Transcriptional regulation of type I interferon responses of myeloid antigen presenting cells

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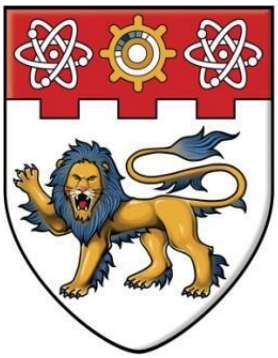

NANYANG TECHNOLOGICAL UNIVERSITY

TRANSCRIPTIONAL REGULATION OF TYPE I INTERFERON RESPONSES OF MYELOID ANTIGEN PRESENTING CELLS

SIN WEI XIANG

SCHOOL OF BIOLOGICAL SCIENCES

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A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of Doctor of Philosophy

2016

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ABBREVIATIONS

AIM-2,	absent in melanoma-2
ANOVA,	analysis of variance
APC,	Ag-presenting cell
ATP,	adenosine triphosphate
bp,	base pair (only with numbers)
BMDC,	bone marrow-derived dendritic cell
BMDM,	bone marrow-derived macrophage
BSA,	bovine serum albumin
CaMKII,	calmodulin kinase II
CARD,	caspase activation and recruitment domain
CCL,	CC chemokine ligand
CCR,	CC chemokine receptor
cDC,	conventional dendritic cell
cDNA,	complementary DNA
C/EBP,	CCAAT/enhancer-binding protein
CLP,	cecal ligation and puncture
CLR,	C-type lectin receptor
CNS,	central nervous system
CpG,	cytosine guanine dinucleotide
CREB,	cAMP response element binding protein
CSF,	colony-stimulating factor
CXCL,	CXC chemokine ligand
DAMP,	damage-associated molecular pattern
DC,	dendritic cell
DMEM,	Dulbecco's modified Eagle's medium
DMSO,	dimethylsulfoxide
DNA,	deoxyribonucleic acid
ds,	double-stranded (as dsDNA)
DSS,	dextran sodium sulfate
EAE,	experimental autoimmune encephalomyelitis
EBI3,	Epstein-Barr virus-induced gene 3
ECL,	enhanced chemiluminescence

EDTA,	ethylenediaminetetraacetic acid
ELISA,	enzyme-linked immunosorbent assay
ERK,	extracellular signal-regulated kinase
FBS,	fetal bovine serum
g,	gram (only with numbers)
GAPDH,	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF,	granulocyte-macrophage CSF
gp,	glycoprotein (e.g., gp100)
h,	hour (only with numbers)
HEPES,	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP,	horseradish peroxidase
IFN,	interferon (e.g., IFN-γ)
IKK,	I-κB kinase
IL,	interleukin (e.g., IL-2)
iNOS,	inducible nitric oxide synthase
i.p.,	intraperitoneal
IRAK,	IL-1 receptor-associated kinase
IRF,	interferon regulatory factor
ISG,	interferon-stimulated gene
ITAM,	immunoreceptor tyrosine-based activation motif
i.v.,	intravenous
JAK or Jak,	Janus kinase
JNK,	c-Jun N-terminal kinase
kb,	kilobase (only with numbers)
kDa,	kilodalton (only with numbers)
LBP,	lipopolysaccharide binding protein
LPA,	lipid A
LPS,	lipopolysaccharide
LRR,	leucine rich repeat
2-ME,	2-mercaptoethanol
Mal/TIRAP,	MyD88 adaptor-like/TIR domain-containing adaptor protein
MAPK,	mitogen-activated protein kinase
M-CSF,	macrophage CSF
MD2,	myeloid differentiation factor 2

mDC,	myeloid dendritic cell
mg,	milligram (only with numbers)
MHC,	major histocompatibility complex
min,	minute (only with numbers)
ml,	milliliter (only with numbers)
mRNA,	messenger RNA
μg,	microgram (only with numbers)
μl,	microliter (only with numbers)
MyD88,	myeloid differentiating factor 88
n,	number in study or group
NBD,	nucleotide-binding domain
ND,	not determined
NF-κB,	nuclear factor κB
NK cell,	natural killer cell
NLRP,	NBD, LRR-containing family, pyrin domain-containing
NO,	nitric oxide
NOD,	nucleotide-binding oligomerization domain
PAGE,	polyacrylamide gel electrophoresis
PAMP,	pathogen-associated molecular pattern
PBS,	phosphate-buffered saline
PCR,	polymerase chain reaction
pDC,	plasmacytoid dendritic cell
PI3K,	phosphatidylinositol 3-kinase
PRR,	pattern recognition receptor
Pyk2,	proline-rich tyrosine kinase 2
r,	recombinant, (e.g., rIFN-γ)
R,	receptor (e.g., IL-2R)
RANTES,	regulated upon activation, normal T cell expressed and secreted
RIG-I,	retinoic acid-inducible gene-I
RNA,	ribonucleic acid
ROS,	reactive oxygen species
rpm,	revolutions per minute
RT-PCR,	reverse transcriptase polymerase chain reaction
SARM,	sterile α- and armadillo-motif-containing protein

SD,	standard deviation
SDS,	sodium dodecyl sulfate
SEM,	standard error of the mean
SOCS,	suppressor of cytokine signaling
ss,	single-stranded (e.g., ssDNA)
STAT,	signal transducer and activator of transcription
Syk,	spleen tyrosine kinase
TBK,	TANK-binding kinase
TBS,	Tris-buffered saline
TBST,	TBS with Tween 20
TGF,	transforming growth factor
Th cell,	T helper cell
TIR,	Toll-IL-1 receptor
TLR,	Toll-like receptor
TNBS,	2,4,6-trinitrobenzene sulfonic acid
TNF,	tumor necrosis factor
TRAF,	TNF receptor-associated factor
TRAM,	TRIF-related adaptor molecule
TRIF,	TIR domain-containing adapter inducing interferon-β
Tris,	tris(hydroxymethyl)aminomethane
U,	unit (only with numbers)

ABSTRACT

Macrophages, as crucial mediators of an innate immune response, exhibit functional plasticity by playing an essential role in both the initiation and resolution of inflammation. Production of the pro-inflammatory cytokine IL-1β, via caspase-11 activation during Gram-negative bacterial infections, and of the pro-regulatory cytokine IL-10, are dependent on signaling by the pleiotropic type I interferon (IFN), IFN-β. The transcription factor IRF7 is thought to be primarily involved in the regulation of type I IFN responses in viral infections. Here we show IRF7 also regulates IFN-β responses to bacterial lipopolysaccharide in bone marrow-derived macrophages (BMDMs) but not in bone marrow-derived dendritic cells (BMDCs). In BMDMs, IRF7 co-operated with IRF3 to elicit robust IFN-β responses to endotoxin exposure, whereas BMDCs depended on IRF3 alone to mediate this response and thus displayed blunted IFN-β expression. IRF7-mediated IFN-β production is necessary for efficient expression of pro-caspase-11 in BMDMs. Accordingly, Irf7$^{-/-}$ mice exhibited substantially reduced serum levels of type I IFN and IL-1β, and were resistant to lethal endotoxin shock. We found that, unlike BMDCs, BMDMs constitutively expressed IRF7 protein. The high basal IRF7 expression in steady-state BMDMs was maintained by constitutive IFN-β signaling, which was in turn dependent on tonic signaling by IL-27p28. Accordingly, in response to TLR4 ligation, BMDMs but not BMDCs depended on IL-27p28 to induce IFN-β synthesis. IL-27p28-mediated IFN-β production, and not IL-27 cytokine itself, is required to restrain inflammatory responses to endotoxin exposure, since BMDMs deficient in IL-27p28 displayed reduced IL-10 synthesis and impaired STAT3-mediated anti-inflammatory responses, which were reversed by addition of exogenous IFN-β. Our data identified a tonic IL-27p28-IFN-β signaling axis as a novel cell type-specific regulator of TLR4-mediated IFN-β induction through the regulation of constitutively expressed IRF7.

1 INTRODUCTION

1.1 Macrophages and dendritic cells in innate immunity

The innate immune response acts as the body's first line of defense against microbial pathogen infections, and subsequently shapes the adaptive immune response. Cells of the innate immune system, such as macrophages and dendritic cells (DCs), function as sentinel cells by sensing invading microbial pathogens and mounting anti-microbial responses. The coordinated and regulated expression of a plethora of cytokines, chemokines, interferons (IFNs), IFN-stimulated genes (ISGs), as well as co-stimulatory molecules by these cells orchestrates the host innate immunity, and further shapes the subsequent development of cellular and humoral adaptive immunity [1].

Macrophages and DCs are cells of the mononuclear phagocyte system (MPS) derived from myeloid progenitors in the bone marrow, spleen or fetal liver [2]. During inflammation, circulating monocytes can also differentiate into monocyte-derived macrophages and DCs, such as inflammatory macrophages and tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS)-producing DCs (TipDCs) [3, 4]. Macrophages were discovered in 1882 by Élie Metchnikoff. They are defined as F4/80^{hi} CD11b^{+} cells in mice, and traditionally recognized as scavenger cells with effector killing functions, proficient in the phagocytosis of pathogens, apoptotic cells and cellular debris, as well as in the killing of microbes, infected cells and tumor cells [5]. Conventional DCs (cDCs) were first described in 1973 by Steinman and Cohn. They are defined as CD11c^{hi} MHC class II^{+} cells in mice, and typically recognized as professional antigen presenting cells (APCs), well versed in antigen uptake, processing, presentation, and activation of the T cell response [5]. Macrophages and DCs display divergent responses to microbial stimuli and differentially contribute to the ensuing immune response [5]. Upon recognition of pathogenic stimuli, macrophages first initiate an inflammatory response, by producing high levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12 and IL-23, as well as NO and ROS, which lead to efficient phagocytosis, effector killing functions and Th1 responses, as well as chemokines, such as CCL5, CXCL9 and CXCL10, which recruit CD8^{+} T cells and NK cells for anti-bacteria and anti-tumor immunity. Following this initial inflammatory response, macrophages remain in the inflamed tissue and can switch to an anti-

inflammatory phenotype with pro-regulatory and recovery functions, by producing large amounts of IL-10, which dampens inflammatory responses and protects against LPS toxicity. This anti-inflammatory response results in wound healing, tissue repair, resolution of inflammation, and restoration of tissue homeostasis [5]. On the other hand, DCs also produce cytokines, such as type I IFNs, IL-12, IL-15 and IL-18, which can prime NK cell responses, such as secretion of IFN-γ, which in turn promotes T cell activation as well as NK cell cytotoxicity. For example, DC-derived IL-12 drives Th1 responses, which play a key role in tissue immunity. However, in contrast to macrophages, antigen presentation capacity is relatively higher in DCs: after antigen recognition and uptake, DCs migrate to draining lymph nodes, and up-regulate the expression of MHC and co-stimulatory molecules to efficiently present antigens to T cells to trigger the adaptive immune response, after which they die by apoptosis [5].

Macrophages and DCs express a wide repertoire of germline-encoded pattern recognition receptors (PRRs) to sense evolutionarily-conserved microbial components, known as pathogen-associated molecular patterns (PAMPs), as well as host-derived danger molecules, known as damage-associated molecular patterns (DAMPs). These PRRs include cell surface and endosomal Toll-like receptors (TLRs), and intracellular cytosolic PRRs, such as retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and absent in melanoma (AIM)-2-like receptors (ALRs), as well as other classes of PRRs, such as C-type lectin receptors (CLRs) [6] (**Figure 1.1**). In general, CLRs, such as dectin-1 and dectin-2, sense β-glucans from fungi to mediate antifungal immunity [6]; RLRs, such as RIG-I / MDA5 and LGP2, are RNA helicases that sense viral double-stranded RNA (dsRNA) and 5'-triphosphated single-stranded RNA (5'ppp-ssRNA) in the host cell cytosol to mediate mainly antiviral immunity [7]; NLRs, such as NOD1 and NOD2, are cytosolic molecules that sense peptidoglycan (PGN) and muramyl dipeptide (MDP) from intracellular bacteria to mediate mainly antibacterial immunity [8]; and AIM2 and the recently identified cyclic GMP-AMP synthase (cGAS) sense the presence of double-stranded DNA (dsDNA) in the cytosol [9]. Many PRRs are commonly expressed on both macrophages and DCs, but they can lead to different responses and functions in these two cell types. For example, following dectin-1-mediated recognition of zymosan, DCs secrete pro- and anti-inflammatory cytokines, such as IL-2 and IL-10, but dectin-1 stimulation alone does not result in cytokine production in macrophages [5].

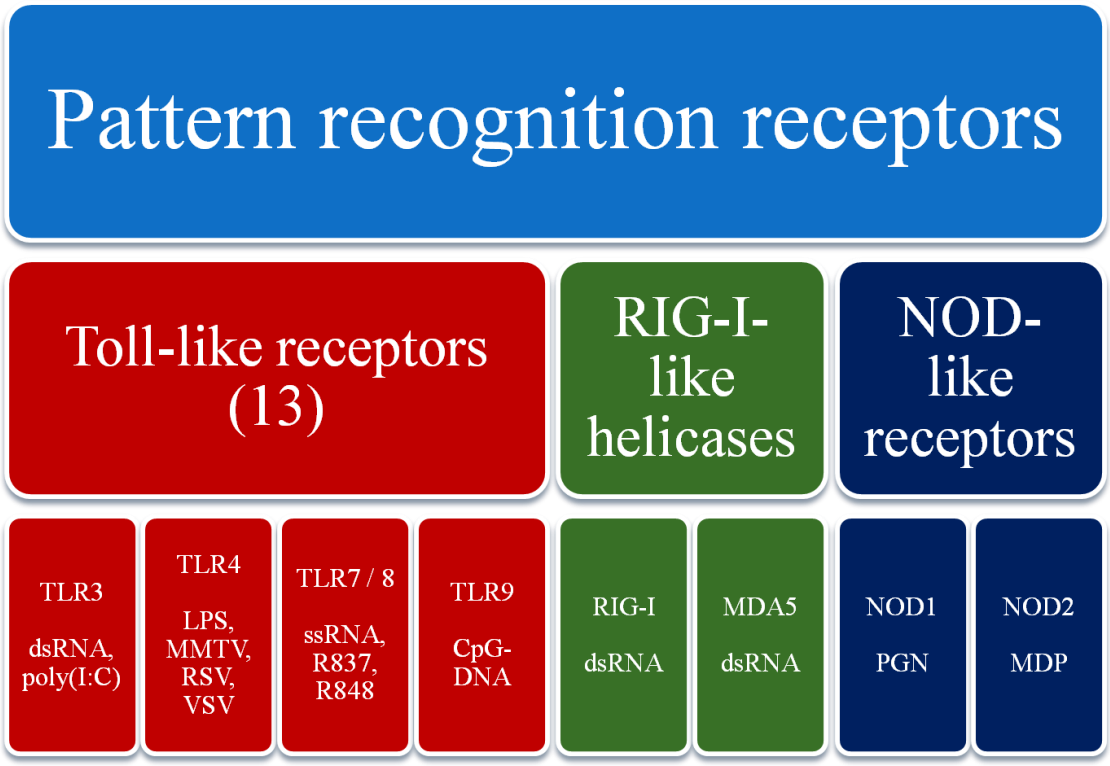

Figure 1.1: Pattern recognition receptors that lead to type I IFN induction.

1.2 Toll-like receptors in innate immunity

Among the classes of PRRs, the TLRs are the most extensively studied to date. TLR research began with the discovery in 1996 by Jules Hoffmann and colleagues of the antifungal response mediated by the Toll protein in *Drosophila melanogaster*, originally found to be involved in fruit fly development [10]. As it turned out, it was homologous to a previously identified human protein, which was later named TLR1 [11]. To date, at least 13 mammalian members of the TLR family have been reported [12]. Monocytes/macrophages and myeloid DCs (mDCs) express most TLRs [13], whereas plasmacytoid DCs (pDCs) exclusively express TLR7 and TLR9 [14]. TLRs mediate the recognition of PAMPs that trigger APC production of immunomodulatory cytokines via intracellular signal transduction cascades through one or more Toll-IL-1 receptor (TIR) adaptor proteins: TIR domain-containing adapter inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), myeloid differentiation factor 88 (MyD88), and MyD88 adaptor-like/TIR domain-containing adaptor protein

(Mal/TIRAP) [15]. MyD88 is utilized by all TLRs except TLR3, whereas TRIF is utilized by TLR3 and TLR4 [15].

TLR1-TLR2, TLR2-TLR6, and TLR5 are cell surface TLRs that recognize triacyl lipopeptides, diacyl lipopeptides, or flagellin, respectively, and use the MyD88 adaptor to activate NF-κB and induce pro-inflammatory cytokines [13]. TLR3, TLR7/8, and TLR9 are endosomal TLRs that recognize viral dsRNA and the mimetic poly(I:C), single-stranded RNA (ssRNA) and the mimetic resiquimod (R848), or un-methylated CpG DNA, respectively [13]. TLR3 ligation leads to TRIF-dependent induction of pro-inflammatory cytokines and type I IFNs in DCs and macrophages. TLR7/8 and TLR9 ligation results in MyD88-dependent activation of IRF7 and induction of type I IFNs in pDCs, or MyD88-dependent activation of NF-κB and induction of pro-inflammatory cytokines in macrophages and cDCs [13]. TLR9 ligation also results in MyD88-dependent activation of IRF1 and induction of IFN-β, iNOS, and IL-12p35 in mDCs [16]. TLR2 is expressed in the endosomes of inflammatory monocytes and induce type I IFNs in response to viral infection via MyD88-mediated activation of IRF3 and IRF7 [17]. Thus, it is pertinent to note that, among the TLR family, TLR2, TLR3, TLR4 (see below), TLR7/8, and TLR9 activation culminate in the induction of type I IFNs from endosomal compartments.

1.3 TLR4 recognition of bacterial lipopolysaccharide

The second human TLR, later named TLR4, was discovered in 1997, and, one year later, Bruce Beutler and colleagues identified TLR4 as the signaling receptor for bacterial endotoxin [18, 19]. TLR4 is expressed on macrophages, cDCs, neutrophils, eosinophils and mast cells, and it senses the lipid A (LPA) moiety of lipopolysaccharide (LPS), a key component of the Gram-negative bacterial cell wall which is responsible for many of the pathological effects of microbial infection, although it also recognizes some viral proteins from vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV), and mouse mammary tumor virus (MMTV), as well as mannan from *Candida albicans*, and high mobility group box 1 (HMGB1) from damaged host cells, among other ligands [20-23]. “Wild-type” or “smooth” LPS, is composed of three covalently linked-moieties: a core oligosaccharide, LPA, and O-antigen, while “rough” LPS does not possess the O-antigen [24]. TLR4, which lacks direct LPS-binding activity,

recognizes LPS in conjunction with CD14 and myeloid differentiation factor 2 (MD2) co-receptors, together with a plasma protein termed LPS-binding protein (LBP), which mediates ligand delivery by presenting LPS in serum to CD14 on the surface of myeloid cells, which in turn captures and transports LPS to be sensed by the TLR4-MD2 receptor complex [25].

TLR4 is unique among TLR family members in that it is the only receptor to transduce signals via two distinct intracellular pathways, i.e. both the MyD88- and TRIF-dependent pathways, and to utilize all of the known adaptor proteins MyD88, Mal/TIRAP, TRIF, and TRAM. Initial binding of LPS to TLR4 at the plasma membrane recruits the adaptor proteins Mal/TIRAP and MyD88, and leads to the formation of the Myddosome signaling platform comprising MyD88 and IL-1 receptor-associated kinase 4 (IRAK4) [26], which induces the early-phase activation of nuclear factor κB (NF-κB), and also the activation of MAP kinases (MAPKs), such as c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), and p38 kinase, resulting in pro-inflammatory cytokine expression. Subsequent internalization and trafficking of TLR4 into endosomal compartments then initiates a second signaling cascade mediated by the adaptor proteins TRAM and TRIF, which activates TANK-binding kinase 1 (TBK1), I-κB kinase (IKK)-ε, and interferon regulatory factor (IRF)-3, and promotes the type I IFN expression [13, 14, 27-32]. TRAM-TRIF signaling also mediates the late-phase activation of NF-κB, exemplifying a certain degree of crosstalk between the two pathways, which cooperate to elicit maximal inflammatory cytokine expression in macrophages. While both MyD88- and TRIF-dependent pathways are required for sustained activation of NF-κB and pro-inflammatory cytokine production in bone marrow-derived macrophages (BMDMs) [33], bone marrow-derived DC (BMDC) production of pro-inflammatory cytokines is independent of TRIF [24], thus exemplifying one aspect of differential LPS signaling between macrophages and cDCs.

Endocytosis of TLR4 from the plasma membrane into endosomes following LPS recognition in macrophages and DCs is mediated by CD14, by immunoreceptor tyrosine-based activation motif (ITAM) signaling via spleen tyrosine kinase (Syk), by phospholipase C γ2 (PLCγ2)-inositol 1,4,5-trisphosphate (IP3)-Ca^{2+} signaling, and by the p110δ isoform of phosphatidylinositol-3-OH kinase (PI3K) [28, 29, 34]. TLR4 endocytosis is required for IRF3 phosphorylation and IFN-β expression [32].

Accordingly, CD14 is required for IRF3 dimerization and IFN-β production in LPS-stimulated macrophages and DCs [28], and PLCγ2 and p110δ are separately shown to be required for IRF3 phosphorylation and IFN-β expression in LPS-challenged BMDMs and BMDCs, respectively [29, 34]. Thus, the current paradigm suggests that PRRs, including TLR4, induce type I IFNs exclusively from intracellular compartments instead of from the plasma membrane. In the case of endosomal TLR7/8 and TLR9, MyD88-dependent IRF7 activation leads to type I IFN expression in pDCs [35, 36]. In the case of endosomal TLR3 and TLR4, TRIF-dependent IRF3 activation results in type I IFN expression in macrophages and mDCs [37, 38]. This has been attributed to the cytosolic localization of TNF receptor-associated factor 3 (TRAF3), which is one of the key determinants for type I IFN induction by intracellular PRRs [39, 40]. Interestingly, the ability of CD14 to activate Ca^{2+}/calcineurin and NFAT pathways in BMDCs but not in BMDMs has been suggested to account for DC terminal differentiation and apoptotic death [24], thus exemplifying another aspect of cell type-specific signaling.

Endotoxin from Gram-negative bacteria is one of the causative agents of sepsis, which is defined as a systemic inflammatory response to a severe microbial infection associated with multiple organ dysfunction, and is a major cause of morbidity and mortality in hospital intensive care units worldwide [41]. Although the molecular mechanisms responsible for the pathogenesis of sepsis are complex and still incompletely understood, APCs, such as macrophages and DCs, and TLRs are believed to play a key role in the dysregulated innate immune response to microbial infection during sepsis [42]. Macrophage and DC responses are tightly regulated to rapidly and effectively mount antimicrobial defenses to pathogenic invasion but at the same time avoid immunopathologies associated with excessive inflammation, through feedforward mechanisms, such as those induced by IFN-γ and mediated by STAT1 to promote macrophage activation, and feedback mechanisms, such as those induced by IL-10 and mediated by STAT3 to prevent excessive macrophage activation [43]. TLR responses are tightly regulated to activate sufficient antimicrobial immunity while at the same time minimizing bystander immunopathology, by many mechanisms that fine-tune the magnitude and duration of the inflammatory response [1]. For example, TLR4-induced gene transcription can be modulated by positive regulators, such as the transcription factor FoxO1 [44], as well as by negative regulators, including soluble factors such as IL-10, signaling inhibitors such as A20, and transcriptional repressors such as activating

transcription factor 3 (ATF3) [43, 45]. The dysregulation of these positive and negative regulatory networks can contribute to the development of sepsis, characterized by a hyperinflammatory phase followed by an immunosuppressive phase [41]. Animal models of sepsis, such as that of intraperitoneal (i.p.) or intravenous (i.v.) LPS-induced endotoxemia, and cecal ligation and puncture (CLP)-induced peritonitis, recapitulate some of the features of bacterial peritonitis and septic shock in humans, and are used to investigate the mechanisms of the LPS response. Mice deficient in proteins involved in LPS recognition, i.e. TLR4, CD14, and MD2 [19, 46]; signaling proteins including MyD88 [47], Mal/TIRAP [48, 49], TRIF [50], and TRAM [51]; cytokines such as TNF-α [52]; and, importantly, IFN-β [53], type I IFN receptor IFNAR1 [54], and type I IFN signaling molecules Tyk2 [53], and STAT1 [53, 55], are resistant to the lethal effects of LPS, indicating the importance of type I IFN signaling, in addition to pro-inflammatory cytokine production, in septic shock mortality [56].

1.4 Type I interferons

Interferons (IFNs) are glycoproteins first discovered more than 50 years ago to "interfere" with influenza virus replication in chick chorio-allantoic membranes [57]. Since then, about 10 mammalian members of the IFN family have been identified, of which seven are found in humans [58]. They are classified into type I IFNs (IFN-α, -β, -ω, -ε and -κ), type II IFNs (IFN-γ), and type III IFNs (IFN-λ, also known as IL-28/29) (**Figure 1.2**) [59]. Since the initial discovery of their antiviral activity, IFNs have been demonstrated to be multi-functional cytokines exerting a wide range of biological activities in the human immune system, including anti-angiogenic and anti-proliferative (hence anti-tumor), and anti-inflammatory properties, as well as directly influencing the differentiation and maturation of certain leukocytes [60-65] (**Figure 1.3**). These have permitted their clinical use as therapeutics in viral infections (e.g. IFN-α2 for treatment of Hepatitis C Virus infections), in oncology (e.g. IFN-α2 for treatment of hairy cell leukemia), and in auto-immune diseases (e.g. IFN-β for treatment of multiple sclerosis) [66]. In addition, recent studies have announced unprecedented efficacy of DC-based vaccines in the presence of type I IFN, which may accentuate DC activation [63, 67, 68]. As a result, type I IFN is now recognized as a vaccine adjuvant, and it may even be possible to predict vaccine efficacy by assessing the levels of type I IFN induced [69].

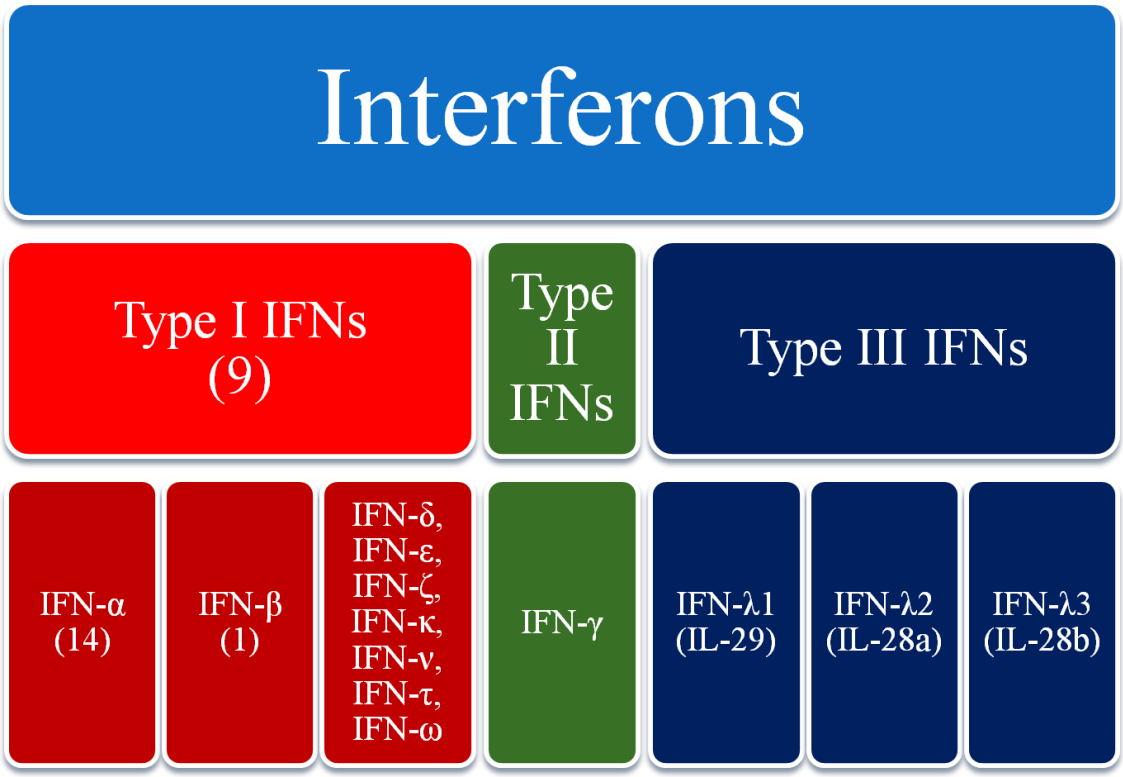

Figure 1.2: Members of the interferon family that are found in mammals.

1.5 Induction of type I interferons by viruses

Type I IFNs (IFN-α and IFN-β) are expressed by almost all cell types in response to diverse pathogens [70, 71], and are thought to play a key role in the restraint of host inflammatory responses under homeostatic conditions [72]. Type I IFNs have long been known to be expressed ubiquitously by many cell types, including immune cells, such as macrophages and DCs and non-immune cells, such as fibroblasts), in response to viral infections [70]. This occurs following the recognition of viral PAMPs by PRRs, including cell surface and endosomal TLRs as well as cytosolic PRRs. In pDCs, the prototypical "IFN-producing cells" (IPCs), the MyD88-IRF7 pathway mediates the induction of high levels of IFN-α and IFN-β following activation of TLR9 by CpG-A DNA [35, 36]. However, in cDCs, IFN-β induction following recognition of CpG-B DNA by TLR9 is mediated by the MyD88-IRF1 pathway [73, 74]. This once again exemplifies cell type-specific signaling pathways leading to type I IFN induction.

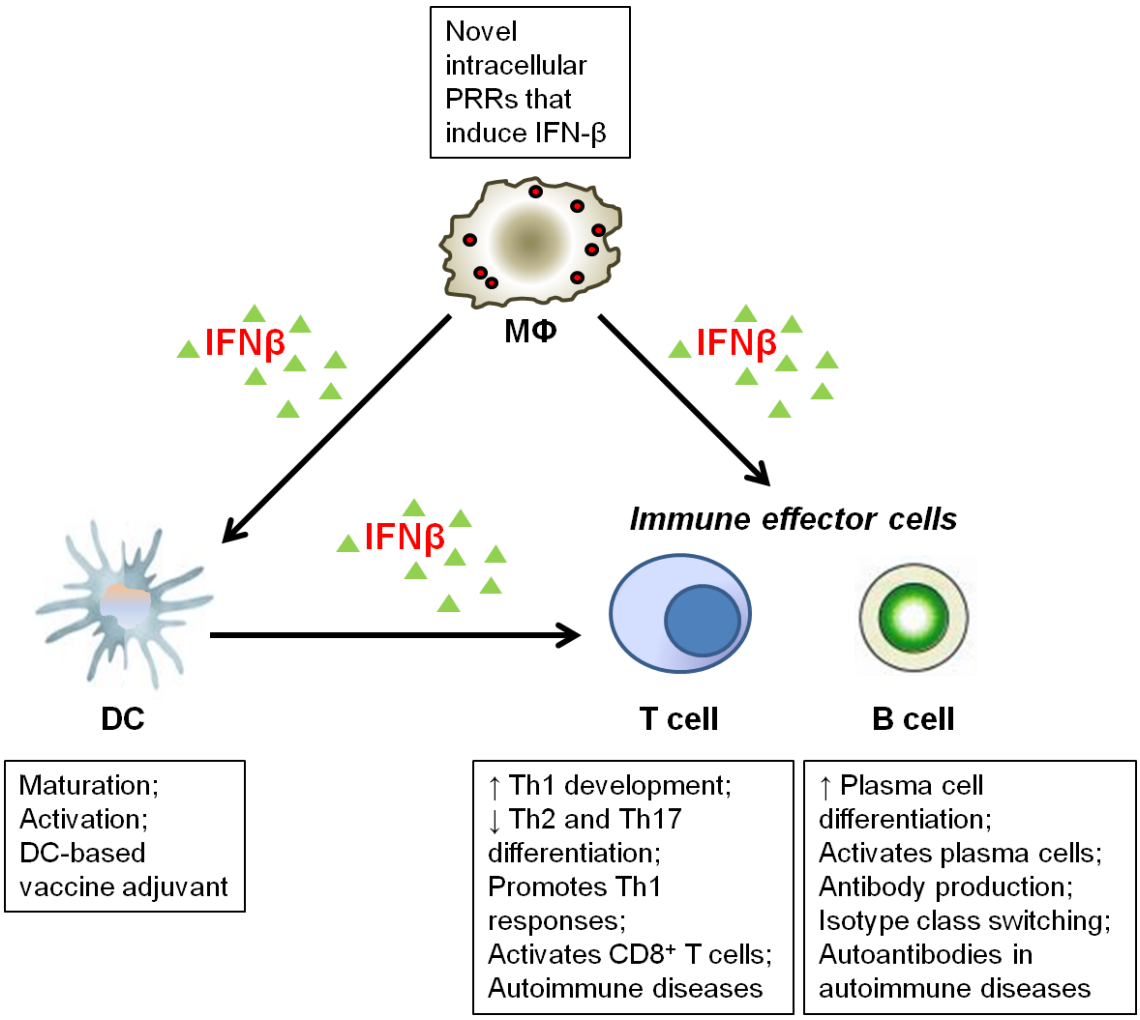

Figure 1.3: Actions of IFN-β on different immune cells.

In fibroblasts and DCs, the "classical pathway" involves the sensing of viral infection by the cytosolic PRRs RIG-I and MDA-5. Host cell sensing of viruses stimulates phosphorylation of IRF3 and IRF7, resulting in the formation of complexes that translocate to the nucleus and elicit the early phase production of primary response genes and rapid, low-level secretion of IFN-β. Subsequent binding of IFN-β to the type I IFN receptor (IFNAR) then triggers downstream Janus kinase (Jak)-signal transducer and activator of transcription (STAT) signaling pathways, and promotes up-regulation of IRF7 synthesis and amplification of the IFN-β response, culminating in the later phase production of high levels of type I IFNs and numerous secondary response genes [16, 75-78]. This constitutes a type I IFN-dependent, IRF7-mediated autocrine/paracrine positive feedback loop, which amplifies the expression of type I IFNs and IFN-stimulated genes (ISGs), including antiviral genes that confer an antiviral state [70]. In

pDCs and cDCs, virus-induced IFN-β responses exhibits biphasic kinetics with two distinct peaks, and the "second phase" of IFN-β transcription depends on IRF8 [79]. Through this IFN-β autocrine/paracrine positive feedback loop, an optimal level of IFN-β induction in DCs is shown to be functionally important for the expression of downstream cytokine genes, such as IL-12p70 [80] and TNF-α [81], as well as for the expression of co-stimulatory molecules, such as CD40 and CD86 [81, 82], which play a role in bridging innate and adaptive immunity. In contrast, the mechanisms that regulate macrophage and DC production of type I IFNs (e.g. whether an autocrine/paracrine type I IFN positive feedback loop is operational) in the context of endotoxin challenge are poorly defined, despite a likely critical role for these responses in regulating inflammatory responses to bacterial infection. It has been proposed that a constitutive low level of IFN-α/β expression and weak IFN-α/β signaling in uninfected cells (e.g. in splenocytes) contributes to the massive and effective type I IFN response upon encounter with viral infection [83, 84]. Autocrine priming of anti-viral immunity via the constitutive release of small quantities of type I IFNs has been well documented in non-immune cells such as fibroblasts [83, 85]. In contrast, constitutive type I IFN production by myeloid antigen presenting cells (mAPCs) remains a poorly characterized phenomenon with unknown molecular basis.

1.6 Induction of type I interferons by bacteria

Apart from viral infections, it is now known that bacteria (e.g. *Escherichia coli*) and bacterial components (e.g. LPS) can also induce type I IFN production [71]. Type I IFNs can mediate host protection against bacteria, but chronic activation of type I IFN signaling can also drive pathological inflammation in disorders such as sepsis [70, 71]. One of the most well-characterized mechanisms that occurs in many cell types is the LPS-induced type I IFN production via TLR4 [86]. IFN-β is the primary type I IFN that is induced in macrophages following LPS stimulation [87]. In macrophages, LPS stimulation of TLR4 induces IFN-β and downstream ISGs predominantly via the TRIF-dependent (MyD88-independent) pathway, which originates from endosomal vesicles after receptor endocytosis, through the activation of IRF3 phosphorylation and nuclear translocation [28, 37, 50, 88]. IFN-β autocrine/paracrine signaling also exists in LPS-stimulated macrophages to induce downstream secondary response genes [37]. For instance, neutralizing antibodies against IFN-β (but not IFN-α) reduced LPS-induced

STAT1 tyrosine phosphorylation in primary murine macrophages [89], while cytokine gene expression of e.g. IL-12p40, iNOS and IP-10/CXCL10, as well as cytokine secretion of e.g. IL-12p70 and IP-10/CXCL10, were suppressed in IFN-$\beta^{-/-}$ murine peritoneal macrophages [90]. However, IFN-$\beta^{-/-}$ mice/cells do not allow us to conclude whether the induction of IFN-β itself is directly dependent on type I IFN signaling via the IFN-α/β receptor (IFNAR). Upon stimulation with lipid A/LPS, BMDMs deficient in IFNAR exhibit comparable levels of IFN-β expression to wild-type BMDMs [91], but Tyk2-deficient peritoneal macrophages exhibit reduced transcription of IFN-β mRNA [53]. These data suggest that adaptor molecule Tyk2 acts downstream of IFNAR in support of the IFN-β response, but since Tyk2 signal transduction occurs downstream of multiple different cytokine receptors [92, 93], the mechanism and potential significance of IFN-β autocrine/paracrine amplification in myeloid antigen presenting cells (mAPCs) remains controversial.

Apart from promoting the production of pro-inflammatory cytokines and chemokines, such as IL-12p70, iNOS and IP-10/CXCL0, the anti-inflammatory actions of IFN-β are increasingly recognized. For instance, type I IFN autocrine/paracrine signaling is involved in the induction of SOCS-1 and SOCS-2 by LPS-stimulated macrophages and DCs [94, 95]. The SOCS family of proteins negatively regulate JAK-STAT signaling, e.g. by blocking STAT1 activation. IFN-β-induced SOCS1 reduces MHC class II and CD40 expression, and thereby inhibits antigen presentation and T cell activation [96]. One of the most compelling examples of the anti-inflammatory effects of IFN-β is its clinical use in the treatment of relapsing-remitting multiple sclerosis (MS) [96]. The mechanisms of action of IFN-β therapy for relapsing-remitting MS are under continuing investigation, but have been suggested to include suppression of T cell activation, inhibition of pro-inflammatory cytokine production (e.g. IL-12 and TNF-α), and stimulation of anti-inflammatory cytokine production (e.g. IL-4 and IL-10) [96]. It has been proposed that IFN-β decreases T cell activation by increasing the prevalence and inhibitory capacities of naturally-occurring regulatory T cells (nTregs) [97, 98], and by increasing the production of IL-27 and IL-10 by DCs and $CD4^+$ T cells, to constrain Th17-mediated autoimmune inflammation [99, 100]. The suppression of Th17 development and IL-17 secretion have been attributed to TRIF-dependent, IFN-β-mediated, IL-27 production in macrophages and DCs [101]. Recently, the elucidation of the signaling pathway leading to LPS-induced IL-10 expression in macrophages,

involving the sequential induction of IFN-β production and signaling followed by IL-27 production and signaling, further sheds light on the immunomodulatory functions of IFN-β [102, 103].

1.7 Activation and regulation of interferon-β

IFN-β, which was first purified and characterized in the late 1970s and early 1980s [104-106], is transcribed from a single, intronless gene in human and mouse. Extensive studies on the chromatin structure and promoter architecture of the *IFNB* gene have revealed that IFN-β transcriptional activation requires the enhancer region located immediately upstream of the core promoter [107]. Using a model of virus-infected human epithelial HeLa cells, Maniatis and colleagues have identified the component transcription factors of the IFN-β "enhanceosome", namely NF-κB RelA/p50, IRF3/7 and ATF-2/c-Jun, that act at the IFN-β enhancer to activate IFN-β transcription [108]. On the other hand, in LPS-stimulated human monocytes, we have found that, in addition to TRIF-dependent IRF3 activation, constitutive binding of the myeloid-specific transcription factor IRF8 to the IFN-β promoter region is also required for induction of IFN-β transcription [109] (**Figure 1.4**).

The production of type I IFN by host cells in response to pathogen exposure is critical in innate and adaptive immunity [reviewed in [110]]. However, dysregulated expression of type I IFN can be detrimental to the host, and systemic overproduction of type I IFN can lead to septic shock syndrome in Gram negative sepsis [111]. Furthermore, type I IFN and members of the IRF family have also been implicated in the induction of autoimmune responses and in the pathogenesis of diseases such as SLE and Sjogren's syndrome [112-114]. Levels of type I IFN thus need to be carefully regulated during the course of infection. Type I IFN production differs in kinetics and magnitude between cell types [53, 89, 91, 109, 115]. We and others have previously reported that, upon viral infection, relatively more rapid and more robust IFN-β transcription occurred in human blood monocytes (as early as 1 h post-infection) compared with non-myeloid cells, such as HeLa cells and HEK293 cells (more than 6 h post-infection) [109, 115-117]. This fast and strong IFN-β transcription is similarly observed upon LPS stimulation of human blood monocytes and murine macrophages, reflecting the remarkably quick monocyte/macrophage response to a range of

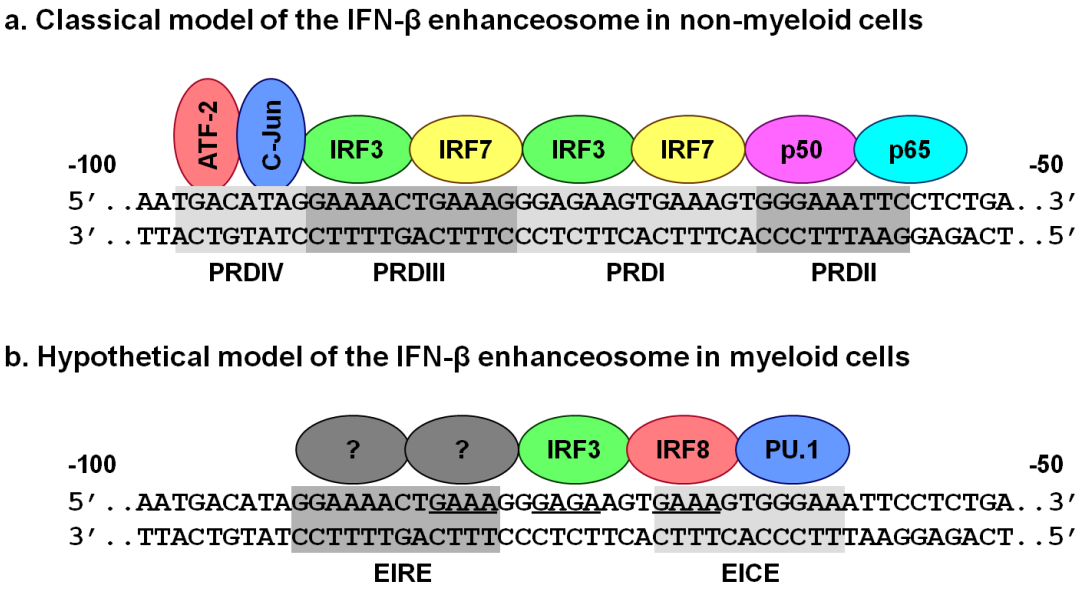

Figure 1.4: IFN-β enhanceosome in non-myeloid and myeloid cells.

pathogenic stimuli [53, 89, 91, 109]. Therefore, we hypothesize that cell type-specific regulation of IFN-β induction might account for the distinct magnitude and/or kinetics of IFN-β responses in distinct cell types.

It has been proposed that a constitutive low level of IFN-β expression and weak IFN-β signaling is operational in uninfected murine M-CSF-derived macrophages ("BMDM") compared with GM-CSF-derived macrophages ("GM-BMM") [87]. This endogenous IFN-β autocrine loop contributes to a basal level of expression of some type I IFN target genes such as STAT1, and "primes" the cells for a more dramatic increase in activation of the TRIF-IRF3 pathway leading to amplified ISG expression in response to LPS stimulation [87]. However, the molecular mechanisms (i.e. the mediators involved) and the functional significance of the constitutive versus LPS-induced IFN-β production and signaling in LPS-stimulated macrophages versus DCs are not comprehensively studied, despite emerging evidence of divergent responses of these two cell types to LPS-TLR4 activation [5, 24], such as the cell type-specific role of CD11b as a positive regulator of LPS-TLR4-induced signaling in mDCs but not in macrophages [118]. The overarching objective of this research is to elucidate the molecular mechanisms that regulate IFN-β induction in macrophages and DCs to permit such a unique cell type-specific response to the same pathogenic stimulus.

1.8 Type I interferons and inflammasomes

Inflammasomes are multi-protein complexes that act as cytosolic signaling platforms for the activation of caspase-1, which processes IL-1β and IL-18 from the immature forms into the active inflammatory cytokines, as well as for the induction of pyroptosis, which is a novel form of lytic inflammatory cell death having characteristics of both apoptosis and necrosis [119-121]. IL-1β is critical for antibacterial host defenses, and its super-induction is a marker of sepsis [122]. IL-1β and IL-18 also play key roles in the regulation of adaptive immune responses by modulating Th1, Th2, and Th17 differentiation and activation in various contexts [119]. Inflammasome function has been broadly characterized in innate immune cells, such as monocytes and macrophages, DCs, and neutrophils. The most extensively studied inflammasome complex to date is the NLRP3 inflammasome [123]. NLRP3 is part of the NLR family of proteins, which comprises three subfamilies NLRP, NOD and ice protease-activating factor/neuronal apoptosis inhibitory protein (IPAF/NAIP), characterized by a central nucleotide-binding domain (NBD), a N-terminal PYD or caspase activation and recruitment domain (CARD), and a C-terminal leucine-rich repeat (LRR) domain [124]. NLRP3 is highly expressed in splenic neutrophils, monocytes, macrophages, and conventional DCs, but lowly expressed in lymphoid cells, eosinophils, and plasmacytoid DCs [125]. NLRP3 expression in macrophages is induced by MyD88- and TRIF-dependent pathways via NF-κB in response to a wide array of inflammatory stimuli, including microbial PAMPs, such as LPS [125-128]. In the canonical model, NLRP3 inflammasome assembly and activation require two signals. The first signal is the priming of NLRP3 and pro-IL-1β expression by TLR agonists or other inflammatory stimuli, such as TNF-α and IL-1 cytokine itself, via the MyD88 pathway and NF-κB activation. The second signal is provided by activators, such as extracellular ATP, nigericin, and pore-forming toxins, among others, which are proposed to trigger potassium efflux, ROS generation, or lysosomal destabilization and cathepsin release, to result in inflammasome complex formation, caspase-1 activation and IL-1β processing [120, 129].

Some inflammasome components, such as AIM2, are IFN-inducible genes up-regulated by type I IFN autocrine/paracrine signaling, thus suggesting the possibility of

a positive feedback loop between type I IFN signaling and inflammasome activation [126]. Importantly, TRIF-dependent type I IFN production and signaling was found to be necessary for murine caspase-11 (ortholog of human caspase-4 and caspase-5) induction and activation, and subsequent caspase-1-dependent IL-1β production and caspase-1-independent macrophage cell death, in the non-canonical NLRP3 inflammasome pathway in Gram-negative bacterial infection, [130-133]. Recently, it has been further elucidated that caspase-11 is crucial for innate immunity and inflammasome activation in response to lysis of pathogen-containing vacuoles and escape of bacteria into the cytosol, mediated by IFN-inducible guanylate-binding proteins (GBPs) [134-136], as well as in response to cytoplasmic LPS, by directly binding to LPS independent of TLR4 [137-139]. LPS-, IFN-β- and IFN-γ-induced caspase-11 gene expression are reportedly dependent on NF-κB and STAT1 [140, 141].

On the other hand, negative regulation of inflammasome activity by type I IFN signaling, as well as vice versa, have also been discovered. Type I IFN treatment has been found to inhibit IL-1 production by two mechanisms: by repressing NLRP1 and NLRP3 inflammasome activation and caspase-1 processing, and by reducing pro-IL-1 expression via the induction of IL-10, which is likely to happen during the late phase after infection for the resolution of inflammation [142]. Also, type I IFN signaling was found to suppress NLRP3 inflammasome activation, caspase-1 activation and IL-1β release in macrophages through SOCS1-mediated inhibition of Rac1-GTP activation and ROS generation [143], or through Tyk2-mediated inhibition of IL-1β translation [144]. Thus, it is thought that the timing of IFN-β exposure determines the positive or negative effect of IFN-β signaling on inflammasome activation: while LPS-induced IFN-β is required for caspase-11 expression and NLRP3 inflammasome activation in Gram-negative bacterial infection [130], IFN-β inhibits NLRP3 inflammasome activation when it is present and sensed by the cells before being stimulated by a TLR agonist [142]. Conversely, inflammasome components have been discovered to negatively regulate type I IFN signaling. For instance, NLRC5 has been demonstrated to inhibit RLR-mediated type I IFN responses by interacting with RIG-I and MDA5 [145], and NLRP4 has been found to inhibit dsRNA- and dsDNA-mediated type I IFN activation by targeting TBK1 for ubiquitination and degradation, and hence inhibiting IRF3 activation [146].

Dysregulated activation of inflammasomes have been implicated in several inflammatory diseases and autoimmune disorders, such as sepsis, colitis, and multiple sclerosis [121]. NLRP3 inflammasomes confers protection against inflammatory bowel disease (IBD), and DSS- or TNBS-induced colitis [119]. Nlrp3$^{-/-}$ mice, Asc$^{-/-}$ mice and caspase-1$^{-/-}$ mice showed increased susceptibility, and increased morbidity and mortality to colitis [119]. On the other hand, IL-1β and IL-18 contribute to exacerbated Th17-driven EAE, and Nlrp3$^{-/-}$ mice, Asc$^{-/-}$ mice and caspase-1$^{-/-}$ mice were protected against EAE, attributed to reduced Th1 and Th17 responses [119]. Interestingly, the ability of IFN-β therapy to suppress EAE has been demonstrated to act through the inhibition of Rac1-GTP activation and ROS generation, which in turn inhibit NLRP3 activation and EAE severity [143]. In sepsis, the roles of individual inflammasome components remain controversial with several conflicting reports [147]. Asc$^{-/-}$ mice are resistant, whereas Nlrp3$^{-/-}$ mice are only partially protected, while IL-1β$^{-/-}$ mice are susceptible to lethal endotoxemia [120, 148, 149]. On the contrary, another report showed that IL-1β$^{-/-}$ or IL-18$^{-/-}$ single knockouts result in partial protection, whereas IL-1β$^{-/-}$ IL-18$^{-/-}$ double knockout results in complete protection against LPS-induced mortality [147]. In addition, IL-1R1$^{-/-}$ mice are resistant to lethal endotoxin shock, indicating that IL-1R signaling is important for mediating the effects of lethal endotoxemia [120, 150, 151]. These apparent discrepancies may be attributed to the overlapping effects of IL-1β and IL-18 in LPS-induced lethality [147], or to the effects of other amplifiers/mediators of endotoxin shock, such as HMGB1 [120, 152]. Nevertheless, it was recently reported that caspase-11$^{-/-}$ mice, like TLR4$^{-/-}$ mice, are protected from lethal endotoxemia, but caspase-1$^{-/-}$ mice are susceptible to lethal septic shock, suggesting that caspase-11-mediated pyroptosis instead of caspase-1-mediated IL-1β and IL-18 secretion plays a central role in lethal endotoxin shock [131, 137, 138]. Of note, Stat1$^{-/-}$ mice failed to produce serum IL-1β in response to LPS, and are resistant to lethal septic shock, whereas neutralization of IFN-β decreased serum IL-1β levels after LPS treatment, thus supporting the notion that IFN-β is implicated in the regulation of LPS-induced IL-1β expression *in vivo* [122]. This was attributed to the role of STAT1 downstream of IFN-β signaling in mediating the activation of caspase-1 and the processing IL-1β [122]. In short, given that type I IFN influences caspase-11 expression and IL-1β production in response to bacterial endotoxin exposure, we explored the functional implications of type I IFN signaling on caspase-11 expression and IL-1β production in this study.

1.9 Interleukin-27

IL-27 is a cytokine of the IL-6 superfamily/IL-12 family of type I cytokines [153]. The IL-12 family is a group of heterodimeric cytokines including IL-12, IL-23, IL-27 and IL-35, which share many molecular partners among themselves and arise out of unique pairings between these subunits [154]. For example, p40 partners p35 to form IL-12, and pairs with p19 to form IL-23 [154]. IL-27 cytokine comprises p28, a p35-related molecule, and Epstein-Barr virus-induced gene 3 (EBI3), a p40-related molecule [155, 156]. Apart from IL-27, the p28 subunit can also reportedly heterodimerize with a soluble cytokine receptor called Cytokine-Like Factor 1 (CLF1) to form a p28/CLF cytokine, which is produced by DCs, and signals through IL-6Rα on NK and T cells to regulate NK and T cell functions, such as increasing IFN-γ production by NK cells, and inducing IL-17 and IL-10 secretion by $CD4^+$ T cells [157]. EBI3 is also known to heterodimerize with p35 to form another cytokine called IL-35, which is an anti-inflammatory cytokine secreted by Treg cells, and signals through the IL-12Rβ2-gp130 receptor complex on T cells to suppress T cell proliferation and mediate Treg cell functions [158-160]. In addition, the p28 subunit itself, known as IL-30, can also be independently secreted as a monomeric protein by APCs. IL-27p28 (IL-30) reportedly acts as a natural antagonist of gp130 signaling [161], and has signaling properties on its own to exert anti-inflammatory effects, such as decreasing pro-inflammatory cytokine production in LPS-stimulated macrophages [162], and inhibiting IL-12-, IFN-γ-, and concanavalin A-induced hepatotoxicity [163].

IL-27 is mainly produced by classical APCs, including monocytes/ macrophages and DCs, when stimulated with PAMPs and TLR agonists including LPS, or other inflammatory stimuli such as TNF-α, and type I and II IFNs [158, 164, 165]. In monocytes/macrophages and DCs, EBI3 is induced in response to diverse stimuli, including LPS stimulation, CD40 ligation, or other inflammatory cytokine stimuli, such as IFN-β [153]. The same inflammatory stimuli also induce the expression of p28, hence promoting the secretion of the heterodimeric cytokine [153]. TLR2, TLR4 and TLR9 ligand-induced EBI3 expression in DCs was found to be dependent on MyD88 and NF-κB-p50/p65, while basal EBI3 mRNA levels in DCs were also found to be dependent on NF-κB p50 [166]. IL-27 protein production is primarily controlled at the

level of IL-27p28 mRNA expression [167, 168]. IL-27p28 expression in macrophages and DCs was found to be dependent on both MyD88 and TRIF signaling, and mediated by type I IFN signaling. In studies using murine macrophages, the transcription factors NF-κB c-Rel, IRF1 and IRF8 downstream of MyD88 signaling was found to be critical for IL-27p28 gene induction in response to TLR4 activation [167, 168]. In studies using murine myeloid DCs, a two-step activation process involving autocrine/paracrine type I IFN production and signaling was found to be necessary for the amplification of TLR4-induced IL-27p28 synthesis, in which the initial induction of p28 depends on the recruitment of IRF3 downstream of TRIF activation (in addition to IRF1), and the subsequent amplification of p28 transcription depends on the recruitment of the ISGF3 complex (as well as IRF1) mediated by autocrine/paracrine type I IFN production and signaling [169, 170]. The involvement of type I IFN signaling and IRF1 in LPS-induced IL-27p28 gene expression was also observed in studies using human monocyte-derived DC [171] and human monocyte-derived macrophages [172]. IL-27p28 production following TLR4 activation by LPS is defective in Tyk2$^{-/-}$, IFN-β$^{-/-}$ and IFNAR1$^{-/-}$ murine macrophages, consistent with the notion that IL-27p28 induction is dependent on type I IFN signaling [173].

IL-27 signals through its cognate receptor complex IL-27R, which consists of a unique subunit IL-27Rα (also termed WSX-1 or T-cell cytokine receptor [TCCR]) and a signal transducing subunit gp130, which is common with the IL-6R receptor complex [155]. While the expression of IL-27R on T cells is well known and the effects of IL-27 on T cells are well studied, IL-27R is also expressed on innate immune cells, including monocytes/macrophages, DCs, Langerhan's cells and NK cells [174, 175]. Upon IL-27 binding to its cognate receptor, JAK-STAT signaling is initiated: Jak1 and Jak2 kinases are activated, and STAT1 and STAT3 transcription factors are phosphorylated [155]. For instance, it has been reported that IL-27-mediated IL-10 production in T cells, and the associated immunosuppressive functions of IL-27, require STAT1 and STAT3 activation [176]. It has also been proposed that IL-27-mediated IL-10 production in murine macrophages is dependent on STAT1 and STAT3 activation and recruitment to the IL-10 promoter [102].

IL-27 is a pleiotropic cytokine up-regulated in septic peritonitis and other inflammatory diseases [164, 177]. IL-27 signaling can promote either pro-inflammatory

or immunosuppressive effects depending on the context in which signaling occurs. This has been studied in great detail in the modulation of many classes of T cell responses in various disease settings. The pro-inflammatory effects of IL-27 are mediated by its ability to promote Th1 commitment, induce Th1 cell proliferation and differentiation, and promote Th1 responses, as well as through the inhibition of inducible Treg (iTreg) development and suppressive function [155, 178]. On the other hand, IL-27 also induces anti-inflammatory responses through the inhibition of Th2 and Th17 differentiation and IL-17 production, the suppression of IL-2 and pro-inflammatory cytokine production, as well as by promoting the development of IL-10-producing regulatory Tr1 cells [158, 179]. While ability of IL-27 to modulate T-cell responses has now been studied in detail, it is currently unknown how IL-27 influences TLR4 signaling in the mAPC populations that first prime these responses. Some studies have suggested that IL-27 also plays dual roles in influencing the responses of APCs. For example, in murine macrophages, IL-27 exerted a pro-inflammatory role by enhancing iNOS expression and promoting LPS-induced nitric oxide production [180]. On the other hand, IL-27 can also exert immunosuppressive effects by inhibiting TNF-α and IL-12 production by activated or *Mycobacterium tuberculosis* infected murine macrophages [181].

Mice deficient in IL-27 production or signaling are usually characterized by an over-production of pro-inflammatory cytokines. For instance, IL-27p28$^{-/-}$ mice and WSX-1$^{-/-}$ mice show exacerbated EAE pathology, and this is due in part to impaired induction of IL-10 in the CNS in the resolution phase of the disease [182]. Both IFN-β and IL-27 induce human IL-10, and suppress human Th17 responses and murine EAE [183]. Notably, IL-27 is a biomarker of sepsis, and appears to play a detrimental role [184]. EBI3$^{-/-}$ mice were resistant to septic peritonitis induced by CLP, due to enhanced bacterial clearance and successful control of infection [177]. IL-27RA$^{-/-}$ mice with defective IL-27 signaling also showed reduced mortality from endotoxic shock induced by i.p. injection with LPS [173]. Interestingly, IL-27 signaling in T cells has recently been demonstrated to be important for eliciting T cell responses to innate immune receptor agonist-based adjuvants in subunit vaccines [185]. This is reminiscent of the immunostimulatory role of the IFN response in the adjuvant effects of TRIF-biased TLR4 agonists [186, 187], suggestive of a close relationship between IL-27 and type I IFNs. In summary, from the current literature, IL-27 exerts diverse effects on lymphocyte activation and function, but the autocrine effects of IL-27 on TLR4

signaling in the mAPC populations that first prime these responses are largely unknown. Since TLR4-expressing mAPC are the primary source of IL-27 production in bacterial infections, and given that this cytokine exerts a potent influence on the balance of pro-inflammatory versus regulatory responses, we hypothesized that autocrine/paracrine effects of IL-27 on macrophages and DCs can alter the outcome of endotoxin exposure.

1.10 Interleukin-10

IL-10 is one of the most potent anti-inflammatory cytokines, initially identified as a "cytokine synthesis inhibitory factor" (CSIF) produced by Th2 cells, that acts directly on T cells to inhibit Th1-cell effector functions and cytokine production, such as IL-2, IFN-γ and TNF-α [188, 189]. IL-10 is the first member of the IL-10 family of cytokines which also comprise IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 [190]. The immunosuppressive effects of IL-10 are important to restrain inflammation during the resolution phase of infections. Consistent with this immuno-regulatory role, the appropriate spatio-temporal tuning of the timing and degree of IL-10 production in response to infection is critical for the proper balance between effective pathogen clearance and resolution of infection [191]. Accordingly, dysregulation of IL-10 responses is associated with either an increased susceptibility to infections on the one hand, or an increased predisposition to auto-immune diseases on the other hand [192].

IL-10 is expressed by innate immune cells, including monocytes/macrophages, DCs, NK cells and neutrophils, as well as by adaptive immune cells, such as various T cell subsets, including Tr1, Treg, Th1, and Th2 cells, and B cells [193]. Myeloid cells, such as macrophages and myeloid DCs, but not plasmacytoid DCs, are a major source of IL-10 upon TLR stimulation during infection [194]. Optimal levels of LPS-induced IL-10 expression in macrophages have been shown to require both MyD88- and TRIF-dependent signaling, as well as type I IFN signaling [103, 193]. IL-27 can induce IL-10 expression in macrophages and T cells via STAT1 and STAT3, and a STAT3-binding motif was found at the human IL-10 gene promoter [102, 176, 195]. Apart from type I IFN signaling and IL-27 signaling, multiple signal transduction pathways, including NF-κB, and ERK, JNK and p38 MAPK pathways, as well as multiple transcription factors, such as Sp1, Sp3, CREB/ATF, c-Maf, NF-κB1 (p50), C/EBP-α, -β, and -δ have also been implicated in LPS-induced IL-10 expression in murine macrophages [196-

200]. In addition, IL-10 expression is also under negative and positive feedback regulation by IL-10 itself. Negative feedback can occur by IL-10-induced activation of dual-specificity protein phosphatase 1 (DUSP1), which inhibits p38 MAPK activation required for IL-10 production [201]. Positive feedback can occur by IL-10-induced up-regulation of tumor progression locus 2 (TPL2), which is an upstream activator of ERK signaling required for IL-10 production [202]. Furthermore, it has been reported that in human monocytes, IL-10 can inhibit LPS-activated IL-10 mRNA synthesis in a negative feedback manner [203], whereas in human monocyte-derived macrophages, IL-10 alone can induce IL-10 in an autocrine manner dependent on STAT3 activation [204]. It has been demonstrated in DCs that IFN-β-mediated IL-10 production is mediated by Jak1 and PI3K/Akt signaling pathways, which suppress the activation of the negative regulator Glycogen Synthase Kinase 3 (GSK3-β), and thus promote augmented IL-10 production [205]. On the other hand, in macrophages, IFN-γ can inhibit IL-10 production by interfering with the PI3K/Akt signaling pathway, as well as the p38 and ERK MAPKs [206]. In addition to the multiple signaling molecules and transcription factors regulating IL-10, chromatin modifications at the IL-10 promoter are also required for efficient IL-10 induction in murine macrophages [207].

IL-10 signals via its cognate receptor complex, comprising IL-10 receptor 1 (IL-10R1) and IL-10R2. IL-10R2 is ubiquitously expressed, whereas differential IL-10R1 expression in various cell types determines cellular responsiveness toward IL-10 [208]. IL-10R1 expression and IL-10 sensitivity are higher in human blood monocytes and macrophages, and lower in myeloid DCs [208]. IL-10R activation propagates signals through Jak1 and Tyk2 to culminate in the anti-inflammatory response, which is recognized to be primarily mediated by STAT3 [209, 210]. This is effected through the transcriptional activation of STAT3-responsive genes, such as SOCS-1 and SOCS-3, that execute the anti-inflammatory response, for example by down-regulating LPS-mediated TNF-α, IL-6, and iNOS production, and inhibiting IFN-γ signaling [188, 189, 209, 211-213]. IL-10 can inhibit the activity of Th1 cells and enhance NK cell proliferation and activation, but it is believed to mainly act on APCs, such as macrophages and DCs, in which the expression of the IL-10R receptor complex is highest, to regulate their pro-inflammatory activities [192]. It has been described to inhibit the TLR-induced expression of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6; Th1-inducing cytokines, such as IFN-γ, IL-2, IL-12 and IL-18;

inflammatory chemokines, such as CCL2-5, CXCL8 and CXCL10; and cell surface molecules, such as MHC class II and CD80/86 co-stimulatory molecules, and therefore inhibits antigen presentation and the subsequent activation of Th1-type responses, thereby promoting Th2 cell proliferation and Th2-type responses [188, 192, 193, 211]. Many mechanisms mediating the suppressive effects of IL-10 have been reported. It is well recognized that IL-10 inhibits the expression of multiple, diverse pro-inflammatory cytokines at the level of gene transcription, such as via posttranscriptional mRNA destabilization and degradation [214]. IL-10 has also been described to dampen MyD88-dependent signaling in DCs, by inducing the ubiquitination and degradation of IRAK4 and TRAF6 [215]. It has also been found to inhibit the NF-κB, p38 MAPK, JNK, and PI3K/Akt pathways of pro-inflammatory cytokine production downstream of TLR4, such as via inhibition of IKK activation (and hence NF-κB activation, nuclear translocation and/or DNA binding) [216, 217]. IL-10 also inhibits the expression of several ISGs in monocytes, by suppressing IFN-induced STAT1 phosphorylation [218]. Interestingly, it was reported that IL-10 can inhibit LPS-induced IFN-β expression by affecting IRF3 phosphorylation in RAW264.7 cell macrophages [219].

From the above, it is evident that IL-10 restrains inflammatory reactions in response to endotoxin, such as in colitis or endotoxin shock. IL-10R signaling in innate immune cells, such as macrophages, is important for mucosal immune tolerance [220, 221]. Macrophage-specific IL-10R deletion led to greater production of NO and ROS, and resulted in more severe DSS-induced colitis [222]. In sepsis, the immune-regulatory functions of IL-10 are essential for the control of systemic inflammatory responses to LPS exposure, and protects against lethality from endotoxin shock. Accordingly, mice deficient in IL-10, particularly in myeloid cells, exhibit uncontrolled inflammatory responses to endotoxin exemplified by unrestrained pro-inflammatory cytokine production, which can result in mortality [192]. Therefore, through multiple mechanisms, IL-10 plays a key role in the termination of inflammatory responses to down-regulate inflammation after the clearance of infections. Given that type I IFN and IL-27 signaling influences IL-10 expression in response to bacterial endotoxin exposure, we explored the biological implications of type I IFN and IL-27p28 signaling on the IL-10/STAT3 anti-inflammatory response in this study.

1.11 Scientific question

What mechanisms regulate the differential type I IFN response to bacterial lipopolysaccharide (LPS) in myeloid antigen presenting cells (mAPC)?

In the context of divergent responses of macrophages and DCs to microbial stimuli, including bacterial LPS, molecules, such as CD11b, which confer cell type-specific regulation of LPS-TLR4 signaling have been recently identified [5, 24, 118], but a gap exists in the understanding of whether and how type I IFN responses are differentially regulated in macrophages and DCs. The research presented in this thesis compares the type I IFN response to bacterial LPS in primary murine bone marrow-derived macrophages (BMDMs) and DCs (BMDCs). This will be achieved through a systematic genetic loss-of-function approach using relevant knockouts under controlled experimental conditions, to uncover novel signaling mediators and transcription factors, and to elucidate the molecular mechanisms responsible for the unique phenomenon of fast and high level of IFN-β production in TLR4-stimulated macrophages. The biological importance of this rapid and robust IFN-β production in LPS-stimulated macrophages in IL-1β production and IL-10 expression were also studied.

In this report, we observed that BMDMs exhibit more robust IFN-β induction and stronger type I IFN responses to LPS exposure compared with BMDCs, and asked whether an IFN-β autocrine/paracrine positive feedback loop exists in LPS-stimulated BMDMs. Using IFNAR1-deficient BMDMs, we clearly demonstrate that an IFN-β amplification loop is operational in LPS-stimulated macrophages. However, in contrast to viral-infected fibroblasts and DCs, in which the IFN-β amplification loop operates after virus infection, type I IFN signaling operates in steady-state BMDMs to prime high-level expression of a preexisting pool of IRF7, which we identified as a novel transcription factor critical for robust IFN-β induction after LPS exposure in BMDMs but not in BMDCs, because steady-state BMDCs express negligible amounts of IRF7 protein, due to weak constitutive type I IFN signaling. This IRF7-mediated IFN-β expression is required for pro-caspase-11 induction in LPS-challenged BMDMs and for IL-1β production *in vivo*. Further, using IL-27p28-deficient BMDMs, we determined that IL-27p28 regulates constitutive production of type I IFN in order to support basal IRF7 expression and rapid amplification of the IFN-β response to later endotoxin

exposure. This IL-27p28-mediated IFN-β expression, and not IL-27p28 itself, is required for optimal IL-10/STAT3-mediated anti-inflammatory response in LPS-challenged BMDMs, because the defect in IL-10 expression in the absence of IL-27p28 can be restored by the addition of exogenous IFN-β, thus contesting the current proposed model that a sequential induction of IFN-β followed by IL-27 leads to IL-10 production. In summary, we report that a novel axis of constitutive IL-27p28/ IFN-β/IRF7 signaling amplifies the IFN-β response to LPS specifically in macrophages, suggesting that IL-27p28/IFN-β/IRF7 determines the magnitude of the type I IFN response to endotoxin exposure in a lineage-restricted fashion and is thus likely to critically influence the outcome of bacterial infections.

2 MATERIALS AND METHODS

2.1 Chemicals

Table 2.1 lists the sources of chemicals used in this study.

Table 2.1: Sources of chemicals.

Chemical	**Source**
Trizol	Ambion, Life Technologies (Carlsbad, California, USA)
Chloroform	Merck Millipore (Temecula, CA, USA)
2-Propanol	Merck Millipore (Temecula, CA, USA)
Ethanol	Merck Millipore (Temecula, CA, USA)
Diethyl pyrocarbonate (DEPC)-treated water	Ambion, Life Technologies (Carlsbad, California, USA)
30% Acrylamide/Bis Solution, 37.5:1	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
Ammonium persulfate	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
Bromophenol blue	Sigma-Aldrich (St. Louis, MO, USA)
Glycerol	Invitrogen, Life Technologies (Carlsbad, California, USA)
2-Mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
Sodium dodecyl sulfate (SDS)	Merck Millipore (Temecula, CA, USA)
10X Tris Glycine-Sodium Dodecyl Sulfate (TG-SDS) Buffer	Axil Scientific Pte Ltd (Singapore)
Tris-HCl	Axil Scientific Pte Ltd (Singapore)
Glycine	Axil Scientific Pte Ltd (Singapore)
Methanol	Merck Millipore (Temecula, CA, USA)
10X Phosphate Buffered Saline (PBS) Buffer	Axil Scientific Pte Ltd (Singapore)
10X Tris Buffered Saline (TBS) Buffer	Axil Scientific Pte Ltd (Singapore)

Polyoxyethylene sorbitan monolaurate (Tween 20)	Promega Corporation (Madison, WI, USA)
Skim milk for immunoassay	Nacalai-Tesque (Kyoto, Japan)
Bovine serum albumin	Sigma-Aldrich (St. Louis, MO, USA)
Sodium azide	Sigma-Aldrich (St. Louis, MO, USA)
Pierce Restore Western Blot Stripping Buffer	Thermo Fisher Scientific Inc. (Rockford, IL, USA)
Sulfuric acid	BDH, VWR International (Radnor, PA, USA)
Formaldehyde solution	Sigma-Aldrich (St. Louis, MO, USA)
Lithium chloride anhydrous	Sigma-Aldrich (St. Louis, MO, USA)
Ethylenediaminetetraacetic acid disodium hydrate (EDTA)	Axil Scientific Pte Ltd (Singapore)
Phenol: chloroform: isoamyl alcohol	Ambion, Life Technologies (Carlsbad, California, USA)

2.2 Buffers

Buffers for Western blotting, ELISA and ChIP were prepared as per **Table 2.2**.

Table 2.2: Preparation of buffers.

Lower gel buffer	1.5M Tris-HCl pH8.8 + 0.4% SDS
Upper gel buffer	0.5M Tris-HCl pH6.8 + 0.4% SDS
1X Transfer buffer (Bjerrum Schafer-Nielsen Buffer with SDS)	48 mM Tris + 39 mM glycine + 1.3 mM SDS + 20% methanol
FA cell lysis buffer	10 mM Tris-HCl pH 8.0 + 0.25% Triton X-100 + 10 mM EDTA + 0.1 M NaCl in sterile water
1% SDS nuclear lysis buffer	50 mM HEPES pH 7.5 + 150 mM NaCl + 2 mM EDTA + 1% Triton X-100 + 0.1% NaDOC (sodium deoxycholate) + 1% SDS in sterile water
ChIP elution buffer	50 mM Tris-HCl pH 7.5 + 10 mM EDTA + 1% SDS in sterile water

2.3 Tissue culture reagents

Table 2.3 lists the sources of tissue culture reagents used for cell line (e.g. L929) and primary cell (e.g. BMDM and BMDC) cultures. Tissue culture media were prepared as shown in **Table 2.4**.

Table 2.3: Sources of tissue culture reagents.

Tissue culture reagent	Source
Dulbecco's Modified Eagle's Medium (DMEM) + 4500 mg/L glucose + 110 mg/L sodium pyruvate	Biopolis Shared Facilities, A*STAR (Singapore)
Roswell Park Memorial Institute (RPMI) 1640 + 10 mM HEPES	Biopolis Shared Facilities, A*STAR (Singapore)
Dulbecco's Phosphate Buffered Saline (DPBS)	Biopolis Shared Facilities, A*STAR (Singapore)
0.125% trypsin/versene	Biopolis Shared Facilities, A*STAR (Singapore)
Heat inactivated fetal bovine serum (FBS)	Sigma-Aldrich (St. Louis, MO, USA)
Hybri-Max dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St. Louis, MO, USA)
HyClone defined fetal bovine serum (FBS)	HyClone Laboratories, Inc. (South Logan, Utah, USA)
Iscove's Modified Dulbecco's Medium (IMDM)	Gibco, Life Technologies (Grand Island, NY, USA)
10,000 U/ml penicillin + 10,000 μg/ml streptomycin	Gibco, Life Technologies (Grand Island, NY, USA)
10 mg/ml gentamicin	Gibco, Life Technologies (Grand Island, NY, USA)
Recombinant mouse macrophage colony-stimulating factor (M-CSF)	ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel)
Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF)	ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel)

Table 2.4: Preparation of tissue culture media.

Tissue culture media	Components
DMEM complete medium	90% DMEM + 4500 mg/L glucose + 110 mg/L sodium pyruvate supplemented with 10% heat inactivated FBS and 100 U/ml penicillin + 100 μg/ml streptomycin
RPMI complete medium	90% RPMI 1640 + 10 mM HEPES supplemented with 10% heat inactivated FBS and 100 U/ml penicillin + 100 μg/ml streptomycin
BMDM differentiation medium	50% DMEM + 4500 mg/L glucose + 110 mg/L sodium pyruvate supplemented with 20% HyClone defined FBS and 30% LCCM, and 100 U/ml penicillin + 100 μg/ml streptomycin
BMDC differentiation medium	90% RPMI 1640 + 10 mM HEPES supplemented with 10% HyClone defined FBS and 20 ng/ml GM-CSF, and 100 U/ml penicillin + 100 μg/ml streptomycin
Freezing medium	80% heat inactivated FBS + 20% Hybri-Max DMSO
Red blood cell (RBC) lysis buffer	155 mM ammonium chloride + 10 mM potassium bicarbonate + 0.1 mM EDTA in sterile water

2.4 Receptor agonists, recombinant cytokines and pharmacological inhibitors

Table 2.5 lists the sources of receptor agonists and recombinant cytokines used for *in vitro* stimulation experiments.

Table 2.5: Sources of receptor agonists and recombinant cytokines.

Agonist / cytokine	Concentration used to stimulate cells	Source
Lipopolysaccharides (LPS) from Escherichia coli 0127:B8	100 ng/ml unless indicated otherwise	Sigma-Aldrich (St. Louis, MO, USA)
Monophosphoryl Lipid A from Salmonella minnesota R595	100 ng/ml unless indicated otherwise	Invivogen (San Diego, CA, USA)

Recombinant mouse IFN-α	250 U/ml or 1 U/ml as indicated	Merck Millipore (Temecula, CA, USA)
Recombinant mouse IFN-β	250 U/ml or 1 U/ml as indicated	Merck Millipore (Temecula, CA, USA)
Recombinant mouse IL-27	10 ng/ml unless indicated otherwise	R&D Systems, Inc. (Minneapolis, MN, USA)
Recombinant mouse IL-10	10 ng/ml unless indicated otherwise	R&D Systems, Inc. (Minneapolis, MN, USA)

Table 2.6 lists the sources of pharmacological inhibitors used for *in vitro* inhibition experiments.

Table 2.6: Sources of pharmacological inhibitors.

Inhibitor	**Concentration used to treat cells**	**Source**
CaM kinase II Inhibitor (KN-93)	40 μM	Merck Millipore (Temecula, CA, USA)
Pyk2 Inhibitor (Tyrphostin A9 / AG 17)	10 μM	Merck Millipore (Temecula, CA, USA)
NF-κB Inhibitor (BAY 11-7085)	10 μM	Merck Millipore (Temecula, CA, USA)

2.5 Mice

All mice were derived from a C57BL/6 genetic background. **Table 2.7** lists the sources of all mice used in this study. IRF3-IRF7 double knockout mice were generated in-house by intercross of $IRF3^{-/-}$ and $IRF7^{-/-}$ mice. Homozygous $IRF3^{-/-}$-$IRF7^{-/-}$ mice were generated by intercross of heterozygous $IRF3^{+/-}$-$IRF7^{+/-}$ F1 mice, and identified by genotyping of tail biopsies. All mice were bred and maintained by the SIgN Mutant Mouse Collection Core Service at the A*STAR Biological Resource Centre (BRC)

under specific pathogen-free (SPF) conditions. All animal experimental procedures were conducted within the parameters of our Institutional Animal Care and Use Committee (IACUC)-approved protocol, in compliance with the National Advisory Committee For Laboratory Animal Research (NACLAR) Guidelines.

Table 2.7: List of mutant mice used in this study.

Strain	Description	Source	Reference
MyD88$^{-/-}$	MyD88-deficient	OrientalBioService, Inc. (Kyoto, Japan)	[223]
Ticam1^{Lps2}/J	TRIF-deficient	The Jackson Laboratory (Bar Harbor, Maine, USA)	[224]
Irf1^{tm1Mak}/J	IRF1-deficient	The Jackson Laboratory (Bar Harbor, Maine, USA)	[225]
IRF3$^{-/-}$	IRF3-deficient	RIKEN BioResource Center (Ibaraki, Japan)	[78]
IRF7$^{-/-}$	IRF7-deficient	RIKEN BioResource Center (Ibaraki, Japan)	[35]
IRF3$^{-/-}$-IRF7$^{-/-}$	IRF3-IRF7 double knockout	Generated by intercross of IRF3$^{-/-}$ and IRF7$^{-/-}$ mice	
Ifnar1^{tm1Agt}/Mmjax	IFNAR1-deficient	Mutant Mouse Regional Resource Centers (MMRRC), National Institutes of Health (NIH) (Bethesda, Maryland, USA)	[226]
Stat1^{tm1Rds}	STAT1-deficient	Taconic Biosciences, Inc. (Hudson, NY, USA)	[227]

2.6 Preparation of murine bone marrow cells

Mice were euthanized by carbon dioxide followed by cervical dislocation to ensure death. After euthanasia, femurs and tibias were dissected from each mouse using scissors and forceps, and the bones were placed into a petri dish containing DMEM

complete medium. Both epiphyses were removed from each bone using scissors and forceps, and bone marrow cells were flushed into a 50-ml polypropylene tube using a 25-G needle and a 10-ml syringe containing DMEM complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in 3 ml Red Blood Cell (RBC) lysis buffer for 3 min at room temperature. RBC lysis was stopped by adding 10 ml DMEM complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in freezing medium. Bone marrow cells were aliquoted into cryogenic vials, and then frozen in liquid nitrogen [228].

In addition, **Table 2.8** lists the sources of additional frozen bone marrow cells obtained for this study. STAT3 is required for viable embryogenesis, and targeted disruption of mouse STAT3 is embryonic lethal [229]. Hence, conditionally mutant mice generated using the loxP-Cre recombinase system driven by the IFN-inducible Mx promoter were utilized, in which injection of the IFN-inducer poly(I:C) leads to the induction of Cre and the deletion of $STAT3^{f/f}$ [230]. Bone marrow cells were obtained from littermate $STAT3^{f/f}$ mice with (knockout) or without (control) the Mx-Cre transgene, treated equivalently with poly(I:C) [230].

Table 2.8: List of mutant bone marrow cells used in this study.

Strain	Description	Source	Reference
$STAT2^{m/m}$ (P117)	STAT2 hypomorphic mutant	Chien-Kuo Lee (National Taiwan University College of Medicine, Taiwan, Republic of China)	[231]
$STAT3^{f/f}$	Control mice lacking the Mx-Cre transgene	Chien-Kuo Lee (National Taiwan University College of Medicine, Taiwan, Republic of China)	[230]
MxCre-$STAT3^{f/f}$	STAT3 conditional knockout	Chien-Kuo Lee (National Taiwan University College of Medicine, Taiwan, Republic of China)	[230]
IL-$27p28^{-/-}$	IL-27p28-deficient	Hiroki Yoshida (Saga University, Saga, Japan)	

2.7 Preparation of L929 cell-conditioned medium

L929 (NCTC clone 929) (ATCC, Manassas, VA, USA) is a murine fibroblast cell line that constitutively secrete macrophage-colony stimulating factor (M-CSF) into the culture medium [232]. Hence, L929 cell-conditioned medium (LCCM) was used as a source of murine M-CSF, a method well-established in the scientific literature [233]. 2.5×10^6 L929 cells were cultured in each 150-mm dish containing 20 ml DMEM complete medium. LCCM was harvested after 48 h, filtered through a 0.2-μm filter unit, aliquoted into 50-ml polypropylene tubes, and then stored at –20°C.

2.8 Differentiation of murine bone marrow-derived macrophages

Frozen bone marrow cells were thawed in a 37°C water bath and transferred to a 15-ml polypropylene tube containing 10 ml DMEM complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in BMDM differentiation medium. Bone marrow cells were counted using trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) and a hemocytometer. For analysis of RNA and culture supernatants, 0.5×10^6 BM cells were cultured in each well of a 6-well plate containing 1.5 ml BMDM differentiation medium. For protein experiments, 1.5×10^6 BM cells were cultured in each 60-mm dish containing 2.5 ml BMDM differentiation medium. For analysis of nuclear extracts, $6.0–7.0 \times 10^6$ BM cells were cultured in each 100-mm dish containing 10.0 ml BMDM differentiation medium. For ChIP experiments, 20.0×10^6 BM cells were cultured in each 150-mm dish containing 20.0 ml BMDM differentiation medium. On Day 3, an equivalent volume of fresh BMDM differentiation medium was added to the culture. On Day 5 and Day 6, the BMDM differentiation medium was aspirated and fresh BMDM differentiation medium was added to the adherent cells. Successful differentiation of bone marrow progenitors into BMDMs was confirmed by flow cytometric analysis of F4/80 and CD11b surface marker expression. On Day 7, BMDMs were used for experiments, and samples were harvested for downstream analysis [233].

2.9 Differentiation of murine bone marrow-derived dendritic cells

Frozen bone marrow cells were thawed in a 37°C water bath and transferred to a 15-ml polypropylene tube containing 10 ml RPMI complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in BMDC differentiation medium. Bone marrow cells were counted using trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) and a hemocytometer. 1.5×10^6 BM cells were cultured in each well of a 24-well plate containing 1.0 ml BMDC differentiation medium. On Day 2, an equivalent volume of fresh BMDC differentiation medium was added to the culture. On Day 4, 1.0 ml BMDC differentiation medium was aspirated and 1.0 ml fresh BMDC differentiation medium was added to the culture. On Day 5, the non-adherent cells were collected and re-plated in suspension culture plates for experiments. For analysis of RNA and culture supernatants, 0.5×10^6 BMDCs were cultured in each well of a 24-well suspension culture plate containing 1.5 ml BMDC differentiation medium. For protein experiments, 1.5×10^6 BMDCs were cultured in each well of a 6-well suspension culture plate containing 2.5 ml BMDC differentiation medium. On Day 6, 1.0 ml BMDC differentiation medium was aspirated and 1.0 ml fresh BMDC differentiation medium was added to the culture. Differentiation of bone marrow progenitors into BMDCs was confirmed by flow cytometric analysis of CD11c surface marker expression. On Day 7, BMDCs were used for experiments, and samples were harvested for downstream analysis [234].

2.10 Gene expression analysis by real-time quantitative-PCR (qRT-PCR)

Total RNA were harvested using TRIzol Reagent (Ambion, Life Technologies, Carlsbad, California, USA) and isolated by acid guanidinium thiocyanate-phenol-chloroform extraction, followed by purification using the PureLink RNA Mini Kit (Ambion, Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. RNA yield and quality were analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from 1 μg total RNA per sample by mRNA-specific reverse transcription using Oligo(dT)12-18 Primer and SuperScript III Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The first-strand cDNA synthesis reaction is shown in

Table 2.9. cDNA yield and quality were analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The cDNA was used as a template for amplification in qRT-PCR in duplicate. qRT-PCR analysis was performed by SYBR Green (Kapa Biosystems, Inc., Boston, MA, USA) detection using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA, USA). The qRT-PCR reaction is shown in **Table 2.10**, and the qRT-PCR cycling conditions are shown in **Table 2.11**. qRT-PCR primers for gene expression analysis are provided in **Table 2.12**. Primer specificity was confirmed using entire genome BLAST searches to exclude cross-reactivity, as well as using dissociation curve analysis to identify the presence of a single amplification product. Each sample was analyzed in duplicate. Results were analyzed by relative quantification using comparative CT (the ΔΔCT method [235]), and either (i) presented relative to *Gapdh* expression, or (ii) normalized to *Gapdh* expression and presented relative to control sample (e.g. untreated wild-type at time zero), as indicated in the figure legends.

Table 2.9: First-strand cDNA synthesis reaction.

Components	**Volume**
1 μg total RNA	11 μl
oligo(dT)12-18	1 μl
10 mM dNTP Mix	1 μl
5X First-Strand Buffer	4 μl
0.1 M DTT	2 μl
SuperScript III RT	1 μl

Table 2.10: qRT-PCR reaction.

Components	**Volume**
2X KAPA SYBR FAST qPCR Master Mix ABI Prism	5 μl
0.5 μM Forward Primer	2 μl
0.5 μM Reverse Primer	2 μl
200 ng/μl Template cDNA	1 μl

Table 2.11: qRT-PCR cycling conditions.

Step	Temperature	Duration	Cycles
Enzyme activation	95 ºC	10 min	1
Denaturation	95 ºC	30 s	40
Annealing	60 ºC	30 s	
Extension/ data acquisition	72 ºC	1 min	

Table 2.12: qRT-PCR primers for gene expression analysis.

Primer	Sequence (5' to 3')
m-Gapdh Forward	ATCTTCTTGTGCAGTGCCAGCCTCGTCCC
m-Gapdh Reverse	TTGACTGTGCCGTTGAATTTGCCGTGAGTG
m-Ifnb1 Forward	CCCTATGGAGATGACGGAGA
m-Ifnb1 Reverse	TCCCACGTCAATCTTTCCTC
m-Il27 Forward	CTCTGCTTCCTCGCTACCAC
m-Il27 Reverse	GGGGCAGCTTCTTTTCTTCT
m-Il10 Forward	AAGGACCAGCTGGACAACAT
m-Il10 Reverse	TTTTCACAGGGGAGAAATCG
m-Ccl5 Forward	CCCTCACCATCATCCTCACT
m-Ccl5 Reverse	CCACTTCTTCTCTGGGTTGG
m-Cxcl10 Forward	AAGTGCTGCCGTCATTTTCT
m-Cxcl10 Reverse	TTTTTGGCTAAACGCTTTCAT
m-Cxcl11 Forward	AACAGGAAGGTCACAGCCATAG
m-Cxcl11 Reverse	TTTGTCGTTTATGAGCCTTCATAGT
m-Stat1 Forward	TGCTACTGTTCCTTCATATGCAGTATTTCT
m-Stat1 Reverse	ATCTCTTGGTCTTTGTTTACAAAATCCATT
m-Irf1 Forward	GCCCTCCTGAGTGAGTTAGGCCTTGGCATC
m-Irf1 Reverse	CCTAGGAGTCAGGGCCAGCTTCACCTCACA
m-Irf7 Forward	GCATTTCGGTCGTAGGGATCTGGATGAAGA
m-Irf7 Reverse	CGTACACCTTATGCGGATCAACTGGA
m-Nlrp3 Forward	ATGCTGCTTCGACATCTCCT
m-Nlrp3 Reverse	AACCAATGCGAGATCCTGAC
m-Casp1 Forward	TAAATGGATTGTTGGATGAACTTTT

m-Casp1 Reverse	CCAGGTAGCAGTCTTCATTACAAAT
m-Casp4 Forward	ACAATGCTGAACGCAGTGAC
m-Casp4 Reverse	CTGGTTCCTCCATTTCCAGA
m-Il1b Forward	TGCAAGTGTCTGAAGCAGCTATGGCAACTG
m-Il1b Reverse	GAAGCTCTTGTTGATGTGCTGCTGCGAGAT

2.11 Nuclear and cytoplasmic extraction

Cytoplasmic and nuclear protein fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Life Technologies, Thermo Fisher Scientific Inc., Rockford, IL, USA). Briefly, 6.0–7.0 × 10^6 cells were scraped, pelleted, washed with PBS, and then pelleted again. The cell pellet was incubated with 200 µl CER I buffer, followed by 11 µl CER II buffer to lyse the cells and obtain the cytoplasmic extract. Intact nuclei were pelleted, washed with PBS, and then pelleted again. The nuclei pellet was incubated with 100 µl NER buffer to lyse the nuclei and obtain the nuclear extract. Cytoplasmic and nuclear extracts were stored at -80°C until analysis by Western blotting.

2.12 Protein expression analysis by Western blotting

Total cell lysates were harvested by lysing cells in Radio Immunoprecipitation Assay (RIPA) buffer (25mM Tris-HCl, pH7.6, 150mM NaCl (sodium chloride), 1% NP-40, 1% SDS (sodium dodecyl sulfate), 1% sodium deoxycholate) with protease and phosphatase inhibitors (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Dubai, UAE; Pierce Phosphatase Inhibitor Tablets, Thermo Fisher Scientific Inc., Rockford, IL, USA) for 1 h at 4°C. Whole cell lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C. Protein concentrations were determined by the Bradford assay using Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Tecan Infinite M200 Microplate Reader (Tecan Trading AG, Switzerland) according to the manufacturer's instructions. Protein concentrations were normalized, and sample lysates were denatured by addition of Sodium Dodecyl Sulfate (SDS) loading buffer with β-mercaptoethanol and then boiling for 5 min at 95°C. Equal amounts of sample lysates were separated by 9% Sodium

Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) under reducing and denaturing conditions (Amersham, GE Healthcare Bio-Sciences, Sweden), and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, GE Healthcare Bio-Sciences, Sweden). Blots were blocked in 5% milk or BSA solution (for phospho-proteins) to prevent non-specific background binding, and probed with specific antibodies in 5% milk or BSA solution (for phospho-proteins). Antibodies used for Western blotting are shown in **Table 2.13**. Chemiluminescence detection was performed using Pierce SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL USA) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Sweden), and CL-XPosure X-ray autoradiography film (Thermo Fisher Scientific Inc., Rockford, IL USA) and Kodak X-Omat 2000 Film Processor (Rochester, NY, USA).

Table 2.13: Antibodies used for Western blotting.

Antibody	**Source**
Actin (MAB1501)	Merck Millipore (Temecula, CA, USA)
α Tubulin (B-7) (sc-5286)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
TATA binding protein TBP [1TBP18] (ab818)	Abcam (Cambridge, MA, USA)
Stat1 (pY701) (612132)	BD Transduction Laboratories (Franklin Lakes, New Jersey, USA)
Stat1 (N-Terminus) (610115)	BD Transduction Laboratories (Franklin Lakes, New Jersey, USA)
STAT2 (phospho-Tyr689) (07-224)	Merck Millipore (Temecula, CA, USA)
Stat2 (L-20) (sc-950)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
Stat3 (phospho-Tyr705) (D3A7) (9145)	Cell Signaling Technology, Inc. (Danvers, MA, USA)
Stat3 (9132)	Cell Signaling Technology, Inc. (Danvers, MA, USA)
IRF-1 (C-20) (sc-497)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
IRF-3 (FL-425) (sc-9082)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
IRF-7 (51-3300)	Invitrogen (Life Technologies, Carlsbad, California, USA)

IRF-7 (H-246) (sc-9083)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
IRF-7 (Y-19) (sc-15993)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
IRF-7 (C-20) (sc-15994)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
IRF-7 (F-1) (sc-74471)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
IRF-7 (C-Terminus) (LS-B577)	LifeSpan BioSciences, Inc. (Seattle, WA, USA)
IRF-7 (Ser471 + Ser472) (bs-3196R)	Bioss Inc. (Woburn, Massachusetts, USA)
IRF-7 (Ser437/438) (14767)	Cell Signaling Technology, Inc. (Danvers, MA, USA)
caspase-1 p10 (M-20) (sc-514)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
Caspase-4 /11 (p20) (Flamy-1) (AG-20B-0060-C100)	Adipogen International, Inc. (San Diego, CA, USA)
NLRP3/NALP3 (Cryo-2) (AG-20B-0014-C100)	Adipogen International, Inc. (San Diego, CA, USA)
IL-1β (M-20) (sc-1251)	Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)
AffiniPure Donkey anti-rabbit HRP (711-035-152)	Jackson ImmunoResearch Inc. (West Grove, PA, USA)
AffiniPure Donkey anti-mouse HRP (715-035-150)	Jackson ImmunoResearch Inc. (West Grove, PA, USA)
AffiniPure Donkey anti-goat HRP (705-035-147)	Jackson ImmunoResearch Inc. (West Grove, PA, USA)

2.13 Cytokine secretion analysis by enzyme-linked immunosorbent assay (ELISA) and Luminex multiplex assay

Cytokine levels in culture supernatants were assayed using VeriKine Mouse Interferon Beta ELISA Kit (PBL Assay Science, Piscataway, NJ, USA), Mouse IL-27 Ready-SET-Go ELISA Set (eBioscience, Inc., San Diego, CA, USA), and Mouse IL-10 Ready-SET-Go ELISA Set (eBioscience, Inc., San Diego, CA, USA), according to the manufacturer's instructions.

Additionally, cytokine levels of IL-10 and pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-12, in culture supernatants were analyzed by Bio-Plex Pro magnetic bead-based multiplex assays (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a 12-plex mouse cytokine panel. Fluorescence intensity was acquired and analyzed using the Bio-Plex 200 System (V6.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.14 Chromatin immunoprecipitation (ChIP) analysis

DNA and proteins in cells were cross-linked using 1% formaldehyde for 10 min at room temperature and quenched using 200 mM glycine for 1 min at room temperature to terminate the cross-linking reaction. Cells were scraped and collected into a 50-ml polypropylene tube, and centrifuged at 3000 rpm for 5 min at 4°C. Cells were lysed with FA cell lysis buffer with protease inhibitor, and nuclei were lysed with 1% SDS nuclear lysis buffer with protease inhibitor. Cross-linked chromatin and associated proteins were sonicated using the Bioruptor sonication device (Diagenode Inc., Denville, NJ, USA) to generate chromatin fragments of an average fragment size of 500 bp. Chromatin fragments were immunoprecipitated overnight at 4°C using control IgG or anti-STAT1 antibodies (sc-345, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) bound to Dynabeads Protein A/G magnetic beads (Life Technologies, Carlsbad, California, USA). Immunoprecipitated chromatin fragments were dissociated from the antibody-bound beads using ChIP elution buffer, cross-links were reversed by incubation with 20 mg/ml pronase for 2 h at 42°C followed by 6 h at 67°C, and DNA was purified using phenol-chloroform extraction followed by ethanol precipitation. Isolated DNA was analyzed to determine the fold enrichment of target DNA sequences relative to input chromatin. The isolated DNA was quantified by qRT-PCR analysis using SYBR Green (Kapa Biosystems, Inc., Boston, MA, USA) detection using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA, USA). qRT-PCR primers for ChIP analysis are provided in **Table 2.14**. ChIP data were normalized to and expressed as percent of input.

Table 2.14: qRT-PCR primers used to quantitate ChIP-enriched DNA.

Primers	Sequence (5’ to 3’)
m-Irf7 Enhancer Forward	CCCTAAAGGTCTACCCACTGC
m-Irf7 Enhancer Reverse	CTCCACAGTCAAGGGTTGTGT

2.15 LPS challenge septic shock

Mice were injected i.p. with LPS from *Escherichia coli* (0111:B4) in sterile PBS (30 μg/g body weight). Serum was obtained via retro-orbital bleeding after 3h, and stored at -80°C until analysis by ELISA. In separate experiments, survival was monitored for 72 h thereafter. Six to 8-week-old gender- and age-matched mice were used in all experiments.

2.16 Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, California, USA). Student’s t test or One-way ANOVA or Paired t tests were used as indicated in the figure legends to determine differences in mean values between groups. Results are expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), as indicated in the figure legends. $P<0.05$ was considered significant.

3 RESULTS (CHAPTER 1)

IRF7 is a novel cell type-specific transcription factor regulating TLR4-mediated IFN-β induction in macrophages but not DCs

3.1 Macrophages exhibit more robust IFN-β induction and stronger type I IFN responses to LPS exposure compared with DCs

Production of type I IFNs by macrophages and DCs following endotoxin exposure is a key component of host homeostasis and critical regulator of inflammatory responses to Gram-negative bacterial infection. We previously reported more robust and rapid induction of IFN-β in primary human blood monocytes after pathogenic stimulation compared with non-myeloid cell types [109]. In order to utilize a systematic genetic loss-of-function approach to compare and unravel the gene regulatory network controlling LPS-mediated IFN-β induction in myeloid cell types, we decided to use primary murine BMDMs and BMDCs as a model to compare and study the molecular mechanisms underlying the activation and regulation of IFN-β in macrophages and DCs. We first stimulated wild-type BMDMs with LPS, and profiled the magnitude and kinetics of IFN-β induction. In agreement with previous studies [53, 89, 90], LPS elicited rapid yet transient IFN-β expression in BMDMs (**Figure 3.1**), whereas IFN-α secretion was not detected in the cell culture supernatants at various time points (data not shown), in line with the findings of other studies that IFN-α is not produced following *in vitro* LPS stimulation of macrophages, and confirming that IFN-β is the primary type I IFN produced by macrophages in response to LPS [87, 236]. LPS-stimulated IFN-β transcription was up-regulated as early as 30 min post-stimulation, and peaked at 1–2 h, followed by a rapid decline in IFN-β mRNA levels by 6 h (**Figure 3.1A**). LPS-stimulated IFN-β protein secretion was detected as early as 1 h post-stimulation, and peaked at 2–6 h, followed by a rapid decline in IFN-β cytokine output by 24 h (**Figure 3.1B**).

TLR4 engagement by LPS can also induce expression of IFN-β in DCs [237, 238], hence we also stimulated wild-type BMDCs with LPS, and profiled the magnitude and kinetics of IFN-β induction. Like BMDMs, and in agreement with previous studies [237, 238], LPS also elicited rapid yet transient IFN-β expression in BMDCs (**Figure**

3.2). LPS-stimulated IFN-β transcription and protein secretion in BMDCs displayed similar kinetics to that in BMDMs, in which IFN-β mRNA levels peaked at 2 h post-stimulation, and declined by 6 h (**Figure 3.2A**), and IFN-β cytokine output peaked at 6 h post-stimulation, and declined by 24 h (**Figure 3.2B**).

However, unlike BMDMs, BMDCs appear to exhibit less robust up-regulation of IFN-β transcription (**Figure 3.1A and 3.2A**) and protein secretion (**Figure 3.1B and 3.2B**). To confirm these observations, we stimulated wild-type BMDMs and BMDCs with LPS simultaneously, and directly compared the magnitude of IFN-β induction. LPS-stimulated production of IFN-β was lower in BMDCs compared with BMDMs (**Figure 3.3**). *Ifnb1* mRNA levels (relative to *Gapdh*) were lower in BMDCs (right axis) compared with BMDMs (left axis), in the range of ~100-fold less (**Figure 3.3A and Figure S1 in Appendix A**). Accordingly, lower amounts of IFN-β cytokine was also detected in the cell culture supernatants of BMDCs compared with BMDMs (**Figure 3.3B**). This was reflected in the reduced levels of LPS-induced STAT1 phosphorylation, which is a downstream indicator of type I IFN receptor signaling, in BMDCs compared with BMDMs (**Figure 3.3C and Figure S2 in Appendix B**). Additionally, it was also noted that (i) a small amount of basal STAT1 phosphorylation was seen in unstimulated BMDMs but not in unstimulated BMDCs (**Figure 3.3C and Figure S2 in Appendix B**), and (ii) the levels of total STAT1 protein expression were lower in BMDCs compared with BMDMs, both before and after LPS stimulation (**Figure 3.3C and Figure S2 in Appendix B**). Consistent with the reduced induction of IFN-β and phosphorylation of STAT1, mRNA levels (relative to *Gapdh*) of downstream type I IFN-dependent chemokine genes, such as *Ccl5* and *Cxcl10*, were lower in BMDCs compared with BMDMs (**Figure 3.3D**). Taken together, these results indicate that while BMDMs and BMDCs respond to the same dose of LPS with similar kinetics of IFN-β production, BMDMs show a higher magnitude of IFN-β induction and a more robust type I IFN response to LPS exposure compared with BMDCs (**Figure 3.4**).

3.2 Autocrine/paracrine type I IFN signaling amplifies the IFN-β response in endotoxin-challenged macrophages but not DCs

In the "classical pathway" of virus-mediated IFN-α/β gene induction operational in most cell types, such as fibroblasts and conventional DCs, an

BMDM

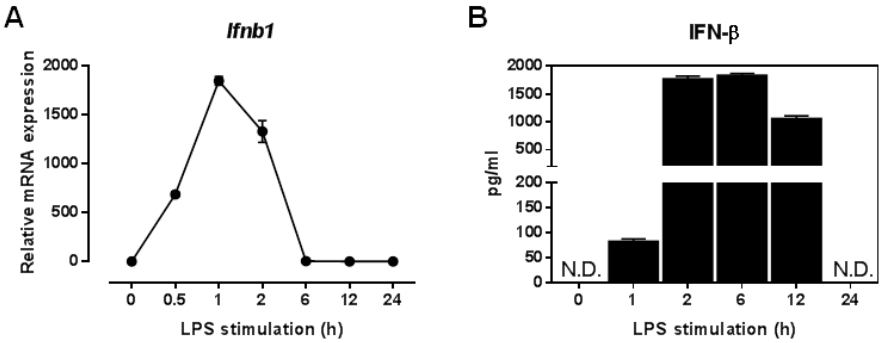

Figure 3.1
IFN-β responses are rapidly and transiently induced by LPS exposure in BMDMs.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of wild-type BMDMs, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments. N.D.: not detected.

BMDC

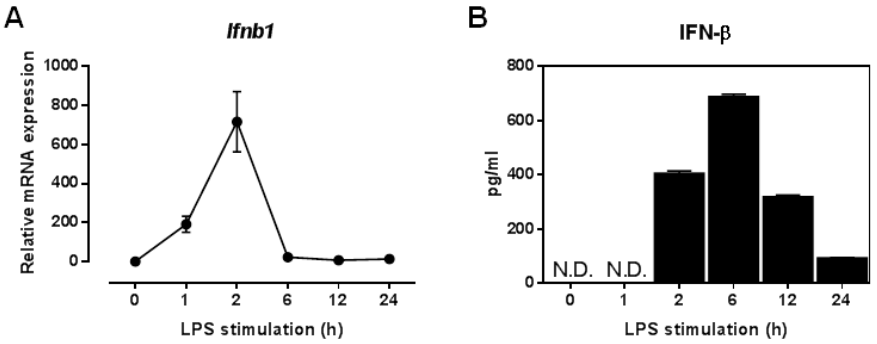

Figure 3.2
IFN-β responses are rapidly and transiently induced by LPS exposure in BMDCs.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of wild-type BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments. N.D.: not detected.

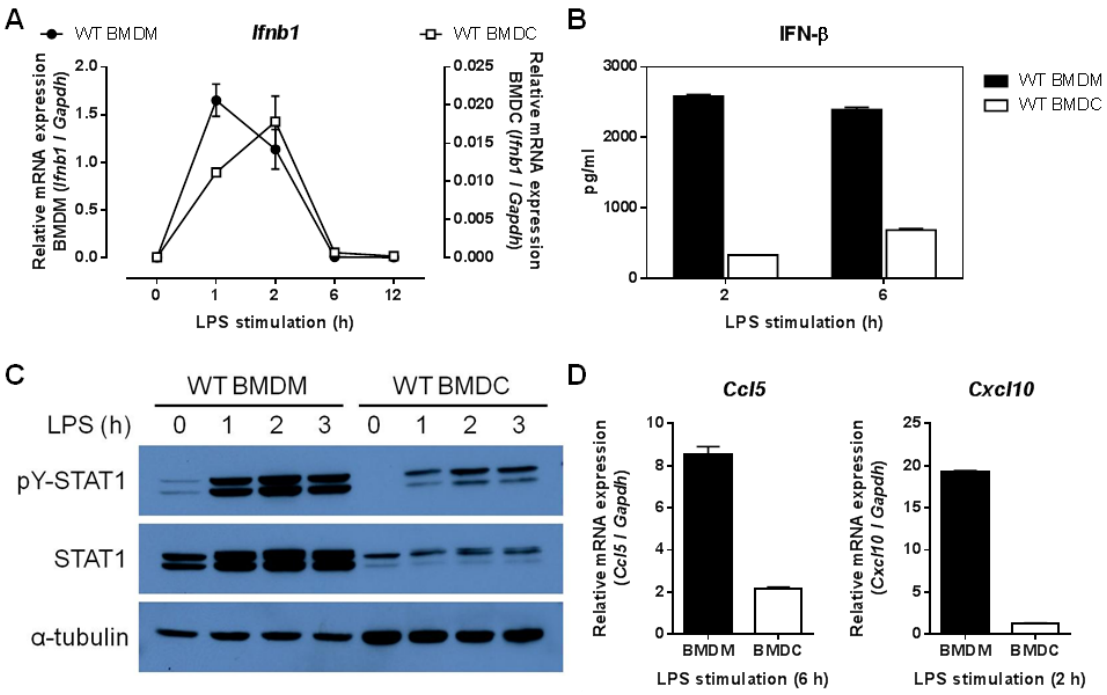

Figure 3.3
DCs exhibit poorer IFN-β induction and weaker type I IFN responses to LPS exposure compared with macrophages.
(A, B) Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* (left axis: $2^{-\Delta CT}$ in BMDMs; right axis: $2^{-\Delta CT}$ in BMDCs). Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.
(C) Western immunoblot analysis of phospho-STAT1 (pY-STAT1) and total STAT1 protein expression in whole cell lysates of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. α-tubulin was used as a loading control. Data are representative of at least two independent experiments.
(D) Real-time PCR analysis of *Ccl5* and *Cxcl10* gene expression in wild-type BMDMs and BMDCs, stimulated with 100ng/ml LPS for 2 h (*Cxcl10*) or 6 h (*Ccl5*). Gene expression was normalized to *Gapdh* and expressed as $2^{-\Delta CT}$ relative to *Gapdh*. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.

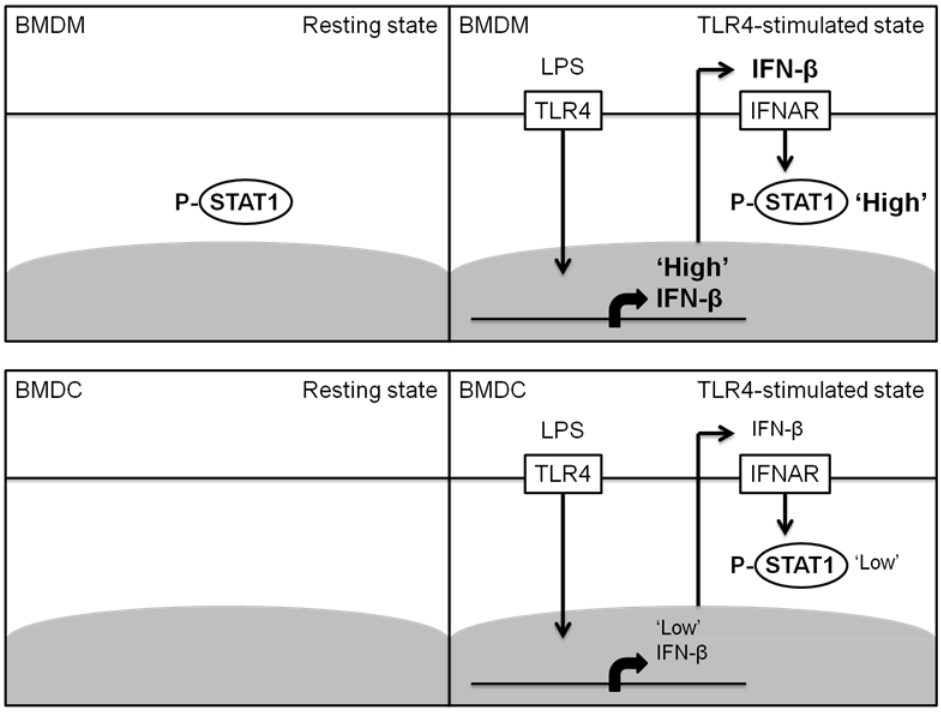

Figure 3.4
Macrophages exhibit more robust IFN-β induction and stronger type I IFN responses to LPS exposure compared with DCs.
BMDMs exhibit more robust up-regulation of LPS-induced IFN-β transcription and secretion relative to BMDCs, resulting in stronger LPS-induced STAT1 phosphorylation in BMDMs. In addition, constitutive STAT1 phosphorylation was detected in un-stimulated BMDMs but not in un-stimulated BMDCs.

autocrine/paracrine type I IFN positive-feedback loop amplifies the second phase of IFN-α/β induction [70, 239]. In macrophages, LPS stimulation induces the primary response gene IFN-β, and autocrine/paracrine type I IFN signaling then activates the expression of secondary response genes, and pro-inflammatory cytokines and chemokines, such as iNOS and CXCL10/IP-10 [37, 89, 90]. However, it is less clear whether LPS-stimulated macrophages require autocrine/paracrine type I IFN signaling for the amplification of IFN-β gene expression. On the one hand, upon stimulation with lipid A, BMDMs deficient in the IFN-α/β receptor (IFNAR) exhibit comparable levels of IFN-β expression to wild-type BMDMs [91]. On the other hand, Tyk2-deficient peritoneal macrophages exhibit reduced IFN-β transcription in response to LPS, suggesting that the adaptor molecule Tyk2 acts downstream of IFNAR in support of the IFN-β response [53]. However, in addition to IFNAR, Tyk2 signal transduction occurs downstream of multiple different cytokine receptors, including IL-6, IL-10, IL-12, IL-13, IL-23 and IL-27 receptors [92, 93]. From these seemingly contradictory reports, together with the fact that Tyk2 is not specific to type I IFN signaling, the mechanism and potential significance of IFN-β autocrine/paracrine amplification in macrophages remain controversial and equivocal. We therefore hypothesized whether differential involvement of autocrine/paracrine type I IFN signaling in macrophages versus DCs could contribute to the characteristically robust type I IFN response observed in LPS-stimulated macrophages versus DCs.

To better define the role played by IFN-β autocrine/paracrine signaling in mediating the macrophage type I IFN response, we analyzed IFN-β mRNA induction and protein secretion using BMDMs and BMDCs derived from IFNAR1-knockout mice. IFNAR1 deletion was verified by stimulating IFNAR1-knockout BMDMs with LPS. LPS-induced STAT1 phosphorylation was completely abolished in IFNAR1-knockout BMDMs compared with wild-type control (**Figure 3.5**), confirming the lack of type I IFN signaling in these cells. Additionally, total STAT1 protein expression was attenuated in IFNAR1-knockout BMDMs compared with wild-type control, both before and after LPS stimulation (**Figure 3.5**), consistent with previous reports that type I IFN signaling is required to maintain appropriate expression of key signaling intermediaries including STAT1 [72, 87, 90].

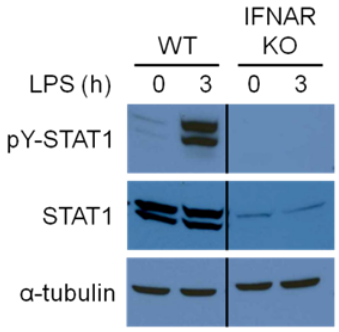

Figure 3.5
IFNAR1 deletion was verified by lack of LPS-induced STAT1 phosphorylation in IFNAR1 knockout BMDMs.
Western immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in whole cell lysates of wild-type and IFNAR1 knockout BMDMs, stimulated or not with 100ng/ml LPS for 3 h. α-tubulin was used as a loading control.

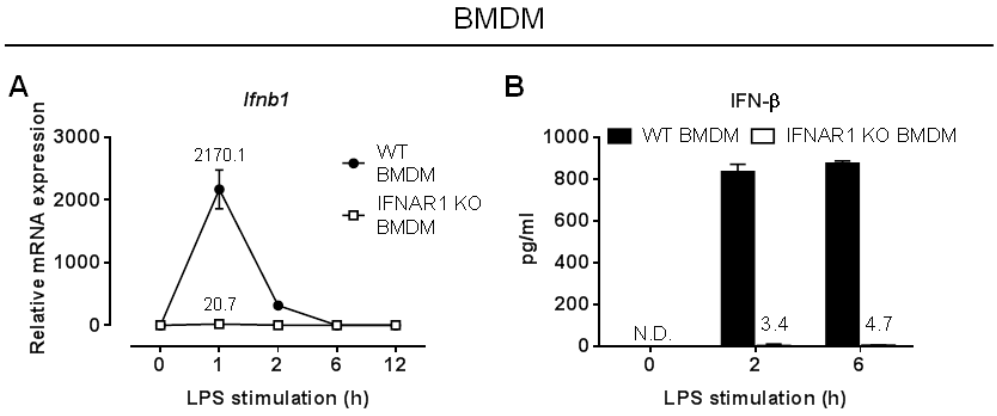

Figure 3.6
Autocrine/paracrine type I IFN signaling amplifies IFN-β gene and protein expression in endotoxin-challenged macrophages.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IFNAR1 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments. N.D.: not detected.

We observed that IFNAR1-deficient BMDMs displayed negligible expression of IFN-β mRNA or protein in response to LPS, whereas wild-type BMDMs exhibited robust IFN-β expression within just 2 h of endotoxin exposure (**Figure 3.6**). These data contrasted with the autocrine amplification of type I IFN responses in virus-challenged fibroblasts and DCs, which occurs only at late time points (> 6 h) after infection [70, 115]. Since we observed that type I IFN signaling is essential for enhanced IFN-β production in LPS-stimulated macrophages, we wondered whether treatment with recombinant IFN-β alone is able to induce IFN-β transcription in the absence of LPS stimulation. While LPS stimulation induced macrophage accumulation of IFN-β mRNA within 2 h, the addition of exogenous IFN-β in the absence of LPS failed to substantially increase IFN-β transcription (**Figure 3.7**), suggesting that both type I IFN signaling and TLR4 activation together are required to amplify IFN-β responses in macrophages. In contrast, IFN-β induction in LPS-stimulated BMDCs was independent of autocrine/paracrine type I IFN signaling (**Figure 3.8**), in agreement with a previous report [237]. Together, these data suggest that the TLR4-induced IFN-β response in macrophages and DCs show differential dependence on autocrine/paracrine type I IFN signaling: autocrine/paracrine type I IFN signaling enhance the IFN-β response to LPS in macrophages but not DCs, and therefore is a cell-type specific positive regulator of IFN-β production in macrophages but not DCs.

3.3 Regulation of the macrophage TLR4-induced IFN-β response by autocrine/paracrine type I IFN signaling is not mediated via the canonical TRIF-IRF3 pathway

Production of type I IFNs in response to bacterial LPS is presently known to be mediated via the TRIF-dependent pathway involving the activation of IRF3 phosphorylation, dimerization, and nuclear translocation [28, 37, 50, 88]. Since macrophage TLR4-mediated IFN-β induction is regulated by autocrine/paracrine type I IFN signaling (**Figure 3.6**), we explored the possibility that this could be due to modulation of the canonical TRIF-IRF3 pathway by autocrine/paracrine type I IFN signaling. To investigate this possibility, we assessed LPS-induced IRF3 phosphorylation and nuclear translocation in IFNAR1-deficient BMDMs. Analysis of total lysates showed that total IRF3 protein expression was essentially normal in IFNAR1-null macrophages. Importantly, we observed significant levels of IRF3

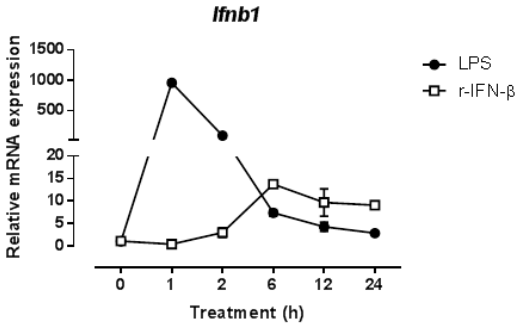

Figure 3.7
Macrophage IFN-β responses are rapidly and transiently induced by LPS exposure but not by exogenous IFN-β cytokine alone.
Analysis of IFN-β gene expression by real-time PCR analysis of wild-type BMDMs, stimulated or not with either 100ng/ml LPS or 250U/ml recombinant murine IFN-β for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated cells of the respective treatments. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

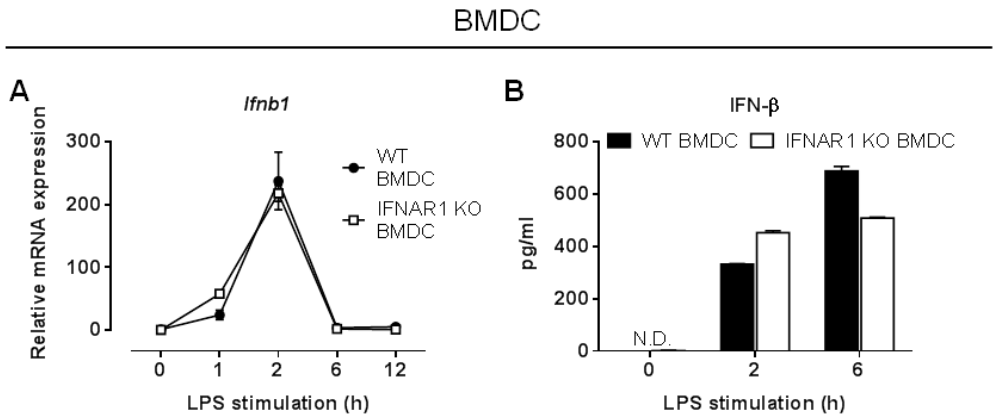

Figure 3.8
Autocrine/paracrine type I IFN signaling does not regulate IFN-β gene and protein expression in endotoxin-challenged DCs.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDCs from IFNAR1 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

phosphorylation in LPS-stimulated IFNAR1-knockout BMDMs comparable to the levels seen in wild-type BMDMs (**Figure 3.9A**). Analysis of nuclear extracts showed that there is a constant pool of unphosphorylated IRF3 present in the nucleus of both unstimulated and LPS-stimulated BMDMs, and it is the phosphorylated form of IRF3 that translocates into the nucleus upon LPS stimulation. Notably, IRF3 nuclear translocation was largely unaltered in LPS-stimulated IFNAR1-knockout BMDMs compared with wild-type BMDMs (**Figure 3.9B**). Purity of nuclear fractionation was confirmed by significant amounts of TATA-binding protein (TBP) (nuclear protein) and trace amounts of α-tubulin (cytosolic protein) in nuclear extracts (**Figure 3.9B**). As expected, IRF3 phosphorylation and nuclear translocation were undetectable in LPS-stimulated TRIF-knockout BMDMs, which served as a positive control (**Figure 3.9**). Thus, despite normal activation of the TRIF-IRF3 pathway, LPS-stimulated IFN-β expression is significantly impaired in BMDMs with defective type I IFN signaling, implying that type I IFN signaling regulates the macrophage TLR4-induced IFN-β response independent of the TRIF-IRF3 pathway.

3.4 Autocrine/paracrine type I IFN signaling through the ISGF3 complex amplifies the IFN-β response in endotoxin-challenged macrophages

In order to identify the molecular mechanisms and transcription factors mediating the regulation of the IFN-β response by autocrine/paracrine type I IFN signaling, we investigated the effects of downstream type I IFN signaling adaptor molecules on LPS-stimulated macrophages. Type I IFN signaling following IFNAR engagement is mediated by (i) the heterotrimeric IFN-stimulated gene factor 3 (ISGF3) complex, comprising STAT1-STAT2-IRF9, which activate the IFN-stimulated response element (ISRE) of downstream ISGs, (ii) the IFN-γ-activated factor (GAF) complex, comprising STAT1-STAT1 homodimers, which activate the IFN-γ-activated site (GAS) of downstream ISGs, or (iii) STAT3 homodimers in other contexts [84, 240]. Hence, we analyzed IFN-β mRNA induction and protein secretion using BMDMs derived from STAT1-knockout, STAT2 hypomorphic mutant, and STAT3 knockout mice. Respective STAT protein deletion was verified by Western blotting with the respective specific antibodies (**Figure 3.10**).

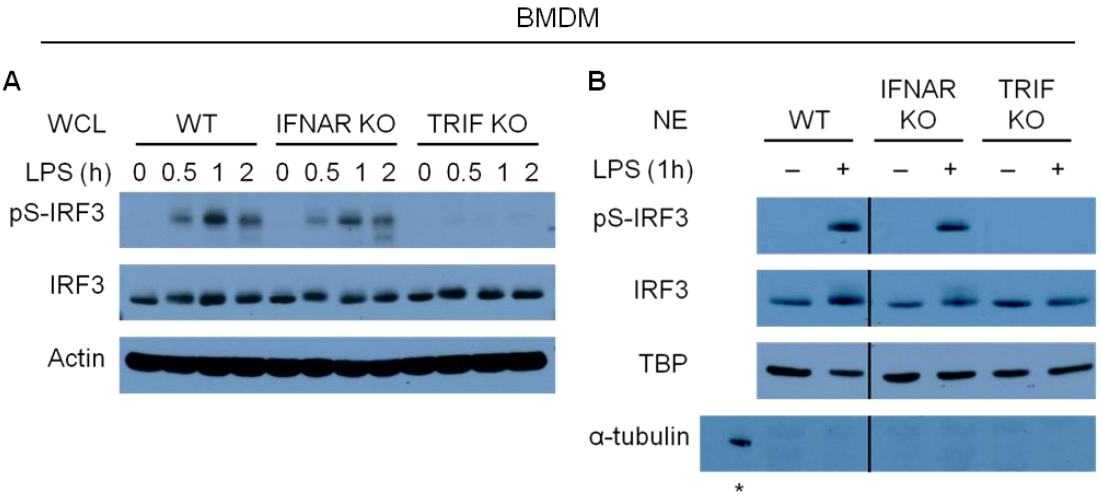

Figure 3.9
Autocrine/paracrine type I IFN signaling does not regulate IFN-β expression via the TRIF-IRF3 pathway in endotoxin-challenged macrophages.
Western immunoblot analysis of phospho-IRF3 and total IRF3 protein expression in whole cell lysates (WCL) (A) or nuclear extracts (NE) (B) of wild-type (WT), IFNAR1 knockout, and TRIF knockout BMDMs, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments. Actin and TATA-binding protein (TBP) were used as loading controls. * indicates α-tubulin detected in WCL of un-stimulated WT BMDMs as a control.

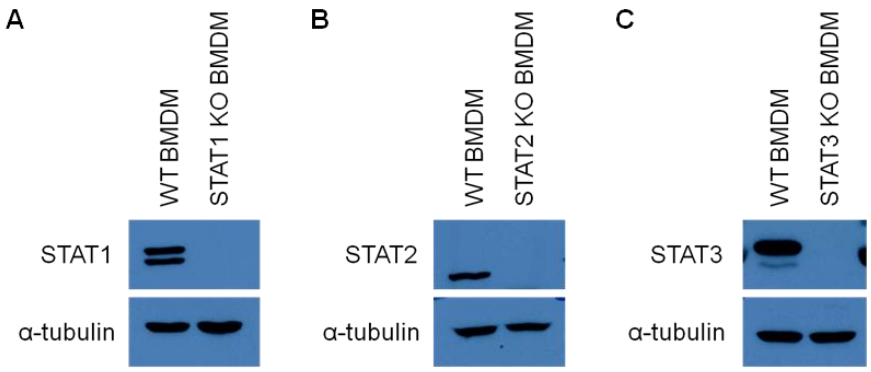

Figure 3.10
STAT1, STAT2, and STAT3 deletion were verified by lack of respective STAT proteins in respective knockout BMDMs.
Western immunoblot analysis of total STAT1, STAT2, or STAT3 protein expression in whole cell lysates of un-stimulated wild-type (WT) and STAT1 knockout, STAT2 hypomorphic mutant, or STAT3 knockout BMDMs.

We observed that LPS-stimulated IFN-β expression was almost entirely abrogated in STAT1-deficient BMDMs (**Figure 3.11A, B**), supporting the concept that autocrine/paracrine type I IFN signaling requires STAT1-containing transcription factor complexes to enhance IFN-β responses in LPS-stimulated macrophages. LPS-stimulated IFN-β expression was similarly ablated in STAT2-mutant BMDMs (**Figure 3.11C, D**), implying that type I IFN signaling, at least in part mediated via ISGF3 complex activation, amplifies IFN-β production upon LPS stimulation of macrophages. In contrast, we observed that IFN-β expression in response to LPS was instead increased in STAT3-knockout BMDMs compared with wild-type BMDMs (**Figure 3.11E, F**), indicating that STAT3 does not play a role in IFN-β induction upon bacterial perturbation. Taken together, examination of the mechanistic basis of the type I IFN signaling that is required for rapid and robust IFN-β induction revealed that IFN-β amplification in TLR4-activated macrophages is driven by a target molecule of which the expression or function depends on both STAT1 and STAT2, but not STAT3.

3.5 Maximal IFN-β expression in endotoxin-challenged macrophages does not depend on IRF1

IRF1 is one of the target molecules known to be regulated by STAT1 homodimers or STAT1-STAT2 heterodimers [240, 241]. It is known to be essential for IFN-β induction in CpG DNA-stimulated myeloid DCs [16, 74], and in TNF-stimulated monocytes and macrophages [242], but its involvement in IFN-β production in LPS-stimulated macrophages is less clear. Thus, we investigated whether IRF1 is necessary for LPS-induced IFN-β production in macrophages. We analyzed IFN-β mRNA induction and protein secretion using BMDMs derived from IRF1-knockout mice, and observed that IFN-β expression was largely unchanged compared with wild-type BMDMs after LPS stimulation (**Figure 3.12**), indicating that IRF1 is not critical for IFN-β transcriptional induction following LPS stimulation in macrophages.

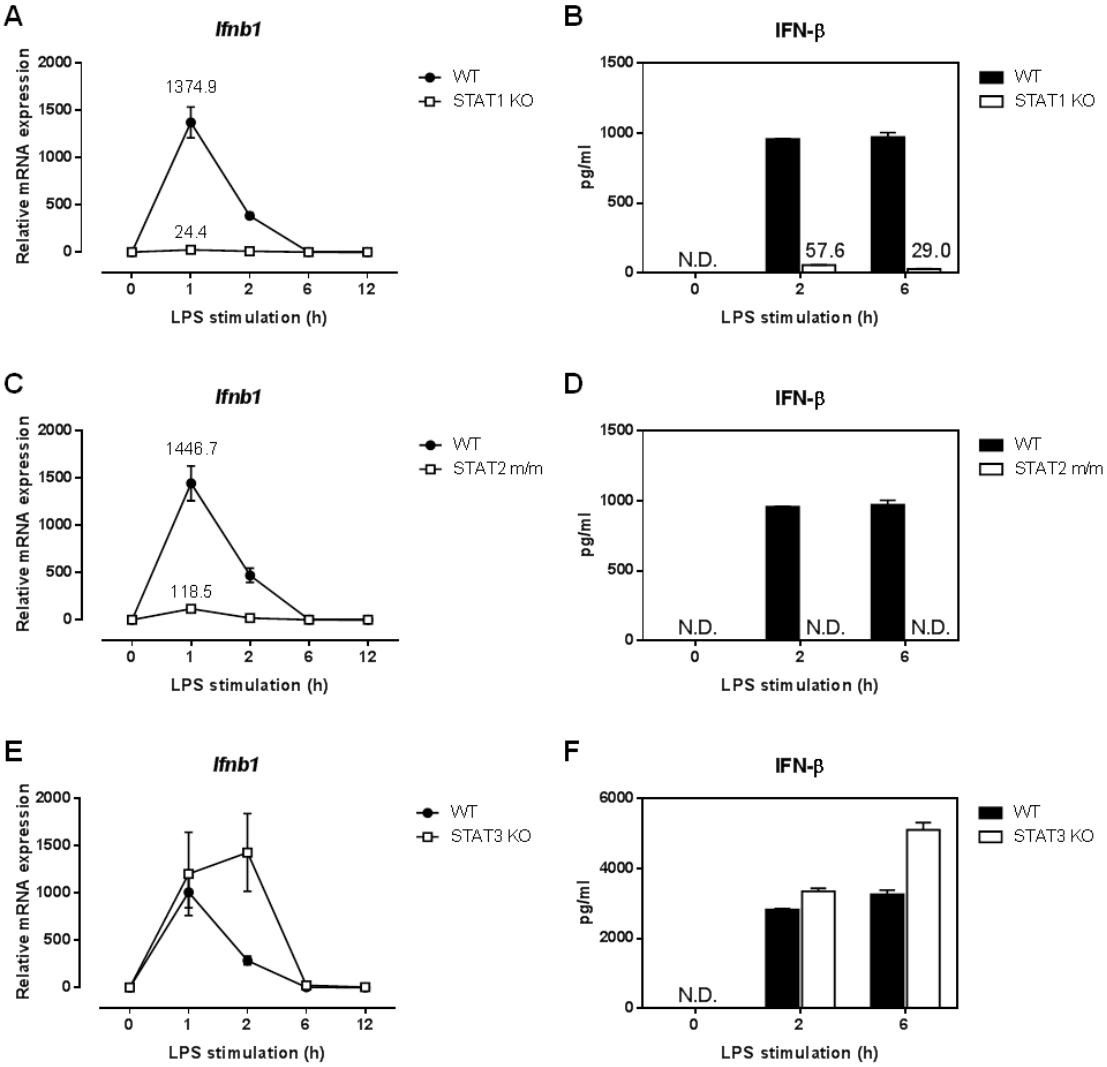

Figure 3.11
Autocrine/paracrine type I IFN signaling via ISGF3, but not STAT3, amplifies IFN-β gene and protein expression in endotoxin-challenged macrophages.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDMs from STAT1 knockout mice (A, B), STAT2 mutant mice (C, D), and STAT3 knockout mice (E, F), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

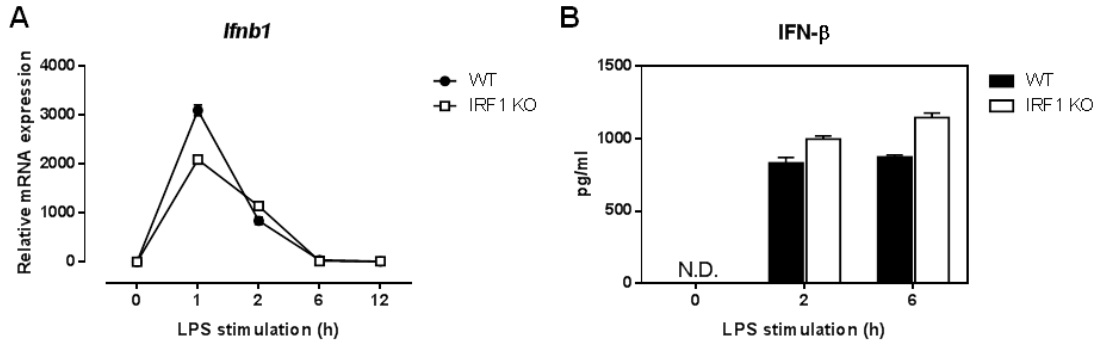

Figure 3.12
Maximal IFN-β expression in endotoxin-challenged macrophages does not depend on IRF1.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IRF1 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

3.6 IRF7 and IRF3 act in concert to induce maximal IFN-β expression and downstream type I IFN responses in LPS-stimulated macrophages

Apart from IRF3, IRF7 is another target molecule known to be regulated by ISGF3 complex activation [240]. It is widely regarded as the 'master regulator' of type I IFN responses in viral infection [16, 35, 75]. It is also essential for CpG-DNA-stimulated IFN-β induction in plasmacytoid DCs [16, 243], but is not required for LPS-stimulated IFN-β responses in conventional DCs [35]. Consequently, IFN-β expression in TLR4-activated macrophages and DCs is thought to be mediated by IRF3 alone [244]. Nevertheless, we asked whether IRF7 is required for LPS-stimulated IFN-β induction in macrophages by analyzing IFN-β mRNA induction and protein secretion using BMDMs derived from IRF7-knockout mice, in conjunction with BMDMs derived from IRF3-knockout mice as a positive control.

Unexpectedly, we observed that LPS-induced IFN-β expression was impaired in IRF7-null BMDMs compared with wild-type cells (**Figure 3.13A, B**), analogous to the expected defect in IRF3-null BMDMs (**Figure 3.13C, D**). To confirm these findings, we measured STAT1 activation and ISG induction downstream of IFNAR signaling. Consistent with the impaired induction of IFN-β, LPS-stimulated phosphorylation of STAT1 and expression of downstream type I IFN-dependent chemokine genes, such as *Ccl5*, *Cxcl10*, and *Cxcl11*, were substantially decreased in IRF7-null BMDMs relative to wild-type cells (**Figure 3.14A, B**), similar to that seen in IRF3-null BMDMs (**Figure 3.14C, D**). Together, these data suggest that IRF7 is a novel transcription factor that regulates TLR4-mediated IFN-β induction in macrophages, through an IFNAR-ISGF3-IRF7 signaling axis. However, it is notable that IFN-β expression is not totally obliterated in IRF7- and IRF3-null BMDMs. Residual IFN-β transcription can be induced and small amounts of IFN-β secretion can be detected in the cell culture supernatants upon LPS stimulation of IRF7- and IRF3-null BMDMs (**Figure 3.13**). This is manifested in the slight levels of STAT1 phosphorylation detected in IRF7- and IRF3-null BMDMs post-stimulation (**Figure 3.14A, C**).

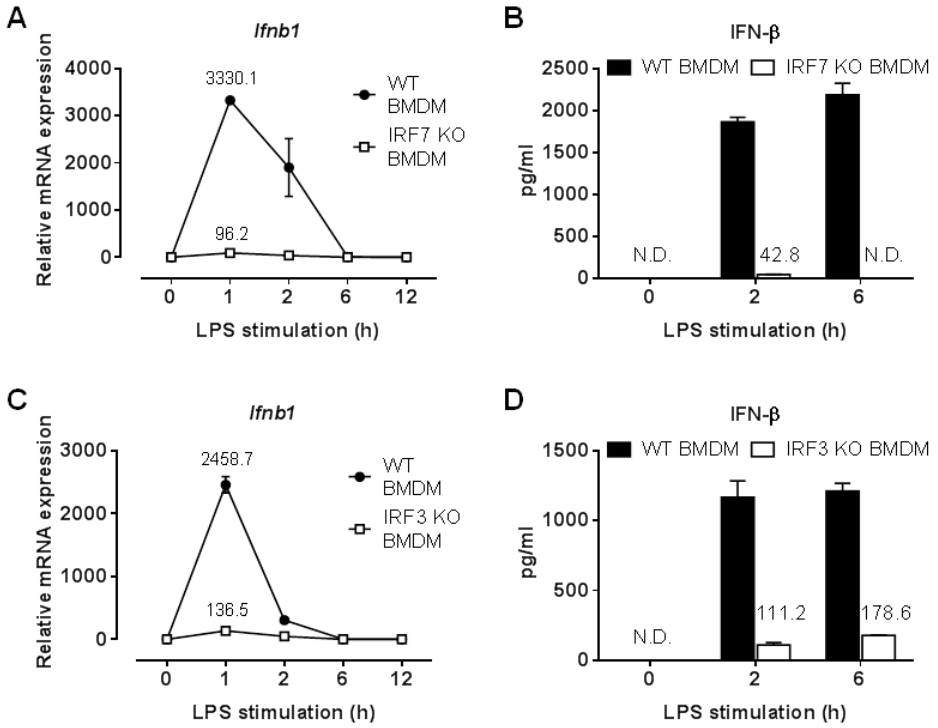

Figure 3.13
Maximal IFN-β expression in endotoxin-challenged macrophages depends on IRF7 and IRF3.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IRF7 knockout mice (A, B), and IRF3 knockout mice (C, D), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments. N.D.: not detected.

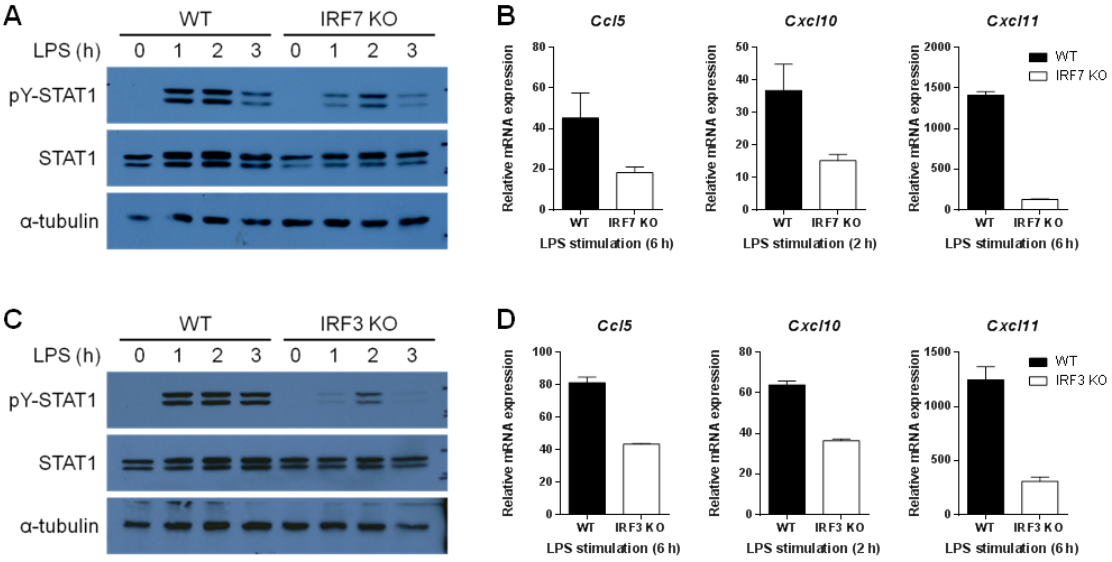

Figure 3.14
STAT1 activation and transcription of IFN-inducible genes are impaired in IRF7- or IRF3-deficient BMDMs.
(A, C) Western immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in whole cell lysates of BMDMs from IRF7 knockout mice (A), and IRF3 knockout mice (B), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.
(B, D) Real-time PCR analysis of *Ccl5*, *Cxcl10* and *Cxcl11* gene expression in BMDMs from IRF7 knockout mice (B), and IRF3 knockout mice (D), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for 2 h (peak level of induction of *Cxcl10*) or 6 h (peak level of induction of *Ccl5* and *Cxcl11*). Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

3.7 Regulation of the macrophage TLR4-induced IFN-β response by IRF7 is not mediated via the canonical TRIF-IRF3 pathway

Since macrophage TLR4-mediated IFN-β induction is regulated by IRF7 (**Figure 3.13A, B**), we again explored whether this could be due to modulation of the canonical TRIF-IRF3 pathway by IRF7. To investigate this possibility, we assessed LPS-induced IRF3 phosphorylation and nuclear translocation in IRF7-deficient BMDMs. Analysis of total lysates showed that total IRF3 protein expression was essentially normal in IRF7-null macrophages (**Figure 3.15A**), and significant levels of IRF3 phosphorylation similar to wild-type levels were observed in LPS-stimulated IRF7-knockout BMDMs (**Figure 3.15A**). Analysis of nuclear extracts showed that IRF3 nuclear translocation was largely unaltered in LPS-stimulated IRF7-knockout BMDMs compared with wild-type BMDMs (**Figure 3.15B**). Purity of nuclear fractionation was confirmed by significant amounts of TATA-binding protein (TBP) (nuclear protein) and trace amounts of α-tubulin (cytosolic protein) in nuclear extracts (**Figure 3.15B**). As a positive control, TRIF-knockout BMDMs showed undetectable levels of IRF3 phosphorylation and nuclear translocation (**Figure 3.15**). Thus, although the TRIF-IRF3 pathway is still operational in BMDMs deficient in IRF7, LPS-stimulated IFN-β expression is significantly impaired in these cells. Taken together with our finding that IRF7 levels are largely unaltered in IRF3-null macrophages (**Figure 3.16**), our results suggest that either IRF3 alone or IRF7 alone is not sufficient for maximal TLR4-mediated IFN-β production in macrophages, indicating a cooperative or synergistic requirement for these two transcription factors. These data reveal that IRF7 and IRF3 are both required in combination to achieve maximal IFN-β production in endotoxin-challenged macrophages (**Figure 3.17**).

3.8 Evidence of IRF7 nuclear translocation in response to LPS

Since we identified IRF7 as a novel transcription factor that regulates TLR4-mediated IFN-β induction in macrophages, we asked whether IRF7 is activated in a similar way as IRF3 after LPS stimulation. First, we investigated whether IRF7 is phosphorylated following LPS stimulation of wild-type macrophages. We assessed LPS-induced IRF7 phosphorylation in wild-type BMDMs, together with IRF7-deficient BMDMs as a negative control, using two commercially available antibodies against

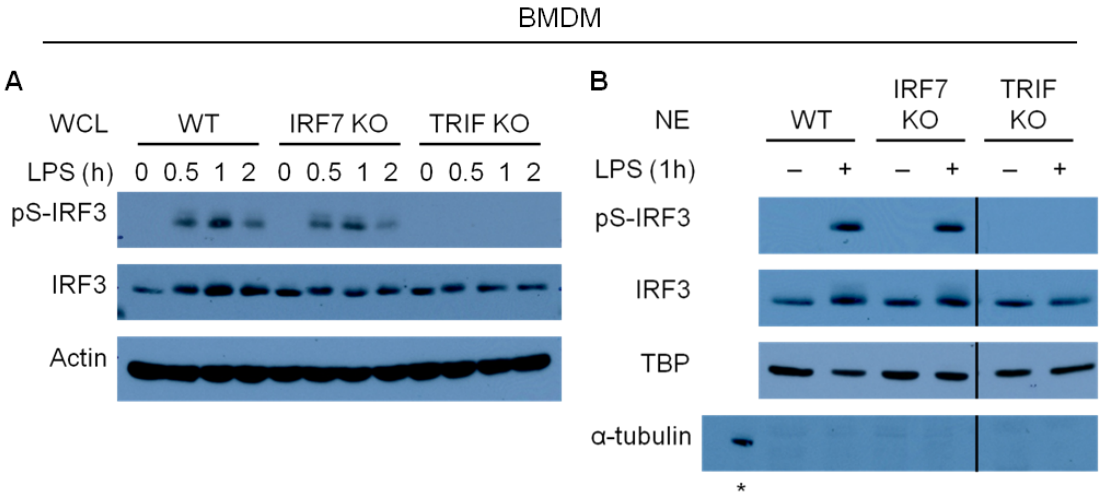

Figure 3.15
IRF7 does not regulate IFN-β expression via the TRIF-IRF3 pathway in endotoxin-challenged macrophages.
Western immunoblot analysis of phospho-IRF3 and total IRF3 protein expression in whole cell lysates (WCL) (A) or nuclear extracts (NE) (B) of wild-type (WT), IRF7 knockout, and TRIF knockout BMDMs, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments. Actin and TATA-binding protein (TBP) were used as loading controls. * indicates α-tubulin detected in WCL of un-stimulated WT BMDMs as a control.

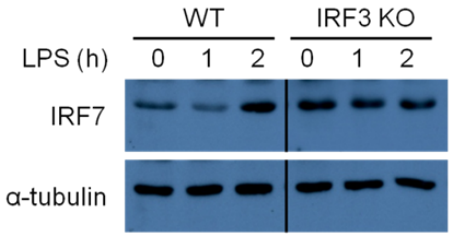

Figure 3.16
IRF7 expression is essentially normal in IRF3-null macrophages.
Western immunoblot analysis of total IRF7 protein expression in whole cell lysates of wild-type and IRF3 knockout BMDMs, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.

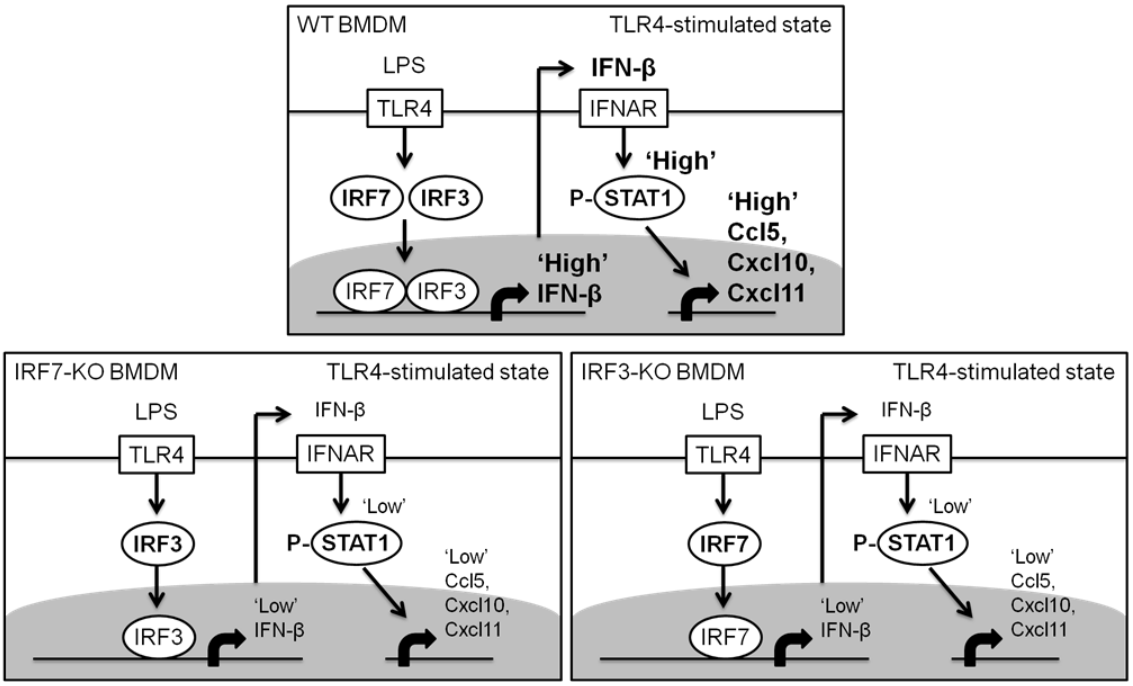

Figure 3.17
IRF7- or IRF3-deficient BMDMs exhibit defective IFN-β induction associated with impaired STAT1 activation and transcription of IFN-inducible genes.
IRF7 knockout BMDMs and IRF3 knockout BMDMs exhibit reduced LPS-induced IFN-β transcription and secretion relative to wild-type BMDMs, resulting in reduced LPS-induced STAT1 phosphorylation and transcription of downstream type I IFN-dependent chemokine genes compared with wild-type BMDMs.

phospho-IRF7. However, both of the tested antibodies did not give a specific band of the correct predicted band size (~ 54 kDa) that is present in wild-type BMDM lysate but absent in IRF7-knockout BMDM lysate (**Figure 3.18**), in contrast to the antibody against total IRF7. Thus, lack of an appropriate antibody suitable for detection of endogenous phospho-IRF7 protein by Western blotting precluded any conclusions about whether IRF7 is phosphorylated in macrophages in response to LPS exposure. Other reagents or approaches are required to investigate this question.

Next, we attempted to assess LPS-induced IRF7 nuclear translocation in wild-type BMDMs, together with IRF7-deficient BMDMs as a negative control, using the antibody against total IRF7. Analysis of nuclear extracts showed that there is a pool of total IRF7 present in the nucleus of unstimulated BMDMs (**Figure 3.19**), analogous to that observed for nuclear IRF3 (**Figure 3.15**). Upon LPS exposure, we observed an increase in the levels of nuclear IRF7 compared with unstimulated wild-type BMDMs (**Figure 3.19**), indicative of IRF7 nuclear translocation in response to LPS exposure. This band was specific because it is present in wild-type BMDM lysate but absent in IRF7-knockout BMDM lysate, and purity of nuclear fractionation was confirmed by significant amounts of TBP protein and trace amounts of α-tubulin protein (**Figure 3.19**). This increase in the levels of total IRF7 in the nucleus was slight, as is the case for total IRF3 (**Figure 3.15**), possibly because, like IRF3, there is a constant pool of unphosphorylated IRF7 present in the nucleus of both unstimulated and LPS-stimulated BMDMs, and it is the phosphorylated form of IRF7 that translocates into the nucleus upon LPS stimulation. Other reagents and tools for the detection of phospho-IRF7 would provide a more accurate assessment of nuclear translocation of phospho-IRF7, and clarify whether this nuclear translocation is dependent on its phosphorylation.

The noncanonical IκB kinase (IKK)-related kinases, TANK-binding kinase-1 (TBK1) and IκB kinase-ε (IKKε), have been reported to directly phosphorylate IRF3 downstream of TRIF in response to viral infection or TLR3 and TLR4 stimulation [245-248]. To address whether TBK1 is an upstream kinase that mediate IRF7 activation in TLR4 signaling, we measured LPS-induced IFN-β gene expression in IRF3 single knockout, IRF7 single knockout, and IRF3-IRF7 double knockout BMDMs, in the absence or presence of the TBK1 inhibitor BX795 (**Figure S3 in Appendix C**). IRF3 single knockout and IRF7 single knockout BMDMs exhibited residual IFN-β

transcription, presumably mediated by IRF7 and IRF3 respectively, compared with the complete abrogation of IFN-β transcription in IRF3-IRF7 double knockout BMDMs. As expected, BX795 further inhibited the IRF3-mediated residual IFN-β transcription in IRF7 knockout BMDMs, to levels seen in IRF3-IRF7 double knockout BMDMs. Interestingly, BX795 further inhibited the IRF7-mediated residual IFN-β transcription in IRF3 knockout BMDMs, to levels seen in IRF3-IRF7 double knockout BMDMs, suggesting that TBK1 also mediates IRF7 activation in TLR4 signaling. Additionally, BX795 inhibited IFN-β transcription in WT BMDMs to levels seen in IRF3-IRF7 double knockout BMDMs, and not just to levels seen in IRF3 knockout BMDMs, suggesting that TBK1 mediates the phosphorylation of both IRF3 and IRF7 in TLR4 signaling. Inhibition of IRF3 phosphorylation by BX795 was confirmed by Western blot (**Figure S3 in Appendix C**). However, we encountered technical difficulties in the direct analysis of IRF7 phosphorylation or nuclear translocation upon TBK1 inhibition (**Figure S3 in Appendix C**). Nevertheless, previous reports indicated that recombinant TBK1 [249], as well as TBK1 in whole cell extracts of LPS-stimulated primary human macrophages [247], can directly phosphorylate IRF7 in an *in vitro* kinase assay. Taken together with our data, these results indicate that TBK1 is an upstream kinase of both IRF3 and IRF7 in TLR4 signaling.

In pDCs, IRF7 activation was found to be mediated by IκB kinase-α (IKKα) downstream of MyD88 in response to TLR7/9 stimulation [250]. To address whether IKKα also mediates IRF7 activation in TLR4 signaling, we measured LPS-induced IFN-β gene expression in BMDMs from IKKα$^{AA/AA}$ knock-in mice, which express a mutant form of IKKα (Ser176 and Ser180 phosphorylation sites were replaced by Ala) that cannot be activated. LPS-induced IFN-β transcription was partially attenuated in IKKα$^{AA/AA}$ BMDMs compared with WT BMDMs, without affecting constitutive IFN-β production and basal IRF7 expression (**Figure S4 in Appendix D**). Early expression of downstream type I IFN-dependent chemokine genes, such as *Ccl5* and *Cxcl10* at 2 h post-stimulation, were also decreased in IKKα$^{AA/AA}$ BMDMs compared with WT BMDMs (**Figure S4 in Appendix D**). Impaired IFN-β production in response to LPS was reported in IKKα$^{AA/AA}$ BMDCs compared with WT BMDCs [251]. However, it has been reported that IKKα may also regulate IRF3 activity downstream of IKKε/TBK1 by direct phosphorylation of IRF3 at Ser402/404/405 [251]. Hence, the contribution of IKKα to IRF7 activation vis-à-vis IRF3 activation remains to be investigated in future

studies, when reagents and tools for the reliable analysis of IRF7 phosphorylation and/or nuclear translocation become available.

In a human pDC cell line, IFN-β production was also found to be mediated by IκB kinase-β (IKKβ) in response to TLR7/9 stimulation [252]. To address whether IKKβ also mediates IRF7 activation in TLR4 signaling, we measured LPS-induced IFN-β gene expression in BMDMs from IKKβ knockout mice. Similar to IKKα$^{AA/AA}$ BMDMs, LPS-induced IFN-β transcription was partially attenuated in IKKβ knockout BMDMs compared with WT BMDMs (**Figure S5 in Appendix E**). Early expression of downstream type I IFN-dependent chemokine genes, such as *Ccl5* and *Cxcl10* at 2 h post-stimulation, were also decreased in IKKβ knockout BMDMs compared with WT BMDMs (**Figure S5 in Appendix E**). However, constitutive IFN-β production and basal IRF7 expression seem to be also impaired in IKKβ knockout BMDMs compared with WT BMDMs (**Figure S5 in Appendix E**). IKKβ was unable to phosphorylate IRF3 in an *in vitro* kinase assay [246]. Hence, the contribution of IKKβ to IRF7 activation vis-à-vis IRF7 expression remains to be investigated in future studies, when reagents and tools for the reliable analysis of IRF7 phosphorylation and/or nuclear translocation become available.

Taken together with the results of previous studies showing strong phosphorylation of IRF7 by TBK1 and much weaker phosphorylation of IRF7 by IKKβ in an *in vitro* kinase assay [252], our data suggest that TBK1 is a major upstream kinase that phosphorylate IRF7, while minor contributions from IKKα/β cannot be ruled out. The development and optimization of methods to analyze IRF7 activation would allow the determination of the relative contributions of TBK1 and IKKα/β in the activation of IRF7 in TLR4 signaling in future studies.

TLR4-induced IFN-β expression is currently thought to be mediated by IRF3 homodimer formation and binding to the IFN-β promoter [76, 253]. However, it has been reported that exogenous over-expression of IRF7 can activate IFN-β promoter reporter in response to LPS exposure, to a level higher than that of IRF3 alone or of co-transfection of both IRF3 and IRF7 [244]. Furthermore, pretreatment of IRF3-deficient BMDMs with exogenous IFN-β up-regulated IRF7 protein and increased IFN-β secretion to levels comparable to that of untreated wild-type macrophages (**Figure S6 in**

Appendix F), suggesting that increased levels of IRF7 homodimers can compensate for IRF3, and also efficiently transactivate the IFN-β gene upon LPS exposure, even in the absence of IRF3 homodimers and/or IRF3-IRF7 heterodimers. Therefore, the relative contributions of IRF3 homodimers, IRF3-IRF7 heterodimers and/or IRF7 homodimers in TLR4-induced IFN-β expression are far from straightforward. A series of approaches, such as dimer formation assays, electrophoretic mobility shift assays, and ChIP-ReChIP assays, which would require an extensive period of method development and optimization, could form the basis of future studies.

3.9 IRF7-mediated regulation of the macrophage TLR4-induced IFN-β response is independent of MyD88 and dependent on TRIF

Notwithstanding the mode of activation of IRF7 in LPS-stimulated macrophages, we next proceeded to investigate the upstream mechanisms regulating the activation of IRF7 in TLR4-induced macrophages. IRF7 has been established to be essential for CpG-DNA-induced IFN-β production in plasmacytoid DCs [16, 35, 76], in which IRF7 mediates robust type I IFN induction in response to virus infection and TLR7/9 activation by directly interacting with the MyD88 adaptor protein [243, 254]. However, production of type I IFNs in response to bacterial LPS is presently believed to be mediated via the MyD88-independent, i.e. TRIF-dependent pathway [28, 37, 50, 88]. In order to clarify whether MyD88 is involved in the signal transduction of IRF7-dependent IFN-β induction pathway in TLR4-activated macrophages (**Figure 3.13A, B**), we examined IFN-β mRNA induction and protein secretion using BMDMs derived from MyD88-knockout mice. In line with the anticipated phenotype of MyD88-knockout cells, pro-inflammatory cytokine gene expression, such as those of TNF-α and IL-6, were decreased compared with wild-type BMDMs after LPS stimulation (**Figure 3.20**), but IFN-β expression was not impaired in MyD88-deficient BMDMs compared with wild-type BMDMs after LPS stimulation (**Figure 3.21A, B**). Consistent with these data, Western blot analyses revealed that LPS-stimulated STAT1 phosphorylation was comparable in MyD88-null and wild-type BMDMs (**Figure 3.21C**). Taken together, these results indicate that, unlike the requirement for MyD88 in TLR7/9-activated pDCs, IFN-β amplification in TLR4-activated macrophages is dependent on IRF7 but independent of MyD88.

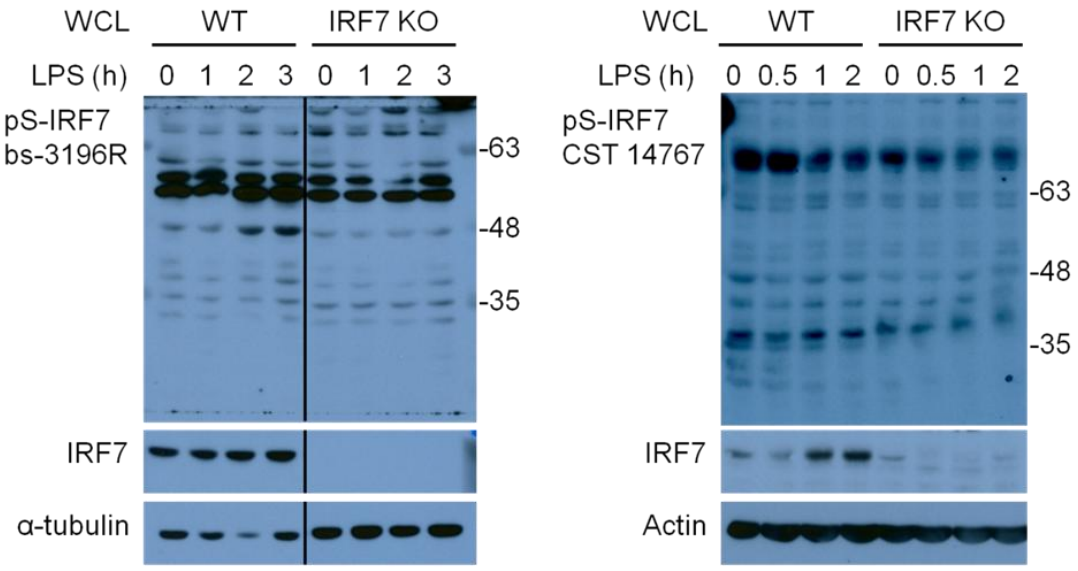

Figure 3.18
Screening of phospho-IRF7 antibodies for investigation of IRF7 phosphorylation using wild-type and IRF7 knockout BMDMs.
Western immunoblot analysis of phospho-IRF7 protein expression in whole cell lysates of wild-type and IRF7 knockout BMDMs, stimulated or not with 100ng/ml LPS for the indicated times. Two commercially available phospho-IRF7 antibodies were screened, but both were not specific for the detection of murine IRF7 protein.

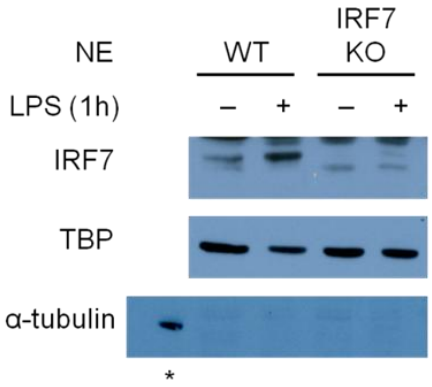

Figure 3.19
Evidence of IRF7 nuclear translocation in endotoxin-challenged macrophages.
Western immunoblot analysis of total IRF7 protein expression in nuclear extracts of wild-type and IRF7 knockout BMDMs, stimulated or not with 100ng/ml LPS for 1 h. Data are representative of at least two independent experiments. * indicates α-tubulin detected in whole cell lysates of un-stimulated wild-type BMDMs as a control.

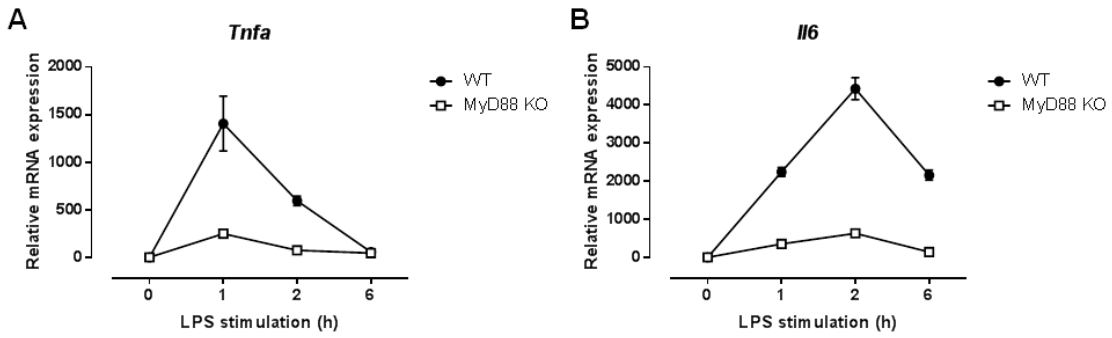

Figure 3.20
MyD88 deletion was verified by reduced transcription of pro-inflammatory cytokine genes in MyD88 knockout BMDMs.
Real-time PCR analysis of *Tnfa* and *Il6* gene expression in BMDMs from MyD88 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

Analysis of IFN-β mRNA induction and protein secretion using BMDMs derived from TRIF-knockout mice confirmed that IFN-β expression in TLR4-activated macrophages is completely dependent on TRIF (**Figure 3.21D, E**). It is notable that, from cycle threshold (CT) values (data not shown) and fold induction values (**Figure 3.21D**), IFN-β transcription is totally abrogated in TRIF-null BMDMs, but not in IRF7- or IRF3-null BMDMs (**Figure 3.13A, C**). This is reflected in residual IFN-β secretion in LPS-stimulated IRF7- or IRF3-deficient BMDMs (**Figure 3.13B, D**), but not in TRIF-deficient BMDMs (**Figure 3.21E**). In agreement with the above observations, LPS-stimulated phosphorylation of STAT1 can be detected at low levels in IRF7- and IRF3-null BMDMs, reflecting the limited production of IFN-β in these cells (**Figure 3.14A, C**), while TRIF-deficient BMDMs, which entirely lack the ability to produce IFN-β, displayed negligible STAT1 phosphorylation after LPS exposure (**Figure 3.21F**). Taken together, these data suggest that IRF7 and IRF3 play partially redundant roles downstream of TRIF to initiate IFN-β induction, and that IRF7 cooperates with IRF3 downstream of TRIF to promote optimal IFN-β synthesis and robust type I IFN responses in endotoxin-challenged macrophages.

3.10 Analysis of IRF3/7 DKO suggest that both IRF3 and IRF7 act in concert downstream of TRIF to mediate optimal IFN-β induction and type I IFN responses to LPS

In order to obtain further evidence that both IRF7 and IRF3 are required in combination downstream of TRIF to achieve maximal IFN-β production in endotoxin-challenged macrophages, we generated IRF3-IRF7 double knockout mice. BMDMs were generated and analyzed for LPS-stimulated IFN-β mRNA induction, protein secretion, and STAT1 phosphorylation in parallel with IRF3 single knockout, IRF7 single knockout, and TRIF knockout BMDMs. We observed that the TLR4-mediated IFN-β response in IRF3-IRF7 double knockout BMDMs phenocopies that in TRIF knockout BMDMs, whereby IFN-β transcription and secretion were entirely abrogated when compared with wild-type BMDMs (**Figure 3.22A, B**). As shown previously, IFN-β transcription and secretion were significantly attenuated but not completely abolished in IRF3 and IRF7 single knockout BMDMs when compared with wild-type BMDMs (**Figure 3.22A, B**). Residual IFN-β transcription and secretion following LPS stimulation of IRF3- or IRF7-deficient BMDMs were reflected in low levels of STAT1

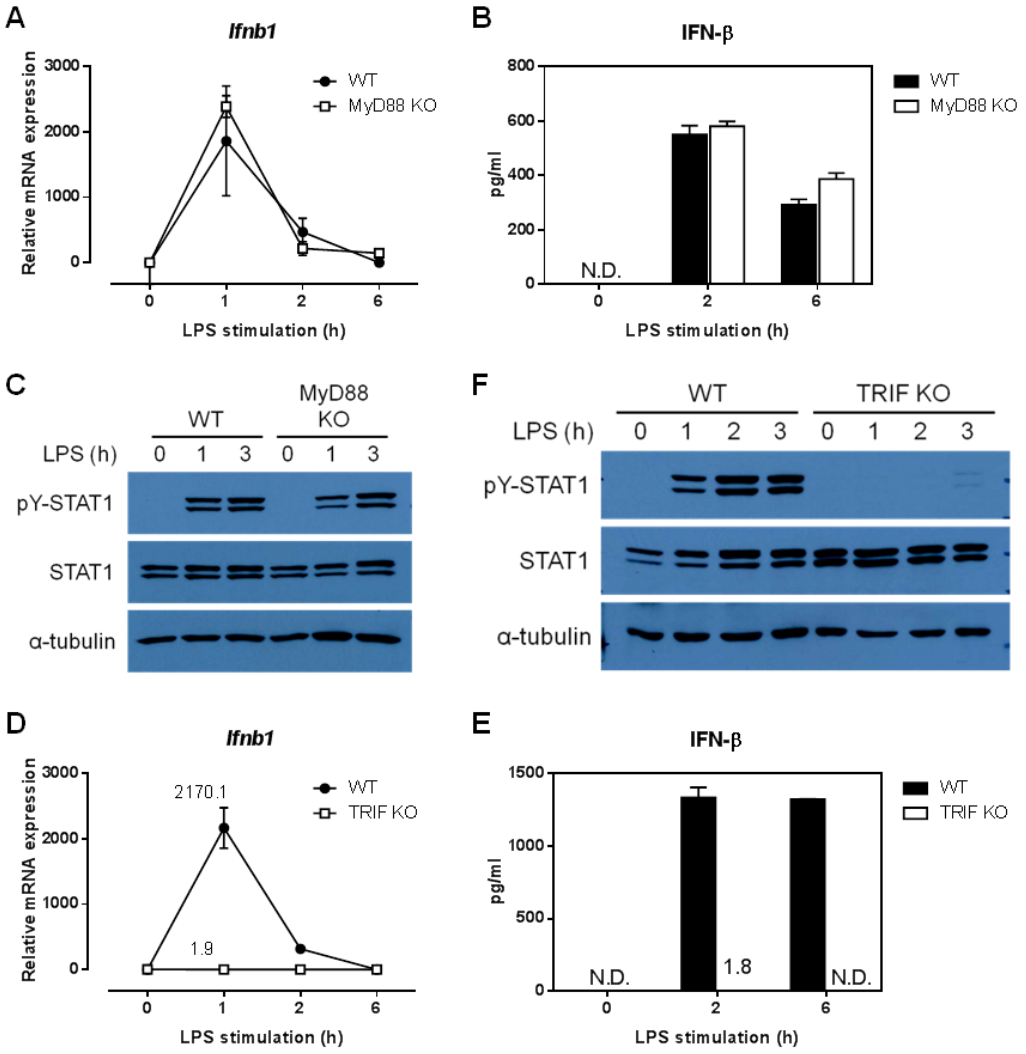

Figure 3.21
IRF7-mediated IFN-β expression in endotoxin-challenged macrophages is independent of MyD88 and completely dependent on TRIF.
(A, B, D, E) Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDMs from MyD88 knockout mice (A, B), and TRIF knockout mice (D, E), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not determined. (C, F) Western immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in whole cell lysates of BMDMs from MyD88 knockout mice (C), and TRIF knockout mice (F), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.

phosphorylation in IRF3- or IRF7-null BMDMs (**Figure 3.22C**). In contrast, total lack of IFN-β transcription and secretion following LPS stimulation of IRF3-IRF7 double knockout BMDMs and TRIF knockout BMDMs were reflected in the total absence of STAT1 phosphorylation in IRF3-IRF7 double knockout BMDMs and TRIF knockout BMDMs (**Figure 3.22C**). Western blotting of IRF3 and IRF7 proteins confirmed the phenotype of these cells. In summary, the relative degree of IFN-β mRNA induction, protein secretion, and STAT1 phosphorylation in the various BMDM phenotypes is: WT > IRF7 > IRF3 > IRF3-IRF7 double knockout (undetectable) = TRIF knockout (undetectable). This is indicative of the relative importance of signaling adaptors and transcription factors in the TLR4-mediated IFN-β response, supporting the notion that both IRF7 and IRF3 act in concert downstream of TRIF to achieve maximal IFN-β production in endotoxin-challenged macrophages (**Figure 3.23**).

3.11 IRF7 promotes type I IFN responses to endotoxin exposure *in vivo*

We next assessed the broader physiological relevance of our findings by verifying the requirement for IRF7 in supporting IFN-β production *in vivo*. Intra-peritoneal LPS injection is widely used as an experimental murine model of septic shock [255]. We sought to determine whether the novel IRF7 transcription factor identified in our experiments had functional consequences for host responses to endotoxin exposure *in vivo* by utilizing a murine model of endotoxin shock that is typically used to characterize the host response to bacterial LPS, and which is mediated by multiple different cytokines including IFN-α/β. Wild-type mice exhibited substantial quantities of type I IFN in serum after intra-peritoneal LPS administration, whereas serum levels of IFN-β cytokine ($n=7$, $p<0.05$ compared with wild-type mice; **Figure 3.24A**) and IFN-α cytokine ($n=4$, $p<0.05$ compared with wild-type mice; **Figure 3.24B**) were below the limit of detection in $IRF7^{-/-}$ mice or $IRF3^{-/-}$ mice. These data indicate that IFN-β responses to LPS *in vivo* also require both the 'master regulator' IRF7 and the transcription factor IRF3.

Previous studies in gene-targeted mice have proposed that IFN-β is a significant contributor to LPS-induced lethality *in vivo* [53, 90, 244]. Hence, we assessed the mortality rate of IRF7 and IRF3 knockout mice together with that of wild-type mice over 3 days following intra-peritoneal LPS challenge. Here we observed that

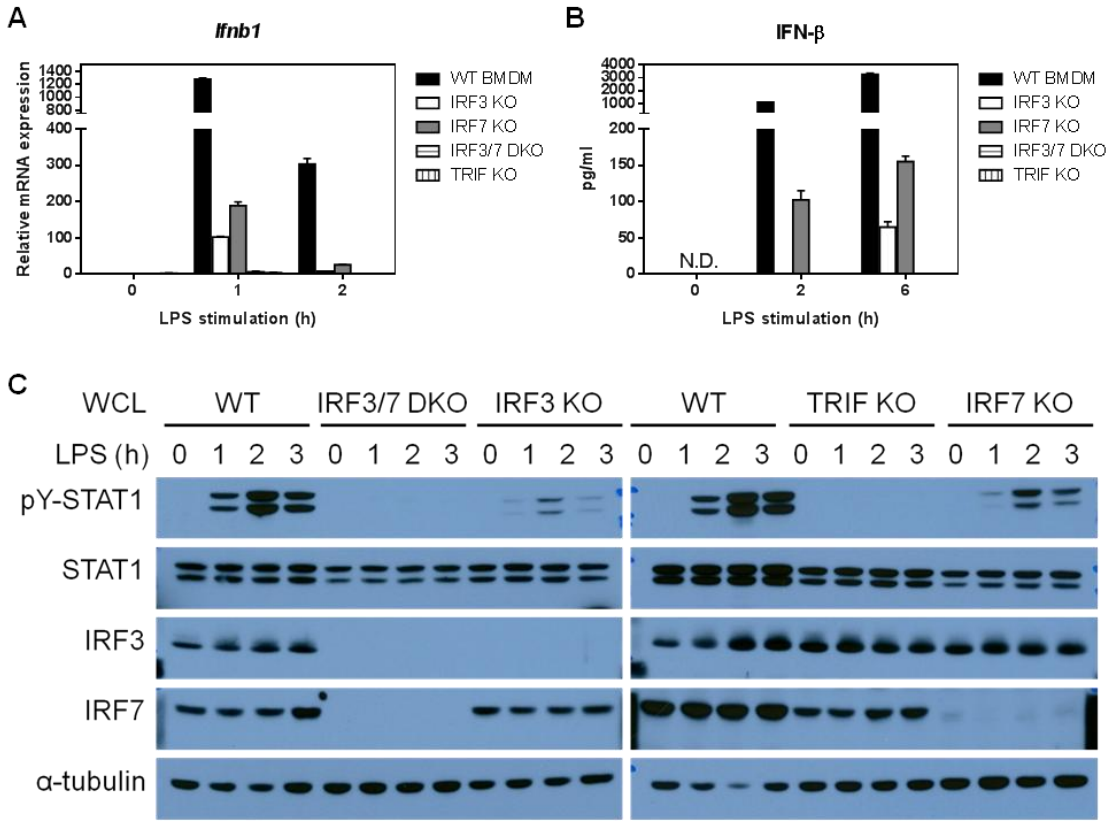

Figure 3.22
Both IRF7 and IRF3, downstream of TRIF, are required for maximal IFN-β expression in endotoxin-challenged macrophages.
(A, B) Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IRF3, IRF7, and TRIF single knockout mice, and IRF3-IRF7 double knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.
(C) Western immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in whole cell lysates of BMDMs from IRF3, IRF7, and TRIF single knockout mice, and IRF3-IRF7 double knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.

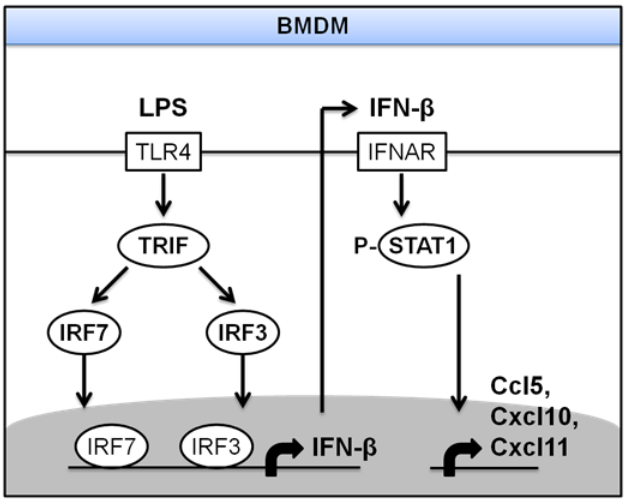

Figure 3.23
IRF7 and IRF3, downstream of TRIF, cooperatively regulate robust IFN-β induction in endotoxin-challenged macrophages.
Loss of either IRF7 alone or IRF3 alone lead to partial impairment of LPS-stimulated IFN-β transcription, secretion, and STAT1 phosphorylation compared with wild-type BMDMs, while loss of either TRIF or both IRF7 and IRF3 result in total impairment of LPS-stimulated IFN-β transcription, secretion, and STAT1 phosphorylation compared with wild-type BMDMs.

both IRF7$^{-/-}$ and IRF3$^{-/-}$ mice exhibited improved survival after LPS challenge when compared with wild-type animals (n=21, $p<0.05$; **Figure 3.25**), thus affirming that IRF7$^{-/-}$ mice exhibited increased resistance to LPS-induced endotoxin shock. Interestingly, IRF3$^{-/-}$ mice are more protected from LPS-induced lethality than IRF7$^{-/-}$ mice (n=21, $p<0.05$; **Figure 3.25**), reflecting the greater contribution of IRF3 compared with IRF7 in the TLR4-mediated IFN-β response *in vitro* (**Figure 3.22**). Together, these data indicate that IRF7-dependent synthesis of IFN-β by TLR4-activated macrophages exerts a significant influence on host protection against endotoxin-induced pathology *in vivo*.

3.12 IRF7 is required for IL-1β responses to LPS *in vivo*

It was recently reported that TLR4-TRIF signaling and the downstream type I IFN response are necessary for *in vivo* IL-1β processing and production in response to Gram-negative bacterial infection [130]. IL-1β is another cytokine that has been proposed to play a detrimental role in the inflammatory response and mortality in mouse models of septic shock [147]. Seeing improved survival following endotoxin exposure in IRF7 and IRF3 knockout compared with wild-type mice, we also measured serum IL-1β levels in IRF7 and IRF3 knockout mice, which show improved survival following endotoxin exposure compared with wild-type mice. Reminiscent of serum IFN-β levels (**Figure 3.24**), serum IL-1β levels were also significantly diminished in IRF7 or IRF3 knockout mice after intra-peritoneal LPS administration (n=6, $p<0.05$ compared with wild-type mice; **Figure 3.26**). These results suggest that serum IFN-β levels correlated with serum IL-1β levels, and required both IRF7 and IRF3, which together could contribute to LPS-induced lethality in these animals.

3.13 IRF7-mediated IFN-β induction is required for caspase-11 mRNA and protein expression in LPS-stimulated macrophages *in vitro*

Processing of pro-IL-1β into the mature form is mediated by the activation of inflammasome complexes, among which the canonical NLRP3 inflammasome is the most extensively studied to date. Recently, activation of a non-canonical inflammasome complex, which consist of caspase-11 (in mice) or caspase-4/5 (in humans) upstream of caspase-1, has been reported to contribute to mature IL-1β processing, secretion and

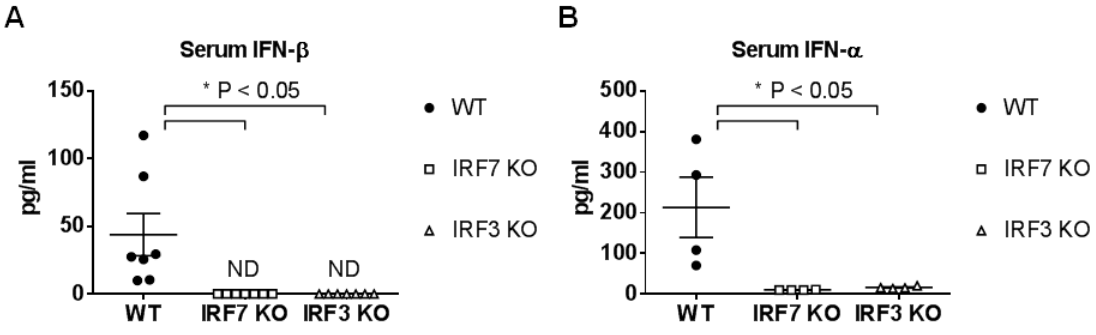

Figure 3.24
IRF7 facilitates type I IFN responses to LPS *in vivo*.
ELISA analysis of IFN-β (A) and IFN-α (B) levels in serum from IRF7 or IRF3 knockout mice, compared with wild-type control littermates, 3 h after i.p. injection of 30 μg/g LPS in sterile PBS. Data are presented as mean ± SEM of n=7 (A) or n=4 (B) mice. N.D.: not detected. (*p<0.05 compared with wild-type mice by one-way ANOVA).

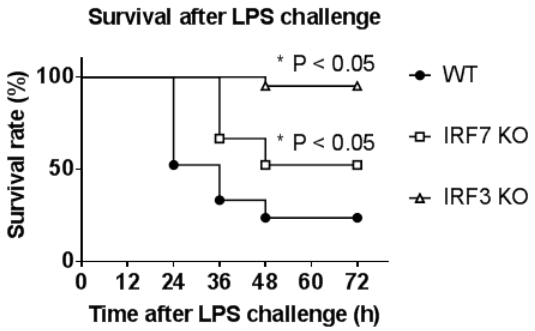

Figure 3.25
IRF7 knockout mice are protected from LPS-induced endotoxin shock mortality *in vivo*.
Survival of IRF7 and IRF3 knockout mice, compared with wild-type control littermates, following i.p. injection of 30 μg/g LPS in sterile PBS. (n=21 mice, *p<0.05 compared with wild-type mice by log-rank test).

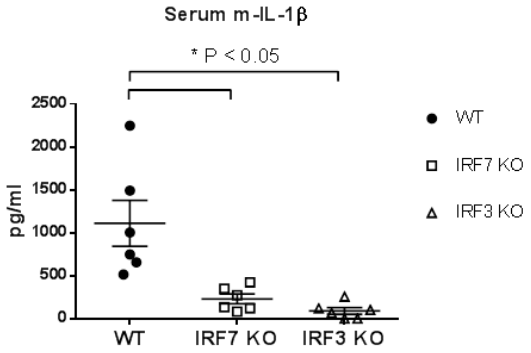

Figure 3.26
IRF7 facilitates IL-1β responses to LPS *in vivo*.
ELISA analysis of IL-1β cytokine levels in serum from IRF7 or IRF3 knockout mice, compared with wild-type control littermates, 3 h after i.p. injection of 30 μg/g LPS in sterile PBS. Data are presented as mean ± SEM of n=6 mice. (*p<0.05 compared with wild-type mice by one-way ANOVA).

septic shock lethality in response to Gram negative bacterial infection or cytoplasmic LPS [130, 137, 138]. Caspase-11 is a type I IFN-dependent gene that is induced downstream of autocrine/paracrine type I IFN signaling in LPS-stimulated macrophages [130, 133, 256]. Since we identified IRF7 as a novel positive regulator of TLR4-mediated IFN-β production in macrophages, and LPS-induced serum IL-1β is reduced in mice lacking IRF7, we asked whether this could be attributed to the effects of IRF7-mediated IFN-β production on caspase-11 expression. We analyzed caspase-11 gene expression in BMDMs derived from IRF3, IRF7, IRF3/7, TRIF knockout mice together with wild-type control cells. In wild-type BMDMs, caspase-11 gene expression was up-regulated as early as 1 h and reached maximal levels at 6 h after LPS stimulation (**Figure 3.27A**). In TRIF knockout BMDMs, which show a defunct IFN-β response (**Figure 3.21D, E, F**), caspase-11 mRNA levels were markedly attenuated at 6 h post-stimulation but not prior to 2 h post-stimulation (**Figure 3.27A**), implying a biphasic response whereby the first phase (up to 2 h post-stimulation) is independent of TRIF, and the second phase requires type I IFN signaling downstream of TRIF to sustain higher levels of caspase-11 induction at 6 h post-stimulation and beyond. In IRF7 knockout BMDMs, caspase-11 transcript levels at 6 h after LPS stimulation were decreased to a similar extent to that seen in IRF3 knockout BMDMs, compared with wild-type BMDMs (**Figure 3.27B**), indicating that IRF7-dependent IFN-β production is necessary for the optimal induction of caspase-11 transcription. Caspase-11 mRNA levels were further reduced in IRF3/7 double knockout BMDMs, to a level comparable to or even lower than that seen in TRIF knockout BMDMs (**Figure 3.27B**), reflecting the more drastic reduction in IFN-β production in IRF3/7 double knockout and TRIF deficient BMDMs compared with that in IRF3 or IRF7 single knockout BMDMs (**Figure 3.22**). The requirement for both IRF7 and IRF3 for maximal IFN-β production in turn promoted optimal caspase-11 induction in endotoxin-challenged BMDMs.

The above gene expression data is supported by protein expression levels of pro-caspase-11. In wild-type BMDMs, pro-caspase-11 protein was undetectable in unstimulated BMDMs, up-regulated at 3 h post-stimulation, and peaked at 6 h post-stimulation (**Figure 3.27C**). In TRIF knockout BMDMs, pro-caspase-11 protein levels at 6 h post-stimulation were severely impaired (**Figure 3.27C**), indicating that pro-caspase-11 protein expression is dependent on type I IFN signaling. Pro-caspase-11 expression was partially impaired in IRF7 knockout BMDMs, and more severely

impaired in IRF3 knockout and IRF3/7 knockout BMDMs, in line with the relative importance of IRF3 and IRF7 in IFN-β induction [(WT > IRF7 > IRF3 > IRF3-IRF7 double knockout (undetectable) = TRIF knockout (undetectable)] (**Figure 3.27C**). The lower levels of pro-caspase-11 in IRF3 knockout BMDMs compared with IRF7 knockout BMDMs correlate with the higher survival rate of IRF3 knockout mice compared with IRF7 knockout mice (**Figure 3.25**), consistent with the role of caspase-11-mediated pyroptosis in lethal endotoxin shock [257]. In contrast, the canonical inflammasome effector pro-caspase-1 was constitutively expressed in unstimulated wild-type BMDMs, and its expression remained largely unchanged following LPS stimulation (**Figure 3.27C**). Importantly, pro-caspase-1 expression in TRIF knockout BMDMs as well as the various knockouts were similar to wild-type control (**Figure 3.27C**), indicating that pro-caspase-1 protein expression is not regulated by type I IFN signaling. NLRP3 and pro-IL-1β protein expression were absent in unstimulated BMDMs, and up-regulated upon endotoxin exposure, but their protein levels were generally unaffected in TRIF knockout BMDMs as well as the other knockout cells (**Figure 3.27C**), confirming the notion that NLRP3 and pro-IL-1β induction are TRIF-independent and regulated by MyD88 [130]. Taken together, these results indicate that IRF7, together with IRF3, is required for optimal pro-caspase-11 expression in LPS-stimulated macrophages, which is likely to be necessary for its effective activation and processing of pro-IL-1β into mature IL-1β, which is in turn a contributing factor in inflammation and lethality in septic shock.

3.14 IFN-β induction in BMDCs is dependent on IRF3 but not on IRF7

We have shown that the robust IFN-β response in endotoxin-challenged macrophages is dependent on the transcription factor IRF7, and that this IRF7-mediated IFN-β response is critical for optimal pro-caspase-11 expression in LPS-stimulated macrophages, which in turn has functional consequences in IL-1β production and LPS-induced lethality in the murine model of septic shock. These findings are intriguing because IFN-β expression in TLR4-activated DCs is thought to be mediated by IRF3 alone [244], based on the previous report that IFN-β transcription is still largely preserved in LPS-stimulated IRF7-deficient DCs [35]. We sought to confirm these findings by analyzing IFN-β mRNA induction and protein secretion using BMDCs derived from IRF7-knockout mice, in conjunction with BMDCs derived from IRF3-

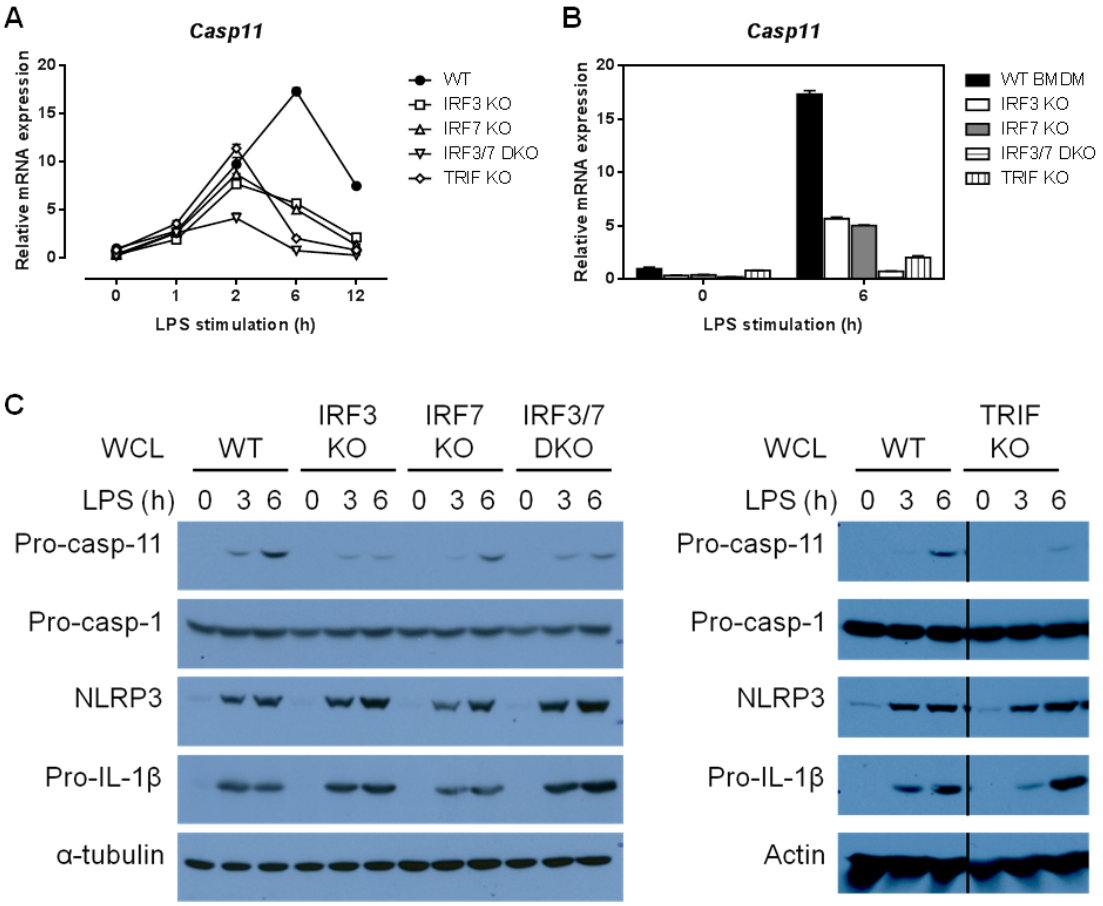

Figure 3.27
Both IRF7 and IRF3 are required for optimal pro-caspase-11 expression in endotoxin-challenged macrophages.
(A, B) Analysis of caspase-11 gene expression by real-time PCR analysis of BMDMs from IRF3, IRF7, and TRIF single knockout mice, and IRF3-IRF7 double knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Casp11* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.
(C) Western immunoblot analysis of pro-caspase-11, pro-caspase-1, NLRP3, and pro-IL-1β protein expression in whole cell lysates of BMDMs from IRF3, IRF7, and TRIF single knockout mice, and IRF3-IRF7 double knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.

knockout mice as a positive control. Indeed, IFN-β gene induction as well as protein secretion was abolished in LPS-stimulated IRF3-knockout BMDCs, but retained in IRF7-knockout BMDCs (**Figure 3.28**), consistent with previous reports that IRF3 alone is sufficient to mediate the type I IFN response in endotoxin-challenged DCs [35, 244]. In contrast, macrophage IFN-β responses employed both IRF3 and IRF7 (**Figure 3.13**). These data demonstrate that IRF7 is an essential component of TLR4-medated type I IFN responses in macrophages but not in DCs.

3.15 IRF7 is constitutively expressed in macrophages but not in DCs

The differential dependence on IRF7 for TLR4-mediated IFN-β responses in macrophages versus DCs may be due to several possibilities: (i) equivalent expression of IRF7 in macrophages and DCs, but differential involvement of IRF7 due to e.g. IRF7 being activated or recruited to the IFN-β promoter in macrophages but not in DCs, or (ii) differential expression of IRF7 protein itself in macrophages versus DCs. We probed IRF7 expression in BMDMs versus BMDCs, to investigate if this could explain the cell-type-specific requirement of IRF7 for LPS-induced IFN-β production.

We explored the possibility that IRF7 may be differentially expressed in macrophages versus DCs by Western blotting for IRF7 protein in these cell types in the absence and presence of LPS stimulation. Surprisingly, we observed that wild-type BMDCs express IRF7 protein at only trace levels, in contrast to the high levels detected in BMDMs, both at the resting state as well as within the window of IFN-β transcriptional induction (at 1–2 h after LPS stimulation), whereas IRF3 protein is markedly expressed in both cell types (**Figure 3.29 and Figure S7 in Appendix G**). Previous studies have suggested that IRF7 is itself an IFN-inducible protein which is up-regulated by autocrine signaling through IFNAR early after viral infection to amplify the second phase of IFN-β production. However, in contrast to the effects of viral infection [16, 35, 75, 258], endotoxin exposure did not significantly enhance IRF7 expression in either mAPC subset within the window of IFN-β transcription (assessed over a 2 h period after LPS stimulation) (**Figure 3.29**). These data suggested that a pre-existing pool of IRF7 protein supports TLR4-mediated IFN-β responses in LPS-challenged macrophages.

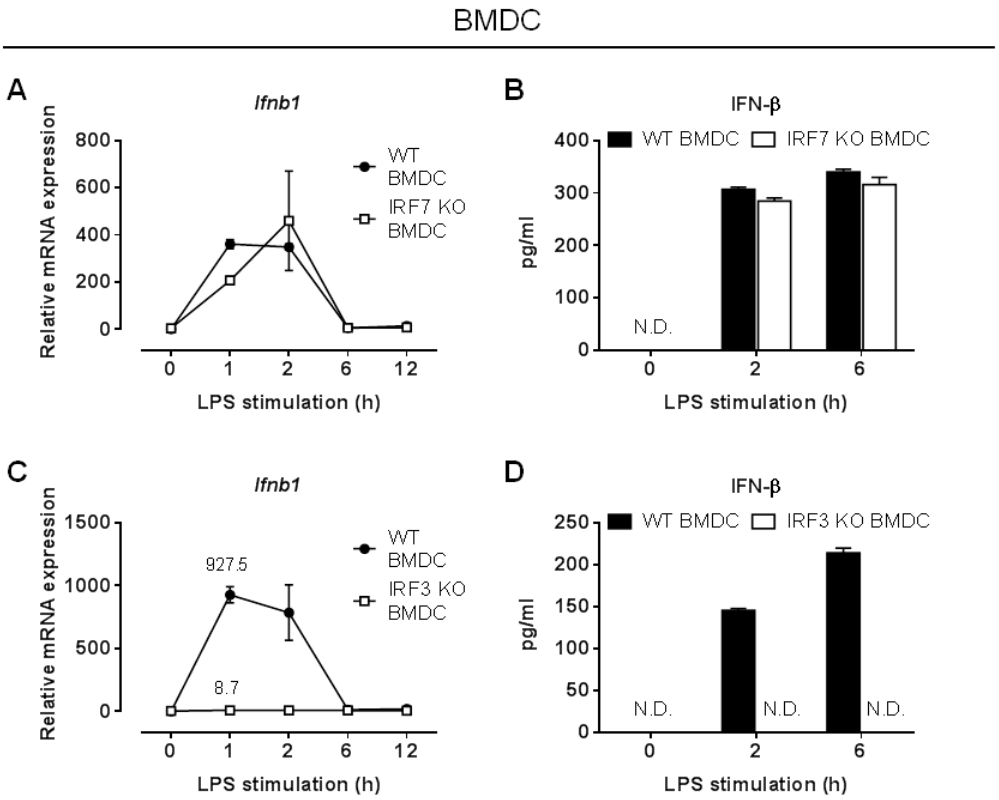

Figure 3.28
Maximal IFN-β expression in endotoxin-challenged BMDCs depends on IRF3 but not IRF7.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDCs from IRF7 knockout mice (A, B), and IRF3 knockout mice (C, D), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

Several reports have shown that IFN-α/β priming can increase IRF7 expression and enhance LPS-induced IFN-α/β transcription and secretion in conventional DCs, monocytes and macrophages [244, 247, 258, 259]. To test the effects of raising IRF7 expression on LPS-induced IFN-β production in BMDCs compared with BMDMs, we pretreated BMDCs with recombinant IFN-α for 12 h. Pretreatment with IFN-α cytokine for 12 h prior to LPS exposure up-regulated IRF7 protein to levels comparable with that in steady-state wild-type macrophages (**Figure 3.30A**). While LPS-stimulated production of IFN-β was curtailed in BMDCs (**Figure 3.3**), pretreatment with IFN-α cytokine for 12 h prior to LPS exposure increased IFN-β secretion to levels comparable with that in untreated wild-type macrophages (**Figure 3.30B**). The ability of IFN-α cytokine to enhance IFN-β responses in LPS-stimulated BMDCs required IRF7, since this effect was not observed in IRF7-knockout BMDCs (**Figure 3.30B**), thus attributing the rescue to the restoration of IRF7 levels in wild-type BMDCs. Together, these data suggest that IRF7 is a lineage-specific regulator of LPS-induced IFN-β production which is active in steady-state macrophages but not DCs.

3.16 BMDMs exhibit higher constitutive IFN-β production than BMDCs

Numerous studies have suggested that basal expression of IRF7 depends on constitutive IFN-β-ISGF3 signaling [83, 84, 87, 90]. The ability of IFN-α cytokine to up-regulate IRF7 expression in BMDCs implies that there is no qualitative defect in type I IFN signaling downstream of IFNAR in BMDCs (**Figure 3.30**). We therefore explored the possibility of differential constitutive IFN-β production in BMDMs and BMDCs. We observed that basal IRF7 mRNA and protein levels (but not IRF3 protein levels) were significantly reduced in IFNAR1-deficient and STAT1-deficient BMDMs, whereas basal IRF7 mRNA and protein levels were largely unaltered in BMDMs that lacked MyD88, TRIF or IRF3 (**Figure 3.31 and Figure S8 in Appendix H**), indicating that the presence of constitutive IFN-β-ISGF3 signaling is responsible for basal IRF7 expression in resting macrophages. We then sought to determine whether the absence of IRF7 protein in resting BMDCs was a consequence of reduced constitutive IFN-β expression in these cells. Indeed, basal production of IFN-β was significantly lower in resting wild-type BMDCs than in BMDMs (**Figure 3.32A and Figure S9 in Appendix I**), suggesting that DCs exhibit comparatively weaker constitutive signaling via IFN-β

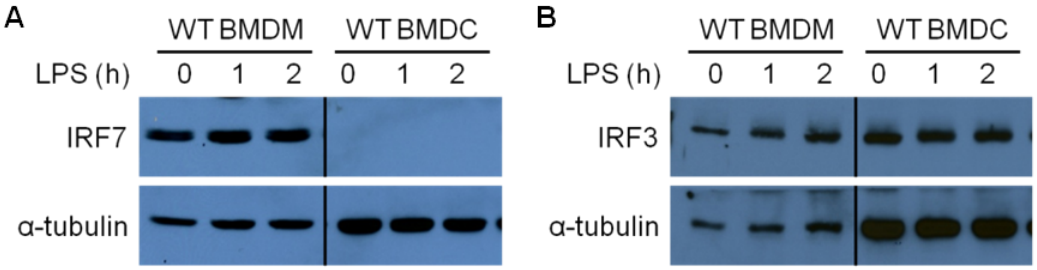

Figure 3.29
Distinct IFN responses of macrophages and DCs are associated with differential expression of IRF7.
Western immunoblot analysis of total IRF7 and total IRF3 protein expression in whole cell lysates of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.

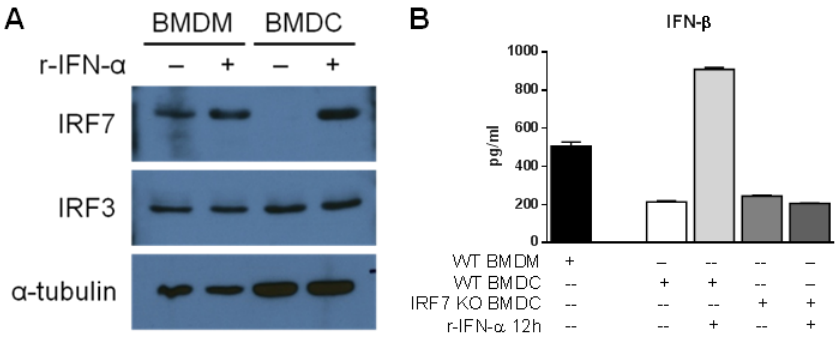

Figure 3.30
IFN-β responses in LPS-challenged BMDCs are enhanced by pre-treatment with IFN-α cytokine and up-regulation of IRF7 protein.
(A) Western immunoblot analysis of total IRF7 protein expression in whole cell lysates of wild-type BMDMs and BMDCs, treated or not with 500U/ml recombinant murine IFN-α for 12 h. Data are representative of at least two independent experiments.
(B) ELISA analysis of IFN-β protein expression in wild-type BMDMs, wild-type BMDCs, and IRF7 knockout BMDCs, treated or not with 500U/ml recombinant murine IFN-α for 12 h and then stimulated with 100ng/ml LPS for 6 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

and thus express only low levels of IRF7 relative to resting macrophages. Indeed, we observed that resting wild-type BMDCs expressed only trace levels of IRF7 mRNA (more than 200-fold lower than BMDMs), whereas BMDMs displayed high levels of IRF7 transcripts (**Figure 3.32B and Figure S9 in Appendix I**). These data suggest that basal expression of *Irf7* in mAPC is regulated at the transcriptional level via lineage-specific differences in constitutive IFN-β signaling. This finding of a comparatively weak constitutive IFN-β signaling in BMDCs is consistent with our earlier finding of a much lower basal STAT1 phosphorylation in unstimulated BMDCs than in unstimulated BMDMs (**Figure 3.3C**). Also, consistent with previous reports that constitutive IFN-β signaling is required to maintain appropriate expression of key signaling intermediaries including STAT1 [72, 87, 90], the levels of total STAT1 protein expression were lower in BMDCs compared with BMDMs, both before and after LPS stimulation (**Figure 3.3C**). Furthermore, constitutive expression of ISGs, including classical antiviral genes such as *Isg15*, *Isg54*, *Isg56*, *Mx1*, *Oas1a* and *Viperin*, were lower in unstimulated BMDCs compared with unstimulated BMDMs (**Figure S10 in Appendix J**). Together, these data suggest that a constitutive IFN-β-IRF7 signaling axis in steady-state macrophages positively regulates the LPS-induced IFN-β response in TLR4-activated macrophages, which is absent in resting DCs, resulting in blunted IFN-β responses to LPS exposure.

3.17 Constitutive IFN-β signaling and STAT1 binding to the IRF7 enhancer sustains basal IRF7 expression in resting macrophages

To understand the mechanistic basis of how basal expression of *Irf7* in mAPC is regulated at the transcriptional level via lineage-specific differences in constitutive IFN-β signaling, we explored the role of STAT1 as a possible mediator of IRF7 regulation. The murine *Irf7* enhancer contains a GAS sequence, which binds STAT1, at 1.1 kb upstream of the transcription start site (TSS) [260]. We performed chromatin immunoprecipitation (ChIP) experiments of STAT1 binding at this upstream GAS enhancer, and detected constitutive STAT1 association at the IRF7 enhancer in resting wild-type macrophages, because STAT1 ChIP positively enriched for IRF7 enhancer DNA region above the background levels produced by control IgG ChIP (**Figure 3.33A**). Constitutive binding of STAT1 to the IRF7 enhancer region was significantly abrogated in resting IFNAR knockout BMDMs, which lack constitutive type I IFN

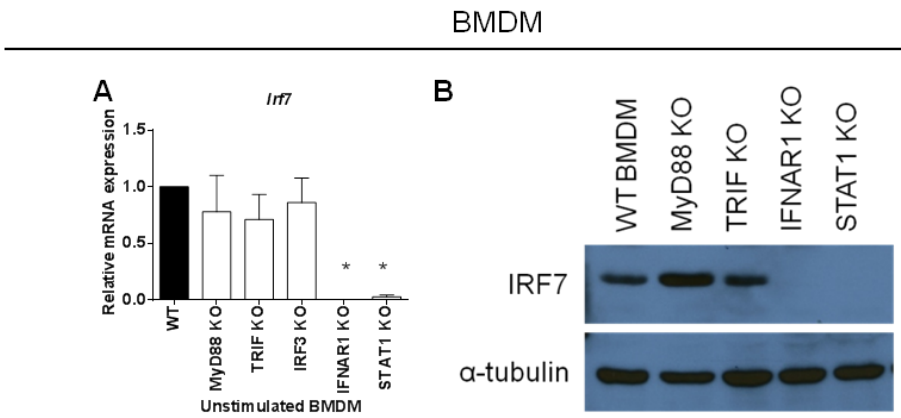

Figure 3.31
High basal expression of IRF7 in resting macrophages is sustained by constitutive IFN-β-STAT1 signaling, independent of MyD88, TRIF, and IRF3.
(A) Real-time PCR analysis of *Irf7* gene expression in resting BMDMs from MyD88, TRIF, IRF3, IFNAR1, and STAT1 knockout mice, compared with wild-type control littermates. *Irf7* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SEM of at least three independent experiments (*p<0.05 with respect to WT BMDM by one-way ANOVA).
(B) Western immunoblot analysis of total IRF7 protein expression in whole cell lysates of resting BMDMs from MyD88, TRIF, IFNAR1, and STAT1 knockout mice, compared with wild-type control littermates. Data are representative of at least two independent experiments.

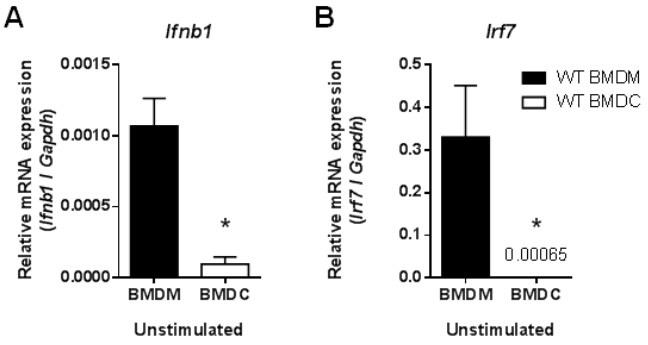

Figure 3.32
Poor IFN-β responses in LPS-challenged BMDCs are associated with weak IFN-β signaling and negligible IRF7 expression at steady-state.
Real-time PCR analysis of *Ifnb1* and *Irf7* gene expression in un-stimulated wild-type BMDMs and BMDCs. Gene expression was normalized to *Gapdh*. Data are presented as mean ± SEM of at least three independent experiments (*p<0.05 by paired t test).

signaling, similar to that seen in STAT1 knockout BMDMs, which served as a background control (**Figure 3.33B**). In contrast, in resting BMDCs, STAT1 binding is not detected at the IRF7 enhancer region, because STAT1 ChIP did not enrich for IRF7 enhancer DNA region compared to control IgG ChIP (**Figure 3.33C**). These data are in line with the negligible basal IRF7 mRNA expression in BMDCs compared with BMDMs (**Figure 3.32B**). Taken together, these results indicate that constitutive IFN-β production and signaling in steady-state macrophages lead to constitutive STAT1 binding to the IRF7 enhancer region, which sustains basal IRF7 transcription and subsequent protein expression and play a critical role in the LPS-induced IFN-β response in macrophages (**Figure 3.34**).

3.18 Constitutive IFN-β production in macrophages is independent of MyD88, TRIF, IRF3 and IRF7

We next attempted to investigate the mechanism of constitutive IFN-β production in resting macrophages. Since basal IRF7 expression is downstream of constitutive IFN-β production and signaling, normal basal IRF7 expression in BMDMs deficient in MyD88, TRIF, and IRF3 (**Figure 3.31**) imply that MyD88, TRIF and IRF3 have no impact on constitutive IFN-β expression. Indeed, constitutive IFN-β transcript levels were also relatively unchanged in resting-state MyD88-, TRIF-, IRF3- as well as IRF7-null BMDMs (**Figure 3.35**), in contrast to LPS-induced IFN-β transcription, which is dependent on TRIF, IRF3, and IRF7 (**Figure 3.13 and 3.21**). These data indicate that constitutive IFN-β expression and LPS-induced IFN-β expression are differentially regulated by different signal transduction pathways and/or transcription factors. In addition, constitutive IFN-β controls basal IRF7 expression (**Figure 3.31**), but not *vice versa* (**Figure 3.35**), placing IRF7 downstream of constitutive IFN-β-ISGF3 signaling. In this regard, the levels of basal IRF7 expression can be used as a proxy indicator of the levels of constitutive IFN-β production and signaling.

3.19 Constitutive IFN-β production in BMDMs depends on constitutive NF-κB

It was reported that the Jak-STAT signaling downstream of type I IFN receptor engagement can be modulated by calcium-dependent kinases, such as calmodulin kinase

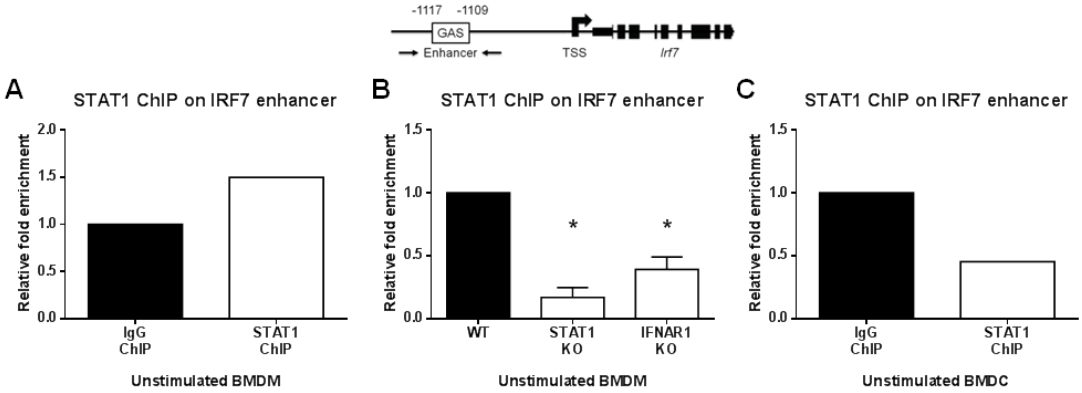

Figure 3.33
High-level IRF7 expression in resting macrophages is sustained by constitutive STAT1 binding to the IRF7 enhancer.
ChIP analysis of STAT1 binding at the IRF7 enhancer in resting wild-type BMDMs (A), in resting BMDMs from STAT1 and IFNAR1 knockout mice compared with wild-type control littermates (B), and in resting wild-type BMDCs (C). ChIP-enriched DNA was normalized to input DNA and expressed relative to the levels observed in control IgG ChIP (A, C), or in STAT1 ChIP in un-stimulated wild-type control cells. Data shown in (B) are presented as mean ± SEM of at least three independent experiments (*$p<0.05$ by one-way ANOVA).

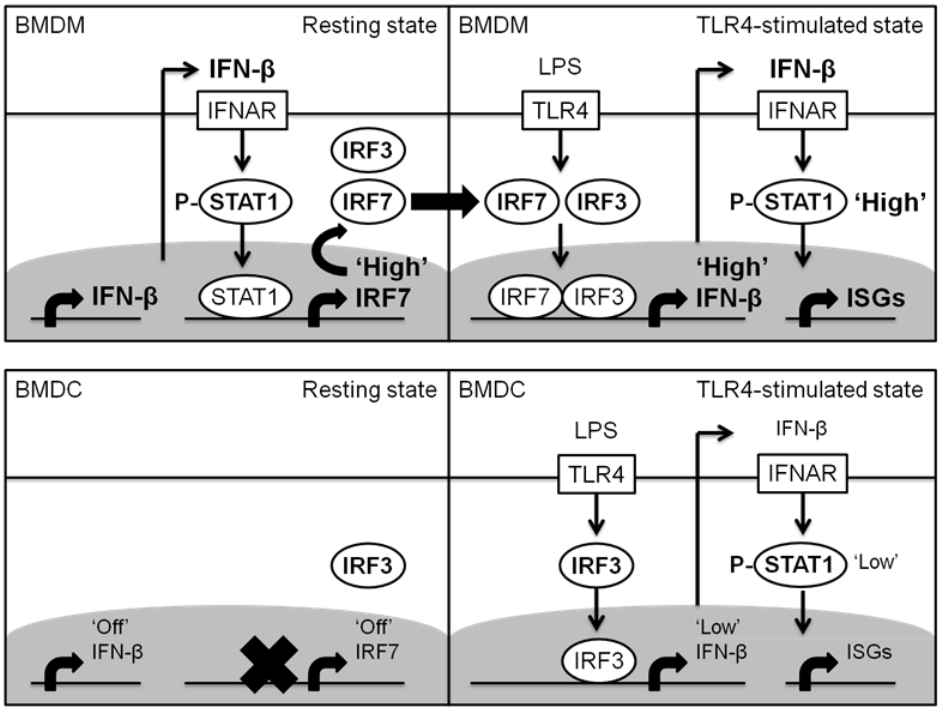

Figure 3.34
Constitutive IFN-β production and signaling, and basal IRF7 expression, present in macrophages but absent in DCs, explains the more robust IFN-β induction and stronger type I IFN responses to LPS exposure in macrophages compared with DCs.
Resting BMDMs exhibit constitutive expression of IFN-β, evidenced by constitutive STAT1 phosphorylation, and basal expression of IRF7, which are absent in resting BMDCs. The presence of IRF7 result in the more robust up-regulation of LPS-induced IFN-β transcription and secretion, evidenced by stronger LPS-induced STAT1 phosphorylation, in macrophages compared with DCs.

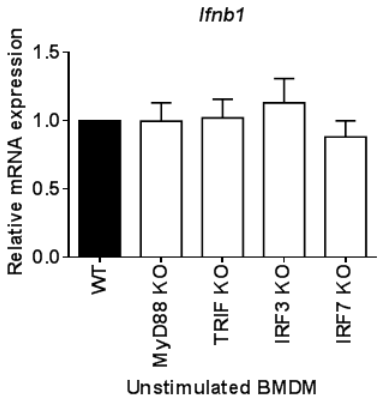

Figure 3.35
Constitutive expression of IFN-β in resting macrophages is not dependent on MyD88, TRIF, IRF3, and IRF7.
Real-time PCR analysis of *Ifnb1* gene expression in resting BMDMs from MyD88, TRIF, IRF3, and IRF7 knockout mice, compared with wild-type control littermates. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SEM of at least three independent experiments.

II (CaMKII) and proline-rich tyrosine kinase 2 (Pyk2), downstream of ITAM-associated receptors [261]. However, it is not known whether constitutive type I IFN signaling can also be regulated by these molecules. Therefore, we asked whether inhibition of CaMK and Pyk2 would affect constitutive type I IFN signaling by assaying basal IRF7 protein expression in resting BMDMs. Inhibition of CaMK did not affect constitutive STAT1 phosphorylation and basal IRF7 protein expression (**Figure 3.36A**). Consistent with this observation, the levels of constitutive IFN-β transcripts were not impaired in macrophages treated with CaMK inhibitor (**Figure 3.36B**), indicating that CaMK does not regulate the constitutive IFN-β-IRF7 signaling axis. Inhibition of Pyk2 attenuated both constitutive STAT1 activation and basal IRF7 protein expression (**Figure 3.36A**). However, the levels of constitutive IFN-β transcripts were not impaired in macrophages treated with Pyk2 inhibitor (**Figure 3.36B**), indicating that Pyk2 inhibition does not act at the level of constitutive IFN-β production, but rather act at the level of constitutive type I IFN signaling downstream of IFNAR to modulate basal IRF7 expression. These results indicate that the calcium-dependent tyrosine kinase Pyk2, but not calmodulin kinase CaMK, potentially regulates constitutive IFN-β signaling downstream of IFNAR and is thus one of the determinants of basal IRF7 expression in resting macrophages.

It was suggested that in fibroblasts, transcription factor RelA can regulate constitutive IFN-β expression [72]. Thus, we also investigated RelA as one of the candidate transcription factors that regulate constitutive IFN-β production. We observed that NF-κB inhibition led to a dramatic down-regulation of constitutive STAT1 phosphorylation and basal IRF7 expression in resting macrophages (**Figure 3.36C**), suggesting that NF-κB is one of the mediators of constitutive IFN-β production. Indeed, constitutive IFN-β transcription is also down-regulated with NF-κB inhibition compared with untreated control (**Figure 3.36D**). Taken together, these results indicate that regulation of constitutive IRF7 expression in BMDMs can occur at two levels: (i) at the level of constitutive IFN-β signaling downstream of IFNAR (without affecting constitutive IFN-β production), as in the case of Pyk2, and/or (ii) at the level of constitutive IFN-β production (which affects downstream constitutive IFN-β signaling), as in the case of NF-κB, which we identified as a key regulator of constitutive IFN-β production and basal IRF7 expression in steady-state macrophages.

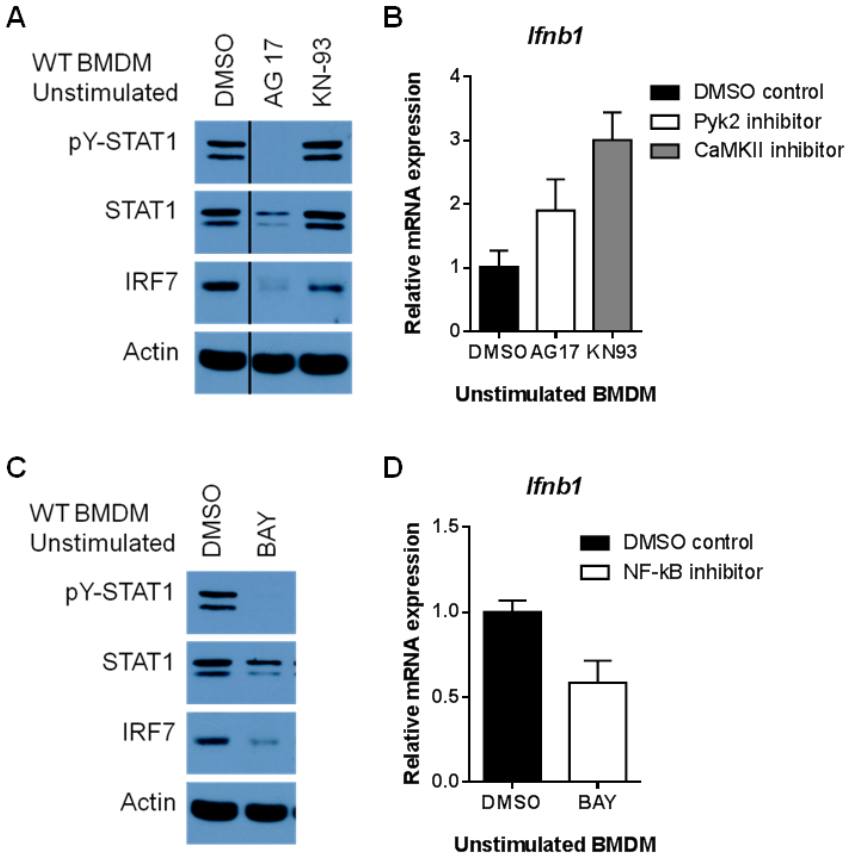

Figure 3.36
Constitutive expression of IRF7 in resting macrophages is dependent on Pyk2 and NF-κB.
(A, C) Western immunoblot analysis of phospho-STAT1, total STAT1, and total IRF7 protein expression in whole cell lysates of resting BMDMs, treated or not with Pyk2 inhibitor (AG 17), CaMKII inhibitor (KN-93), or NF-κB inhibitor (BAY 11-7085), compared with DMSO control. Data are representative of at least two independent experiments.
(B, D) Real-time PCR analysis of *Ifnb1* gene expression in resting BMDMs, treated or not with Pyk2 inhibitor (AG 17), CaMKII inhibitor (KN-93), or NF-κB inhibitor (BAY 11-7085), compared with DMSO control. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated DMSO control cells. Data are presented as mean ± SD of one representative from at least two independent experiments.

4 RESULTS (CHAPTER 2)

IL-27p28 regulates IFN-β induction and type I IFN responses to LPS through constitutive IRF7 in macrophages

4.1 IL-10 is an early type I IFN response gene which is induced via type I IFN signaling through the ISGF3 complex in macrophages

IL-10 is a potent anti-inflammatory cytokine that suppresses inflammatory responses during infection by restricting mAPC expression of pro-inflammatory mediators including TNF-α, IL-6 and IL-12 [211]. It has been previously suggested that the production of IFN-β and IL-10 are coordinately regulated after TLR4 stimulation [40, 103, 202]. In agreement with previous studies which have shown that IL-10 production from LPS-stimulated macrophages is dependent on type I IFN production and signaling [103], we verified that LPS-induced IL-10 mRNA and cytokine expression is substantially decreased in IFNAR1-knockout BMDMs (**Figure 4.1A, B**), and diminished in STAT1-deficient and STAT2-mutant BMDMs compared with wild-type control (**Figure 4.1C-F**). Collectively, these data indicate that, like IFN-β (**Figure 3.6 and 3.11**), TLR4-mediated IL-10 induction requires type I IFN production and signaling at least in part via ISGF3 complex activation.

4.2 BMDCs exhibit lower IL-10 production and STAT3-mediated anti-inflammatory response than BMDMs

Since we confirmed a contribution from type I IFN/ISGF3 signaling in the regulation of IL-10 expression in TLR4-stimulated macrophages, we wondered whether the magnitude of IL-10 induction is correlated with the magnitude of type I IFN production in BMDCs. We stimulated wild-type BMDMs and BMDCs with LPS simultaneously, and directly compared the magnitude of IL-10 induction. The relatively weak IFN-β response in BMDCs (**Figure 3.3**) was associated with restraint of LPS-stimulated IL-10 expression compared with BMDMs (**Figure 4.2**). *Il10* mRNA levels (relative to *Gapdh*) were ~100-fold lower in BMDCs (right axis) compared with BMDMs (left axis), (**Figure 4.2A and Figure S11 in Appendix K**), and lower amounts of IL-10 cytokine was detected in the cell culture supernatants of BMDCs compared

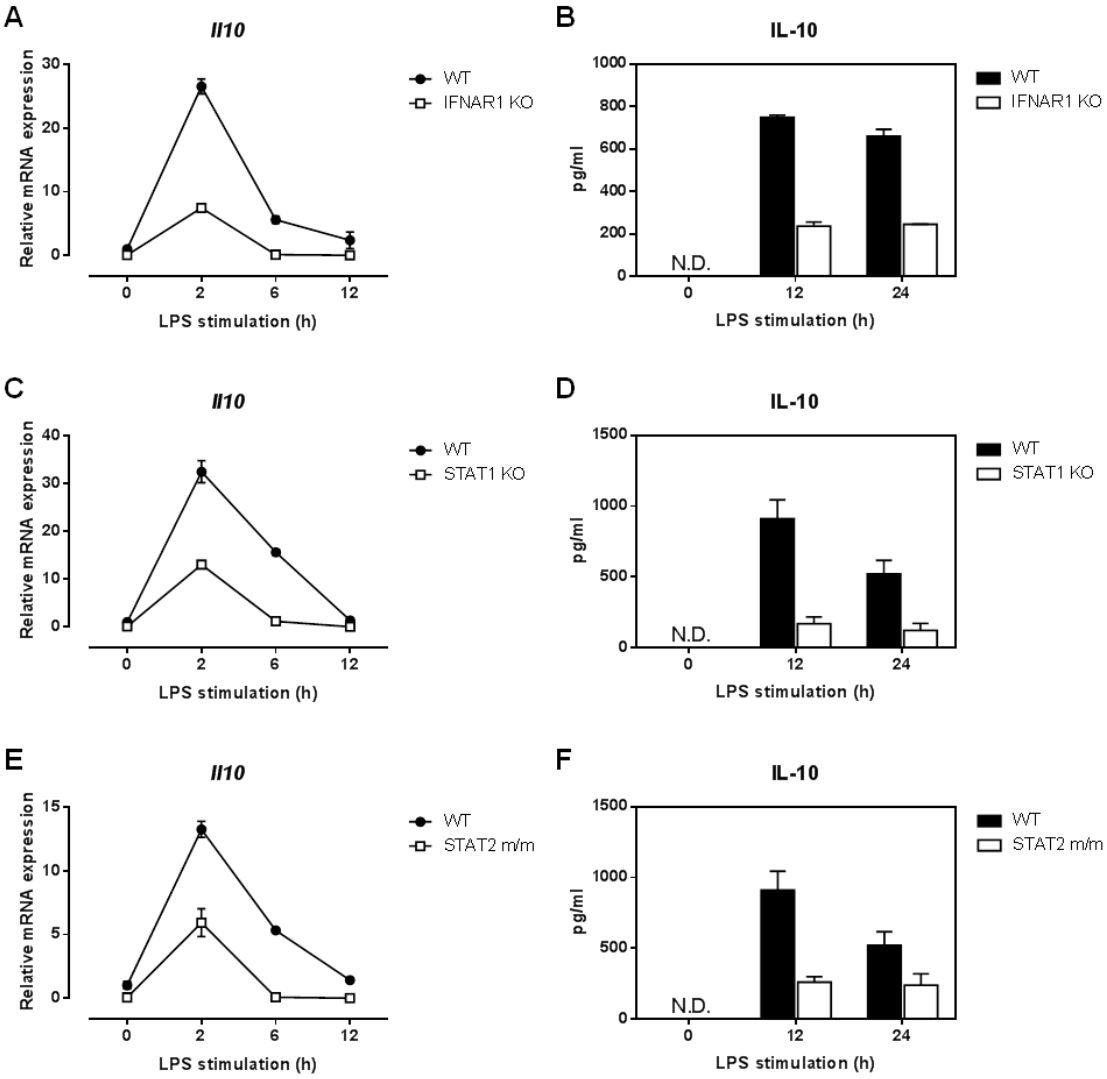

Figure 4.1
Macrophage IL-10 responses require type I IFN signaling mediated by the ISGF3 complex.
Analysis of IL-10 gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IFNAR1 knockout mice (A, B), STAT1 knockout mice (C, D), and STAT2 mutant mice (E, F), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Il10* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

with BMDMs (**Figure 4.2B**). Previous studies have shown that IL-10 suppresses the expression of pro-inflammatory cytokines including TNF-α, IL-6, and IL-12p40 via effects on STAT3 [211]. Accordingly, Western blot analyses revealed that BMDCs exhibit little STAT3 phosphorylation compared with BMDMs (**Figure 4.2C and Figure S12 in Appendix L**), and release higher quantities of TNF-α, IL-6, and IL-12p40 after LPS exposure (**Figure 4.2D**), in line with another report which showed that BMDCs are more potent producers of TNF-α, IL-6, and IL-12p40 than BMDMs in response to LPS [262]. Taken together, these results indicate that the relatively poorer type I IFN response in BMDCs is associated with a lower magnitude of IL-10 induction and a weaker STAT3-mediated anti-inflammatory response to LPS exposure compared with BMDMs (**Figure 4.3**).

4.3 Autocrine/Paracrine type I IFN signaling mediated by ISGF3 complex is required for IL-27p28 gene expression and cytokine production in LPS-stimulated macrophages

It has been reported that LPS-induced IL-10 expression in macrophages requires the sequential induction of type I IFN production and signaling followed by IL-27 production and signaling, and that the latter directly regulates IL-10 transcription [102, 103]. IL-27p28 has been identified as a LPS-inducible gene in macrophages and DCs, dependent on both the TLR4-MyD88-NF-κB/c-Rel pathway and the TLR4-TRIF-IRF3 pathway for initial induction, and on the autocrine/paracrine type I IFN signaling and ISGF3 complex activation for transcriptional amplification, and optimal production and release [102, 167, 169, 170]. We checked IL-27p28 mRNA and cytokine levels in LPS-stimulated IFNAR1-knockout BMDMs, and confirmed that they are decreased compared with wild-type control (**Figure 4.4A, B**), consistent with previous reports that IL-27 induction is dependent on intermediate IFN-β production and IFNAR signaling in BMDMs [102]. We further ascertained that IL-27p28 gene expression and cytokine production are similarly diminished in LPS-stimulated STAT1-deficient and STAT2-mutant BMDMs (**Figure 4.4C-F**), suggesting that, like IFN-β and IL-10 genes, type I IFN signaling, at least in part via ISGF3 complex activation, is required for the induction of IL-27p28 in macrophages, as has been proposed in DCs [170].

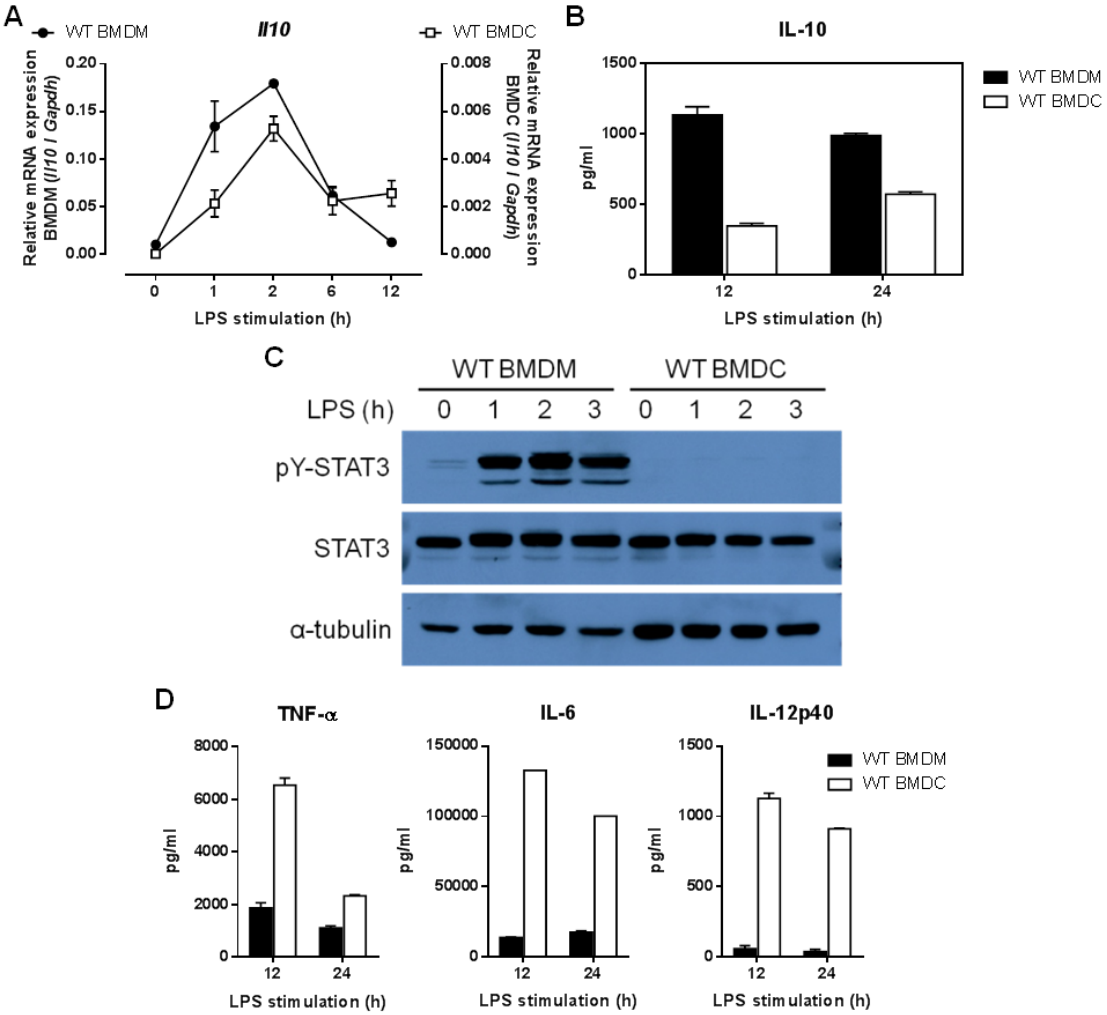

Figure 4.2
Weak IL-10 responses in endotoxin-challenged DCs are associated with a weak STAT3-mediated anti-inflammatory response.
(A, B) Analysis of IL-10 gene and protein expression by real-time PCR and multiplex analysis of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. *Il10* expression was normalized to *Gapdh* (left axis: fold induction in BMDMs; right axis: fold induction in BMDCs). Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.
(C) Western immunoblot analysis of phospho-STAT3 and total STAT3 protein expression in whole cell lysates of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.
(D) Multiplex analysis of TNF-α, IL-6 and IL-12p40 production in wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for 12 h or 24 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

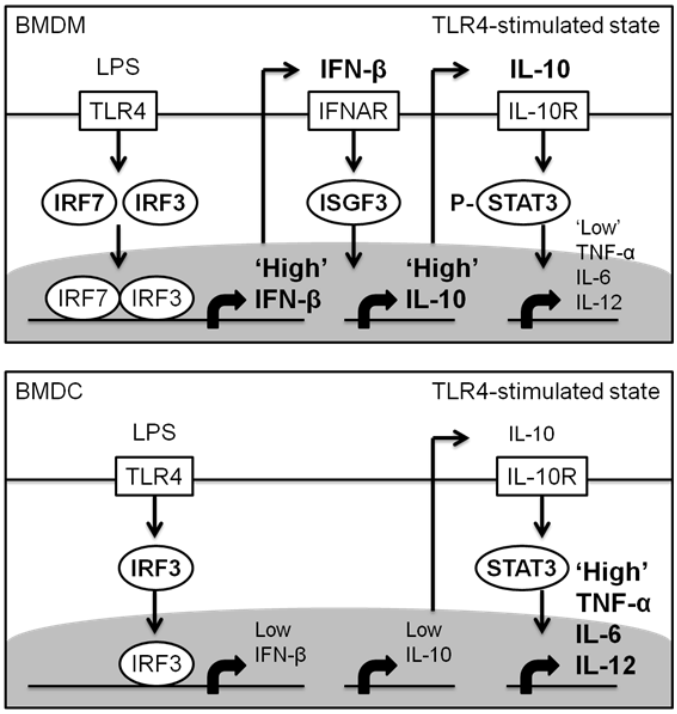

Figure 4.3
Poor IFN-β responses in LPS-challenged DCs are associated with weak IL-10 responses and a weak STAT3-mediated anti-inflammatory response.
DCs exhibit poorer IFN-β induction and weaker type I IFN responses to LPS exposure compared with macrophages. This phenomenon is associated with lower LPS-induced IL-10 production, negligible STAT3 activation, and higher pro-inflammatory cytokine production in DCs compared with macrophages.

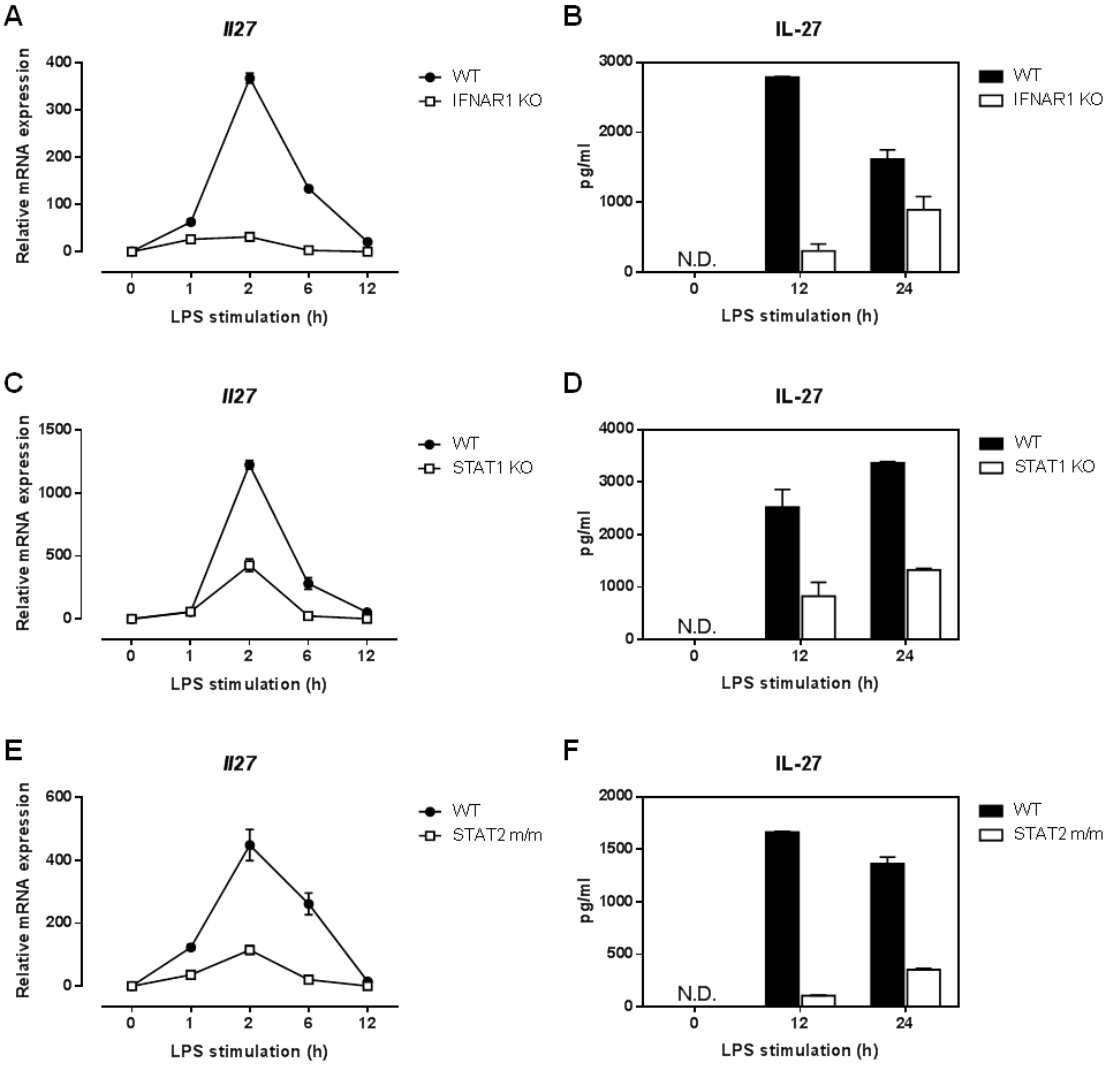

Figure 4.4
Induction of IL-27p28 in endotoxin-challenged macrophages requires type I IFN signaling through the ISGF3 complex.
Analysis of IL-27 gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IFNAR1 knockout mice (A, B), STAT1 knockout mice (C, D), and STAT2 mutant mice (E, F), together with their respective wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Il27p28* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

4.4 IL-27p28 is required for maximal IL-10 production and STAT3-mediated suppression of pro-inflammatory responses in LPS-stimulated macrophages

In the light of the differential IL-10 and STAT3-mediated anti-inflammatory response to LPS in DCs versus macrophages (**Figure 4.2**), we decided to investigate the role of IL-27 in regulating IL-10 responses to TLR4 ligation in both BMDMs and BMDCs using cells derived from IL-27p28-deficient mice. We analyzed their production of IL-10 and pro-inflammatory cytokines in response to LPS stimulation. We observed substantial decreases in production of IL-10 in IL-27p28 knockout BMDMs (**Figure 4.5A**), consistent with that observed in BMDMs lacking IL-27R signaling [211], implying that the STAT3-mediated anti-inflammatory response downstream of IL-10 may be impaired in IL-27p28 knockout BMDMs. Indeed, Western blot analyses revealed that STAT3 phosphorylation was considerably attenuated in IL-27p28-knockout BMDMs compared with wild-type control, despite normal STAT3 expression levels (**Figure 4.5B**), consistent with the concept that sustained STAT3 phosphorylation in endotoxin-challenged macrophages depends on IL-10 [103]. Noting that the inducibility of IL-10 mRNA and secretion of IL-10 cytokine are not totally obliterated in macrophages lacking IL-27p28, it is important to ask whether this partial reduction in IL-10 levels translates functionally into biological consequences. Consistent with previous studies showing that inhibited IL-10 levels led to increased pro-inflammatory cytokine production in LPS-stimulated macrophages [103], induction of the MyD88-dependent cytokines TNF-α, IL-6 and IL-12p40 in response to LPS was strongly up-regulated in IL-27p28$^{-/-}$ as compared to wild-type BMDMs (**Figure 4.5C**), in line with the phenotype of increased pro-inflammatory cytokine secretion in STAT3-deficient BMDMs (**Figure 4.6**). Therefore, striking but incomplete loss of LPS-induced STAT3 phosphorylation in IL-27p28-knockout BMDMs is reflected in the trend of increased pro-inflammatory cytokine production analogous to that seen in STAT3-deficient BMDMs. These data indicated that IL-27p28 does not directly mediate the MyD88-dependent expression of pro-inflammatory cytokines in BMDMs, but rather it is the impaired IL-10 synthesis in IL-27p28$^{-/-}$ BMDMs that permits increased inflammatory responses to LPS exposure. Indeed, addition of exogenous IL-10 to IL-27p28$^{-/-}$ BMDMs was sufficient to prevent LPS-induced up-regulation of the pro-inflammatory cytokines (**Figure 4.7**).

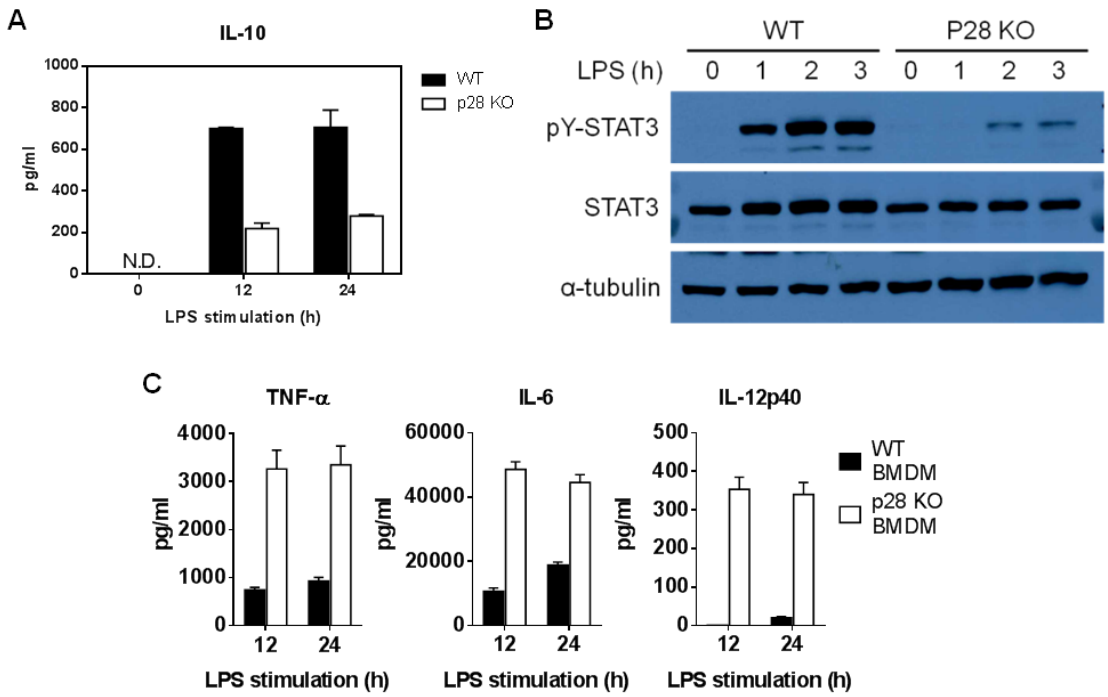

Figure 4.5
IL-27p28 production and signaling is required for maximal IL-10 expression and STAT3-mediated anti-inflammatory responses in macrophages.
(A) Multiplex analysis of IL-10 production in BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for 12 h or 24 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.
(B) Western immunoblot analysis of phospho-STAT3 and total STAT3 protein expression in whole cell lysates of BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.
(C) Multiplex analysis of TNF-α, IL-6 and IL-12p40 production in BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for 12 h and 24 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.

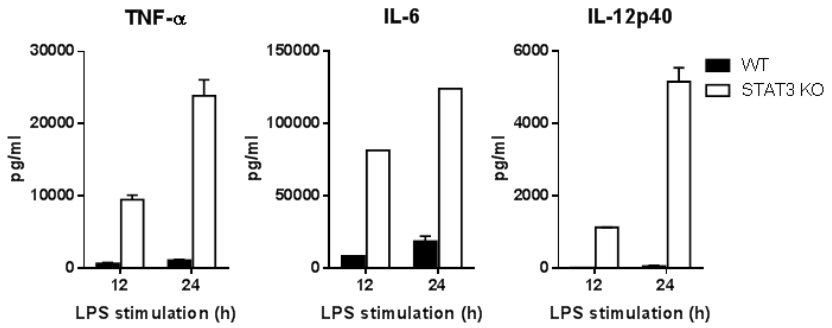

Figure 4.6
Super-induction of pro-inflammatory cytokines in STAT3-deficient BMDMs.
Multiplex analysis of TNF-α, IL-6 and IL-12p40 production in BMDMs from STAT3 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for 12 h and 24 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

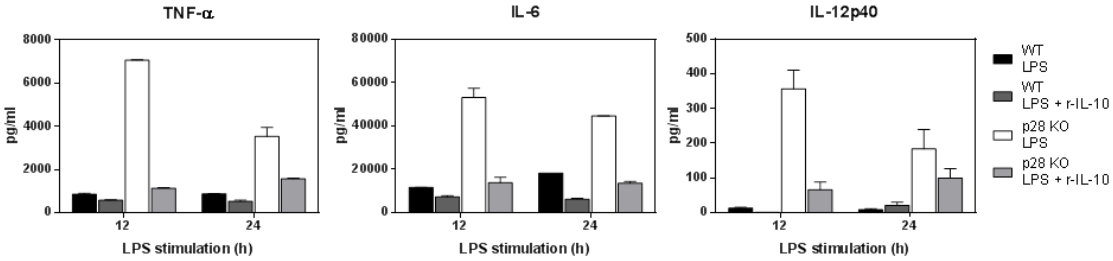

Figure 4.7
IL-10 cytokine restores STAT3-mediated anti-inflammatory responses in IL-27p28-deficient macrophages.
Multiplex analysis of TNF-α, IL-6 and IL-12p40 production in BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, treated or not with 100ng/ml LPS either alone or in combination with 10ng/ml recombinant murine IL-10 for 12 h and 24 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

Taken together, macrophages lacking IL-27p28 are characterized by lower IL-10 production, which translates functionally into decreased STAT3 activation and increased pro-inflammatory cytokine production in response to LPS stimulation, thus supporting the view that maximal IL-10 induction and secretion is necessary for optimal STAT3 activation and effective suppression of pro-inflammatory cytokines in LPS-stimulated macrophages. These data are consistent with the current paradigm of the regulation of IL-10 production in LPS-challenged macrophages, in which type I IFN regulates LPS-mediated IL-10 transcription via the intermediate induction of IL-27 downstream of type I IFN production and signaling.

4.5 IL-27p28 signaling is not required for IL-10 production and suppression of pro-inflammatory cytokines in BMDCs

Because the magnitude of the IL-10 response is different between macrophages and DCs, we next assessed whether IL-27p28 could promote IL-10 production in LPS-stimulated BMDCs. However, when we asked whether IL-27p28 impacts upon IL-10 levels and the STAT3-mediated anti-inflammatory response in DCs, we observed that IL-10 secretion by LPS-challenged BMDCs was comparable in both IL-27p28-deficient and wild-type cells (**Figure 4.8A**), indicating that IL-27p28 is not involved in TLR4-mediated IL-10 induction in DCs, unlike the requirement for IL-27p28 in macrophages (**Figure 4.5**). Accordingly, pro-inflammatory cytokine secretion by LPS-stimulated IL-27p28 knockout BMDCs were similar to wild-type BMDCs (**Figure 4.8B and Figure S13 in Appendix M**). These data indicated that unlike BMDMs, TLR4-mediated IL-10 induction and the IL-10-mediated anti-inflammatory response in BMDCs does not require IL-27p28.

4.6 STAT3 is not required for IL-27p28-mediated IL-10 gene expression and cytokine production in LPS-stimulated BMDMs

As shown above, LPS-stimulated IL-10 induction is differentially dependent on IL-27p28 cytokine in macrophages versus DCs (**Figure 4.5 and 4.8**), and LPS-stimulated STAT3 phosphorylation is also differentially activated in macrophages versus DCs (**Figure 4.2C**). STAT3 is reportedly activated downstream of IL-27R signaling pathway to promote production of IL-10 in T cells [176], as well as act

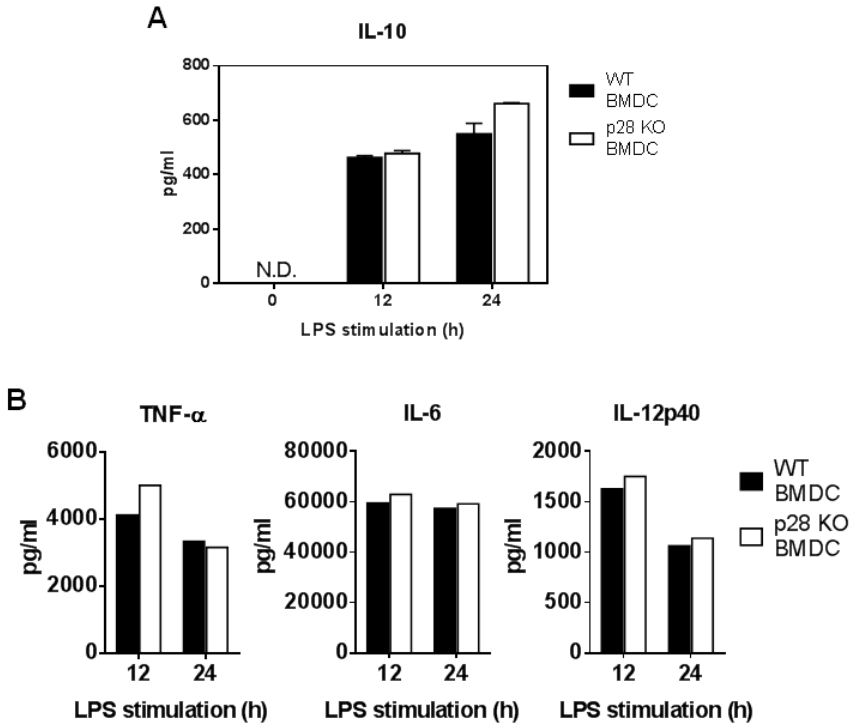

Figure 4.8
IL-27p28 production and signaling is not required for maximal IL-10 expression and STAT3-mediated anti-inflammatory responses in DCs.
(A) Multiplex analysis of IL-10 production in BMDCs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. (B) Multiplex analysis of TNF-α, IL-6 and IL-12p40 production in BMDCs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for 12 h and 24 h. Data are from one representative of at least two independent experiments.

downstream of IFN-α to induce IL-10 production in a B cell line [263]. However, the requirement of STAT3 for LPS-induced IL-10 activation in macrophages has been controversial [102, 204, 264]. We then investigated whether STAT3 is required for IL-27p28-dependent IL-10 induction in LPS-stimulated macrophages. Our data revealed that STAT3-deficient BMDMs exhibited unchanged or even enhanced IL-10 responses (**Figure 4.9**), indicating that STAT3 is not involved in TLR4-mediated IL-10 induction in macrophages, in contrast to IL-27-induced IL-10 expression in T cells [176]. These data indicate that, like IFN-β induction, LPS-stimulated IL-10 induction does not require STAT3, and confirms that STAT3 activation does not regulate IL-10 induction and is downstream of IL-10 production and signaling in BMDMs. This observation, that both LPS-induced IL-10 production (**Figure 4.9**) and IFN-β production (**Figure 3.11E, F**) are independent of STAT3 fits the model that IFN-β production and signaling regulates IL-10 induction in LPS-stimulated macrophages. However, this also raises questions about the relative importance of IFN-β and IL-27 in the regulation of LPS-induced IL-10 production in macrophages.

4.7 IL-27p28-mediated IL-10 induction is independent of IRF1

Further examination of the mechanistic basis of IL-27p28-mediated IL-10 induction in LPS-challenged macrophages led us to investigate IRF1, because LPS-stimulated IL-27p28 production was clearly reduced in IRF1-deficient macrophages [167], and in IRF1-silenced DCs [171]. Indeed, analysis of both IL-27p28 mRNA induction and IL-27 cytokine secretion in IRF1 knockout BMDMs confirmed that IL-27 production were substantially reduced in the absence of IRF1 compared with wild type control cells (**Figure 4.10A, B**). However, analysis of IL-10 gene and protein expression in IRF1 knockout BMDMs showed that production of IL-10 was largely unchanged in IRF1-knockout macrophages (**Figure 4.10C, D**). In addition, it has been shown that IL-10 production is enhanced significantly in IRF1-deficient splenic DCs [265]. Thus, IL-10 induction does not correlate with IL-27 expression in IRF1 knockout BMDMs, as well as DCs. While IL-27p28 induction requires IRF1, IFN-β and IL-10 expression are independent of IRF1, thus raising the possibility that either low levels of IL-27p28 production are sufficient for normal IL-10 induction, or IL-27p28 contributes but do not directly account for IL-10 production.

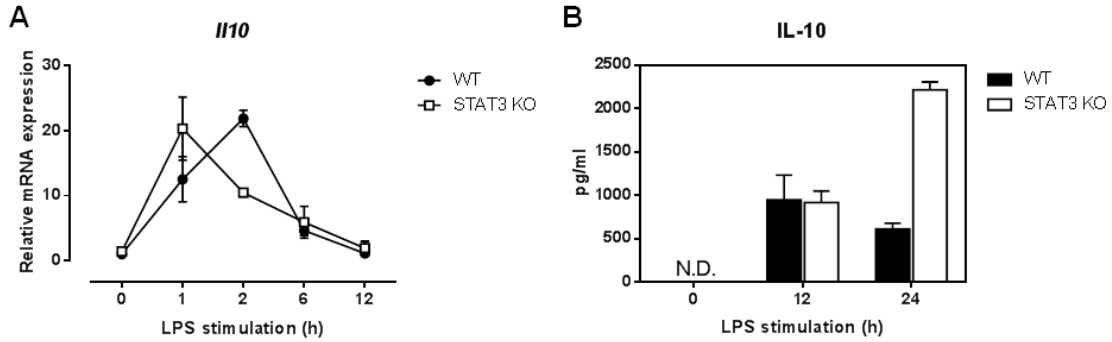

Figure 4.9
IL-27p28-mediated IL-10 expression in LPS-stimulated macrophages does not depend on STAT3.
Analysis of IL-10 gene and protein expression by real-time PCR and multiplex analysis of BMDMs from STAT3 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Il10* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

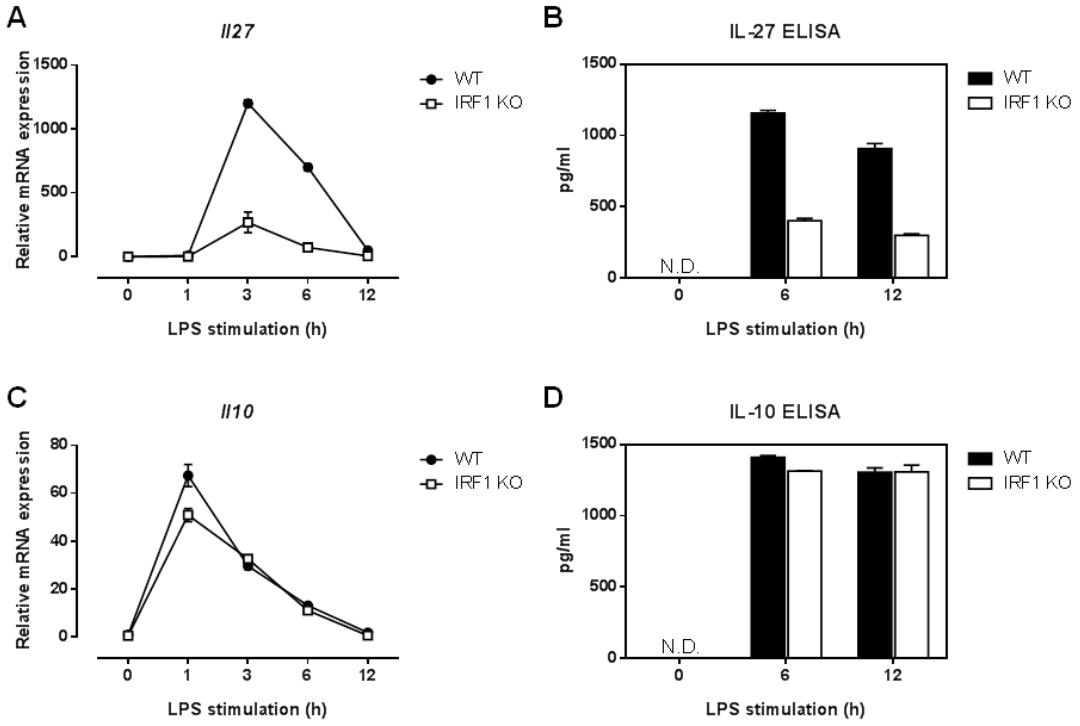

Figure 4.10
Macrophage LPS-stimulated IL-27p28 transcription and IL-27 secretion are reduced in IRF1-deficient BMDMs, while IL-10 production is normal.
Analysis of IL-27 and IL-10 gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IRF1 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

4.8 IFN-β and IL-10 gene expression are coordinately induced in LPS-stimulated macrophages

The above lines of evidence, that (i) IL-10 induction in DCs is independent of IL-27p28 signaling, (ii) IL-10 (and IFN-β) induction in macrophages is independent of STAT3 and IRF1, while IL-27p28 induction in macrophages requires IRF1, suggest that the regulation of IL-10 by IFN-β and IL-27 is more complex than anticipated. We reexamined the transcription profile of IFN-β, IL-27p28, and IL-10 in response to LPS in wild-type macrophages by performing a detailed kinetic analysis of IFN-β, IL-27p28 and IL-10 expression in LPS-stimulated macrophages, and found that IL-10 gene expression closely follows that of IFN-β, being transcriptionally up-regulated as early as 30 min post-stimulation. IL-27p28 mRNA is not yet significantly induced at this time point, and it is only substantially up-regulated at 1 h post-stimulation (**Figure 4.11A**). Measurement of cytokine levels showed that IFN-β and IL-10 is detected in cell culture supernatants as early as 1 h post-stimulation (**Figure 3.1B and 4.11B**), while IL-27 cytokine is only first detected at 2 h post-stimulation (**Figure 4.11C**). The short lag time between IFN-β and IL-10 expression effectively means that the production of these two cytokines closely parallel each other, and are coordinately regulated following LPS stimulation of macrophages. The temporal profile of IL-27p28 induction appears to be relatively delayed, while the kinetics of IL-10 mRNA expression coincided strongly with that of IFN-β transcription, instead of IL-27 transcription.

4.9 IL-27p28 cytokine supports amplification of IFN-β responses in LPS-stimulated macrophages

The above observations led us to hypothesize about the relative roles of IFN-β vis-à-vis IL-27 in the regulation of IL-10 production in LPS-challenged macrophages. Because both IL-27p28 and IFN-β share similarities of being co-regulated downstream of IFNAR-ISGF3 signaling, while IFN-β has been shown to regulate TLR-mediated IL-27p28 gene expression in macrophages and DCs [102, 170], we hypothesized whether IL-27p28 could reciprocally regulate LPS-mediated IFN-β induction in activated macrophages. We therefore sought to determine whether IL-27p28 influenced IFN-β responses in endotoxin-challenged macrophages. Indeed, we observed that IFN-β expression was impaired in LPS-stimulated IL-27p28-knockout BMDMs (**Figure 4.12**).

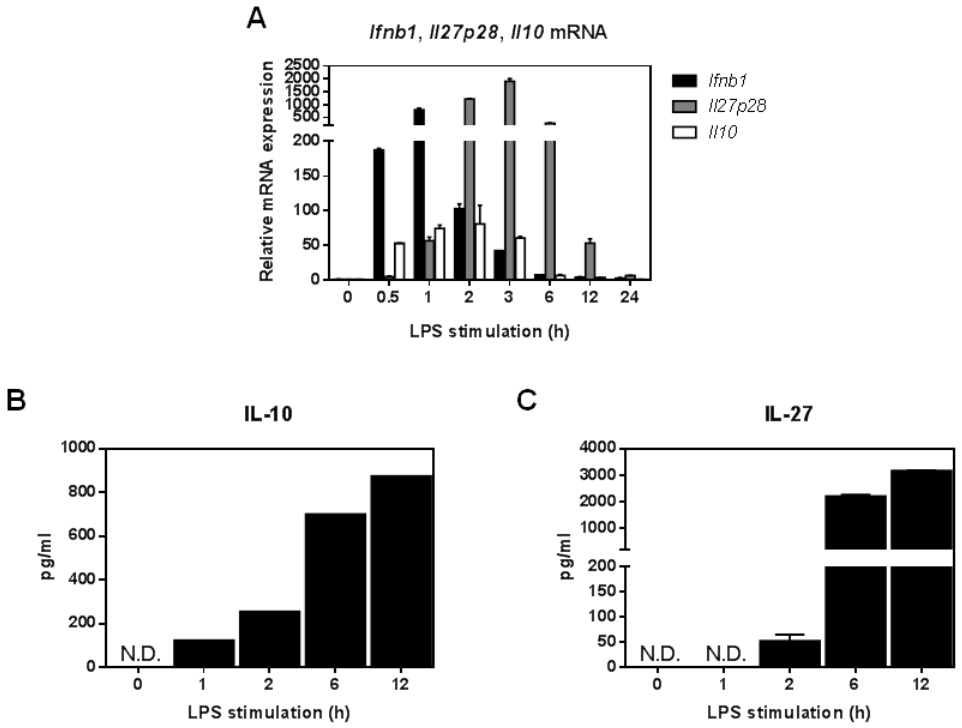

Figure 4.11
IL-10 mRNA expression coincides with induction of IFN-β transcription and precedes that of IL-27p28 in LPS-stimulated macrophages.
(A) Real-time PCR analysis of the kinetics of *Ifnb1*, *Il27p28*, and *Il10* gene expression in wild-type BMDMs, following stimulation with 100ng/ml LPS for the indicated times. Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.
(B, C) ELISA analysis of IL-10 and IL-27 protein expression in wild-type BMDMs, following stimulation with 100ng/ml LPS for the indicated times. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

These results indicated that IL-27p28 production and signaling are required for the amplification of IFN-β responses in LPS-stimulated macrophages. To assess the biological significance of the decreased IFN-β production in IL-27p28-deficient macrophages, we probed STAT1 activation, downstream of IFNAR engagement, and the expression of LPS-induced STAT1-dependent genes. Analysis of STAT1 phosphorylation by Western blotting showed that early STAT1 activation at 1 h post-LPS stimulation is impaired in IL-27p28-deficient BMDMs compared to wild-type control, and its activation at 2 h and 3 h post-LPS stimulation were also partially reduced (**Figure 4.13A**). To assess the functional significance of the decreased IFN-β production and STAT1 activation in IL-27p28-knockout BMDMs, gene expression analysis further revealed that LPS-inducible type I IFN-dependent chemokine genes *Ccl5*, *Cxcl10* and *Cxcl11* [37, 87, 90] were down-regulated in IL-27p28-knockout BMDMs (**Figure 4.13B**). These results indicate that defective IFN-β production and signaling in macrophages lacking IL-27p28 production and signaling functionally influences the induction of certain ISGs. Together, these data suggested that LPS induction of type I IFN-response genes depends on high levels of IFN-β production achieved only in IL-27p28-sufficient macrophages.

However, it is noteworthy that IFN-β responses to LPS are completely abolished in TRIF-deficient BMDMs (**Figure 3.21D, E**), whereas IFN-β synthesis is not entirely ablated in IL-27p28-knockout BMDMs (**Figure 4.12**). In agreement with the above observations, LPS-stimulated phosphorylation of STAT1 can be detected at low levels in IL-27p28-knockout BMDMs, reflecting the limited production of IFN-β in these cells (**Figure 4.13A**), while TRIF-deficient BMDMs, which entirely lack the ability to produce IFN-β, displayed negligible STAT1 phosphorylation after LPS exposure, reflecting the greater depletion of IFN-β expression in TRIF-deficient BMDMs (**Figure 3.21F**). It is worthwhile to note that both basal and LPS-induced total STAT1 protein expression are lower in IL-27p28-knockout BMDMs as well (**Figure 4.13A**). Taken together, these data indicate that IL-27p28 production and signaling is necessary for robust TLR4-induced IFN-β production in macrophages, which is functionally important for optimal STAT1 activation and transcriptional induction of a subset of type I IFN-response genes.

BMDM

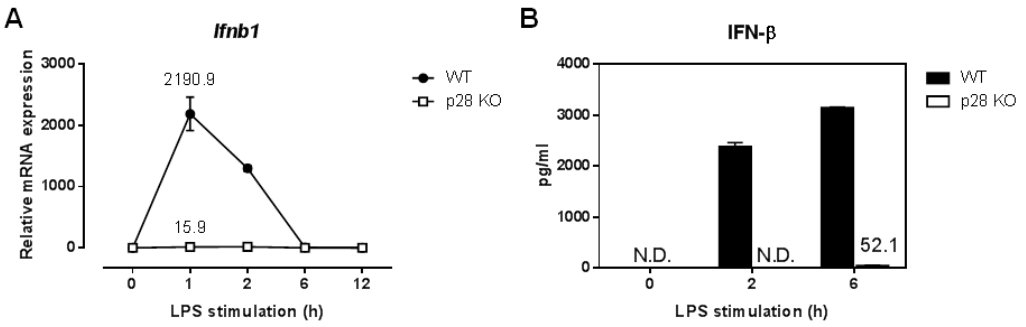

Figure 4.12
IL-27p28 is required for robust type I IFN responses to LPS exposure in macrophages.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments. N.D.: not detected.

BMDM

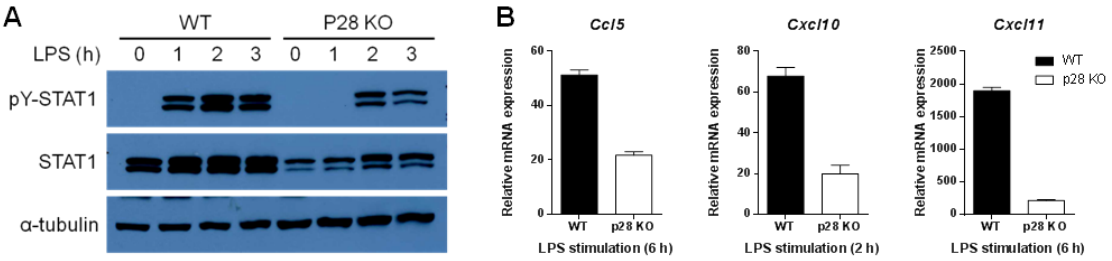

Figure 4.13
Defective IFN-β induction in IL-27p28-deficient BMDMs is associated with impaired STAT1 activation and transcription of IFN-inducible genes.
(A) Western immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in whole cell lysates of BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least two independent experiments.
(B) Real-time PCR analysis of *Ccl5*, *Cxcl10* and *Cxcl11* gene expression in BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for 2 h (*Cxcl10*) or 6 h (*Ccl5* and *Cxcl11*). Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.

4.10 Addition of exogenous recombinant IFN-β rescues IL-10 induction and STAT3-mediated anti-inflammatory response in the absence of IL-27p28 signaling

In the light of our observations that IFN-β production is significantly impaired in IL-27p28-deficient macrophages, the question arises whether the defect in IL-10 production seen in IL-27p28-deficient macrophages is due directly to the lack of IL-27 or is attributable to IFN-β. Our data indicated that IL-27p28 is required for IFN-β induction in TLR4-stimulated macrophages but not for IL-10 induction in TLR4-stimulated DCs, and that STAT3 is dispensable for IL-10 synthesis in LPS-challenged macrophages. We therefore sought to determine whether IL-27p28 effects on macrophage production of IL-10 are attributable to the intermediary functions of IFN-β.

To reexamine the requirement of IL-27 production and signaling vis-à-vis type I IFN production and signaling for LPS-induced IL-10 expression, we performed a rescue experiment involving addition of exogenous recombinant IFN-β together with LPS to IL-27p28-deficient BMDMs. Co-treatment with recombinant IFN-β and LPS rescued STAT1 phosphorylation in IL-27p28-knockout BMDMs, thus attributing the defective LPS-induced STAT1 phosphorylation in IL-27p28-knockout BMDMs to impaired IFN-β production, and suggesting that IL-27p28-mediated IFN-β production and autocrine/paracrine type I IFN signaling are required to support full STAT1 activation in BMDMs (**Figure 4.14A**). Interestingly, total STAT1 protein expression is still lower in IL-27p28-knockout BMDMs treated with recombinant IFN-β together with LPS (**Figure 4.14A**), suggesting that IL-27p28 production and signaling control total STAT1 protein expression independent of IFN-β/type I IFN signaling.

Importantly, addition of exogenous IFN-β to IL-27p28-knockout macrophages rescued LPS-induced IL-10 synthesis to levels comparable with those observed in wild-type macrophages (**Figure 4.14B**). This implies that there is no obligate requirement of IL-27 production and signaling for LPS-induced IL-10 expression, and that in the absence of IL-27 production and signaling, IFN-β/type I IFN signaling is able to bypass the requirement of IL-27 to induce normal levels of IL-10 in LPS-stimulated macrophages. Addition of exogenous IFN-β also restored LPS-stimulated STAT3 phosphorylation in IL-27p28-deficient BMDMs to wild-type levels (**Figure 4.15A**), and

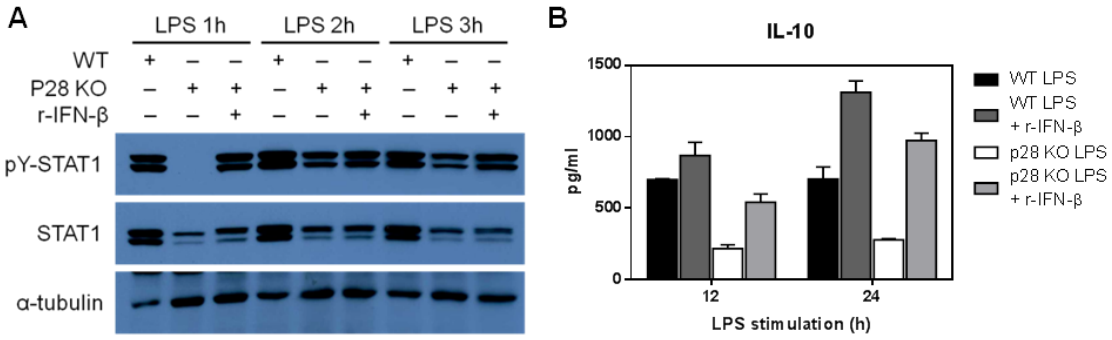

Figure 4.14
IFN-β cytokine rescues STAT1 activation and restores IL-10 production in endotoxin-challenged IL-27p28-deficient macrophages.
(A) Western immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in whole cell lysates of BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, treated or not with 100ng/ml LPS either alone or in combination with 250U/ml recombinant murine IFN-β for 1-3 h. Data are representative of at least two independent experiments.
(B) Multiplex analysis of IL-10 production in BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, treated or not with 100ng/ml LPS either alone or in combination with 250U/ml recombinant murine IFN-β for 12 h and 24 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.

decreased pro-inflammatory mediator concentrations in the culture supernatants with comparable efficiency to co-treatment with IL-10 and LPS (**Figure 4.15B and 4.7**), thus attributing this rescue to a restoration of normal IL-10 levels.

Together, these data suggested that IL-27p28-mediated production of IFN-β augments IL-10 expression in macrophages, but not in DCs, which is required to promote STAT3 activation and suppress pro-inflammatory macrophage responses to LPS. In summary (**Figure 4.16**), we provided new findings that there is no obligate requirement of IL-27 production and signaling for LPS-induced IL-10 expression in macrophages. In the absence of IL-27 production and signaling, the level of IFN-β expression tunes the magnitude of IL-10 induction: residual amounts of IFN-β protein secretion in IL-27p28-deficient BMDMs (**Figure 4.12**) generated minimal IL-10 cytokine secretion, STAT3 activation, and suppression of pro-inflammatory cytokines (**Figure 4.5**) that could be rescued by supplementation with exogenous recombinant IFN-β (**Figure 4.14 and 4.15**).

4.11 IL-27p28 signaling is not required for IFN-β induction in BMDCs

In view of our finding that IL-27p28 is required for LPS-induced IL-10 production in macrophages but not DCs (**Figure 4.5 and 4.8**), we wondered whether TLR4-mediated IFN-β induction also show differential dependence on IL-27p28 in macrophages versus DCs. We found that the induction of IFN-β gene in LPS-stimulated BMDCs was generally comparable between IL-27p28-knockout and wild-type cells (**Figure 4.17**), contrasting strongly with the critical requirement for IL-27p28 in mediating IFN-β responses in endotoxin-challenged macrophages (**Figure 4.12**). This observation, that both LPS-induced IL-10 (**Figure 4.8**) and IFN-β production (**Figure 4.17**) in BMDCs are independent of IL-27p28 fits the current understanding that IFN-β and IL-10 are typically co-regulated in LPS-stimulated macrophages and DCs, and we conclude that while IL-27p28 is essential for both IFN-β and IL-10 production in macrophages, IL-27p28 is redundant for both IFN-β and IL-10 production in DCs.

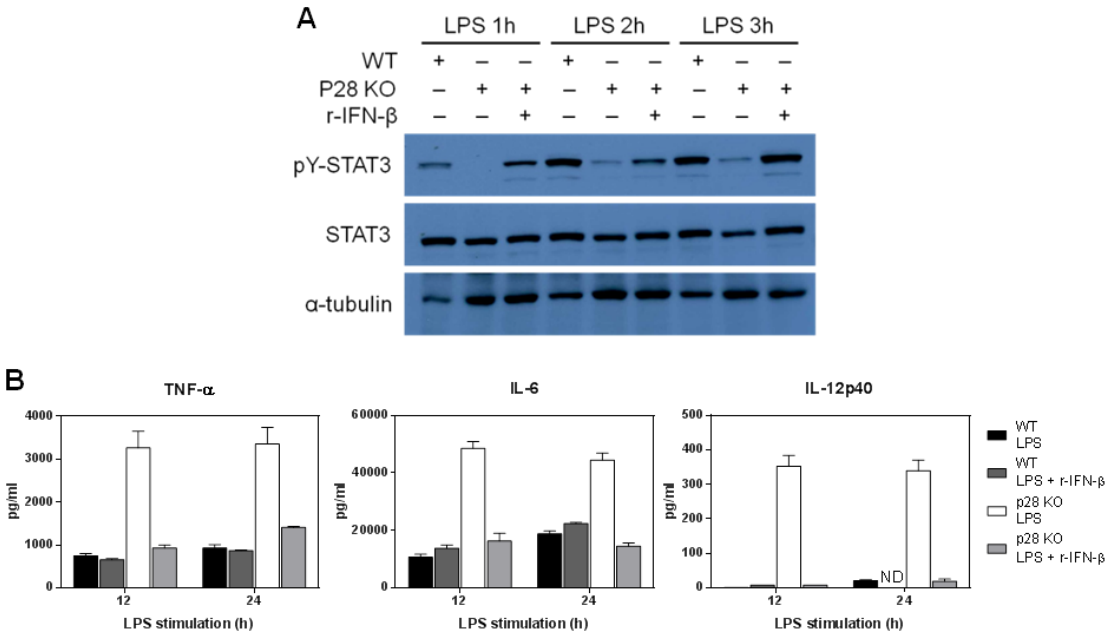

Figure 4.15
IFN-β cytokine restores STAT3 activation and suppression of pro-inflammatory responses in IL-27p28-deficient macrophages.
(A) Western immunoblot analysis of phospho-STAT3 and total STAT3 protein expression in whole cell lysates of BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, treated or not with 100ng/ml LPS either alone or in combination with 250U/ml recombinant murine IFN-β for 1-3 h. Data are representative of at least two independent experiments.
(B) Multiplex analysis of TNF-α, IL-6 and IL-12p40 production in BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, treated or not with 100ng/ml LPS either alone or in combination with 250U/ml recombinant murine IFN-β for 12 h and 24 h. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.

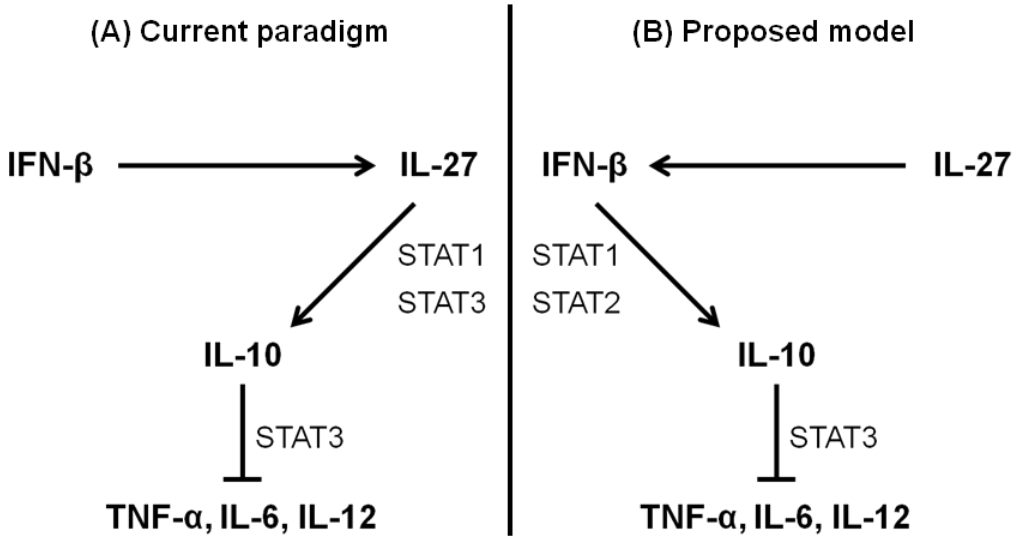

Figure 4.16
IL-27p28-mediated IFN-β production is required for optimal IL-10/STAT3-mediated anti-inflammatory responses in endotoxin-challenged macrophages.
(A) Current paradigm of the regulation of LPS-induced IL-10 production in macrophages by type I IFN via the intermediate induction of IL-27 proposed that type I IFN regulates LPS-mediated IL-10 transcription via the intermediate induction of IL-27 downstream of type I IFN production and signaling.
(B) Our data in Figures 4.12 to 4.15, that (i) IFN-β induction in BMDMs is dependent on IL-27p28, and (ii) exogenous recombinant murine IFN-β is able to rescue IL-10 production, STAT3 activation, and suppression of pro-inflammatory cytokine production in BMDMs that lack IL-27p28, placed IFN-β downstream of IL-27p28 in the regulation of LPS-mediated IL-10 transcription.

4.12 IL-27p28 promotes type I IFN responses to endotoxin exposure *in vivo*

Having shown that IL-27p28 is required for TLR4-mediated IFN-β induction in macrophages but not DCs (**Figure 4.12 and 4.17**), we then assessed the broader physiological relevance of our findings by verifying the requirement for IL-27p28 in supporting IFN-β production *in vivo*. IL-27p28 knockout mice that had been challenged with LPS via the intra-peritoneal route showed a trend towards reduced serum levels of IFN-β cytokine (n=3, p=0.0582) compared with wild-type mice (**Figure 4.18**). These data suggest that the impaired IFN-β responses in TLR4-activated macrophages lacking IL-27p28 translate into a partial defect (albeit not statistically significant) in type I IFN responses in IL-27p28-deficient mice *in vivo*.

4.13 Regulation of the macrophage TLR4-induced IFN-β response by IL-27p28 is not mediated via the canonical TRIF-IRF3 pathway

We identified IL-27p28 as a novel regulator of macrophage TLR4-mediated IFN-β induction (**Figure 4.12**). To extend this finding, we began to look at the possible molecular basis of this phenomenon of IL-27p28-mediated IFN-β amplification in TLR4-activated macrophages. First, we explored the possibility that this could be due to modulation of the canonical TRIF-IRF3 pathway by IL-27p28. To investigate this possibility, we assessed LPS-induced IRF3 phosphorylation and nuclear translocation in IL-27p28-deficient BMDMs. Analysis of total lysates showed that total IRF3 protein expression was essentially normal in IL-27p28-null macrophages, and the levels of IRF3 phosphorylation in LPS-stimulated IL-27p28-knockout BMDMs was almost comparable to the levels seen in wild-type BMDMs (**Figure 4.19A**). Analysis of nuclear extracts showed that IRF3 nuclear translocation was also similar between LPS-stimulated IL-27p28-knockout and wild-type BMDMs (**Figure 4.19B**). As a positive control, TRIF-knockout BMDMs showed undetectable levels of IRF3 phosphorylation and nuclear translocation (**Figure 4.19**). Thus, despite intact TRIF-IRF3 pathway, LPS-stimulated IFN-β expression was significantly impaired in IL-27p28-deficient BMDMs. We then turned our attention to interrogate the involvement of IRF7 in IL-27p28 regulation of IFN-β expression in macrophages, since our earlier finding identified IRF7 as a novel regulator of TLR4-mediated IFN-β induction in macrophages (**Figure 3.6**).

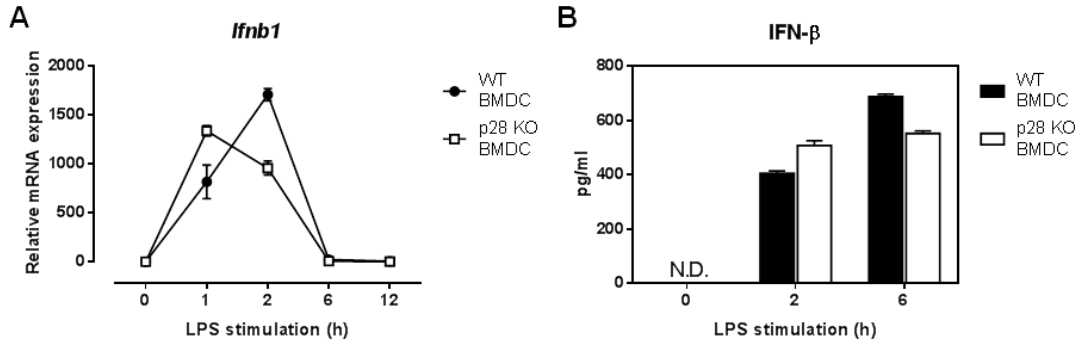

Figure 4.17
IFN-β responses in endotoxin-challenged DCs are independent of IL-27p28.
Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of BMDCs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments. N.D.: not detected.

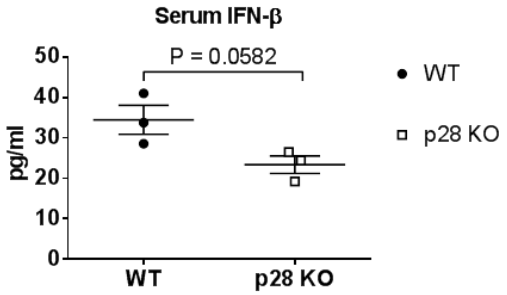

Figure 4.18
IL-27p28 knockout mice show a trend towards decreased type I IFN responses to LPS *in vivo*.
ELISA analysis of IFN-β levels in serum from IL-27p28 knockout mice, compared with wild-type control littermates, 3 h after i.p. injection of 30 µg/g LPS in sterile PBS. Data are presented as mean ± SEM of n=3 mice. (p=0.0582 compared with wild-type mice by unpaired t test).

4.14 IL-27p28 modulates IRF7 expression by regulating constitutive IFN-β production and STAT1 binding to the IRF7 enhancer

We next assessed whether IRF7 is involved in TLR4-activated IFN-β synthesis downstream of IL-27p28 production and signaling. Western blot analyses showed that resting wild-type macrophages intrinsically express high levels of IRF7 protein, but the levels of IRF7 protein were significantly reduced in IL-27p28 knockout macrophages, both at the basal state as well as within the window of IFN-β transcriptional induction up to 2 h after LPS stimulation (**Figure 4.20A**), while total IRF3 protein expression was unaffected in IL-27p28 knockout BMDMs (**Figure 4.19A and 4.20B**). These data implicated IRF7 in the IL-27p28-driven amplification of IFN-β responses in LPS-challenged macrophages.

We earlier found that basal IRF7 expression is controlled by constitutive IFN-β production and signaling in macrophages, Hence, the diminished expression of basal IRF7 protein in IL-27p28 knockout BMDMs suggest that IL-27p28 production/signaling could mediate constitutive IFN-β production in macrophages. Indeed, further examination of resting BMDMs revealed that IFN-β transcript levels were significantly reduced in IL-27p28-deficient cells (**Figure 4.21A**), and that high basal expression of IRF7 mRNA required intact IL-27p28 signaling (**Figure 4.21B**), thus supporting the concept that IL-27p28 regulates constitutive IFN-β production and impacts on basal expression of *Irf7* at the transcriptional level.

It has previously been suggested that constitutive IFN-β signaling maintains basal expression of key signaling intermediaries including STAT1 [72, 87, 90], and here we observed that IL-27p28-deficient macrophages with corresponding defects in constitutive IFN-β signaling displayed reduced basal expression of total STAT1 protein (**Figure 4.13A and 4.14A**). Indeed, our chromatin immunoprecipitation (ChIP) experiments further revealed that the constitutive association of STAT1 with the IRF7 enhancer in IL-27p28-knockout BMDMs is reduced compared to resting wild-type macrophages (**Figure 4.22**). Together, these data indicated that in resting macrophages, IL-27p28 enhances constitutive IFN-β production and signaling which promotes STAT1 recruitment to the IRF7 enhancer, resulting in high basal expression of IRF7 protein, which supports robust IFN-β responses upon subsequent LPS exposure.

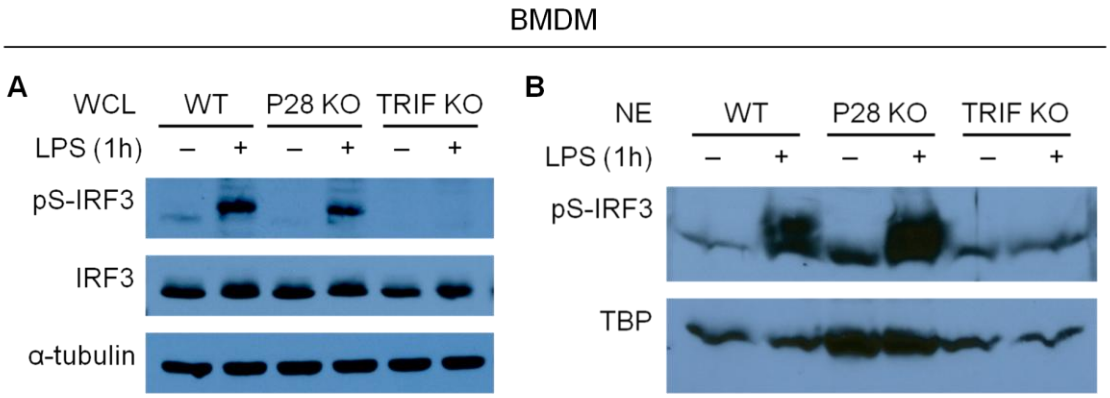

Figure 4.19
IL-27p28 does not regulate IFN-β expression via the TRIF-IRF3 pathway in endotoxin-challenged macrophages.
Western immunoblot analysis of phospho-IRF3 and total IRF3 protein expression in whole cell lysates (A) or nuclear extracts (B) of wild-type and IL-27p28 knockout BMDMs, stimulated or not with 100ng/ml LPS for 1 h.

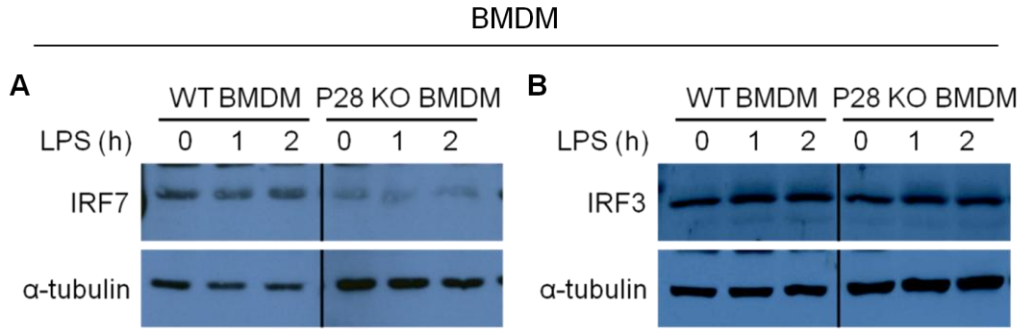

Figure 4.20
Constitutive IL-27p28 production and signaling sustains IRF7 expression in resting macrophages.
Western immunoblot analysis of total IRF7 and total IRF3 protein expression in whole cell lysates of BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times. Data are representative of at least three independent experiments.

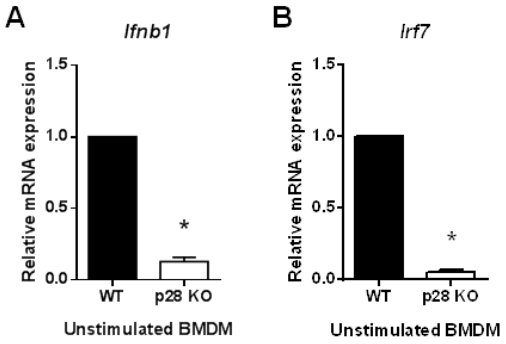

Figure 4.21
IL-27p28 maintains basal Irf7 gene expression via constitutive IFN-β signaling in steady-state macrophages.
Real-time PCR analysis of *Ifnb1* and *Irf7* gene expression in un-stimulated BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates. Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SEM of at least three independent experiments (*p<0.05 by paired t test).

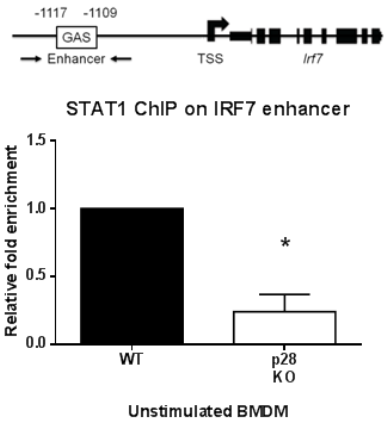

Figure 4.22
High-level IRF7 expression in resting macrophages is sustained by constitutive IL-27p28 signaling and STAT1 binding to the IRF7 enhancer.
ChIP analysis of STAT1 binding at the IRF7 enhancer in resting BMDMs from IL-27p28 knockout mice, compared with wild-type control littermates. ChIP-enriched DNA was normalized to input DNA and expressed relative to the levels observed in in STAT1 ChIP in un-stimulated wild-type control cells. Data are presented as mean ± SEM of at least three independent experiments (*p<0.05 by t test).

4.15 Pre-treatment with IFN-β cytokine up-regulates IRF7 and rescues LPS-induced IFN-β responses in IL-27p28-deficient BMDMs

Having established the relationship that IL-27p28 regulates LPS-induced IFN-β expression via its effects on constitutive IFN-β production and basal IRF7 expression, we asked whether exogenous IFN-β could rescue basal IRF7 expression and LPS-induced IFN-β expression in IL-27p28 knockout BMDMs. We pre-treated IL-27p28 knockout BMDMs with 1U/ml recombinant murine IFN-β (equivalent to about 40pg/ml IFN-β) for 5 h to mimic the concentration of endogenous constitutive IFN-β production [87]. This level of IFN-β cytokine in the cell culture supernatants was confirmed to be below the level of detection of the IFN-β ELISA assay used to quantitate IFN-β secretion in our experiments (data not shown). Pretreatment of IL-27p28-deficient BMDMs with exogenous IFN-β up-regulated both IRF7 mRNA and protein to levels above that of untreated wild-type macrophages (**Figure 4.23A, B**), suggesting that IRF7 up-regulation is highly sensitive to low levels of type I IFN signaling. In parallel, pretreatment with exogenous IFN-β increased IFN-β transcription and secretion to levels above that of untreated wild-type macrophages (**Figure 4.23C, D**). The ability of IFN-β pretreatment to enhance IFN-β responses in LPS-stimulated IL-27p28 knockout BMDMs required IRF7, since this effect was not observed in IRF7-null BMDMs (**Figure 4.23E, F**), thus attributing the rescue to the restoration of IRF7 levels in IL-27p28 knockout BMDMs. Together, these data suggest that IRF7 is an IL-27p28-dependent regulator of LPS-induced IFN-β production.

4.16 BMDMs exhibit higher constitutive IL-27p28 production than BMDCs

Since our earlier finding showed that constitutive IFN-β production in BMDMs is dependent on IL-27p28 production/signaling (**Figure 4.21**), we next asked whether the reduced constitutive IFN-β synthesis (**Figure 3.32**) and basal IRF7 expression (**Figure 3.29**) in resting BMDCs relative to BMDMs could be due to differential IL-27p28 production. We compared the levels of IL-27p28 transcripts in resting wild-type BMDMs and BMDCs, and observed that basal production of IL-27p28 was indeed reduced in BMDCs compared with BMDMs (**Figure 4.24 and Figure S9 in Appendix I**). These data suggest that the decreased activity of the IL-27p28-IRF7 signaling axis in

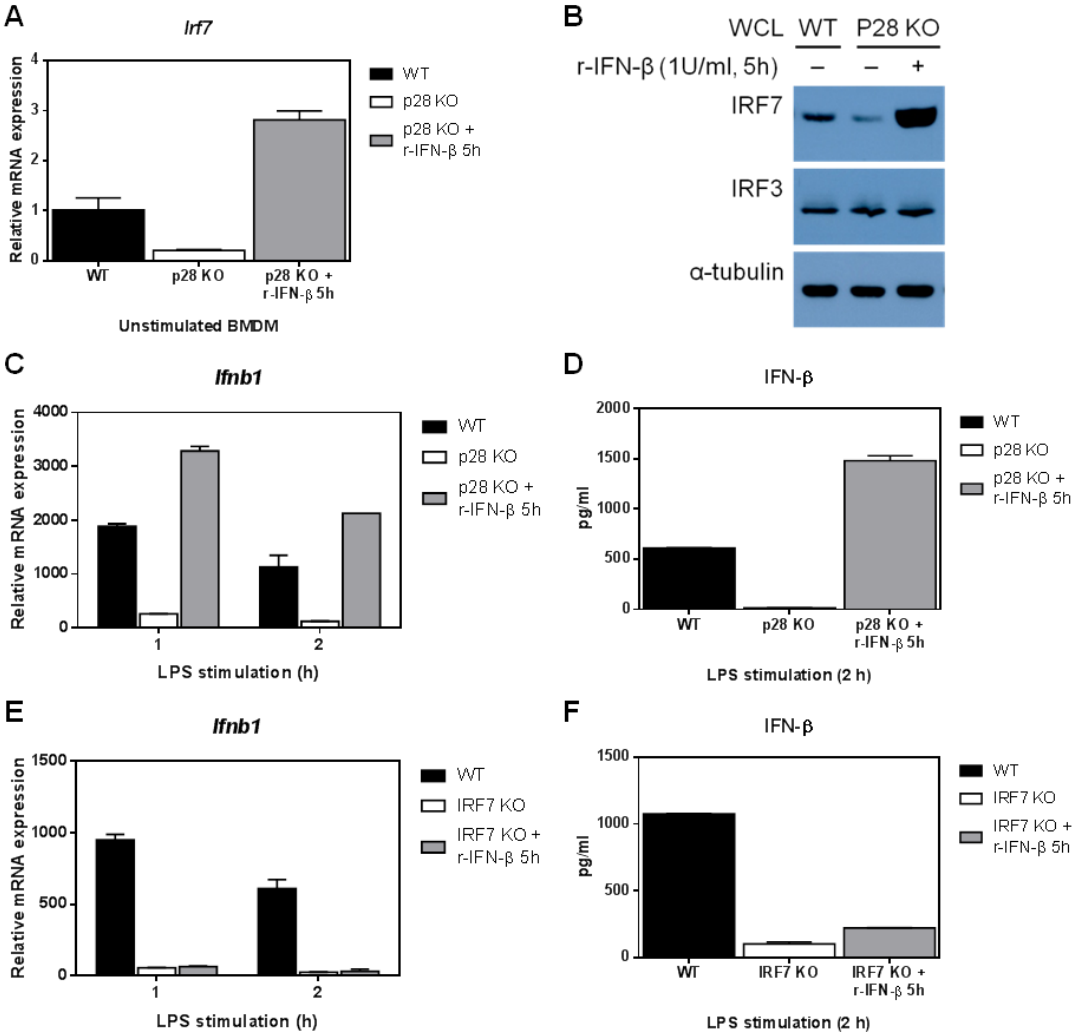

Figure 4.23
IFN-β responses in LPS-challenged IL-27p28-deficient BMDMs are rescued by pre-treatment with exogenous IFN-β cytokine resulting in the up-regulation of IRF7 protein.
(A, B) Analysis of IRF7 gene and protein expression by real-time PCR and western immunoblot analysis in whole cell lysates of wild-type and IL-27p28 knockout BMDMs, treated or not with 1U/ml recombinant murine IFN-β for 5 h. *Irf7* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells.
(C-F) Analysis of IFN-β gene and protein expression by real-time PCR and ELISA analysis of wild-type, IL-27p28 knockout (C, D), and IRF7 knockout (E, F) BMDMs, treated or not with 1U/ml recombinant murine IFN-β for 5 h and then stimulated with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

BMDCs compared with BMDMs, i.e. weaker constitutive IL-27p28-IFN-β signaling and negligible basal IRF7 expression in BMDCs, results in relatively lower levels of LPS-induced IFN-β mRNA transcript and cytokine production in BMDCs, which are largely independent of IRF7 and IL-27p28. Based on our results, we propose a model in which IL-27p28 positively regulates constitutive IFN-β expression and signaling. It is likely that both signals are required to maintain adequate total STAT1 levels to sustain high levels of IRF7 expression in resting macrophages, which is ultimately responsible (together with IRF3) for robust IFN-β production by macrophages in response to TLR4 ligands, which is in turn biologically relevant for optimal STAT1 activation and transcriptional induction of a subset of type I IFN-dependent cytokines and chemokines.

In conclusion, we propose that IL-27p28 is a cell-type specific positive regulator of IFN-β and IL-10 production in macrophages, but not in myeloid DCs. In macrophages, constitutive IL-27p28 controls constitutive IFN-β expression, and positively regulates high basal STAT1 and IRF7 levels. We established an essential role for IRF7 (together with IRF3) for the amplification of IFN-β production in response to LPS. In the light of these findings, we further refine the current knowledge of IL-10 regulation by proposing that there is no obligate requirement of IL-27p28 production and signaling for LPS-induced IL-10 production, and that the level of IFN-β is sufficient to tune the magnitude of IL-10 induction and the STAT3-mediated anti-inflammatory response in the absence of IL-27p28 production and signaling. On the contrary, in DCs, the relative lack of the IL-27p28-IRF7 signaling axis, due to a weaker constitutive IL-27p28-IFN-β signaling and negligible IRF7 expression, results in relatively lower IFN-β production in response to LPS. This in turn results in relatively lower LPS-induced IL-10 production, culminating in a weaker STAT3-mediated anti-inflammatory response in DCs compared with macrophages (**Figure 4.25**).

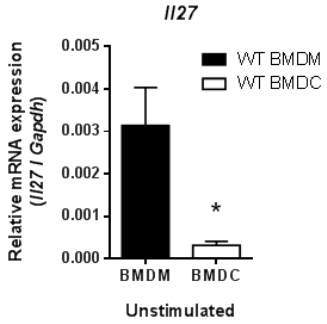

Figure 4.24
Weak IFN-β signaling and negligible IRF7 expression in steady-state DCs are associated with low constitutive IL-27p28 production.
Real-time PCR analysis of *Il27p28* gene expression in un-stimulated wild-type BMDMs and BMDCs. *Il27p28* expression was normalized to *Gapdh*. Data are presented as mean ± SEM of at least three independent experiments (*p<0.05 paired t test).

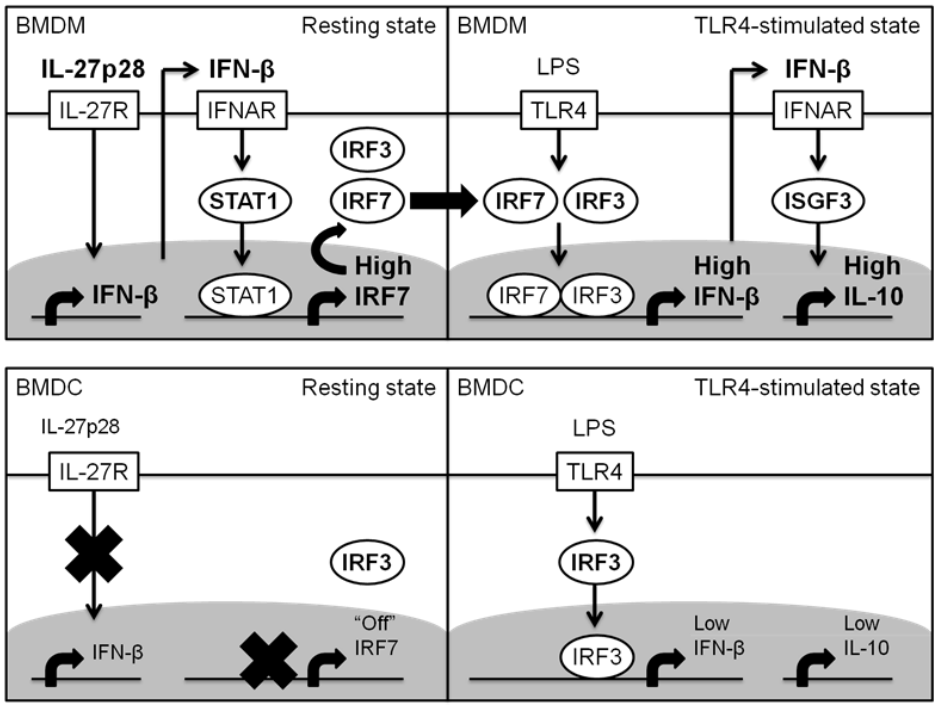

Figure 4.25
Cell type-specific signaling axis comprised of IL-27p28/IFN-β/IRF7 is constitutively active in macrophages but not in DCs.
In macrophages, constitutive IL-27p28 controls constitutive IFN-β production and basal IRF7 expression to amplify IFN-β responses to LPS. In contrast, DCs exhibit weak constitutive signaling via the IL-27p28-IFN-β axis and display negligible basal IRF7 expression, leading to blunted IFN-β responses, reduced IL-10 production, and minimal restraint of inflammatory responses to LPS.

5 DISCUSSION

TLR4, the mammalian homologue of the *Drosophila* Toll gene, was discovered more than 15 years ago by Beutler and Hoffmann *et al.* as the receptor for LPS recognition and signaling, in conjunction with the plasma LBP and the membrane co-receptors CD14 and MD2 [10, 19]. Of all the TLRs known to date, TLR4 is the only TLR that utilizes both MyD88 and TRIF signaling adaptors for signal transduction. The current paradigm is that MyD88 signals early NF-κB activation from the plasma membrane, while TRIF signals IRF3 activation and type I IFN induction, as well as late NF-κB activation, from endosomal compartments upon CD14-dependent TLR4 endocytosis [28, 266]. Among the members of the TLR family, TLR3, TLR4, TLR7/8 and TLR9 sensing of bacterial and viral infections have been reported to result in IFN-β induction. IFN-β expression exhibits different patterns of induction in response to different stimuli in different cell types: in non-immune cells such as fibroblasts (MEFs), HEK cells, and HeLa cells, virus-induced IFN-β expression follows a relatively delayed kinetics (being transcriptionally induced after 6 h) with a relatively low and more sustained level of induction [109, 267]; in BMDCs, virus-induced IFN-β expression follows biphasic kinetics (with the first phase peaking at 2 h and a second higher peak at 7 h post-stimulation) [79]. We have previously reported that in human monocytes, LPS-induced IFN-β expression follows a rapid and transient kinetics (being transcriptionally induced as early as 1 h and declining after 2 h post-stimulation) with a robust and high level of induction [109]. We observed a similar temporal profile in LPS-stimulated murine BMDMs and BMDCs, but BMDMs exhibit more robust IFN-β expression and type I IFN responses compared with BMDCs (**Figure 3.1 to 3.3**). Although mechanisms underlying type I IFN gene induction in response to viral infections have been extensively studied in fibroblasts and some immune cells such as DCs, showing the involvement of positive feedback through IRF7 and IRF8 respectively [35, 79], molecular mechanisms by which monocytes/macrophages express high amounts of type I IFNs in response to LPS have remained relatively poorly characterized, apart from the involvement of the classical TLR4-TRIF-IRF3 pathway [16, 75].

In this study, we used a systematic genetic loss-of-function approach using a broad range of knockout mice, from which BMDMs and BMDCs were generated under controlled experimental conditions, to unravel the gene regulatory network controlling

LPS-mediated IFN-β induction in macrophages, compared with that in DCs. The various knockout cells have been used extensively in previous studies to investigate the roles of signal transducers and transcription factors in cellular responses to various TLR agonists and cytokines *in vitro*, without major confounding from differences in the bone marrow compartment and hematopoiesis, which are more likely to affect observations made *in vivo*. Although IRF7 has been reported to play a role in the differentiation of U937 and HL60 monocytic cell lines to macrophages *in vitro* [268], hematopoietic cell populations, and, specifically, the frequencies of splenic $CD11b^{+}$ macrophages and $CD11c^{hi}$ $MHCII^{hi}$ cDCs were not altered in IRF7 knockout mice [35, 269]. Myeloid development was normal in MyD88, STAT1, or STAT3 knockout mice [47, 223, 227, 230, 270]. To our knowledge, there is no evidence implicating TRIF, IRF1, IRF3, IFNAR, STAT2, or IL-27p28 in macrophage development. Importantly, consistent with the above, no overt differences were observed in macrophage yields in our experiments by morphological observations or flow cytometric analyses for all the knockout cells used in this study.

From this work, we identified IRF7 as a novel cell type-specific transcription factor implicated in LPS-induced IFN-β expression in BMDMs but not BMDCs. We demonstrate for the first time that macrophages constitutively express high levels of IRF7, and this is achieved through constitutive IL-27p28-IFN-β signaling, leading to high basal STAT1 expression and its constitutive binding to the IRF7 enhancer to sustain high levels of IRF7 expression at steady-state. By acting in concert with IRF3, this novel IL-27p28-IFN-β-IRF7 signaling axis supports amplification of the macrophage IFN-β response to TLR4 ligation. Thus, we propose a new paradigm, whereby IRF7, in addition to IRF3, is also critical for robust IFN-β induction following LPS stimulation of macrophages, and the two transcription factors work in a cooperative or synergistic manner to facilitate strong IFN-β amplification in the macrophage TLR4 pathway. On the contrary, in DCs, the relative lack of a tonic IL-27p28-IFN-β-IRF7 signaling axis, as epitomized by negligible basal levels of IRF7 expression, likely due to weaker constitutive IL-27p28-IFN-β signaling and other known or unknown negative regulators, led to relatively lower IFN-β transcript and protein expression in response to TLR4 activation, which are largely independent of IL-27p28, autocrine/paracrine IFNAR signaling, and IRF7 (**Figure 5.1**).

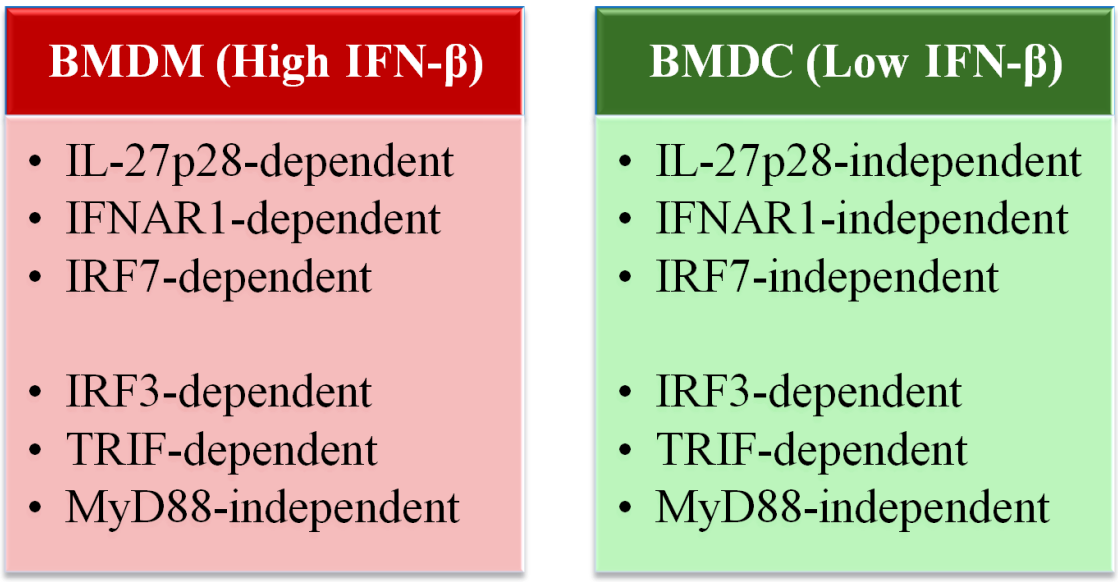

Figure 5.1: Macrophage-restricted role for IL27p28/IFN-β/IRF7 signaling in type I IFN responses to TLR4 ligation.

5.1 Differential dependence of LPS-induced IFN-β expression on type I IFN signaling in BMDMs versus BMDCs

Here, using IFNAR1-deficient BMDMs and BMDCs, we established that autocrine/paracrine type I IFN signaling is required for the amplification of IFN-β gene expression and cytokine production in LPS-stimulated BMDMs but not BMDCs (**Figure 3.6 and 3.8**). This is consistent with the conclusions of Karaghiosoff *et al.*, which showed that LPS-induced IFN-β activation was impaired in peritoneal macrophages lacking Tyk2 (a kinase downstream of IFNAR) [53], and that of Hoshino *et al.*, which showed that LPS-stimulated IFN-β expression was largely normal in IFNAR-null BMDCs [237]. However, our observations were different from the findings of Doyle *et al.*, in which lipid A-stimulated IFN-β expression was only slightly reduced in IFNAR-deficient BMDMs [91], and that of Gautier *et al.*, in which LPS-induced IFN-β up-regulation was reduced in IFNAR-null BMDCs [80]. This may be due to the different TLR4 agonists used (LPS versus lipid A), different methods of DC differentiation from mouse bone marrow progenitors, or other unknown variables in experimental conditions. Nevertheless, our experiments using the same dosage of the same TLR4 agonist (LPS), and standardized downstream analyses of gene expression

and cytokine production in both cell types, conclusively showed that LPS-induced IFN-β expression is dependent on autocrine/paracrine type I IFN signaling in BMDMs but not in BMDCs. This is supported by further evidence that LPS-induced IFN-β up-regulation is almost completely abrogated in STAT1- and STAT2-knockout BMDMs (**Figure 3.11**), but largely retained in STAT1-null BMDCs [237].

In BMDMs, despite normal IRF3 phosphorylation and nuclear translocation, TLR4-mediated IFN-β induction was impaired in the absence of type I IFN signaling (**Figure 3.6 and 3.9**), but type I IFN signaling alone in the absence of TLR4 activation did not substantially up-regulate IFN-β transcription (**Figure 3.7**), indicating that type I IFN signaling does not directly induce IFN-β expression. These observations led us to hypothesize that type I IFN signaling supports TLR4-mediated IFN-β responses in macrophages via an independent mechanism distinct from the canonical TRIF-IRF3 pathway. Amplification of IFN-β responses by autocrine/paracrine type I IFN signaling may potentially occur on two levels [271]: (i) by a type I IFN positive feedback loop *after* pathogenic stimulation, in which initial small amounts of IFN-β lead to later high levels of IFN-β secretion [76], or (ii) by *constitutive* type I IFN signaling in the absence of pathogenic stimulation, which primes the cells for augmented IFN-β responses upon microbial infection [72]. We found that LPS stimulation rapidly induced accumulation of IFN-β mRNA in macrophages within just 2 h of endotoxin exposure (**Figure 3.1**), in contrast to the IFN-β response to viral infection in fibroblasts and cDCs, which exhibit delayed amplification (>6 h post-infection) dependent on IFNAR, and IRF7 or IRF8 respectively [35, 78, 79, 267]. This suggests the higher likelihood of the latter mechanism, whereby *constitutive* type I IFN signaling in steady-state macrophages primes the cells for augmented IFN-β responses almost immediately upon LPS challenge. Indeed, we found that constitutive type I IFN signaling is responsible for establishing the high-level expression of IRF7 in resting macrophages, which in turn is required, in cooperation with IRF3, for robust IFN-β responses in LPS-challenged macrophages. However, it is still unclear whether type I IFN signaling *after* TLR4 stimulation plays a role in the amplification of the IFN-β response, because IFNAR knockout BMDMs do not allow us to dissect the relative roles of type I IFN signaling before and after LPS stimulation. One possible approach to investigate this question may be to use IFNAR-blocking antibodies added simultaneous with LPS treatment compared with before LPS treatment to decipher the role of *LPS-induced* type I IFN

signaling versus *constitutive* type I IFN signaling, respectively. However, to obtain effective blocking, certain conditions such as the duration of antibody treatment, and the kinetics of antibody binding and receptor blocking will have to be optimized. While we showed here that constitutive type I IFN signaling in steady-state macrophages is necessary for the robust IFN-β response upon endotoxin exposure, the subsequent autocrine/paracrine type I IFN signaling in LPS-challenged macrophages is required for the normal gene expression and cytokine production of downstream ISGs, such as IL-27p28 and IL-10 (**Figure 4.1 and 4.4**). In other words, while "constitutive IFN-β" is necessary for the IFN-β response to LPS in macrophages, "induced IFN-β" is required for the downstream ISG (e.g. pro-caspase-11, IL-27p28, and IL-10) response. Constitutive type I IFN signaling has been demonstrated to play a role in homeostasis, such as in augmenting the responses to other cytokines like IFN-γ and IL-6 [72], and in preventing cellular transformation and tumorigenesis [272]. It would be interesting to examine in future studies whether there are other differential roles of "constitutive IFN-β" versus "induced IFN-β" in homeostasis, in the development of myeloid cells, or in the macrophage response to other inflammatory stimuli.

5.2 Differential expression of constitutive and LPS-induced IFN-β in BMDMs versus BMDCs

In contrast to fibroblasts, splenocytes have been reported to have higher constitutive type I IFN production, leading to higher IRF7 expression in uninfected splenocytes and enhanced IFN-β production in viral-infected splenocytes [83]. Splenocytes consist of a variety of immune cells, including macrophages and various subsets of DCs. It was reported that M-CSF-differentiated macrophages displayed higher constitutive IFN-β production, resulting in increased IFN-β activation upon LPS stimulation, compared with GM-CSF-differentiated macrophages [87]. Here, we compared M-CSF-differentiated BMDMs with GM-CSF-differentiated BMDCs, and found that steady-state BMDMs exhibit higher constitutive IFN-β production (**Figure 3.32**) and tonic type I IFN signaling (manifested as higher basal STAT1 phosphorylation, STAT1 binding to the IRF7 promoter, and basal IRF7 expression) (**Figure 3.3, 3.32 and 3.33**) than steady-state BMDCs. Furthermore, constitutive expression of ISGs, including classical antiviral genes such as *Isg15*, *Isg54*, *Isg56*, *Mx1*, *Oas1a* and *Viperin*, were lower in unstimulated BMDCs compared with unstimulated

BMDMs (**Figure S10 in Appendix J**). This diminished constitutive expression of type I IFN and antiviral genes may constitute another mechanism of "enforced viral replication", to allow viral replication in BMDCs, which in turn permits efficient antigen presentation and induction of the adaptive immune response by DCs. This phenomenon of "enforced viral replication" has been previously reported in $CD11c^+$ DCs and $CD169^+$ metallophilic macrophages, which was mediated mechanistically by the selective expression of USP18, an inhibitor of the type I IFN response, in these cells but not in other cell types [273, 274]. Importantly, we identified that constitutive IFN-β production is mediated by tonic IL-27p28 production/signaling (**Figure 4.21**), thus introducing a novel player in cytokine functional cross-talk, even in the absence of acute infection, in steady-state macrophages.

Tonic type I IFN signaling is a prerequisite for robust LPS-induced IFN-β production in macrophages (**Figure 3.6**), but constitutive IFN-β expression and LPS-induced IFN-β expression are mediated by distinct mechanisms — the former is independent of whereas the latter is dependent on TRIF, IRF3, and IRF7. This also allowed us to rule out endotoxin contamination of the BMDM differentiation media for the observed priming of LPS-induced IFN-β responses by tonic type I IFN signaling. In unstimulated fibroblasts, NF-κB RelA and c-Jun binding to the IFN-β promoter has been shown to promote basal IFN-β expression [72, 85, 275, 276]. A similar requirement for NF-κB activation is also observed in our basal IFN-β expression, tonic type I IFN signaling, and hence constitutive IRF7 expression in steady-state BMDMs (**Figure 3.36**). It is highly possible that a certain fraction of NF-κB is active and bound to the IFN-β promoter in steady-state macrophages, since constitutive NF-κB activation has been detected in differentiated macrophages, and is shown to be essential for macrophage survival [277]. Aberrant constitutive NF-κB activation has been implicated in the pathology of many cancers, including Hodgkin lymphoma, mucosa-associated lymphoid tissue lymphoma, diffuse large B-cell lymphoma, multiple myeloma, and colorectal, breast, and prostate cancers [278], whereas constitutive type I IFN signaling is involved in the suppression of cellular transformation [272]. Therefore, it may be interesting to investigate the relationship between dysregulated constitutive NF-κB activation and type I IFN signaling in tumorigenesis. Nevertheless, having identified that constitutive IFN-β production is also mediated by tonic IL-27p28 production/ signaling (**Figure 4.21**), it remains to be further investigated whether basal NF-κB

activation occurs upstream or downstream of tonic IL-27p28 production/signaling, or both. Since NF-κB can mediate LPS-induced IL-27p28 gene expression via NF-κB binding sites on the IL-27p28 promoter [168], it is possible that basal NF-κB activation could also control constitutive IL-27p28 production in steady-state macrophages.

Evidence of modulation of TLR responses and signaling downstream of cytokine receptors by tonic signaling downstream of ITAM-coupled receptors in the regulation of macrophage activation have begun to emerge [206]. It was reported that IFN-α-induced Jak-STAT signaling is regulated by the calcium-dependent kinases CaMK and Pyk2 downstream of ITAM-associated receptors [261]. In addition, calcium signaling mediated by CaMK has been found to be important for TLR4-mediated production of pro-inflammatory cytokines and type I IFNs in macrophages by directly activating TAK1 and IRF3 [279]. However, constitutive production of IFN-β in macrophages is independent of CaMK and Pyk2 (**Figure 3.36**). However, tonic type I signaling is dependent on Pyk2 but not its upstream regulator CaMK (**Figure 3.36**), indicating divergent regulation of constitutive type I IFN signaling by different members of the calcium-dependent pathway. Further investigations are required to understand the potentially complex regulation of constitutive type I IFN signaling by ITAM and calcium-dependent pathways.

Using "homogenous" populations of BMDMs and BMDCs, we found that BMDMs inherently display higher levels of constitutive IFN-β production (**Figure 3.32**) and more robust LPS-induced IFN-β responses (**Figure 3.3**) compared with BMDCs. Indeed, cell type-specific priming of type I IFN responses by autocrine IFNAR signaling has also been reported in coronavirus infection of BMDMs versus BMDCs [280], and subset-specific responses to LPS stimulation have also been described in distinct splenic DC subsets [281]. However, immune cell populations do not exist in isolation *in vivo*, and paracrine signaling most likely influences the responses of neighboring cell types in the local microenvironment. Indeed, we found that the LPS-induced IFN-β responses of BMDCs can be enhanced by the addition of exogenous type I IFN and the restoration of IRF7 expression (**Figure 3.30**). This implies that type I IFN secreted by macrophages or other cell types may prime neighboring unstimulated DCs to mount a strong IFN-β response when subsequently challenged with LPS. Indeed, type

I IFN secreted after TLR stimulation can prime neighboring DCs by paracrine signaling, and amplify DC responses to pathogen infection [81].

5.3 Differential dependence of LPS-induced IFN-β expression on IRF7 in BMDMs versus BMDCs

Both IRF1 and IRF7 genes possess GAS and ISRE sites on their promoters, which bind STAT1-containing transcription factor complexes downstream of type I IFN signaling [240]. Thus, they are candidate transcription factors that could play a role in IFNAR-dependent IFN-β induction in LPS-stimulated macrophages. It is known that IRF1 is important for CpG-DNA-induced IFN-β production in myeloid DCs and for TNF-induced IFN-β production in macrophages [16, 35, 76]. However, it appears not to be involved in LPS-induced IFN-β gene expression and cytokine production in macrophages (**Figure 3.12**). A similar finding was observed for viral-infected fibroblasts, where IRF1 was also found to play no role in the transcription of IFN-β [225, 282]. Interestingly, we observed a partial reduction in IL-27p28 gene expression and cytokine production in LPS-stimulated IRF1-deficient BMDMs (**Figure 4.10**), and IRF1 has been reported to play a role in IL-27p28 gene expression by binding to the IL-27p28 promoter in LPS-stimulated peritoneal macrophages [167, 168]. Importantly, our data showed that IRF7 is required for LPS-stimulated IFN-β induction in BMDMs, but not in BMDCs (**Figure 3.13 and 3.28**). This IRF7-independent IFN-β expression in LPS-stimulated BMDCs was also observed by Honda *et al.* [35]. While dispensable for BMDCs, IRF7 was found to be indispensable for the IFN-β amplification loop in virus-infected fibroblasts, for CpG-stimulated IFN-β induction in plasmacytoid DCs [16, 35, 75], and in our study here for IFN-β responses to TLR4 ligation in macrophages. In contrast to our results, which clearly demonstrate for the first time that IRF7 is required for LPS-induced expression of IFN-β in macrophages using IRF7 knockout BMDMs, a previous report concluded otherwise using shRNA knockdown of IRF7 in BMDMs [283]. This may be due to an incomplete knockdown of IRF7 expression in their approach, or due to an altered state of their BMDMs upon lentivirus transduction, as evidenced by the delayed and much reduced levels of IFN-β expression in response to LPS, compared with our wild-type BMDMs.

In BMDMs, despite normal IRF3 phosphorylation and nuclear translocation, TLR4-mediated IFN-β induction was impaired in the absence of IRF7 (**Figure 3.13 and 3.15**), indicating that IRF7 supports TLR4-mediated IFN-β responses independent of IRF3. Indeed, analysis of IRF3 single knockout and IRF7 single knockout BMDMs, together with IRF3-IRF7 double knockout and TRIF knockout BMDMs, showed that absence of either IRF3 or IRF7 alone results in residual LPS-induced IFN-β responses, while lack of both IRF3 and IRF7 phenocopies the complete absence of LPS-induced IFN-β responses in TRIF knockout BMDMs (**Figure 3.22**), suggesting that both IRF3 and IRF7 are activated downstream of TRIF, and cooperate or synergize with each other to induce maximal IFN-β expression in LPS-challenged macrophages. However, we were unable to conclusively determine whether endogenous IRF7 is phosphorylated downstream of TRIF in BMDMs due to the lack of appropriate reagents. Probing of whole cell lysates from LPS-stimulated macrophages with two different commercially-available phospho-IRF7 antibodies failed to detect a specific band of the expected size corresponding to the band size detected using the total IRF7 antibody (~51 kDa), which corresponds to the predominant full-length isoform of mouse Irf7 [284] (**Figure 3.18**). Nevertheless, it is highly likely that TRIF may serve to recruit and activate IRF7, since, using overexpression studies, TRIF was shown to be able to interact with both IRF7 and IRF3 [285], and using reporter gene assays, IRF7 was shown to be activated by TRIF and TRAM [286]. To further verify, other approaches may have to be explored, such as immunoprecipitating total cellular phosphorylated proteins using antibodies against phospho-serine and/or phospho-threonine residues, and then probing for IRF7, or *vice versa* [287]. These approaches critically depend on the stability of the protein, since IRF7 is a labile protein known to have a short half-life in most cell types [78, 288], except in pDCs, in which the longer half-life of IRF7 protein was partially attributed to autocrine IFN signaling following infection [288]. However, in that report, the stability of IRF7 was not directly compared between different cell types in the absence of stimulation. Hence, it will be interesting to study whether the half-life of IRF7 is different in steady-state BMDMs and BMDCs, and the possibility that this is dependent on differential constitutive type I IFN production/signaling and/or IL-27p28 production/ signaling in BMDMs versus BMDCs. Apparently, IRF7 activity can also be regulated by other post-translational modifications besides phosphorylation, for example, poly-ubiquitination mediated by TRIM21; SUMO modification mediated by PIAS1; and lysine acetylation mediated by p300/CREB-binding protein-associated factor (PCAF)

and general control of amino acid synthesis protein 5 (GCN5) [115, 289]. It remains an open question whether these post-translational modifications may also potentially be involved in IRF7-mediated IFN-β induction in the TLR4 pathway. It is also unclear whether TLR4-activated IFN-β induction is mediated by IRF3-IRF7 heterodimers, or IRF3-IRF3 homodimers and IRF7-IRF7 homodimers, or a combination of the above. Nonetheless, we were able to obtain evidence of IRF7 nuclear translocation in endotoxin-challenged wild-type BMDMs, albeit the increase in the levels of nuclear IRF7 protein upon LPS stimulation was slight, because of the presence of basal levels of nuclear IRF7 protein in steady-state BMDMs (**Figure 3.19**). This is analogous to that of IRF3, whereby a basal level of unphosphorylated IRF3 protein was also detected in the nucleus in the absence of stimulation (**Figure 3.15** and [79]), and it is the phosphorylated form of IRF3 that translocates from the cytosol into the nucleus after LPS stimulation. As such, like that of IRF3, probing with antibodies against phospho-IRF7 instead of total IRF7 may be more specific and provide clearer evidence of IRF7 nuclear translocation, and of whether this process is dependent on TRIF. Alternatively, nuclear localization of IRF7 may also be studied using immunofluorescence staining and confocal microscopy, if a suitable antibody becomes available [287].

There has been an emerging common theme that type I IFN induction is signaled from intracellular organelles instead of from the plasma membrane [290]. In particular, TRAM-TRIF signals IRF3 activation and type I IFN induction from endo-lysosomal compartments following CD14-dependent TLR4 endocytosis [291]. This process is dependent on the tyrosine kinase Syk, phospholipase Cγ2 (PLCγ2), intracellular calcium, p110δ isoform of phosphatidylinositol-3-OH kinase (PI3K), and the ITAM-containing adaptors DAP12 and FcεRγ [28, 29, 34]. Based on this concept, IRF7 activation downstream of TRIF may also be dependent on CD14-mediated TLR4 internalization into endo-lysosomal compartments, and further investigation into this aspect would be interesting. Intriguingly, while both macrophages and DCs required CD14 to mediate TLR4 internalization when treated with LPS, TLR4 endocytosis and IFN-β expression upon phagocytosis of whole *E. coli* bacteria or LPS-coated latex beads are dependent on CD14 in macrophages, but independent of CD14 in DCs [28]. These cell type-specific differences were suggested to be due to the differential "permissiveness" of TLR internalization between DCs and BMDMs [28]. It would be interesting to investigate whether IRF7 is required for IFN-β expression in response to

E. coli or LPS-coated latex beads in BMDMs and BMDCs. In short, further investigation into the relative dependency of IRF3 and IRF7 activation on signaling from the plasma membrane versus signaling from the endosomal compartment may yield novel mechanistic insights into the spatio-temporal regulation of IRF3 and IRF7 activation in the TLR4 pathway.

Importantly, we established that this IRF7-dependent IFN-β response in endotoxin-challenged macrophages is physiologically relevant *in vivo*, as manifested in an impaired systemic type I IFN response to intraperitoneal LPS administration in IRF7-deficient mice (**Figure 3.24**), and also biologically significant *in vivo*, in that IRF7-deficient mice are partially protected from lethal septic shock (**Figure 3.25**). A wide variety of cell types express TLR4 and can respond to LPS *in vivo*, including endothelial cells, and predominantly myeloid cells (monocytes, macrophages, DCs, and granulocytes) [281, 292]. We supported the suggestion by Rehli *et al.* and Salkowski *et al.* that macrophages are the main cell type that respond to endotoxin exposure *in vivo*, since we showed that maximal IFN-β expression requires IRF7 in BMDMs but not BMDCs, and serum IFN-β levels are dependent on IRF7 *in vivo* [292, 293]. Indeed, it was found that splenic macrophages may be a significant source of IL-27p28 in murine sepsis [294]. However, the contribution from DCs, such as after priming by paracrine type I IFN signaling resulting in IRF7 expression (**Figure 3.30** and [81]), or from other subsets of DCs [281], cannot be ruled out. Hence, the examination of LPS-induced IFN-β responses in mice with macrophage-specific or DC-specific conditional knockout of IRF7 would certainly help to clarify this point.

5.4 IRF7-dependent IFN-β responses to LPS in BMDMs is functionally important for IL-1β production *in vivo*

IL-1β plays a protective role in antibacterial inflammatory responses, such as by mediating IFN-γ production, which is critical for immunity against intracellular pathogens, and by enhancing T cell activation [122, 295]. However, excessive production of IL-1β can also contribute to the pathology of septic shock and other inflammatory diseases [122, 151]. Hence, tight control of IL-1β levels is crucial for mounting appropriate antibacterial host defenses, while preventing detrimental hyperinflammatory responses. Recently, emerging evidence point to a type I IFN and

IL-1 crosstalk, in which type I IFN signaling regulates IL-1β production via effects on non-canonical inflammasome activation involving caspase-11 and caspase-1 activation, in response to Gram-negative bacterial infection, such as *S. typhimurium*, entero-hemorrhagic *E. coli* (EHEC), and *C. rodentium* infection [130, 132]. This is reflected in reduced serum IL-1β levels in IFNAR-null mice infected with *E. coli* BL21, as well as in STAT1-null mice injected i.p. with LPS [122, 130]. Consistent with this concept, we observed reduced serum IL-1β levels in IRF7-deficient mice injected i.p. with LPS (**Figure 3.26**), indicating that IRF7-dependent IFN-β expression in macrophages is functionally important in determining circulating IL-1β levels. Pro-caspase-11 protein expression in macrophages stimulated with EHEC or LPS was shown to be dependent on TRIF and type I IFN signaling [130, 140, 296]. Indeed, we found that optimal caspase-11 mRNA induction and pro-caspase-11 protein expression in LPS-challenged macrophages are partially attenuated in the absence of IRF7 alone or IRF3 alone, and almost completely abrogated in the absence of both IRF7 and IRF3, or TRIF (**Figure 3.27**), closely reflecting the relative dependency of LPS-induced IFN-β production on IRF7, IRF3, and TRIF (**Figure 3.22**). Decreased pro-caspase-11 expression in IRF7- and IRF3-deficient BMDMs may at least partly explain the reduced serum IL-1β levels in IRF7- and IRF3 knockout mice, in accordance with previous findings that caspase-11 knockout mice exhibited reduced serum IL-1β levels after LPS injection [131]. Defective pro-caspase-11 expression, but not pro-caspase-1 expression, in IRF7- and IRF3-deficient BMDMs is also consistent with the increased survival of IRF7- and IRF3 knockout mice in the lethal septic shock model (**Figure 3.25**), in line with previous reports that caspase-11 knockout mice, but not caspase-1 knockout mice, were partially protected against LPS-induced lethality [131]. However, we were unable to assay caspase-11 processing into mature caspase-11 in our *in vitro* LPS-stimulated BMDMs, because canonical "two-signal" inflammasome activation, such as by LPS + ATP or LPS + nigericin stimulation, does not involve caspase-11 [130, 131]. *In vitro* caspase-11 activation is only effected either by whole bacteria infection [130-132], or by intracellular LPS delivered into the cytosol by direct transfection, electroporation, or cholera toxin B (CTB) [131, 137, 138]. Hence, using these *in vitro* infection models would aid in determining if caspase-11 processing into mature caspase-11 and IL-1β secretion via the non-canonical inflammasome pathway are perturbed in macrophages lacking IRF7 or other components of type I IFN production and signaling.

5.5 Differential expression of constitutive IRF7 in BMDMs versus BMDCs

We found that the cell type-specific requirement of IRF7 for LPS-induced IFN-β production in macrophages but not DCs is attributed to the differential constitutive expression of IRF7 protein in steady-state BMDMs versus BMDCs, instead of the differential activation of IRF7 by TLR4 stimulation between the two cell types. BMDMs possessed high levels of basal IRF7 expression, in contrast to BMDCs, which exhibited negligible levels of basal IRF7 expression (**Figure 3.29**). In the context of viral infection, pDCs have been described as classical 'IFN-producing cells' (IPCs) since they are the most potent known producers of type I IFNs as they have constitutive high expression of IRF7 [194, 297]. However, this population does not express TLR4 and is thus unresponsive to LPS [194, 297]. We therefore propose that macrophages represent the prototypical population of 'IFN-producing cells' in the context of TLR4 ligation, since they also constitutively express high levels of IRF7. Variable expression of IRF7 may therefore influence the magnitude of the IFN-β response in different host cell lineages [271]. The basal level of IRF7 also correlates with the resistance to virus infection of cells. For example, the differential susceptibility of primary macrophages, J774 cells, and RAW cells to Newcastle Disease Virus (NDV) infection inversely correlated with the basal expression of IRF7 in these cells [298]. This differential ability of various cell types to control viral replication could have implications in the orchestration of antiviral responses by diverse cell types. As such, a comparative analysis of the regulation of IRF7 expression in different cell types by basal constitutive IL-27p28-IFN-β signaling would be warranted.

Accumulating evidence suggests that IRF7 expression and activity is tightly regulated at multiple levels including transcription [299], post-transcription (mRNA stability) [300], translation [301, 302], and post-translation (protein stability) [288, 289]. For example, one study indicated that the transcription factor FOXO3 suppresses basal IRF7 transcription in BMDMs, and that in the absence of FOXO3, basal IRF7 levels in macrophages are even higher [303]. Future studies will therefore be required to determine whether this FOXO3-IRF7 gene regulatory circuit can be influenced by tonic IL-27p28-IFN-β signaling. In addition, IRF7 translation can also be negatively regulated by 4E-BP1, 4E-BP2, and OASL1 [301, 302]. Hence, in addition to the present

finding that constitutive IL-27p28-IFN-β signaling sustains high levels of IRF7 expression in macrophages at the transcriptional level by maintaining high basal STAT1 expression and binding to the IRF7 enhancer, further studies would be needed to address questions related to other possible mechanisms of autocrine IL-27p28-IFN-β signaling in IRF7 regulation at the post-transcriptional level, such as by de-repression and/or up-regulation of IRF7 translation, through the inhibition of negative regulators and/or activation of positive regulators. It would also be useful to determine the endogenous negative regulators that suppress IRF7 expression in cDCs, and to assess whether these restrictions could be counteracted by IL-27p28-IFN-β signaling.

Higher constitutive IFN-β production and enhanced basal STAT1 and IRF7 mRNA levels, as well as increased LPS-induced type I IFN production, have been reported for M-CSF-derived BMDMs (BMDMs) compared with GM-CSF-derived BMDMs (GM-BMMs) [87]. Our novel finding that links IRF7 to LPS-induced IFN-β expression in BMDMs provides a mechanistic explanation for this phenomenon: BMDMs exhibit more robust TLR4-activated IFN-β induction than GM-BMMs [87] possibly because the former possess higher basal IRF7 expression, which synergize with IRF3 activation downstream of TRIF to amplify the IFN-β response. Macrophages have been described to take on polarized functions, termed M1 (classically-activated) or M2 (alternatively-activated) macrophages, depending on their extracellular milieu [304]. It was reported that GM-BMMs have properties similar to M1-polarized (classically-activated) macrophages with a pro-inflammatory TNF-α^{hi}, IL-12p40^{hi} phenotype, whereas BMDMs have properties similar to M2-polarized (alternatively-activated) macrophages with an anti-inflammatory IFN-β^{hi}, IL-10^{hi} phenotype, respectively [87, 305]. The robust IFN-β and IL-10 responses to LPS exposure observed in the BMDMs used in our study are in line with the above description of M2-like macrophages. An IRF5-IRF4 balance for M1-M2 macrophage polarization have been proposed, because the transcription factor IRF5 was found to play a role in M1 macrophage polarization [306], while IRF4 controls M2 macrophage polarization [307]. In this regard, IRF7 may be considered as a transcription factor associated with M2 macrophage polarization. Indeed, it was demonstrated that IRF7 controls the pro- to anti-inflammatory (M1-to-M2) phenotype switch in central nervous system (CNS)-resident microglia: chronic exposure to TGFβ1 down-regulated IRF7 and prevented the acquisition of an anti-inflammatory M2 phenotype [308].

5.6 Differential dependence of LPS-induced IFN-β expression on IL-27p28 signaling in BMDMs versus BMDCs

We extended our findings by discovering that constitutive IFN-β production (**Figure 4.21**), total STAT1 protein expression (**Figure 4.13**), STAT1 binding to the IRF7 enhancer region (**Figure 4.22**), and basal IRF7 protein expression (**Figure 4.20**) in steady-state BMDMs are dependent on tonic IL-27p28 production, which is higher in steady-state BMDMs compared with BMDCs (**Figure 4.24**). Accordingly, LPS-induced IFN-β responses were impaired in IL-27p28 knockout BMDMs (**Figure 4.12**), but not in IL-27p28 knockout BMDCs (**Figure 4.17**), recapitulating the phenotype of IFNAR-deficient BMDMs (**Figure 3.6**) versus BMDCs (**Figure 3.8**), and of IRF7-null BMDMs (**Figure 3.13**) versus BMDCs (**Figure 3.28**), thus supporting our model of a cell type-specific regulation of TLR4-activated IFN-β induction by a constitutive IL-27p28-IFN-β-IRF7 signaling axis. This is reflected in the partial reduction in serum IFN-β levels in IL-27p28 knockout mice injected i.p. with LPS (**Figure 4.18**), albeit not to the significant extent as that observed in IRF7 knockout mice (**Figure 3.24**). This may be explained by the ability of type I IFN secreted by DCs (which is independent of IL-27p28) or other cell types to restore downstream basal IRF7 expression in IL-27p28 knockout macrophages (but not in IRF7 knockout macrophages) by paracrine signaling, enabling IL-27p28 knockout macrophages to mount a strong IFN-β response when subsequently challenged with LPS *in vivo* (**Figure 4.23**). Indeed, consistent with the notion that type I IFN is one of the major contributors to mortality in LPS-induced endotoxin shock [53], mice deficient in the EBI3 subunit of IL-27, as well as mice with disrupted IL-27 signaling, were shown to be protected against lethal septic shock [173, 177], in line with our findings in IRF7 knockout mice (**Figure 3.25**). Interestingly, IL-27 has been identified as a biomarker of human sepsis [309]. Hence, our novel findings of a role for constitutive IL-27p28 production/signaling in determining the steady-state macrophage phenotype and the macrophage response to bacterial LPS could contribute towards a better understanding of the molecular mechanisms underlying the development of sepsis.

IL-27p28-deficient macrophages, with a diminished IFN-β and IL-10 induction (**Figure 4.5 and 4.12**), and enhanced secretion of pro-inflammatory mediators, such as

TNF-α, IL-6 and IL-12p40 (**Figure 4.5**), upon LPS stimulation, is reminiscent of the "GM-BMMs" (GM-CSF-induced BMDMs) or "M1-like" macrophages phenotype [87]. These generally possess a IL-12^{hi}, IL-23^{hi}, IL-10^{lo} pro-inflammatory phenotype, while M-CSF-induced BMDMs or "M2-like" macrophages typically possess a IL-12^{lo}, IL-23^{lo}, IL-10^{hi} anti-inflammatory phenotype [87]. "M1-like" macrophages also have lower constitutive IFN-β production [87], which is in line with the phenotype of IL-27p28 knockout macrophages (**Figure 4.21**). These data point to the potential role of IL-27p28 in controlling macrophage polarization. If this is indeed true, it could be of great biological importance. For instance, macrophages also infiltrate tumors and play pivotal roles in tumor growth and metastasis: classical M1 macrophages produce IL-12 to promote tumoricidal responses, while alternative M2 macrophages produce IL-10 and help tumor progression [310]. In this context, it will certainly be of interest to determine whether targeted disruption of IL-27 production and signaling in "M2-like" tumor-associated macrophages (TAMs) is able to alter their phenotype toward a "M1-like" phenotype with anti-tumor activity.

We also provided evidence that the impaired IFN-β production in IL-27p28- and IRF7-deficient macrophages led to defective STAT1 activation and resulted in diminished induction of various ISGs in response to LPS. IL-27p28 and IRF7 promote the optimal production of the IFN-regulated chemokines CCL5, CXCL10 and CXCL11 (**Figure 3.14 and 4.13**), as well as the inflammatory chemokines CCL3 and CCL4 (data not shown). All of these chemokines can recruit activated effector memory T cells, while CCL3, CCL4 and CCL5 can recruit CCR5-expressing monocytes and DCs [311, 312]. IL-27p28 and IRF7 deficiency could therefore potentially affect the recruitment of other immune cells and impact the development of the innate and adaptive immune response to bacterial infections. Human IRF7 deficiency was recently reported to impair the type I and III IFN response to influenza virus infection [313], and human STAT1 deficiency has been associated with defective responses to mycobacterial and viral infections [314]. Cases of human IL-27 deficiency have not been reported to date, but several IL-27 polymorphisms have been associated with autoimmune diseases, such as inflammatory bowel disease and Crohn's disease [315, 316], and viral diseases, such as chronic hepatitis B virus (HBV) infection [317]. However, the effects of human IL-27p28 and IRF7 deficiency on Gram-negative bacterial infections have remained relatively unexplored to date, and certainly deserve further investigation, since cross-

regulation of IL-27p28 and IFN-β production may represent a mechanism by which type I IFN responses can be fine-tuned to achieve graded transcriptional responses over the natural course of an infection.

The present finding that constitutive IL-27p28 regulates the constitutive weak IFN-β signal in resting macrophages introduces a novel cytokine player in the concept of the "revving-up model" of IFN-β signaling proposed by Taniguchi *et al.*, in which continuous, weak IFN-β signaling primes cells for rapid enhancement of IFN-β responses after pathogen encounter [72, 84]. Thus, incorporating our novel finding of a cross-talk between IL-27p28 and IFN-β production and signaling, and expanding on this model, we propose that steady-state macrophages exhibit constitutive IL-27p28-IFN-β signaling that supports weak basal activation of molecules such as STAT1 and maintains the expression of target genes such as IRF7 under homeostatic conditions. Upon pathogen encounter, the notion that IFN-β is an IL-27p28-dependent gene (**Figure 4.12**), while IL-27p28 is a type I IFN-dependent gene (**Figure 4.4**), effectively means that macrophage activation then leads to mutual up-regulation of IL-27p28 and IFN-β to promote rapid amplification of the IFN-β response, through IRF7.

Although EBI3, a subunit of IL-27, plays an important role in murine septic shock [177], the constitutive and LPS-induced IFN-β expression that we showed to be dependent on the IL-27p28 subunit may not necessarily be interacting with EBI3. Of note, IL-27p28 has also been demonstrated to function independently of EBI3, by antagonizing gp130-mediated signaling and IL-6-mediated production of IL-17 and IL-10 in T cells [161]. The IL-27p28 subunit alone (also known as IL-30) reportedly has anti-inflammatory properties, which can inhibit inflammation-induced liver injury, distinct from IL-27 [163]. Interestingly, a secreted complex, formed by IL-27p28 and the soluble cytokine receptor cytokine-like factor 1 (CLF), which activates IL-6R signaling and induces IL-6-mediated IL-17 and IL-10 secretion in T cells, has also been identified [157]. Therefore, the relationship between IL-27p28, EBI3, and other known and unknown binding partners, and the cross-talk between IL-27R WSX-1, gp130, and other related receptor complexes such as IL-6R, are far from straightforward. Thus, it is still an open question whether the effects of IL-27p28 can also be mediated through another cytokine distinct from the IL-27 heterodimer, or through another receptor distinct from the currently known IL-27R complex.

5.7 IL-27p28-dependent IFN-β responses to LPS in BMDMs is functionally important for IL-10 production

In contrast to our findings whereby IL-27p28 is not required for IL-10 production in BMDCs (**Figure 4.8**), Iyer *et al.* showed that the sequential induction of type I IFNs and downstream IL-27 are required for IL-10 production in BMDMs, i.e. the pathway operates LPS → IFNs → IFNAR → IL27 → IL-27R → IL-10 (**Figure 4.16** and [102]). They showed that IL-27 can directly induce IL-10 through the activation and binding of STAT1 and STAT3 to the IL-10 promoter [102]. However, our data indicated that IL-10 production in BMDMs is independent of STAT3 (**Figure 4.9**), analogous to that of IFN-β (**Figure 3.11**). In addition, our data showed that IL-27 production is reduced to ~30% of wild-type levels in LPS-stimulated IRF1-null BMDMs (**Figure 4.10**), in agreement with findings from an earlier study [167, 168], but IL-10 and IFN-β transcription and secretion remained largely unchanged (**Figure 3.12 and 4.10**). This strongly suggested that IL-10 production is not directly regulated by IL-27 signaling. Moreover, the LPS-induced IL-10 transcriptional up-regulation preceded that of IL-27p28, and more closely correlated with that of IFN-β (**Figure 4.11**). We believed that IL-10 production in BMDMs may be directly regulated by IFN-β rather than by IL-27, since addition of exogenous recombinant IFN-β together with LPS is able to restore IL-10 synthesis in IL-27p28-deficient BMDMs to wild-type levels (**Figure 4.14**). This is further supported by the restoration of STAT3 activation and the suppression of pro-inflammatory cytokine production by exogenous IFN-β in IL-27p28-deficient macrophages (**Figure 4.15**). This clearly demonstrates that there is no obligate requirement for IL-27p28 in mediating the LPS-induced IL-10 response in macrophages, since IFN-β alone in the absence of IL-27 production and signaling is sufficient to support macrophage IL-10 induction and promote STAT3-mediated anti-inflammatory responses in LPS-stimulated macrophages. We conclude that the pathway for IL-10 production in BMDMs operates IL27p28 → IFNs → IFNAR → IL-10.

We further characterized that impaired IL-27p28 signaling in TLR4-stimulated macrophages led to blunted IL-10 production, decreased STAT3 activation, and increased production of pro-inflammatory cytokines (**Figure 4.5**) in an endotoxin response more typical of DCs (**Figure 4.2**). This anti-inflammatory property of

IL-27p28 in the context of LPS stimulation finds similarities in other models of infection. For instance, reduced amounts of IL-10 production and/or over-production of various pro-inflammatory cytokines, such as TNF-α and IL-12p40, resulting in severe inflammation in affected organs, such as lung and liver immunopathology, or exacerbated encephalitis was observed in IL-27R WSX-1-deficient mice infected with *M. tuberculosis*, *T. cruzi*, or *T. gondii*, respectively [155, 158]. Taken together, the requisite roles of IL-27p28 production and signaling in mediating robust IFN-β and inflammatory chemokine induction on the one hand, and effective IL-10-mediated anti-inflammatory response on the other hand, further attest to the pleiotropic nature of this cytokine in the context of macrophage TLR4-induced immunity.

In view of the involvement of IL-27p28 production and signaling in IFN-β regulation in LPS-stimulated macrophages, it is not unexpected that IFN-β shares similarities with IL-27 in terms of its pleiotropic effects. This is exemplified by the positive and negative roles of type I IFNs in different inflammatory and autoimmune diseases, such as in multiple sclerosis (MS) and systemic lupus erythematosus (SLE) respectively [318]. In MS, type I IFNs exert beneficial immunomodulatory functions, and recombinant IFN-β is widely used as a first-line therapy to reduce the frequency and severity of relapsing-remitting MS [318]. It is thought that the therapeutic effects of IFN-β in MS patients are partly mediated by IL-27 induction, since IL-27 suppression of Th17 development is protective murine models of the disease [155, 158, 319]. The mechanisms of action of IFN-β and IL-27 therapy in MS are subject to ongoing investigation, but it has been proposed IL-10 induction is a key component of successful treatment [183, 318, 319]. Thus, the triad of IFN-β, IL-27, and IL-10 appear to exert a range of overlapping immuno-regulatory effects in both human and murine Th1/Th17 inflammatory disorders [183]. However, a recent report that IL-27 signaling is dispensable for successful IFN-β therapy in a murine model of MS raised questions about the functional relevance of IL-27 in the clinical efficacy of IFN-β therapy in MS [183]. In this regard, our finding that IL-10 production in macrophages is IL-27p28-independent but still requires IFN-β may offer a partial explanation of this controversy. Notwithstanding the above, our identification of IL-27p28 production and signaling as a novel regulator of IFN-β induction in macrophages adds to the complexity of interdependent and independent immuno-regulatory mechanisms of IFN-β, IL-27, and IL-10, and poses a challenge to identify the shared and distinct modes of action of each,

in human and murine experimental systems of Th1- and Th17-mediated autoimmune diseases [183]. Finally, our observation that optimal levels of cytokines are critical for their downstream functions (i.e. optimal levels of IFN-β are necessary for adequate IL-10 production, and optimal levels of IL-10 are necessary for appropriate STAT3-mediated anti-inflammatory responses) underscores the importance of a careful orchestration and regulation of the kinetics and magnitude of cytokine production following pathogenic stimulation, as higher or lower amounts result in pathologic consequences. This is especially pivotal in the switching of the macrophage response from an initial pro-inflammatory response to a subsequent anti-inflammatory or suppressive phenotype [320]. Therefore, based on the cell type-specific signaling pathways leading to type I IFN induction outlined in this study, we posit that targeted modulation of IL-27p28 production and signaling in macrophages may represent a novel therapeutic strategy in a range of different infections and inflammatory conditions in which macrophage dysfunction or dysregulation drives disease pathophysiology.

6 CONCLUSION

A novel IL-27p28-IRF7 signaling axis regulates the type I IFN response in endotoxin-challenged macrophages but not in dendritic cells

Type I interferons (IFNs) are constitutively expressed by many different cell types as a means to restrain inflammation under homeostatic conditions, and the up-regulation of this tonic signaling is tightly controlled to ensure efficient immune responses to environmental pathogens while limiting tissue pathology. Previous reports have indicated that type I IFN production in response to microbial infection differs in kinetics and magnitude between cell types, and it remains unclear how the magnitude of type I IFN signaling is regulated over the natural course of an infection. In the current report, using a genetic loss-of-function approach, we defined the unique molecular mechanisms that facilitate the potent IFN-β response to toll-like receptor (TLR)-4 activation in macrophages, influencing the balance of pro- versus anti-inflammatory responses to LPS exposure. This report contains a number of novel findings:

- Robust IFN-β responses to LPS require autocrine/paracrine type I IFN signaling in macrophages but not in DCs.
- Optimal IFN-β responses to LPS require IRF7 in macrophages but not in DCs.
- IRF7 cooperates with IRF3 to promote optimal IFN-β synthesis in macrophages.
- Robust IFN-β responses to LPS require IL-27p28 signaling in macrophages but not in DCs.
- IL-27p28 controls constitutive IFN-β production in resting macrophages.
- IL-27p28 regulates constitutive IRF7 expression via an IFN-β-dependent mechanism in resting macrophages.
- Steady-state BMDMs, but not BMDCs, express high levels of IRF7 protein.
- Maximal levels of IFN-β production, mediated by IL-27p28 and IRF7, are functionally important for normal STAT1 activation, and optimal induction of a subset of type I IFN- and STAT1-dependent genes, such as CCL5 and CXCL10.

- IRF7-dependent IFN-β amplification is required for macrophage expression of pro-caspase-11 and IL-1β production *in vivo*.
- IL-27p28-dependent IFN-β amplification is required for macrophage expression of IL-10.
- IFN-β is sufficient for IL-10 production in IL-27p28-deficient macrophages.
- Maximal levels of IL-10 production, mediated by high levels of IFN-β downstream of IL-27 production and signaling, are functionally important for normal STAT3 activation, and optimal IL-10-mediated suppression of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-12p40.

Our data define a new model of type I IFN regulation that is operational in macrophages but lacking in DCs. Under homeostatic conditions, resting macrophages exhibit constitutive IL-27p28-IFN-β signaling that supports a weak basal activation of STAT1 sufficient to maintain a basal expression of IRF7. Upon pathogen encounter, specifically LPS from Gram-negative bacteria, the pre-existing pool of IRF7, together with IRF3, are required for the induction of type I IFN synthesis in macrophages, both *in vitro* and *in vivo*, whereas DCs depend exclusively on IRF3 to mediate a much weaker response. These data provide novel mechanistic insight into the molecular basis of the divergent roles played by macrophages and DCs in anti-microbial immunity, which will critically inform future studies of their disparate roles in host protection against bacterial pathogens.

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8 APPENDIX A

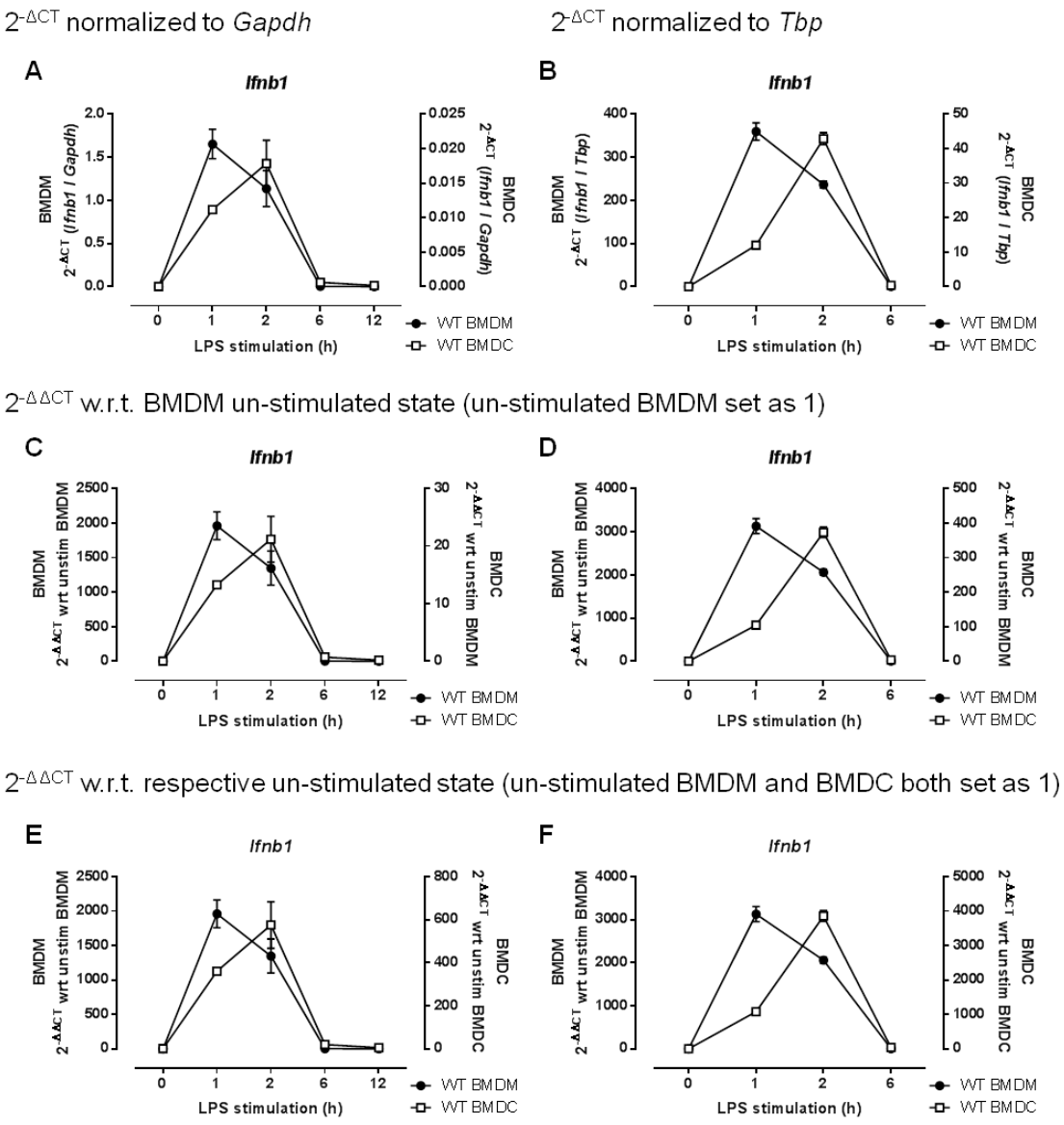

Figure S1
DCs exhibit poorer IFN-β induction in response to LPS exposure compared with macrophages.
Real-time PCR analysis of IFN-β gene expression in wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. In separate experiments, *Ifnb1* expression was normalized to *Gapdh* (A, C, E) or *Tbp* (B, D, F), and expressed as $2^{-\Delta CT}$ (A, B) or $2^{-\Delta\Delta CT}$ relative to the levels observed in un-stimulated BMDMs (C, D) or $2^{-\Delta\Delta CT}$ relative to the levels observed in respective un-stimulated cells (E, F) (left axis: BMDMs; right axis: BMDCs). Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.

APPENDIX B

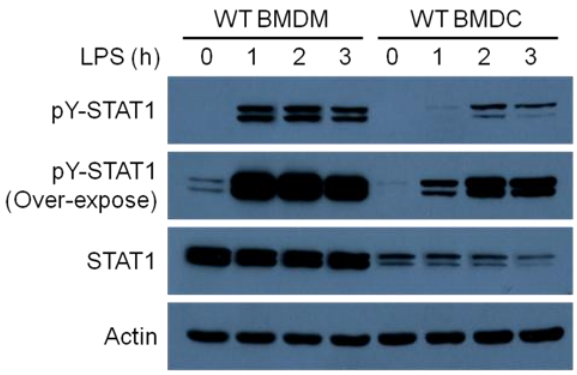

Figure S2
DCs exhibit weaker type I IFN responses to LPS exposure compared with macrophages.
Western immunoblot analysis of phospho-STAT1 (pY-STAT1) and total STAT1 protein expression in whole cell lysates of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. Actin was used as a loading control.

APPENDIX C

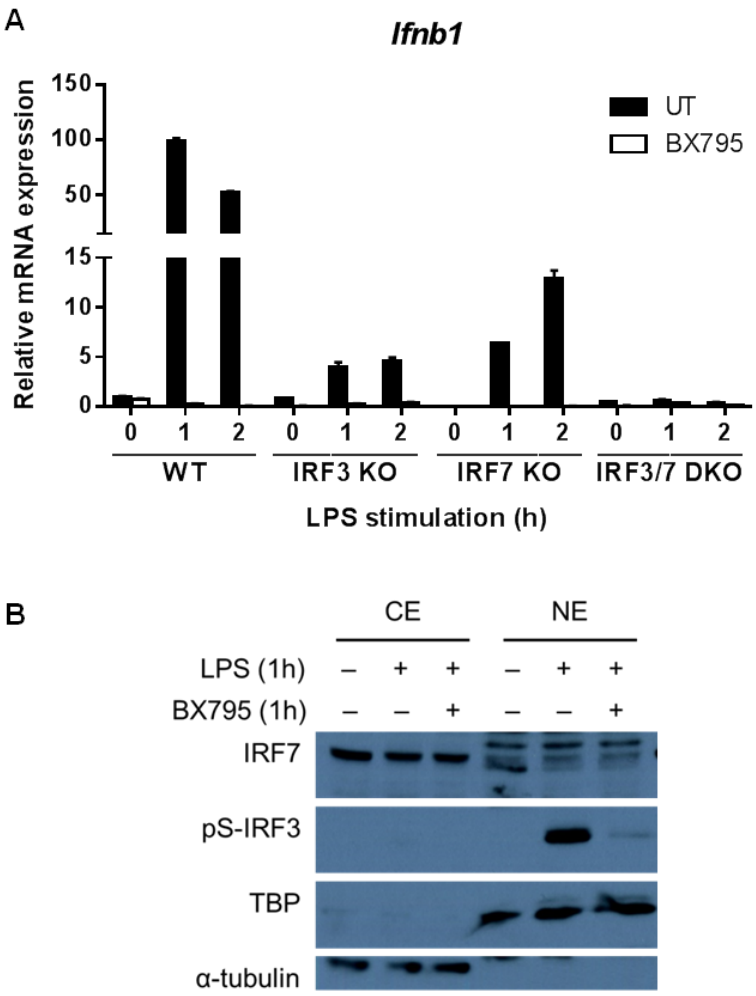

Figure S3
TBK1 is required for the activation of both IRF7 and IRF3 for maximal IFN-β expression in endotoxin-challenged macrophages.
(A) Real-time PCR analysis of IFN-β gene expression in BMDMs from IRF3 single knockout mice, IRF7 single knockout mice, and IRF3-IRF7 double knockout mice, compared with wild-type control littermates, pre-treated or not with 2μM BX795 (TBK1 inhibitor) for 1 h, and then stimulated or not with 100ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-treated and un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations.
(B) Western immunoblot analysis of total IRF7 and phospho-IRF3 protein expression in cytoplasmic extracts (CE) and nuclear extracts (NE) of wild-type BMDMs, pre-treated or not with 2μM BX795 (TBK1 inhibitor) for 1 h, and then stimulated or not with 100ng/ml LPS for 1 h. α-tubulin and TATA-binding protein (TBP) were used as loading controls.

APPENDIX D

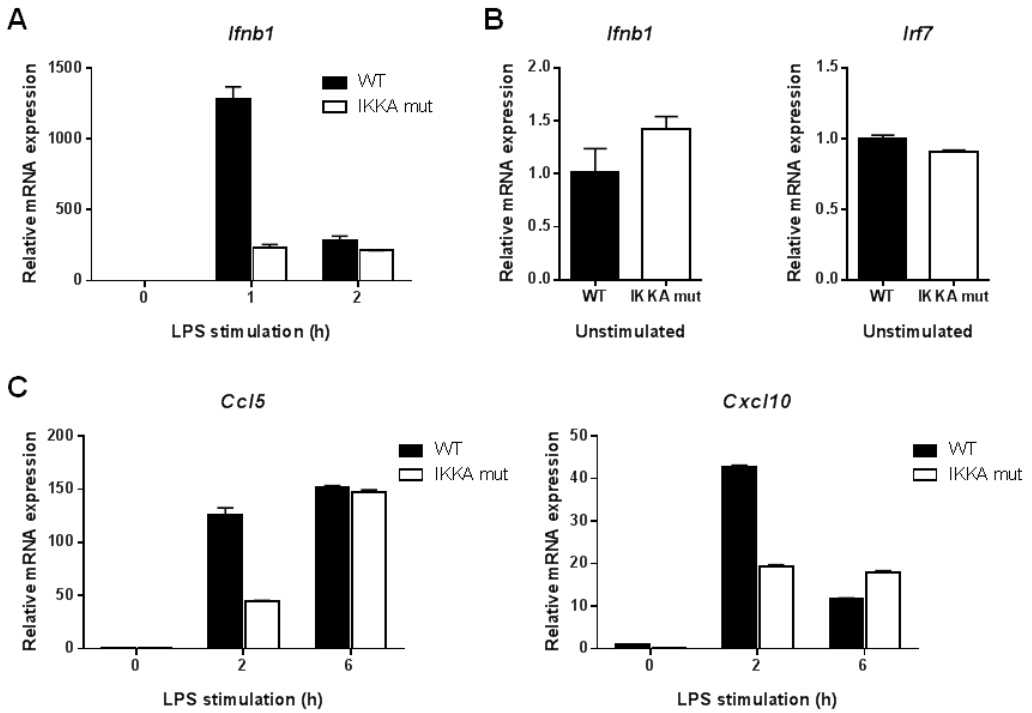

Figure S4
IKKα is required for maximal IFN-β expression in endotoxin-challenged macrophages.
(A, C) Real-time PCR analysis of *Ifnb1*, *Ccl5* and *Cxcl10* gene expression in BMDMs from IKKα mutant mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times.
(B) Real-time PCR analysis of *Ifnb1* and *Irf7* gene expression in un-stimulated BMDMs from IKKα mutant mice, compared with wild-type control littermates. Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations.

APPENDIX E

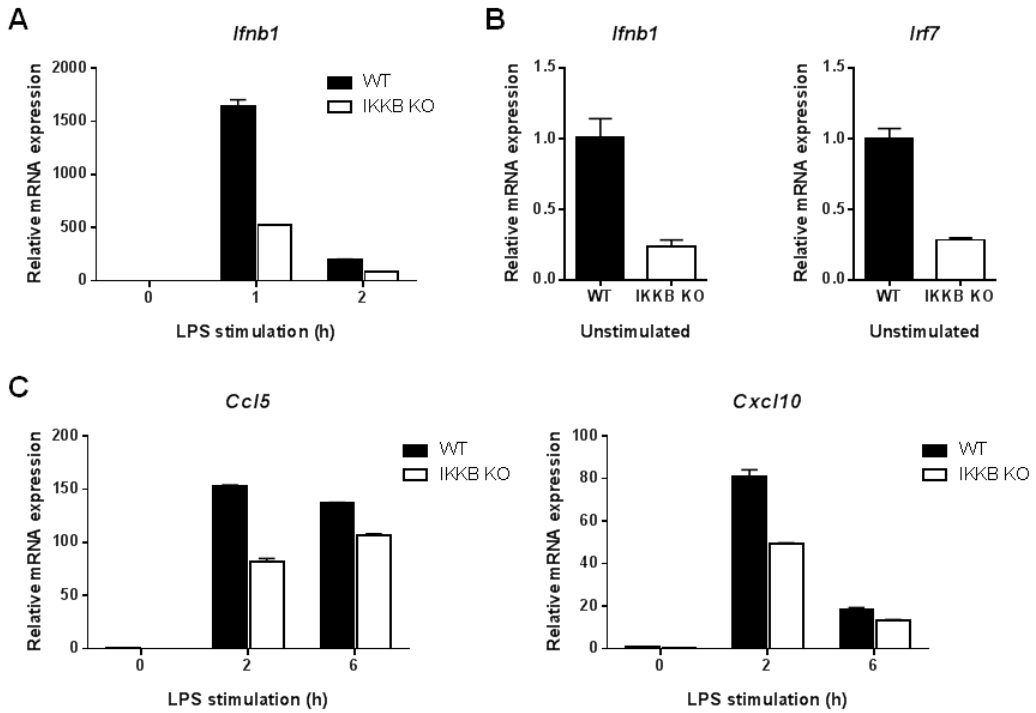

Figure S5
IKKβ is required for maximal IFN-β expression in endotoxin-challenged macrophages.
(A, C) Real-time PCR analysis of *Ifnb1*, *Ccl5* and *Cxcl10* gene expression in BMDMs from IKKβ knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for the indicated times.
(B) Real-time PCR analysis of *Ifnb1* and *Irf7* gene expression in un-stimulated BMDMs from IKKβ knockout mice, compared with wild-type control littermates. Gene expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations.

APPENDIX F

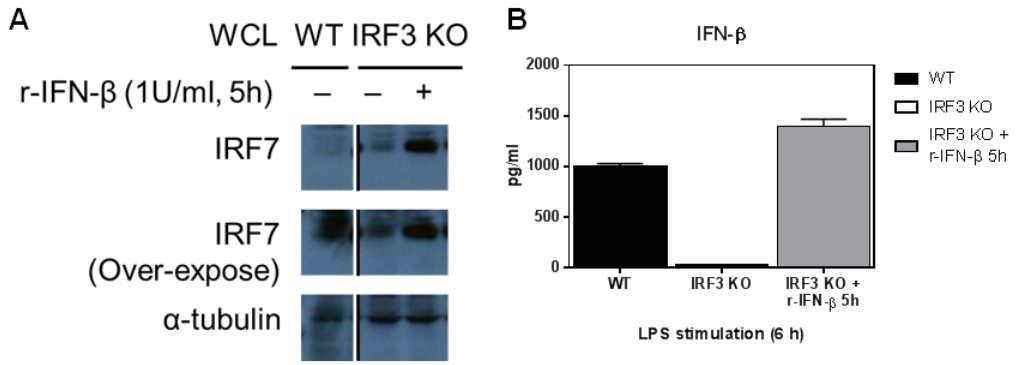

Figure S6
IFN-β responses in LPS-challenged IRF3-deficient BMDMs are rescued by pre-treatment with exogenous IFN-β cytokine resulting in the up-regulation of IRF7 protein.
(A) Western immunoblot analysis of IRF7 protein expression in whole cell lysates of wild-type and IRF3 knockout BMDMs, treated or not with 1U/ml recombinant murine IFN-β for 5 h.
(B) ELISA analysis of IFN-β secretion by wild-type and IRF3 knockout BMDMs, treated or not with 1U/ml recombinant murine IFN-β for 5 h and then stimulated with 100ng/ml LPS for 6 h.

APPENDIX G

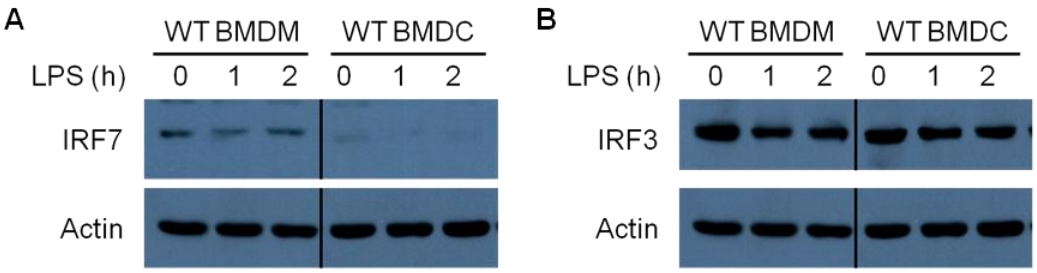

Figure S7
Distinct IFN responses of macrophages and DCs are associated with differential expression of IRF7.
Western immunoblot analysis of total IRF7 and total IRF3 protein expression in whole cell lysates of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times.

APPENDIX H

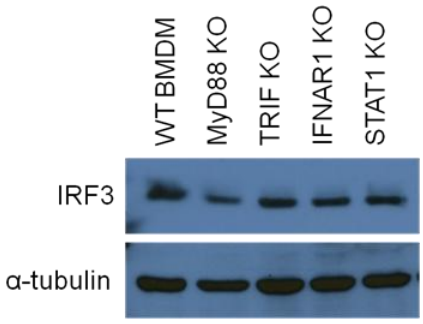

Figure S8
Basal expression of IRF3 in resting macrophages is independent of MyD88, TRIF, and constitutive IFN-β-STAT1 signaling.
Western immunoblot analysis of total IRF3 protein expression in whole cell lysates of resting BMDMs from MyD88, TRIF, IFNAR1, and STAT1 knockout mice, compared with wild-type control littermates.

APPENDIX I

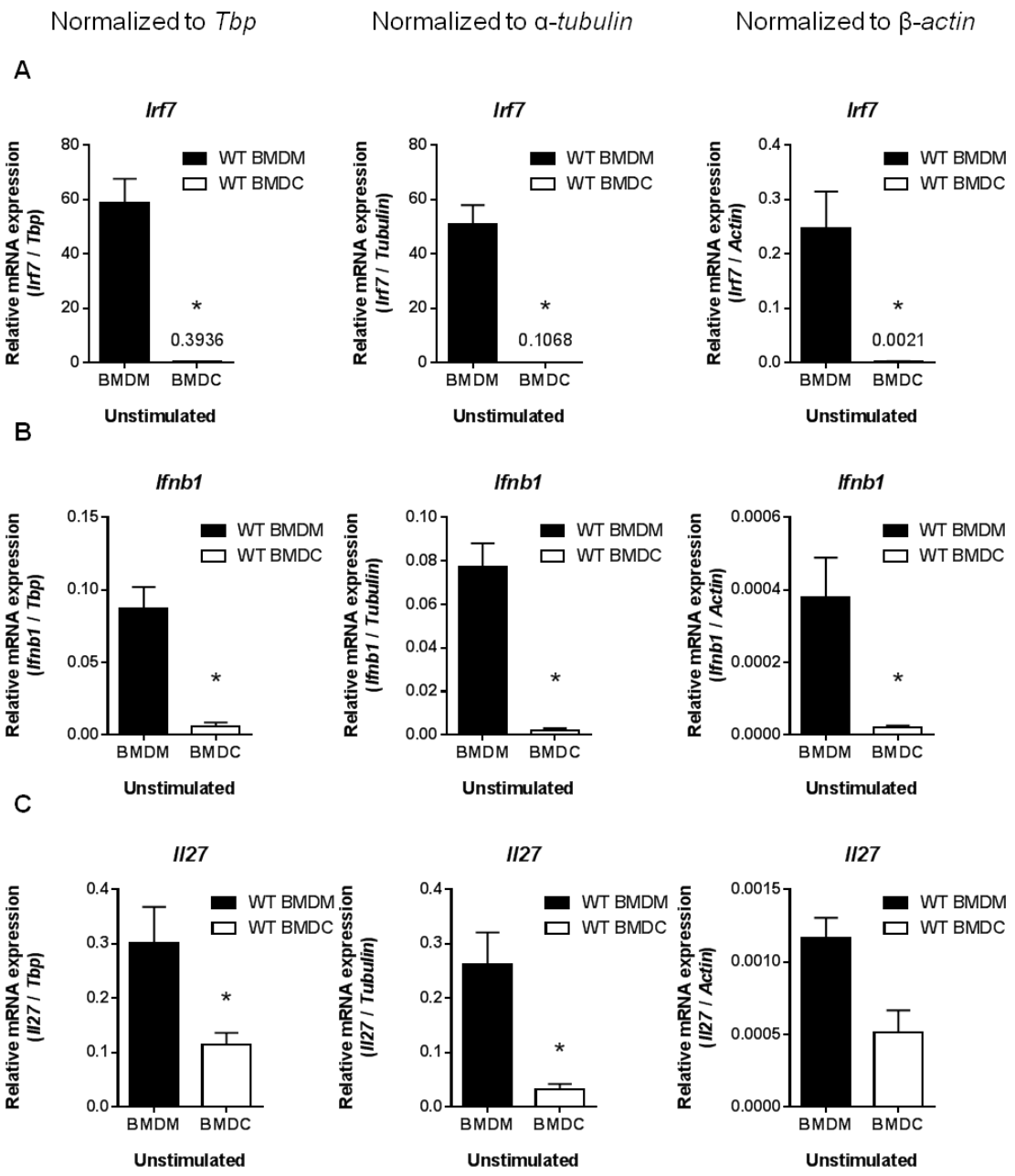

Figure S9
Poor IFN-β responses in LPS-challenged BMDCs are associated with weak IL-27p28-IFN-β signaling and negligible IRF7 expression at steady-state.
Real-time PCR analysis of *Irf7* (A) , *Ifnb1* (B), and *Il27p28* (C) gene expression in un-stimulated wild-type BMDMs and BMDCs. Gene expression was normalized to *Tbp*, α-*tubulin*, or β-*actin*. Data are presented as mean ± SEM of at least three independent experiments (*p<0.05 by paired t test).

APPENDIX J

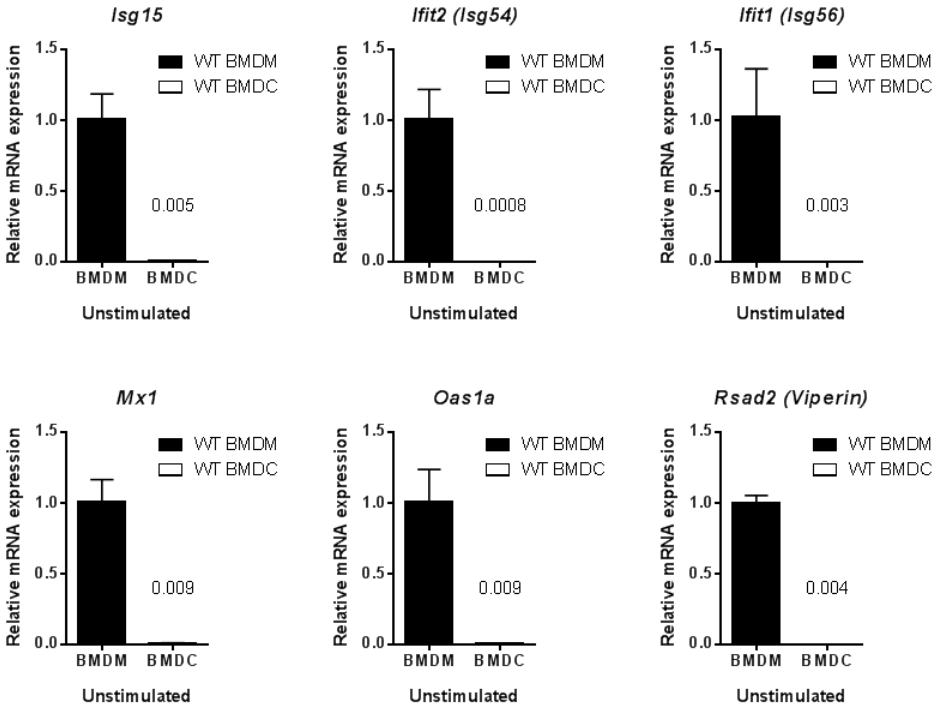

Figure S10
Weak IFN-β signaling and negligible IRF7 expression in steady-state BMDCs are associated with lower constitutive ISG expression.
Real-time PCR analysis of *Isg15*, *Isg54*, *Isg56*, *Mx1*, *Oas1a* and *Viperin* gene expression in un-stimulated wild-type BMDMs and BMDCs. Gene expression was normalized to *Gapdh*. Data are presented as mean ± SD of duplicate determinations from one representative experiment.

APPENDIX K

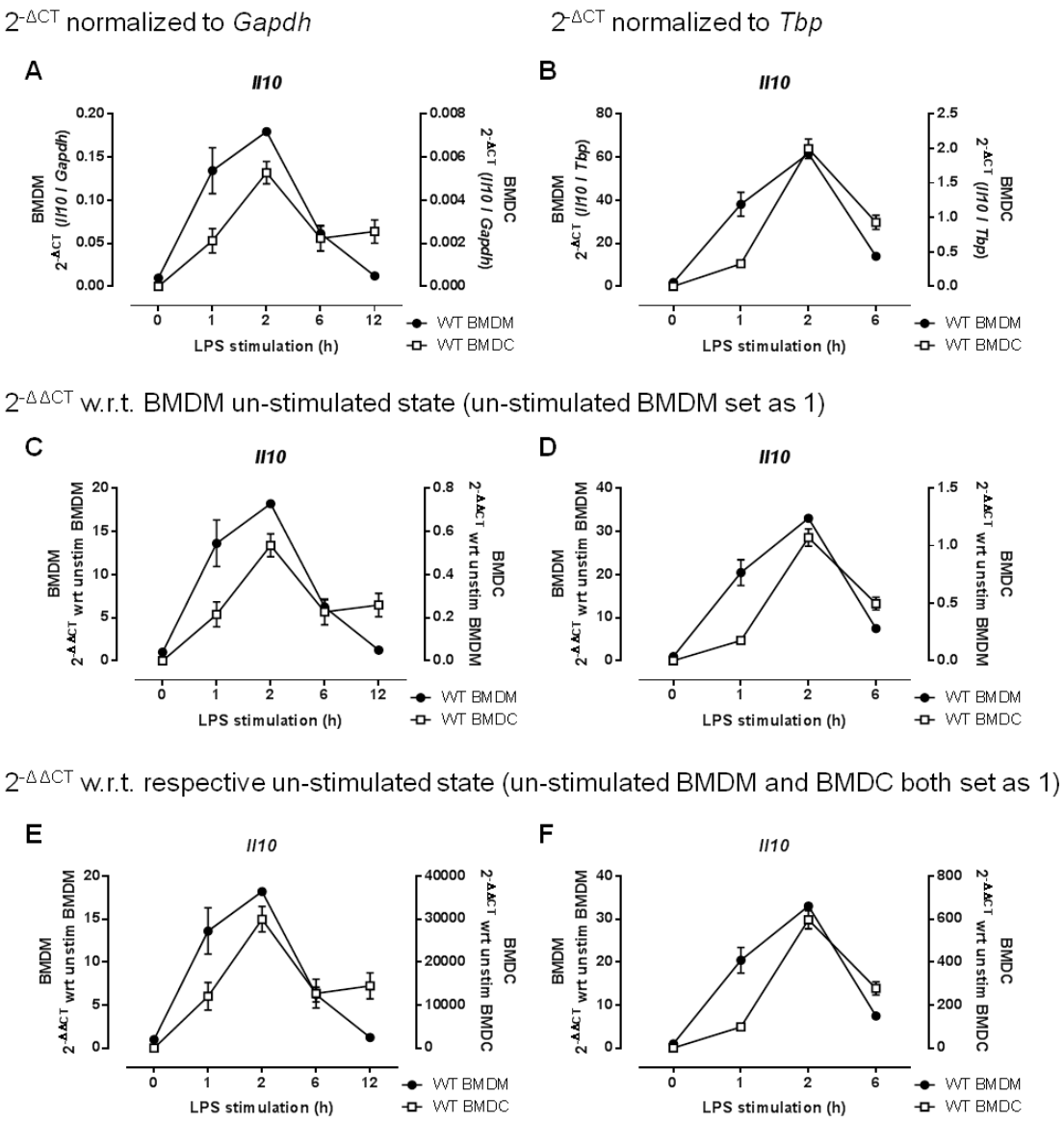

Figure S11
Weak IL-10 responses in endotoxin-challenged DCs.
Real-time PCR analysis of IL-10 gene expression in wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times. In separate experiments, *Il10* expression was normalized to *Gapdh* (A, C, E) or *Tbp* (B, D, F), and expressed as $2^{-\Delta CT}$ (A, B) or $2^{-\Delta\Delta CT}$ relative to the levels observed in un-stimulated BMDMs (C, D) or $2^{-\Delta\Delta CT}$ relative to the levels observed in respective un-stimulated cells (E, F) (left axis: BMDMs; right axis: BMDCs). Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments.

APPENDIX L

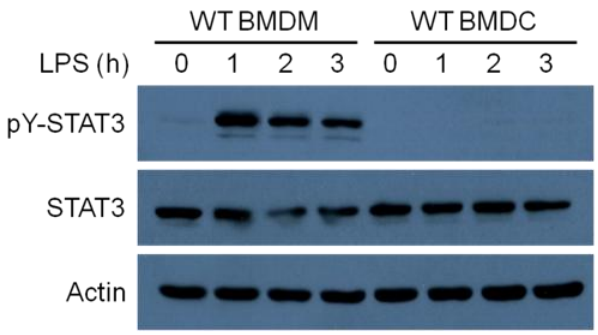

Figure S12
Weak STAT3-mediated anti-inflammatory response in endotoxin-challenged DCs.
Western immunoblot analysis of phospho-STAT3 and total STAT3 protein expression in whole cell lysates of wild-type BMDMs and BMDCs, stimulated or not with 100ng/ml LPS for the indicated times.

APPENDIX M

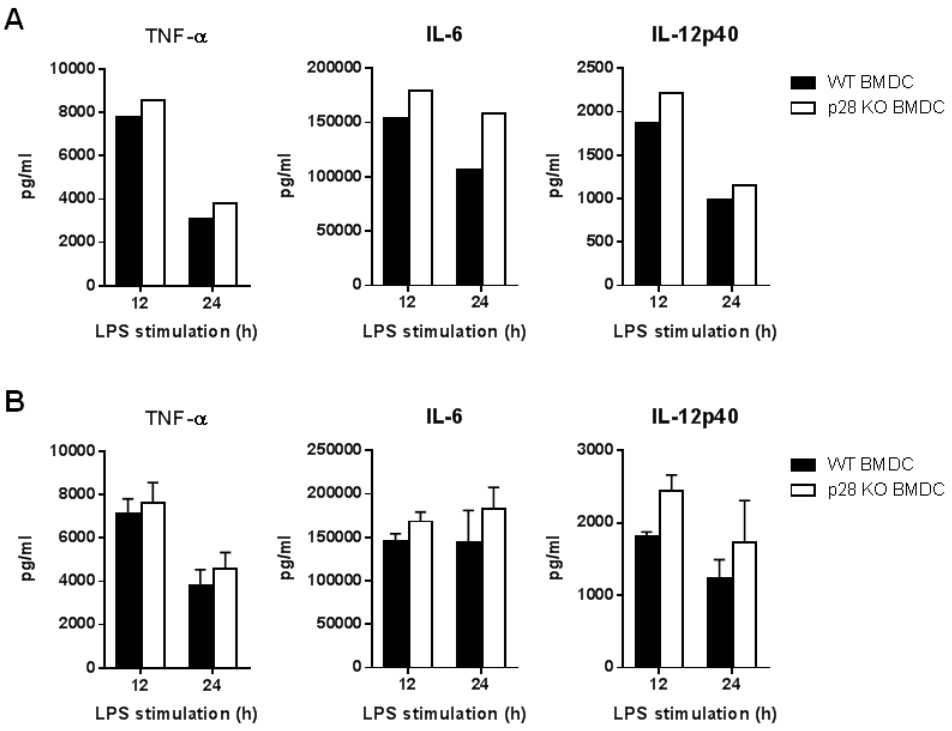

Figure S13
IL-27p28 production and signaling is not required for maximal IL-10 expression and STAT3-mediated anti-inflammatory responses in DCs.
Multiplex analysis of TNF-α, IL-6 and IL-12p40 production in BMDCs from IL-27p28 knockout mice, compared with wild-type control littermates, stimulated or not with 100ng/ml LPS for 12 h and 24 h. (A) Data are from one representative of at least two independent experiments. (B) Data are presented as mean ± SEM of two biological replicates.

9 AUTHOR'S PUBLICATIONS

Li P, Wong JJ, Sum C, **Sin WX**, Ng KQ, Koh MB, Chin KC. IRF8 and IRF3 cooperatively regulate rapid interferon-β induction in human blood monocytes. Blood. 2011;117(10):2847-54.

Sin WX, Li P, Yeong JP, Chin KC. Activation and regulation of interferon-β in immune responses. Immunol Res. 2012;53(1-3):25-40.

Sin WX*, Yeong J*, Chin KC. IRF7 is a novel cell type-specific transcription factor regulating TLR4-mediated type I IFN responses in macrophages but not dendritic cells. (*equal contribution) (manuscript in preparation)

10 POSTERS, AWARDS, INVITED TALKS

Sin WX, Li P, Chin KC. IRF8 and IRF3 cooperatively regulate rapid IFN-β induction in human blood monocytes. Presented at 15th International Congress of Immunology (ICI), 2013; Cytokines (Annual Meeting of the International Cytokine and Interferon Society), 2014.