimental replicates is prone to happen. Also, unique regulation patterns of certain genes with further investigation potential may be buried and difficult to analyze and mine out. For the future, more time points should be sampled, especially in the very early gene induction stage of TLR4 stimulation, in order to generate more instructive data. And, how will

arrestin 2 regulate TLR4 signaled gene expression waning process would also be an interesting future research subject.

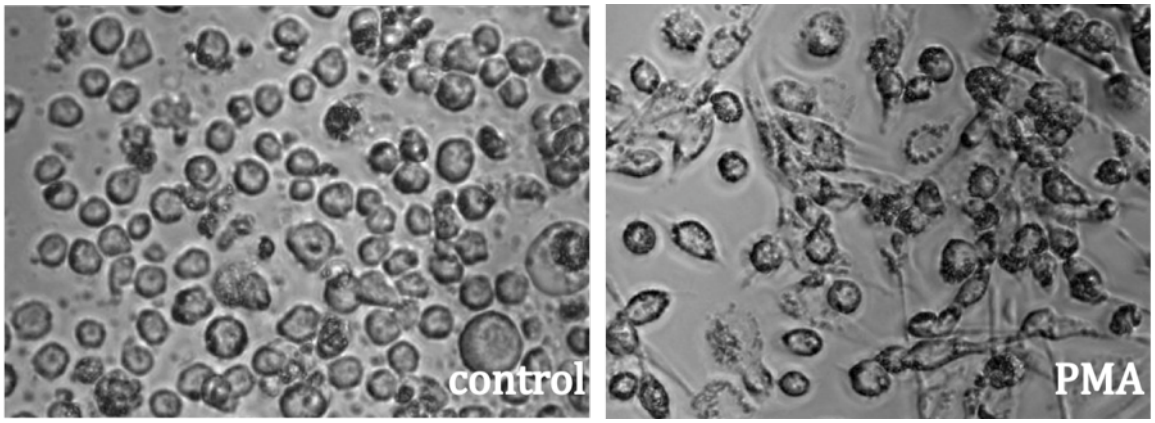
5, arrestins are promiscuous scaffolding proteins that regulate tremendous amount of signaling pathways. Perhaps, they are not good candidates for studying TLR4 signaling with the hope to develop certain therapeutic target. For the future, people may want to also focus on some arrestin associated proteins e.g. other adaptor proteins, arrestin modification proteins, which have more specific function in terms of TLR4 signaling.

And last, I did not investigate arrestin 3's effect on TLR4 signaling, due to the lack of proper antibody. It is known that arrestin 3 exhibits some distinguish functions from arrestin 2. So what is the function of arrestin 3 in TLR4 signaling? This should be an immediate research subject once the proper antibody is available.

APPENDICES

APPENDIX A: Figures

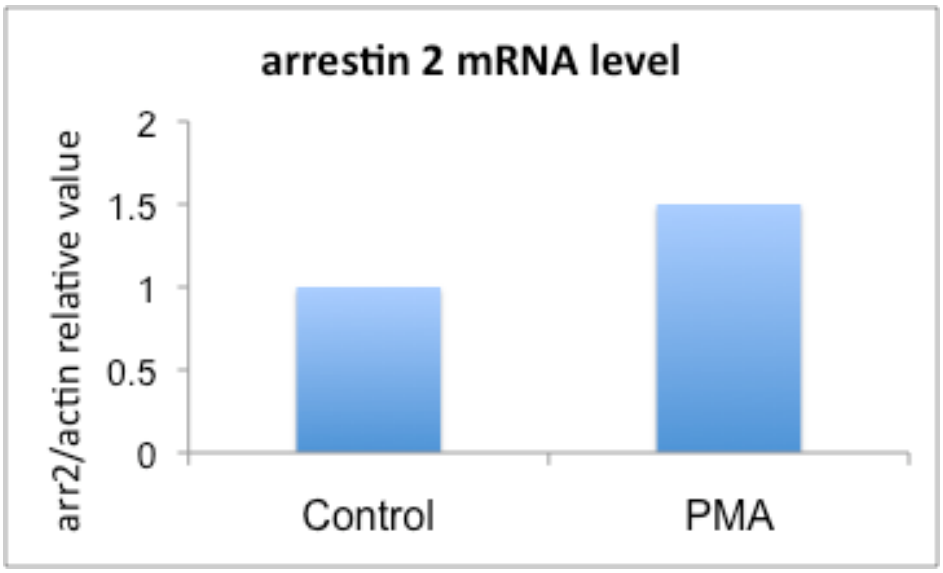
Figure 1: THP1 morphology change during PMA induced macrophage-like differentiation.



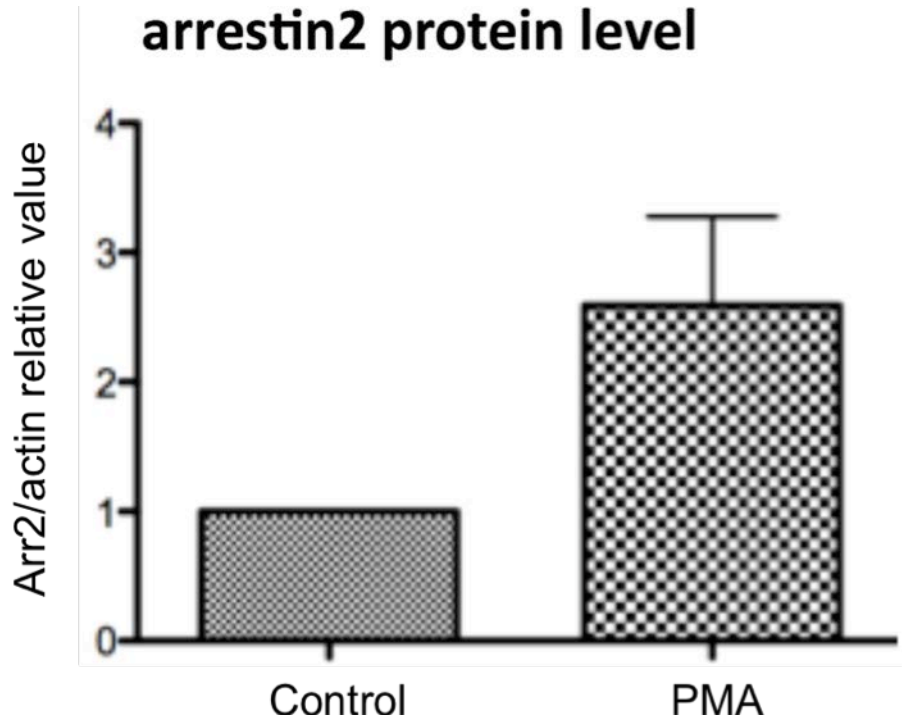
Phase contract microscope image (taken under 10x objective representing 100x magnification) of undifferentiated THP-1 cells (control, left) and differentiated THP-1 cells after 3-day PMA (50ng/ml) induction (PMA, right).

Figure 2: Arrestin 2 expression level increase during THP1 macrophage-like differentiation. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

a



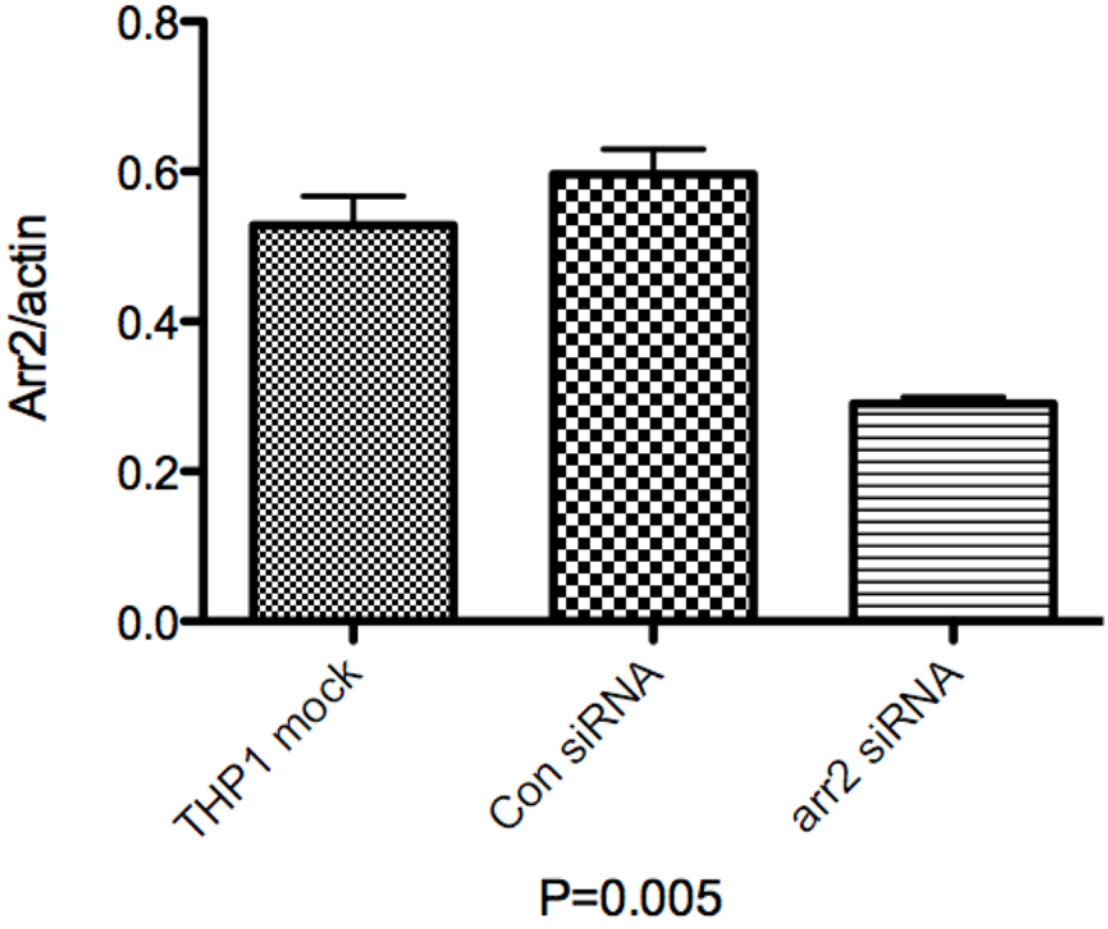
b



(a) Arrestin 2 mRNA expression level in THP-1 cells (control) and differentiated THP-1 cells (PMA) after 2-day PMA (50ng/ml) induction plus 3-day siRNA knockdown, graph was a representative of 2 independent qPCR experiments using two sets of arrestin 2 primers respectively, Y axis represents relative value of arrestin 2 normalized to human

Figure 2 (cont'd).
beta actin gene. (b) Arrestin 2 protein expression level in THP-1 cells (Control) and differentiated THP-1 cells (PMA) after 3-day PMA (50ng/ml) induction. Data are presented as mean±SEM. $P<0.01$. Y-axis represents relative value of arrestin 2 normalized to human beta actin protein.

Figure 3: Arrestin 2 protein level was significantly knocked down by siRNA in PMA differentiated THP-1 cells.



THP-1 was induced by 50ng/ml PMA for 2 days and then treated with siRNA (100μM) for 3 days, then subjected to western blot. Arrestin 2 Protein level were normalized against β-actin, THP1 mock is sample treated with only transfection reagent, Con siRNA is sample treated with control siRNA, arr2 siRNA is sample treated with arrestin 2 siRNA, Data are presented as mean±SEM.

Figure 4: Arrestin 2 and IL6 mRNA levels in 4 biological replicates used for PCR array measured by regular qPCR.

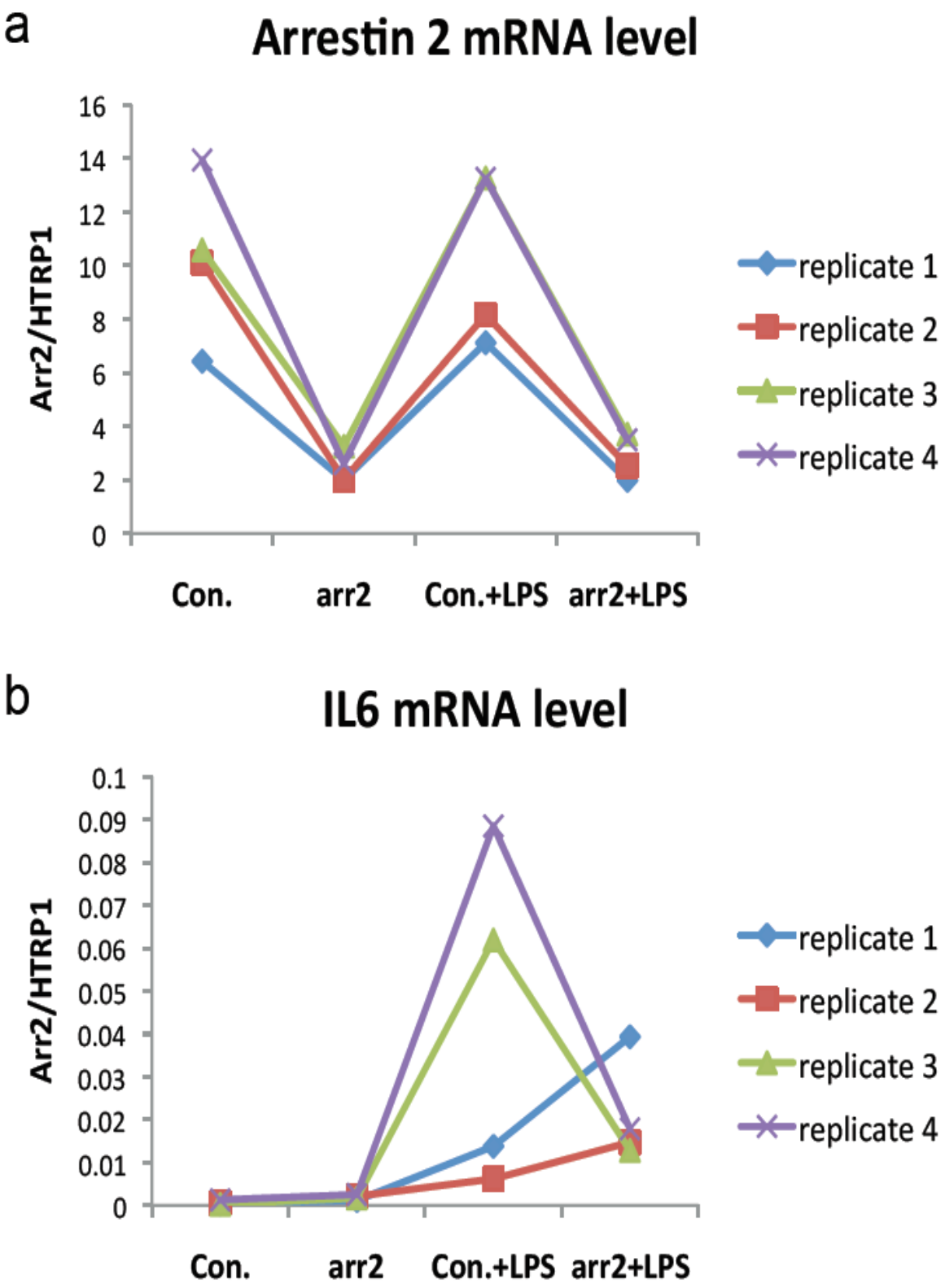


Figure 4 (cont'd).
(a) Arrestin 2 mRNA level relative to human HTRP1 gene. (b) IL6 mRNA relative to human HTRP1. For both graph Con. is samples treated with control siRNA and no LPS stimulation, arr2 is sample treated with arrestin 2 siRNA and no LPS stimulation, Con.+LPS is sample treated with control siRNA and LPS (1μg/ml) stimulation, arr2+LPS is sample treated with arrestin 2 siRNA and LPS (1μg/ml) stimulation.

Figure 5: PCR-array data cluster analysis.

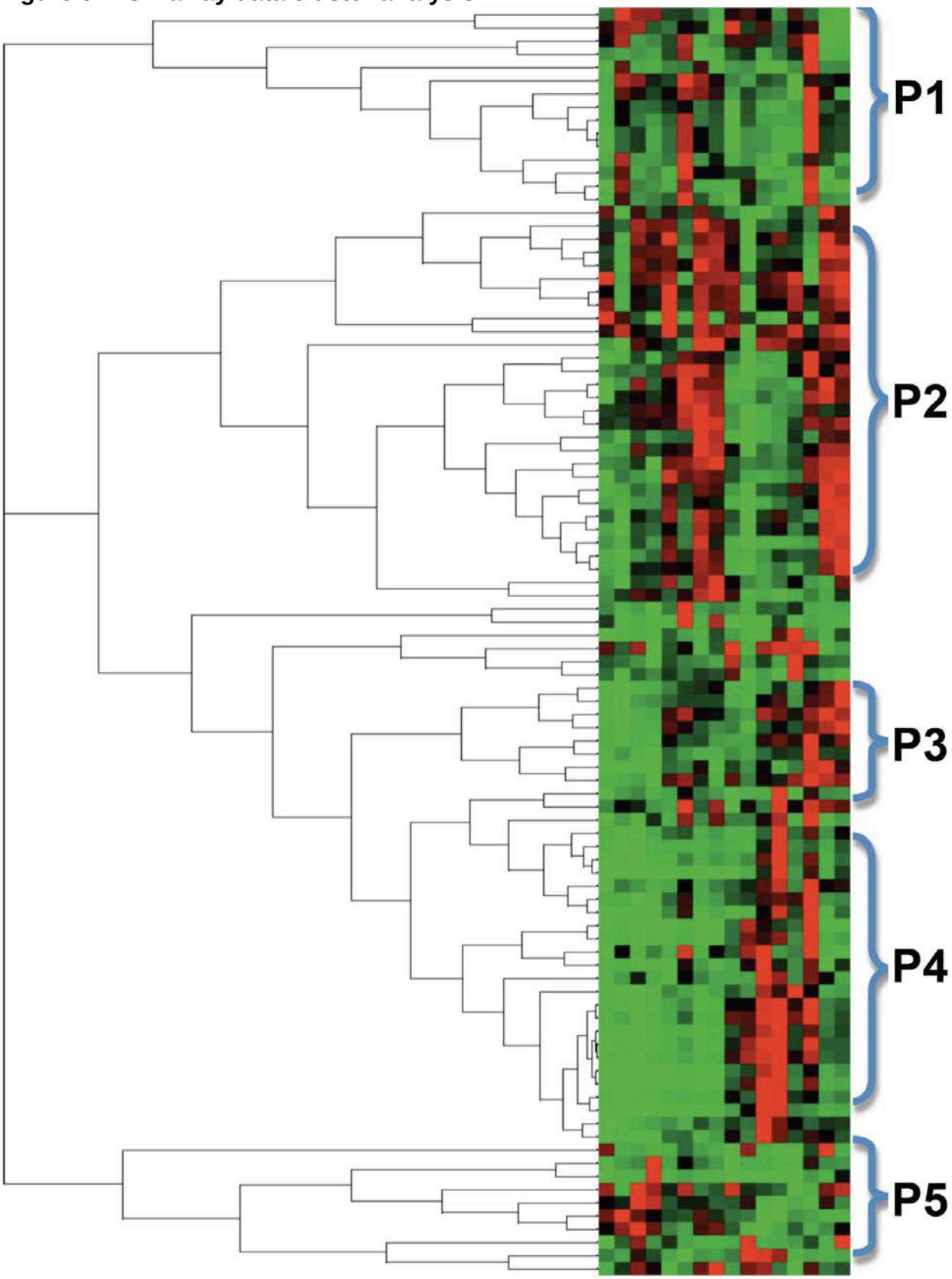
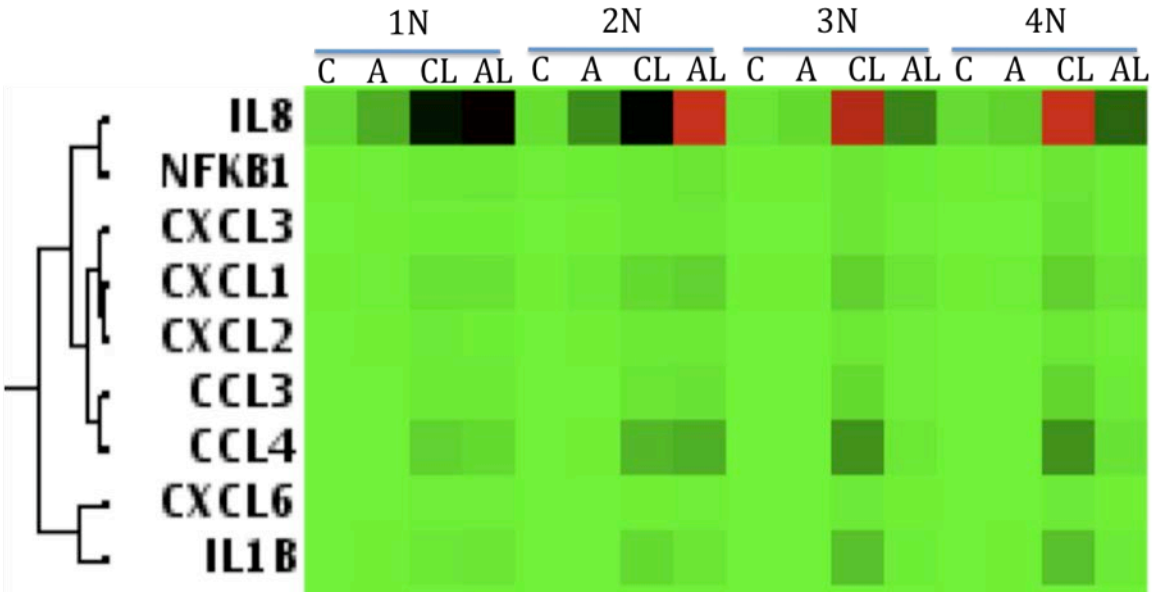


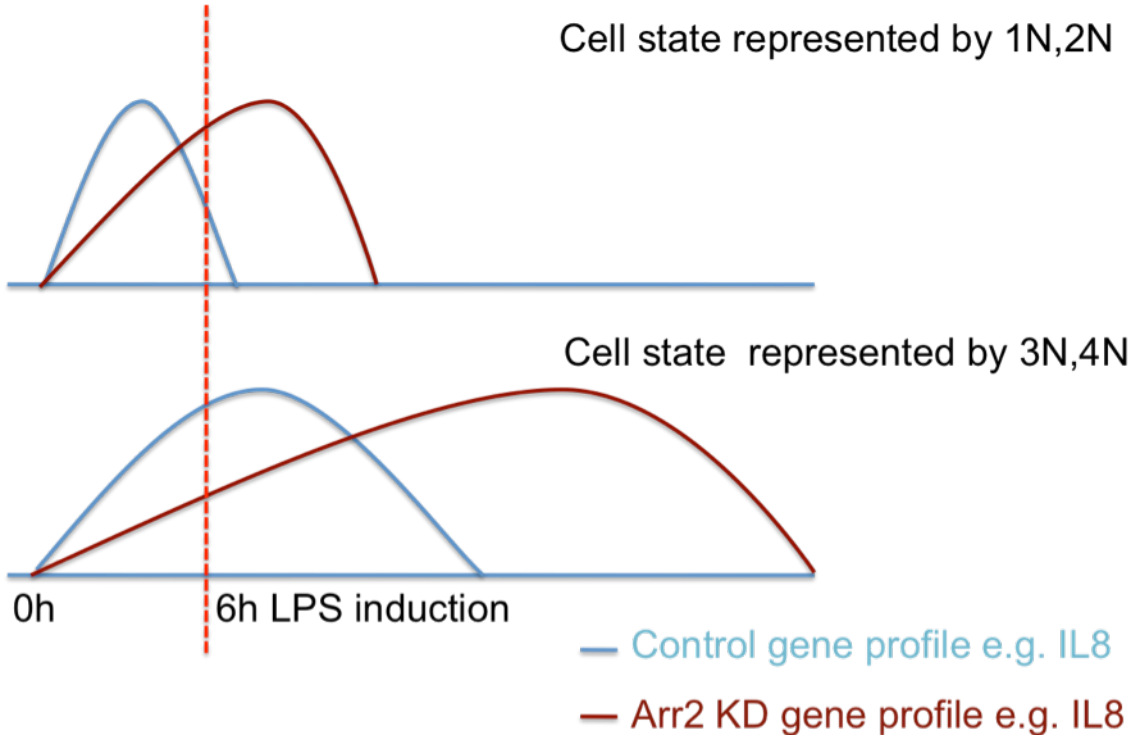
Figure 5 (cont'd).
SABiosciences web-based PCR-array analysis of the influence of arrestin 2 knock down on LPS induced gene expression on differentiated THP-1 cells. Gene expression magnitudes are normalized against the average with in each gene. Green color denotes minimal gene expression, black color denotes average gene expression and red color denotes maximal gene expression. Each row represents one gene. From top to bottom, the genes are CCL21, RPL13A, CCL23, IL9, IL22RA2, TLR4, TIRAP, PPC, PPC, IL22, HGDC, FOS, LTB, HPRT1, PPC, NOS2, IL6R, TLR1, NFATC3, TOLLIP, GAPDH, RTC, RTC, FASLG, RTC, C3AR1, ITGB2, B2M, FLT3LG, HDAC4, CCR1, LY96, CCR3, TLR6, C4A, NR3C1, IL1RAP, IL1RN, C3, IL1R1, TNFSF14, MYD88, TLR3, IL10RB, TLR5, CCL17, CRP, CCL16, TLR7, CCL22, CCL24, CCL7, CXCL5, CEBPB, CSF1, CCL5, RIPK2, CCR4, CXCR4, IL1F10, LTA, CCL13, CXCL10, CCL8, CCL2, IL6, BCL6, CD40, CXCL9, CCL19, TNF, IL10, IL18RAP, IL1A, CCR7, IL8, NFKB1, CXCL3, CXCL1, CXCL2, CCL3, CCL4, CXCL6, IL1B, IL23A, TLR2, IFNG, CCL11, IL8RA, KNG1, CD40LG, CCR2, IL8RB, IL23R, IL18 and ACTB respectively. Each column represents one sample with individual treatment. From left to right the samples are cell treated with control siRNA in replicate 1, 2, 3, 4 respectively, cell treated with arrestin 2 siRNA in replicate 1, 2, 3, 4 respectively, cell treated with control siRNA and stimulated with LPS in replicate 1, 2, 3, 4 respectively, cell treated with arrestin 2 siRNA and stimulated with LPS in replicate 1, 2, 3, 4 respectively. Genes are hierarchically clustered according to the similarity of expression profile. Genes are divided into 5 representative pattern groups, denoted as P1, P2, P3, P4 and P5.

Figure 6: PCR-array cluster including IL8 etc.



This cluster image is excised from the full graph generated by SABiosicences web based PCR-array analysis software, Gene expression magnitude are normalized against the average of all genes used in array analysis. Green denotes minimal expression, black denotes average expression and red denotes maximal expression. 1N, 2N, 3N, 4N represent biological replicates 1, 2, 3, and 4. C, A, CL, AL denotes cells treated with control siRNA, cells treated with arrestin 2 siRNA, cell treated with control siRNA and stimulated with LPS and cells treated with arrestin 2 siRNA and stimulated with LPS.

Figure 7: Possible scenario of how arrestin 2 regulate LPS induced gene expression.



1N, 2N, 3N, 4N denote biological replicates 1,2,3,4 respectively. Blue line represents gene e.g. IL8 expression curve after induction in control cells. Red line represents gene e.g. IL8 expression curve after induction in arrestin 2 knock down cells. Arrestin 2 knock down may expand gene expression kinetic curve. Due to the gene expression kinetic curve time span are different in different cell sub-populations, At certain time point e.g. 6hr after LPS induction, arrestin 2's effect on gene mRNA levels may appear opposite. In cell population represented by 1N, 2N, higher mRNA level exists in arrestin 2 deficient cells. But in cell population represented by 3N, 4N, higher mRNA level exists in wild type cells.

Figure 8: Arrestin 2 exhibits different regulation function on TLR4 signaling in different gene groups.

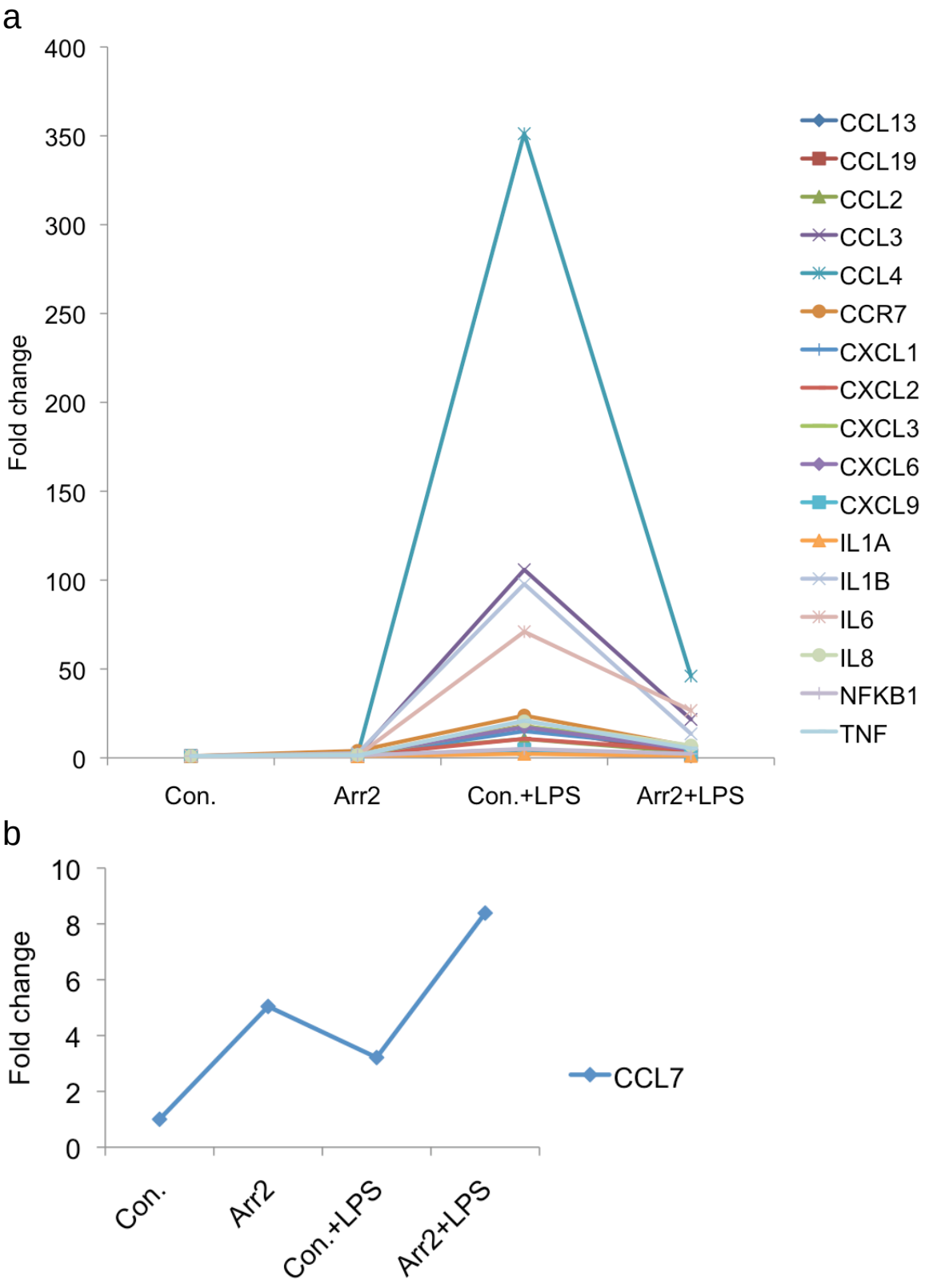
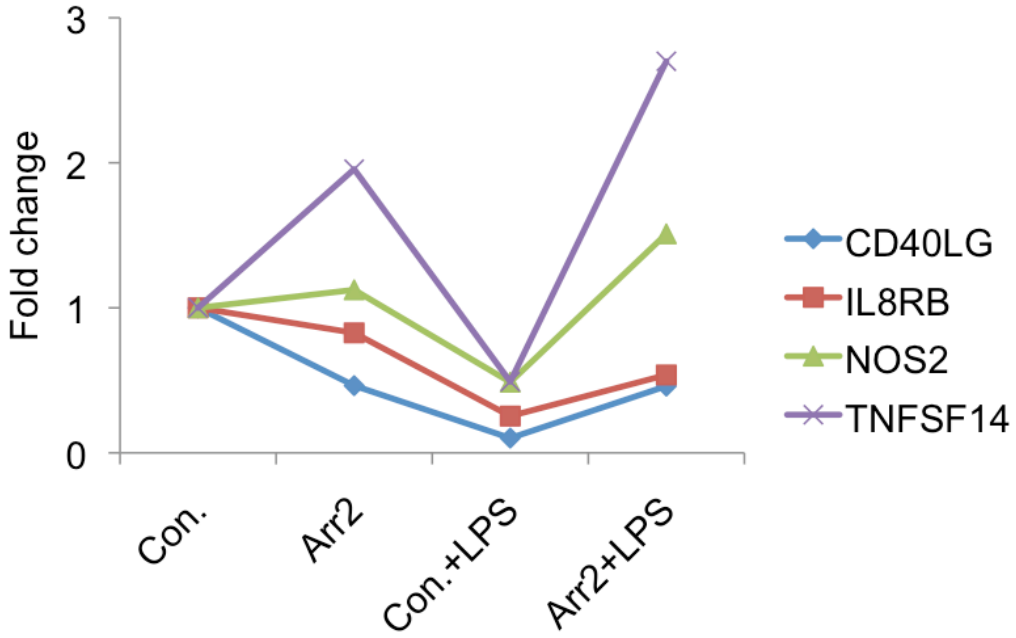


Figure 8 (cont'd).

c



Con., Arr2, Con.+LPS, Arr2+LPS denote cells treated with control siRNA, cells treated with arrestin 2 siRNA, cell treated with control siRNA and stimulated with LPS and cells treated with arrestin 2 siRNA and stimulated with LPS, respectively. Y-axis: fold change represents relative mRNA level (generated by PCR-array) normalized to mRNA level in control cell without LPS stimulation. (a) Genes were highly induced by LPS stimulation but down regulated by arrestin 2 knockdown; (b) Genes were induced by LPS stimulation and further enhanced by arrestin 2 knockdown; (c) Genes were down regulated by LPS stimulation but rescued or enhanced by arrestin 2 knockdown.

APPENDIX B: Tables

Table 1: PCR-ARRAY (Human Inflammatory Response and Autoimmunity) gene table.

Position	Unigene	GeneBank	Symbol	Description
A01	Hs.478588	NM_001706	BCL6	B-cell CLL/lymphoma 6
A02	Hs.529053	NM_000064	C3	Complement component 3
A03	Hs.591148	NM_004054	C3AR1	Complement component 3a receptor 1
A04	Hs.534847	NM_007293	C4A	Complement component 4A (Rodgers blood group)
A05	Hs.54460	NM_002986	CCL11	Chemokine (C-C motif) ligand 11
A06	Hs.414629	NM_005408	CCL13	Chemokine (C-C motif) ligand 13
A07	Hs.10458	NM_004590	CCL16	Chemokine (C-C motif) ligand 16
A08	Hs.546294	NM_002987	CCL17	Chemokine (C-C motif) ligand 17
A09	Hs.50002	NM_006274	CCL19	Chemokine (C-C motif) ligand 19
A10	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2
A11	Hs.57907	NM_002989	CCL21	Chemokine (C-C motif) ligand 21
A12	Hs.534347	NM_002990	CCL22	Chemokine (C-C motif) ligand 22
B01	Hs.169191	NM_005064	CCL23	Chemokine (C-C motif) ligand 23
B02	Hs.247838	NM_002991	CCL24	Chemokine (C-C motif) ligand 24
B03	Hs.514107	NM_002983	CCL3	Chemokine (C-C motif) ligand 3
B04	Hs.75703	NM_002984	CCL4	Chemokine (C-C motif) ligand 4
B05	Hs.514821	NM_002985	CCL5	Chemokine (C-C motif) ligand 5
B06	Hs.251526	NM_006273	CCL7	Chemokine (C-C motif) ligand 7
B07	Hs.271387	NM_005623	CCL8	Chemokine (C-C motif) ligand 8
B08	Hs.301921	NM_001295	CCR1	Chemokine (C-C motif) receptor 1
B09	Hs.511794	NM_001123396	CCR2	Chemokine (C-C motif) receptor 2
B10	Hs.506190	NM_001837	CCR3	Chemokine (C-C motif) receptor 3
B11	Hs.184926	NM_005508	CCR4	Chemokine (C-C motif) receptor 4
B12	Hs.370036	NM_001838	CCR7	Chemokine (C-C motif) receptor 7
C01	Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5
C02	Hs.592244	NM_000074	CD40LG	CD40 ligand
C03	Hs.517106	NM_005194	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
C04	Hs.709456	NM_000567	CRP	C-reactive protein, pentraxin-related

Table 1 (cont'd).

C05	Hs.591402	NM_000757	CSF1	Colony stimulating factor 1 (macrophage)
C06	Hs.789	NM_001511	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
C07	Hs.632586	NM_001565	CXCL10	Chemokine (C-X-C motif) ligand 10
C08	Hs.590921	NM_002089	CXCL2	Chemokine (C-X-C motif) ligand 2
C09	Hs.89690	NM_002090	CXCL3	Chemokine (C-X-C motif) ligand 3
C10	Hs.89714	NM_002994	CXCL5	Chemokine (C-X-C motif) ligand 5
C11	Hs.164021	NM_002993	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
C12	Hs.77367	NM_002416	CXCL9	Chemokine (C-X-C motif) ligand 9
D01	Hs.593413	NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4
D02	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)
D03	Hs.428	NM_001459	FLT3LG	Fms-related tyrosine kinase 3 ligand
D04	Hs.728789	NM_005252	FOS	FBJ murine osteosarcoma viral oncogene homolog
D05	Hs.20516	NM_006037	HDAC4	Histone deacetylase 4
D06	Hs.856	NM_000619	IFNG	Interferon, gamma
D07	Hs.193717	NM_000572	IL10	Interleukin 10
D08	Hs.654593	NM_000628	IL10RB	Interleukin 10 receptor, beta
D09	Hs.83077	NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)
D10	Hs.158315	NM_003853	IL18RAP	Interleukin 18 receptor accessory protein
D11	Hs.1722	NM_000575	IL1A	Interleukin 1, alpha
D12	Hs.126256	NM_000576	IL1B	Interleukin 1, beta
E01	Hs.306974	NM_173161	IL1F10	Interleukin 1 family, member 10 (theta)
E02	Hs.701982	NM_000877	IL1R1	Interleukin 1 receptor, type I
E03	Hs.478673	NM_002182	IL1RAP	Interleukin 1 receptor accessory protein
E04	Hs.81134	NM_000577	IL1RN	Interleukin 1 receptor antagonist
E05	Hs.287369	NM_020525	IL22	Interleukin 22
E06	Hs.126891	NM_052962	IL22RA2	Interleukin 22 receptor, alpha 2
E07	Hs.98309	NM_016584	IL23A	Interleukin 23, alpha subunit p19
E08	Hs.677426	NM_144701	IL23R	Interleukin 23 receptor
E09	Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)

Table 1 (cont'd).

E10	Hs.709210	NM_000565	IL6R	Interleukin 6 receptor
E11	Hs.624	NM_000584	IL8	Interleukin 8
E12	Hs.194778	NM_000634	CXCR1	Chemokine (C-X-C motif) receptor 1
F01	Hs.846	NM_001557	CXCR2	Chemokine (C-X-C motif) receptor 2
F02	Hs.960	NM_000590	IL9	Interleukin 9
F03	Hs.375957	NM_000211	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
F04	Hs.77741	NM_000893	KNG1	Kininogen 1
F05	Hs.36	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
F06	Hs.376208	NM_002341	LTB	Lymphotoxin beta (TNF superfamily, member 3)
F07	Hs.660766	NM_015364	LY96	Lymphocyte antigen 96
F08	Hs.82116	NM_002468	MYD88	Myeloid differentiation primary response gene (88)
F09	Hs.632209	NM_004555	NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3
F10	Hs.654408	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
F11	Hs.709191	NM_000625	NOS2	Nitric oxide synthase 2, inducible
F12	Hs.122926	NM_000176	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
G01	Hs.103755	NM_003821	RIPK2	Receptor-interacting serine-threonine kinase 2
G02	Hs.537126	NM_001039661	TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
G03	Hs.654532	NM_003263	TLR1	Toll-like receptor 1
G04	Hs.519033	NM_003264	TLR2	Toll-like receptor 2
G05	Hs.657724	NM_003265	TLR3	Toll-like receptor 3
G06	Hs.174312	NM_138554	TLR4	Toll-like receptor 4
G07	Hs.604542	NM_003268	TLR5	Toll-like receptor 5
G08	Hs.662185	NM_006068	TLR6	Toll-like receptor 6
G09	Hs.659215	NM_016562	TLR7	Toll-like receptor 7
G10	Hs.241570	NM_000594	TNF	Tumor necrosis factor
G11	Hs.129708	NM_003807	TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14
G12	Hs.368527	NM_019009	TOLLIP	Toll interacting protein

Table 1 (cont'd).

H01	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H02	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H03	Hs.728776	NM_012423	RPL13A	Ribosomal protein L13a
H04	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H05	Hs.520640	NM_001101	ACTB	Actin, beta
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

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