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I kappa B kinase alpha (IKKα) activity is required for functional maturation of dendritic cells and acquired immunity to infection

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Dendritic cells (DC) are required for priming antigen-specific T cells and acquired immunity to many important human pathogens, including *Mycobacteriuim tuberculosis* (TB) and influenza. However, inappropriate priming of auto-reactive T cells is linked with autoimmune disease. Understanding the molecular mechanisms that regulate the priming and activation of naïve T cells is critical for development of new improved vaccines and understanding the pathogenesis of autoimmune diseases. The serine/threonine kinase IKKα (CHUK) has previously been shown to have anti-inflammatory activity and inhibit innate immunity. Here, we show that IKKα is required in DC for priming antigen-specific T cells and acquired immunity to the human pathogen *Listeria monocytogenes*. We describe a new role for IKKα in regulation of IRF3 activity and the functional maturation of DC. This presents a unique role for IKKα in dampening inflammation while simultaneously promoting adaptive immunity that could have important implications for the development of new vaccine adjuvants and treatment of autoimmune diseases.

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Introduction

Our immune system can be broadly divided into innate and adaptive mechanisms, the latter providing immunological memory to protect against secondary infection. In the past two decades, the critical role of the innate immune response in shaping acquired immunity has been established on both a cellular and molecular basis (Iwasaki and Medzhitov, 2010). Two key discoveries have driven developments in this field; the identification of Toll-like receptors (TLRs) expressed on innate immune cells that recognize microbial-associated molecular patterns (Takeuchi and Akira, 2010), and the characterization of dendritic cells (DC) that acquire antigens and prime naïve T cells (Steinman, 2008). T cell-mediated immunity (CMI) is critical for protection against many important infectious diseases in humans including TB and influenza. CMI is maintained by antigen-specific T cells and initiated by DC-mediated T-cell priming (Steinman, 2008; Iwasaki and Medzhitov, 2010). In the case of exogenous antigens, DC process and present peptides on MHC II molecules to naïve $CD4^+$ helper T cells (T_H), endogenous or viral antigens are presented on MHC I molecules to $CD8^+$ cytotoxic T cells (CTLs). T_H1 cells produce interferon (IFN) γ, which is critical for immunity to intracellular pathogens including TB and viruses through the activation of macrophages and CTLs. Whereas T_H2 cells produce interleukin (IL)-4 and promote B cell-mediated antibody responses.

Effective T-cell priming requires three signals: antigen presentation to a specific T-cell receptor (TCR), co-stimulation of the antigen-presenting cell (APC) by T-cell ligands, and cytokine production by the APC (Steinman, 2008; Iwasaki and Medzhitov, 2010). The third signal is usually triggered by pattern recognition receptors (PRRs), including TLRs, expressed by the APC that detect tissue injury or infection (Takeuchi and Akira, 2010), and is required to trigger the functional maturation of the APC (Sporri and Reis e Sousa, 2005). T_H1 cells require IL-12 production by the APC, which is triggered by engagement of specific TLRs that detect bacterial or viral infection (Manicassamy and Pulendran, 2009). There are 12 TLRs in the mouse and 10 in the human, TLR1–9 are conserved in both species (Takeuchi and Akira, 2010). Each TLR responds to a different class of molecule and triggers specific cellular responses, this is attributed to the recruitment of different signalling adaptors (Takeuchi and Akira, 2010). MyD88 is utilized by all TLRs except TLR3, which responds to double-stranded RNA (dsRNA) from virus-infected cells through a different adaptor called TRIF. TLR4 is the only receptor that recruits both MyD88 and TRIF, both of which are required for cytokine production in response to TLR4 signalling. MyD88 drives inflammatory cytokine production through NF-κB activation (Dev *et al*, 2011), while TRIF mediates activation of IFN regulatory factor 3 (IRF3) and expression of type I IFN

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(IFNα/β) (Honda and Taniguchi, 2006). MyD88-dependent signalling is critical for priming T_H1 cell responses (Schnare *et al*, 2001), but the contribution of TRIF-dependent IRF3 activation is less clear. TLR7 and TLR9 also trigger type I IFN production, in response to ssRNA or CpG-DNA, respectively, but through a MyD88-dependent pathway leading to activation of IRF7 or IRF1 (Takeuchi and Akira, 2010), and not IRF3.

Type I IFN production is usually considered in the context of innate anti-viral immunity, however TLR4, 3, 7 and 9 agonists are potent adjuvants for T_H1-mediated immune responses and have the conserved property of inducing type I IFN (Manicassamy and Pulendran, 2009), suggesting a potentially important role for type I IFN in T_H1 cell priming.

NF-κB is the prototypical pro-inflammatory transcription factor (Dev *et al*, 2011), and MyD88-mediated NF-κB activation is dependent on the IκB kinase complex (IKK) (Zandi *et al*, 1997), which leads to phosphorylation and degradation of the endogenous inhibitors of NF-κB (IκBs). The canonical IKK complex consists of three subunits: IKKα (IKK1; CHUK), IKKβ (IKK2) and IKKγ (NEMO). IKKα and IKKβ are serine/threonine kinases, but only IKKβ is required for IκB phosphorylation and canonical NF-κB activation (Hacker and Karin, 2006). IKKγ is an ubiquitin binding protein that links the IKK complex with upstream signalling pathways and is critical for canonical NF-κB activation. The role of IKKα in the canonical IKK complex is still unclear, IKKα is not required for canonical NF-κB activation (Hacker and Karin, 2006), but several studies have shown a role for IKKα in negative regulation of NF-κB activity by a variety of different mechanisms (Lawrence *et al*, 2005; Li *et al*, 2005; Liu *et al*, 2007; Shembade *et al*, 2011). However, IKKα is required for an alternative NF-κB pathway through the phosphorylation and processing of p100 leading to activation of p52/RelB heterodimers (Senftleben *et al*, 2001). Alternative NF-κB signalling is independent of IKKβ and IKKγ but is instead mediated by NF-κB inducing kinase (NIK; MAP3K14), which phosphorylates and activates IKKα (Senftleben *et al*, 2001). Genetic studies in mice clearly show that the NIK-IKKα-p100 pathway is required for lymphoid organogenesis and B-cell development (Bonizzi and Karin, 2004). Components of alternative NF-κB signalling, namely NIK and RelB, have also been implicated in DC development and function (Wu *et al*, 1998; Hofmann *et al*, 2011), although the signalling pathways involved have not been delineated.

Recent studies have shown that IKKα is required for MyD88-dependent type I IFN expression in FLT3-derived plasmacytoid DC (pDC) and conventional DC (cDC), through phosphorylation of IRF7 and IRF1, respectively (Hoshino *et al*, 2006, 2010). But the role of IKKα in DC function and T-cell priming has not been addressed. We previously showed that IKKα attenuates innate immunity through downregulation of NF-κB-mediated transcription in macrophages (Lawrence *et al*, 2005). Here, we evaluated the role of IKKα in acquired immunity and priming antigen-specific T cells. Using knock-in mice that express a mutant form of IKKα that cannot be activated ($IKK\alpha^{AA}$) (Cao *et al*, 2001), we show that IKKα activation in the haematopoietic compartment is critical for acquired immunity to the facultative intracellular pathogen; *Listeria monocytogenes*. Although innate resistance to primary *L. monocytogenes* infection was enhanced in $Ikk\alpha^{AA/AA}$ mice, they failed to develop T-cell memory and showed increased susceptibility to secondary infection. IKKα was required in DC for IRF3-dependent IFNβ and IL-12 expression and priming of naïve $CD4^+$ T cells.

Our studies show that IKKα plays reciprocal roles in innate and adaptive immunity, limiting non-specific inflammation while promoting acquired antigen-specific immunity. This unique role for IKKα in the transition from innate to adaptive immunity may have important implications for vaccine development and treatment of infectious diseases.

Results

IKKα is required for priming antigen-specific T cells in vivo

Delayed-type (IV) hypersensitivity (DTH) is a classic model of CMI. Methylated BSA (mBSA) is commonly used as an antigen in animal models of DTH and provokes a strong T_H1-mediated IFNγ response (Asquith *et al*, 2009). We evaluated the role of IKKα in CMI using a mouse model of mBSA-DTH; $Ikk\alpha^{AA/AA}$ mice (Cao *et al*, 2001) and littermate controls (WT) were immunized with mBSA in Freunds' complete adjuvant (mBSA/CFA), by intradermal (i.d.) injection. Two weeks after immunization, mBSA was injected into the right paw to provoke an antigen-specific immune reaction, PBS was injected into the left paw as a control, paw swelling was measured daily to monitor the inflammatory reaction. Figure 1A shows a substantial decrease in the response to mBSA after immunization of $Ikk\alpha^{AA/AA}$ mice. Due to the requirement for IKKα in lymphotoxin β (LTβ) signalling in radiation-resistant stromal cells, $Ikk\alpha^{AA/AA}$ mice have defects in lymph node (LN) and spleen development (Bonizzi *et al*, 2004). To determine if this role for IKKα was responsible for the defect in DTH, we conducted bone marrow adoptive transfer experiments. C57Bl6/Ly5.1 transgenic mice were lethally irradiated and reconstituted with bone marrow cells from $Ikk\alpha^{AA/AA}$ or WT mice (Ly5.2), LN and spleen micro-architecture were normal in $Ikk\alpha^{AA/AA}$ chimeric mice (data not shown) (Bonizzi *et al*, 2004). However, the mice remained unresponsive to mBSA challenge (Figure 1B). Therefore, IKKα activation in the haematopoietic compartment mediates DTH.

To check the priming of endogenous mBSA-specific T cells in $Ikk\alpha^{AA/AA}$ mice, we performed *ex vivo* antigen re-call assays. Antigen (mBSA)-specific IFNγ and IL-2 production by splenocytes from mBSA/CFA-immunized $Ikk\alpha^{AA/AA}$ chimeric mice was severely impaired, with no effect on IL-10 production or non-specific activation of T cells with concanavalin A (ConA) (Figure 1C). There were no defects in activation of T cells from $Ikk\alpha^{AA/AA}$ mice in response to TCR cross-linking with anti-CD3/CD28 antibodies (Supplementary Figure S1), indicating that IKKα had no intrinsic role in antigen-mediated T-cell activation. To test priming of naïve T-cell responses in $Ikk\alpha^{AA/AA}$ mice, we purified $CD4^+$ T cells from spleen and LNs of mBSA/CFA-immunized mice and co-cultured them with naïve splenic $CD11c^+$ DC or bone marrow-derived DC (BMDC) from either WT or $Ikk\alpha^{AA/AA}$ mice, in the presence of mBSA. DC from WT or $Ikk\alpha^{AA/AA}$ mice were able to trigger IFNγ and IL-2 production by $CD4^+$ T cells from mBSA/CFA-immunized WT mice, but $CD4^+$ T cells from mBSA/CFA-immunized $Ikk\alpha^{AA/AA}$ mice were unresponsive even in the presence of WT DC (Figure 1D

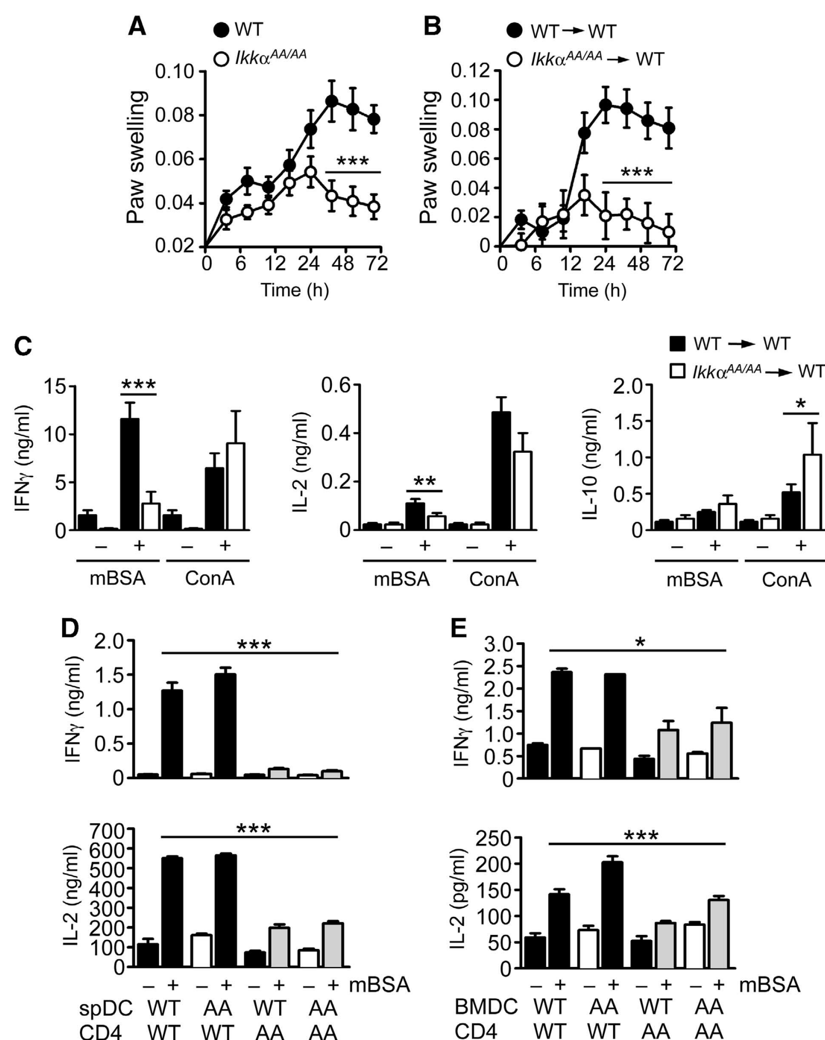

Figure 1 IKKα is required for CD4$^+$ T-cell priming *in vivo*. (**A**) *Ikkα*$^{AA/AA}$ and littermate control mice (WT), and radiation chimeras generated with WT and *Ikkα*$^{AA/AA}$ bone marrow cells (**B**), were immunized intradermally (i.d.) with CFA/mBSA. Fourteen days after immunization, mice were injected with mBSA in the left footpad, PBS was injected into the right footpad as a control. Paw swelling was measured at the indicated time points by plethysmography as an index of antigen (mBSA)-induced inflammation. Data are represented as mean ± s.e.m. of $n = 10$, statistical analysis was performed with Mann–Whitney test; $^{***}P \leqslant 0.001$. (**C**) Splenocytes from CFA/mBSA-immunized WT and *Ikkα*$^{AA/AA}$ chimeric mice were collected after 14 days and re-stimulated *in vitro* with mBSA or concanavalin A (ConA); IFNγ, IL-2 and IL-10 were measured in culture supernatants by ELISA after 72 h. Data are represented as mean ± s.e.m. of $n = 10$, statistical analysis was performed with Mann–Whitney test; $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.005$, $^{***}P \leqslant 0.001$. (**D**) CD4$^+$ T cells were isolated from lymph nodes (LN) and spleen of CFA/mBSA-immunized WT and *Ikkα*$^{AA/AA}$ (AA) mice after 14 days; T cells were co-cultured with CD11c$^+$ DC isolated from spleens of naïve WT and *Ikkα*$^{AA/AA}$ mice (spDC; **D**), or bone marrow-derived DC (BMDC; **E**), in the presence or absence of mBSA. IFNγ and IL-2 were measured in culture supernatants by ELISA after 72 h. Data are represented as mean ± s.e.m. of $n = 6$, statistical analysis was performed with Mann–Whitney test; $^{*}P \leqslant 0.05$, $^{***}P \leqslant 0.001$.

and E). These data suggested a defect in T-cell priming rather than antigen presentation and activation.

IKKα is required for acquired immunity to infection

To investigate the role of IKKα in acquired immunity in the context of infection, we used the human pathogen *L. monocytogenes* (Lm). Lm is a facultative intracellular bacteria that is normally controlled by cell-mediated immune responses but can cause fatal listeriosis in immune-compromised individuals (Lecuit, 2007). Lm infection in mice is widely used as a model to study the cellular and molecular basis of CMI (Pamer, 2004); acquired immunity to Lm requires CD4$^+$ T cell-dependent development of memory CD8$^+$ T cells (Sun and Bevan, 2003). We infected *Ikkα*$^{AA/AA}$ and WT bone marrow chimeric mice intravenously (i.v.) with 10^4 colony forming units (CFU) of a recombinant Lm strain engineered to express chicken ovalbumin (Lm-OVA). *Ikkα*$^{AA/AA}$ chimeras showed increased resistance to primary Lm-OVA infection with reduced bacterial load in spleen and liver after 5 days (Figure 2A and B), however, upon secondary infection after 35 days with a high dose of bacteria (10^6 CFU), *Ikkα*$^{AA/AA}$ chimeric mice showed impaired protection with significantly increased bacterial load in spleen and liver (Figure 2A and B). To test the role of IKKα in the priming and activation of protective Lm-specific CD8$^+$ T cells, we injected mice with naïve CD8$^+$ T cells that specifically recognize OVA peptide in the context of MHC I (OT-I cells) before primary infection with Lm-OVA. We measured the expansion of OT-I cells in the spleen of infected mice 7 days after infection. *Ikkα*$^{AA/AA}$ chimeric mice showed no

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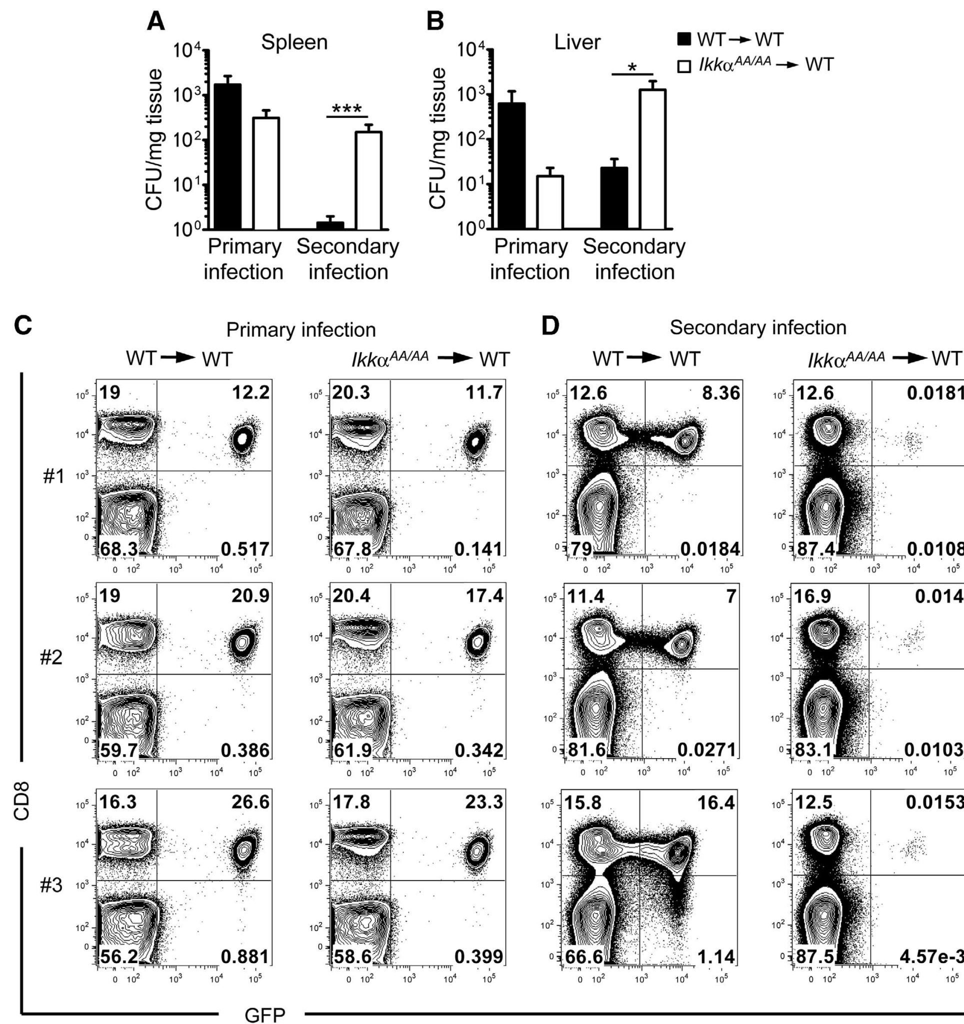

Figure 2 IKKα is required for acquired immunity to *L. monocytogenes* and CD8$^+$ T-cell memory. WT and $Ikk\alpha^{AA/AA}$ chimeric mice were infected intravenously (i.v.) with 10^4 CFU *L. monocytogenes* expressing chicken ovalbumin (Lm-OVA), spleens (**A**) and liver (**B**) were collected after 7 days for CFU measurements (primary infection). In parallel experiments, mice received a second infection with 10^6 CFU Lm-OVA 35 days after primary infection and CFU was determined in spleen and liver after a further 5 days (secondary infection). Data are represented as mean ± s.e.m. of $n = 8$–16, statistical analysis was performed with Mann–Whitney test; $*P \leqslant 0.05$, $***P \leqslant 0.001$. (**C**) 10^4 CD8$^+$ OT-I.EGFP cells were adoptively transferred to WT and $Ikk\alpha^{AA/AA}$ chimeric mice prior to infection with Lm-OVA (10^4 CFU i.v.), expansion of OVA-specific OT-I cells was measured by FACS analysis of spleens 7 days post infection (primary infection; **C**). In parallel experiments, mice received a second infection with 10^6 CFU Lm-OVA after 35 days, spleens were collected after a further 5 days for FACS analysis of OT-I cells (secondary infection; **D**). Representative data are shown from $n = 6$ mice.

impairment in expansion of OT-I cells after primary infection (Figure 2C). However, upon secondary infection 35 days later, OT-I cells failed to re-expand in $Ikk\alpha^{AA/AA}$ chimeric mice compared to mice reconstituted with WT bone marrow (Figure 2D). Re-call assays *ex vivo* with splenocytes and OT-I-specific OVA peptide showed defective expansion of OVA-specific CD8$^+$ T cells correlated with severely reduced numbers of IFNγ producing cells (Supplementary Figure S2). These data suggest that IKKα plays an important role in the development and/or maintenance of memory CD8$^+$ T cells, and thus acquired immunity to infection.

IKKα activation regulates the functional maturation of DC

Priming of naïve antigen-specific T cells is the defining property of DC (Steinman, 2008), whereas both DC and macrophages are able to trigger antigen-specific T-cell activation (Hume, 2008). Our data showed a defect in CD4$^+$ T-cell priming in $Ikk\alpha^{AA/AA}$ mice that is intrinsic to the APC compartment and likely reflects a role of IKKα in DC. It was also shown that DC are critical for priming protective CD8$^+$ T cells and acquired immunity to Lm infection in mice (Jung *et al*, 2002). We next assessed the role of IKKα specifically in DC function. Effective T-cell priming by DC requires three signals (Manicassamy and Pulendran, 2009): (1) Antigen presentation by MHC to a specific TCR. (2) Co-stimulation by cognate ligand-receptor interactions between the DC and T cell. (3) Cytokine production by the DC, usually provoked by TLR stimulation or a 'danger' signal that triggers DC functional maturation. In the case of T_H1-mediated IFNγ responses, the critical cytokine produced by DC is IL-12 (Manicassamy and Pulendran, 2009).

FACS analysis of DC populations in spleen of $Ikk\alpha^{AA/AA}$ mice showed no overt differences in number or phenotype compared with littermate controls (Supplementary Figure S3). Furthermore, LPS-induced maturation of GM-CSF-derived

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BMDC from *Ikkα*$^{AA/AA}$ mice appeared normal based on MHC II and co-stimulatory molecule expression (Figure 3A). Similar results were obtained with FLT3-derived cDC (data not shown). In a functional assay of T-cell priming, using naïve CD4$^+$ T cells from OT-II transgenic mice, LPS-stimulated BMDC from *Ikkα*$^{AA/AA}$ mice showed a profound impairment of T_H1 cell priming in the presence of MHC II-specific OVA peptide, measured by IFNγ production (Figure 3B). However, LPS-independent antigen-induced IL-10 production remained unchanged. To test if the observed defect could prevent T-cell priming *in vivo*, we used an adoptive transfer approach. BMDC derived from WT or *Ikkα*$^{AA/AA}$ mice were loaded with MHC II-restricted OVA peptide (OVAp) in the presence or absence of LPS and injected into the footpad of naïve WT mice. Seven days after injection of antigen-loaded DC the draining popliteal LN and spleen was collected from recipient mice and single-cell suspensions prepared for re-call responses to OVAp *ex vivo*. Mice immunized with *Ikkα*$^{AA/AA}$ BMDC in the presence of LPS showed severely impaired IFNγ responses compared with WT BMDC (Figure 3C), in keeping with an intrinsic defect in DC-mediated T_H1 cell priming. FACS analysis of antigen-loaded DC prior to adoptive transfer confirmed no difference in activation markers and MHC II expression (Supplementary Figure S4). Finally, to confirm that IKKα activity in DC was required for T-cell priming

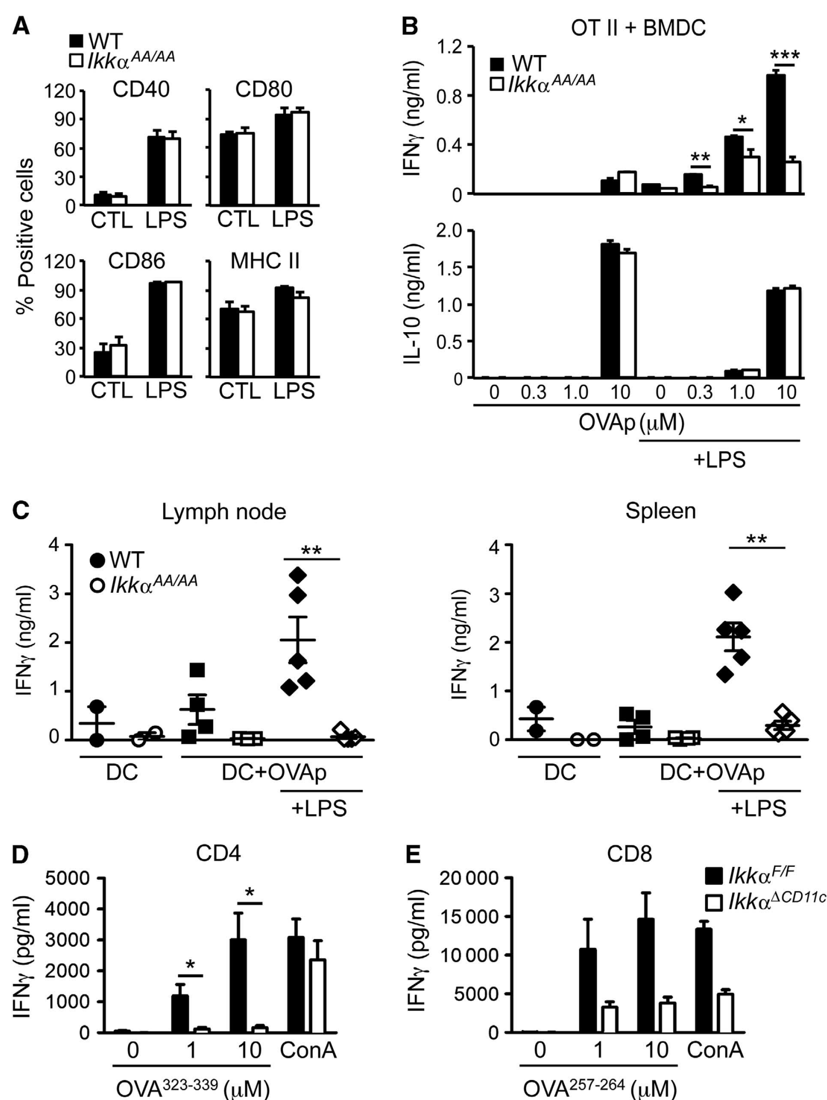

Figure 3 IKKα is required for TLR-induced functional maturation of DC. (**A**) FACS analysis of BMDC from WT and *Ikkα*$^{AA/AA}$ mice after LPS (100 ng/ml)-induced maturation for 24 h. Data are represented as mean ± s.e.m. of $n = 3$. (**B**) BMDC from WT and *Ikkα*$^{AA/AA}$ mice were loaded with MHC II-restricted OVA peptide (OVA$^{323–339}$; OVAp) with and without LPS stimulation for 24 h before co-culture with naïve CD4$^+$ OT-II T cells. IFNγ and IL-10 were measured in culture supernatants after 72 h. Data are represented as mean ± s.e.m. of $n = 4$, statistical analysis was performed with Student's *t*-test; $*P \leqslant 0.05$, $**P \leqslant 0.005$, $***P \leqslant 0.001$. (**C**) BMDC from WT and *Ikkα*$^{AA/AA}$ mice were loaded with OVAp in the presence and absence of LPS for 24 h, 2.5×10^5 DC were injected into the footpad of naïve WT mice. Seven days after DC injection, the popliteal LN and spleen were collected and single-cell suspensions prepared for antigen (OVAp) re-call assay *in vitro*; measured by IFNγ production in culture supernatants after 72 h. Data are represented as mean ± s.e.m. of $n = 5$, statistical analysis was performed with Mann–Whitney test; $**P < 0.005$. (**D**, **E**) 10^6 OVA-specific CD4$^+$ (OT-II; **D**) or CD8$^+$ (OT-I; **E**) T cells were adoptively transferred to *Ikkα*$^{\Delta CD11c}$ and *Ikkα*$^{F/F}$ mice, before immunization with CFA/OVA i.d. The inguinal LN was collected after 5 days and single-cell suspensions prepared for antigen re-call assays with MHC II (OVA$^{323–339}$) and MHC I (OVA$^{257–264}$) specific peptides; IFNγ production was measured in culture supernatants after 72 h. Data are represented as mean ± s.e.m. of $n = 3$–6, statistical analysis was performed with Mann–Whitney test; $*P < 0.05$.

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in vivo, we used mice with a targeted deletion of IKKα in CD11c$^+$ cells. Mice expressing a 'floxed' *Ikkα* gene (*Ikkα*$^{F/F}$) (Liu *et al*, 2008) were bred with transgenic mice expressing Cre recombinase from the CD11c promoter (CD11c-Cre) (Caton *et al*, 2007). FACS analysis of splenic DC populations in *Ikkα*$^{\Delta CD11c}$ mice and littermate controls (*Ikkα*$^{F/F}$) revealed no obvious differences in number or phenotype (Supplementary Figure S5A), in keeping with data from *Ikkα*$^{AA/AA}$ mice. To test T-cell priming *in vivo*, we transferred naïve T cells from OT-I (CD8$^+$) or OT-II (CD4$^+$) TCR transgenic mice, to *Ikkα*$^{\Delta CD11c}$ and *Ikkα*$^{F/F}$ mice before immunization with OVA/CFA by i.d. injection at the base of the tail. Draining inguinal LN was collected after 5 days for antigen re-call assay. LN cell suspensions were re-stimulated with OVAp specific for MHC I or MHC II, respectively, and IFNγ production measured in cell culture supernatants by ELISA. IFNγ production by both CD4$^+$ and CD8$^+$ OVA-specific T cells was significantly impaired in *Ikkα*$^{\Delta CD11c}$ mice compared to controls (Figure 3D and E). These data demonstrate that IKKα expression and activity is required specifically in DC for priming antigen-specific CD4$^+$ and CD8$^+$ T-cell responses *in vivo*.

IKKα regulates IRF3-dependent IFNβ and IL-12 production by DC

We next measured TLR-induced cytokine production by BMDC from *Ikkα*$^{AA/AA}$ and WT mice; *Ikkα*$^{AA/AA}$ DC showed a specific defect in IL-12 and IFNβ production in response to the TLR4-ligand LPS, whereas TNF-α production was unaltered (Figure 4A). This was confirmed at the mRNA level by quantitative real-time PCR analysis (qRT–PCR; Supplementary Figure S6). TLR-induced IL-12 expression by DC is a critical factor for priming T_H1 responses (Manicassamy and Pulendran, 2009); IFNβ expression is also linked to the functional maturation of DC, at least in part through autocrine/paracrine regulation of IL-12 expression (Gautier *et al*, 2005). Another important signal for cytokine production by DC is T cell-mediated co-stimulation; in particular, CD40 activation on DC by CD40L (CD154) expressed by activated T cells is an important signal for IL-12 production and T_H1 cell priming (Schulz *et al*, 2000). CD40 triggers activation of alternative NF-κB signalling through IKKα in B cells (Senftleben *et al*, 2001), therefore we tested the role of IKKα in CD40-induced IL-12 expression in BMDC. However, we found no difference in CD40L-induced IL-12 production by *Ikkα*$^{AA/AA}$ BMDC compared to WT controls (Supplementary Figure S7).

TLR4-induced IL-12 and IFNβ expression in DC is regulated by MyD88-dependent NF-κB activation, and TRIF-mediated IRF3 activation (Yamamoto *et al*, 2003). Despite its critical role in IFNβ expression, the role of IRF3 in the functional maturation of DC is not well established. To determine if IRF3 in DC was required for T-cell priming, we derived BMDC from IRF3 knockout mice (*Irf3*$^{-/-}$) and performed parallel

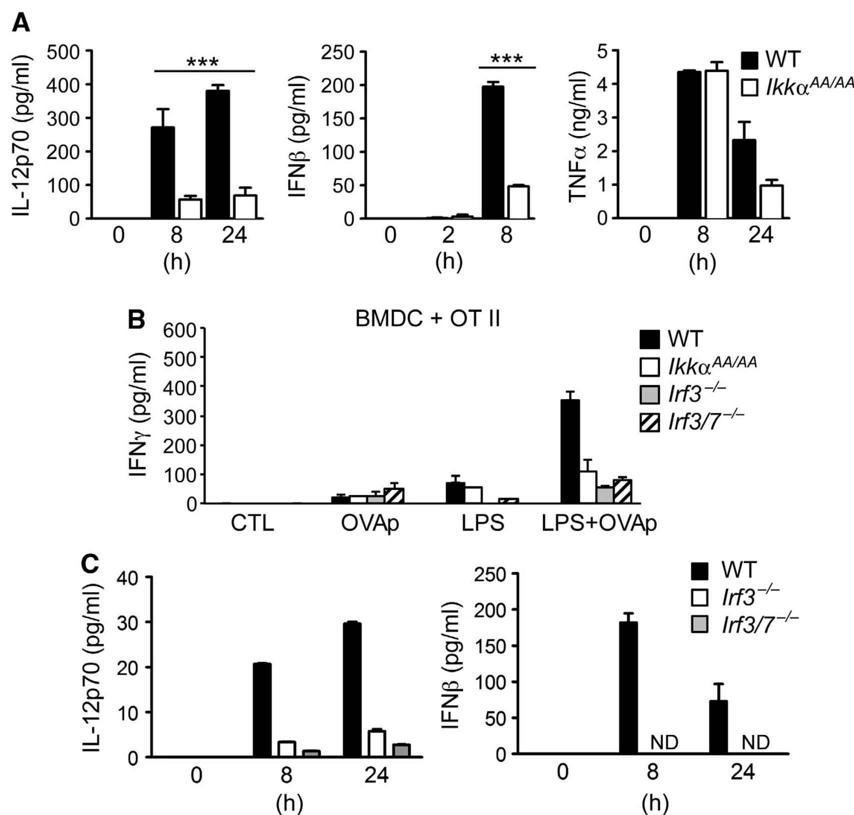

Figure 4 IKKα is required for IRF3-dependent functional maturation of DC. (**A**) BMDC from WT and *Ikkα*$^{AA/AA}$ mice were stimulated with LPS and cytokine production measured in culture supernatants by ELISA at the indicated time points. Data are represented as mean ± s.e.m. of $n = 4$, statistical analysis was performed with Student's *t*-test; $***P \leqslant 0.001$. (**B**) BMDC from WT, IRF3, IRF3 + IRF7 knockout (*Irf3*$^{-/-}$; *Irf3/7*$^{-/-}$) and *Ikkα*$^{AA/AA}$ mice were loaded with MHC II-restricted OVA peptide (OVA$^{323–339}$; OVAp) with and without LPS stimulation for 24 h before co-culture with naïve CD4$^+$ OT-II T cells. IFNγ was measured in culture supernatants after 72 h. (**C**) WT, *Irf3*$^{-/-}$ and *Irf3/7*$^{-/-}$ BMDC were stimulated with LPS and cytokine production measured in culture supernatants by ELISA at the indicated time points. Data are represented as mean ± s.e.m. of $n = 3$.

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experiments as done with $Ikk\alpha^{AA/AA}$ BMDC. Although $Irf3^{-/-}$ BMDC showed no defects in LPS-induced MHC II and co-stimulatory molecule expression (Supplementary Figure S8), they were unable to prime OT-II T cells in the presence of OVAp, measured by IFNγ production (Figure 4B), displaying a similar phenotype to $Ikk\alpha^{AA/AA}$ BMDC. As expected, IFNβ and IL-12 expression was profoundly inhibited in BMDC from IRF3 knockout mice (Figure 4C), confirming that TLR4-induced IFNβ/IL-12 expression is critically dependent on IRF3. Furthermore, there was no additive effect in BMDC from mice deficient in both IRF3 and IRF7 ($Irf3/Irf7^{-/-}$), suggesting a minimal role for IRF7 in this context. These data demonstrate a striking overlap between the functions of IRF3 and IKKα in DC that suggested that IKKα may regulate IRF3 activity.

IKKα-mediated IFNβ and IL-12 expression is independent of NF-κB activation

IKKα is a component of the canonical IKK complex regulating NF-κB activation in response to TLR signalling (Karin and Ben-Neriah, 2000; Takeuchi and Akira, 2010). However, previous studies have established no role for IKKα in TLR4-mediated activation of NF-κB signalling (Senftleben *et al*, 2001; Lawrence *et al*, 2005). In fact, several studies have shown that IKKα can attenuate NF-κB activation (Lawrence *et al*, 2005; Li *et al*, 2005; Liu *et al*, 2007; Shembade *et al*, 2011). LPS-induced NF-κB activation was unaffected in BMDC from $Ikk\alpha^{AA/AA}$ mice, as measured by phosphorylation and degradation of IκBα (Supplementary Figure S9A), as was expression of the canonical NF-κB target gene *Tnfa* (Figure 4A; Supplementary Figure S6). IKKα is critical for the alternative NF-κB signalling pathway, which depends on the processing of p100 (NFκB2) to p52 (Senftleben *et al*, 2001). This pathway requires the inducible degradation of the E3 ligase TRAF3, leading to stabilization of NIK and subsequent phosphorylation of IKKα (Vallabhapurapu *et al*, 2008). The mutation in $Ikk\alpha^{AA/AA}$ mice prevents the phosphorylation of IKKα by NIK in this pathway (Senftleben *et al*, 2001). We found no evidence for activation of alternative NF-κB signalling in LPS-stimulated BMDC, with no changes in TRAF3, NIK or p100/p52 expression in either WT of $Ikk\alpha^{AA/AA}$ cells (Supplementary Figure S9B). Furthermore, BMDC from NIK knockout mice ($Nik^{-/-}$) showed no defects in LPS-induced IFNβ or IL-12 production (Supplementary Figure S9C). To confirm that the phenotype of $Ikk\alpha^{AA/AA}$ DC was not due to defects in alternative NF-κB signalling, we compared LPS-induced IL-12 and IFNβ gene expression in BMDC derived from $Nik^{-/-}$ or $Nfkb2^{-/-}$ mice, and mice that constitutively express active p52 due to a p100 truncation (p100ΔGRR; *Nfkb2*Δ/Δ) (Ishikawa *et al*, 1997). $Nfkb2^{-/-}$, *Nfkb2*Δ/Δ, or $Nik^{-/-}$ BMDC showed no obvious defects in LPS-induced IL-12/IFNβ (Supplementary Figure S10). These data showed that IKKα-mediated IFNβ and IL-12 expression was independent of either canonical or alternative NF-κB signalling.

TRIF-mediated TAK1 activation is required for IFNβ expression in DC

MyD88-dependent TLR4 signalling triggers TAK1-mediated phosphorylation of IKKβ on two serine residues in its activation loop (Ser176/180) (Karin and Ben-Neriah, 2000), that are equivalent to the sites in IKKα (Ser177/181) phosphorylated by NIK (Senftleben *et al*, 2001). The TRIF adaptor protein was previously shown to recruit TAK1 in response to TLR3 signalling (Jiang *et al*, 2004). Since NIK was not required for IFNβ expression in BMDC, we examined the possible involvement of TAK1 in this process. To examine the role of TAK1 in TRIF-mediated IFNβ expression, we stimulated BMDC with LPS or dsRNA (poly(dI:dC)) in the presence or absence of a TAK1 inhibitor; 5Z-7-oxozeaenol (5Z) (Ninomiya-Tsuji *et al*, 2003). In parallel, we used an IKKε/TBK1 inhibitor MRT67307 (MRT) that blocks TRIF-mediated activation of IRF3 (Gleason *et al*, 2011). Treatment of BMDC with 5Z blocked inducible phosphorylation of IKKα/β, detected with an antibody that recognizes phosphorylation of both kinases in their respective activation loops (Figure 5A). As expected, 5Z did not affect Ser396 phosphorylation of IRF3 which is mediated by IKKε/TBK1 (Zhao *et al*, 2007). In contrast, the IKKε/TBK1 inhibitor (MRT) blocked IRF3 phosphorylation but did not affect IKKα/β phosphorylation (Figure 5A and B). However, both 5Z and MRT effectively inhibited LPS or dsRNA-induced IFNβ expression in BMDC (Figure 5C and D). These data suggest a requirement for TAK1 in TRIF-dependent IKKα/β activation and IFNβ expression in BMDC, but not IKKε/TBK1-mediated IRF3 phosphorylation. As expected, IKKε/TBK1-mediated Ser396 phosphorylation of IRF3 was also unaffected in $Ikk\alpha^{AA/AA}$ BMDC (Supplementary Figure S11), despite inhibition of IFNβ expression.

TAK1 is well established to regulate both IKK and JNK activation downstream of TLR4. However, we found no change in LPS or dsRNA-induced JNK activation in $Ikk\alpha^{AA/AA}$ BMDC (Supplementary Figure S12A), indicating that reduced IFNβ expression in $Ikk\alpha^{AA/AA}$ DC is not attributed to effects on JNK activation. Furthermore, a specific JNK inhibitor had only a partial effect on LPS and dsRNA-induced IFNβ expression in BMDC (Supplementary Figure S12B), whereas the TAK1 inhibitor almost completely blocked IFNβ induction (Figure 5B and C). These data suggest that TAK1-mediated JNK activation only partially contributes to IFNβ expression and TAK1-mediated IKKα/β activation also has a significant role.

Next, we treated BMDC with the NEDDylation inhibitor MLN4924 (MLN) to block poly-ubiquitination and degradation of phosphorylated IκBα, preventing activation of NF-κB downstream of TAK1-mediated IKKα/β activation. Despite effectively blocking IκBα degradation (data not shown), MLN did not inhibit IFNβ induction in BMDC stimulated with either LPS or dsRNA (Figure 5C and D). Furthermore, even in the presence of MLN, 5Z effectively inhibited IFNβ expression (Figure 5C and D), demonstrating that TAK1-mediated regulation of IFNβ induction was independent of NF-κB activation. Using a specific IKKβ inhibitor (BI605906) (Pauls *et al*, 2012), we confirmed that IKKβ-mediated NF-κB activation was not required for TRIF-dependent IFNβ expression in BMDC (Supplementary Figure S13). Finally, to confirm the results with the TAK1 inhibitor we generated TAK1 (Map3k7)-deficient BMDC from $Map3k7^{F/F}$ mice expressing tamoxifen-inducible Cre recombinase (CreER) (Wang *et al*, 2012). BMDC from $Map3k7^{F/F}$*-CreER* mice were treated with tamoxifen (4-OHT) to induce deletion of TAK1 followed by stimulation with LPS or dsRNA. TAK1 deletion ($Map3k7^{\Delta}$) in BMDC inhibited the phosphorylation of IKKα/β in response to both LPS and dsRNA, but did not affect Ser 396

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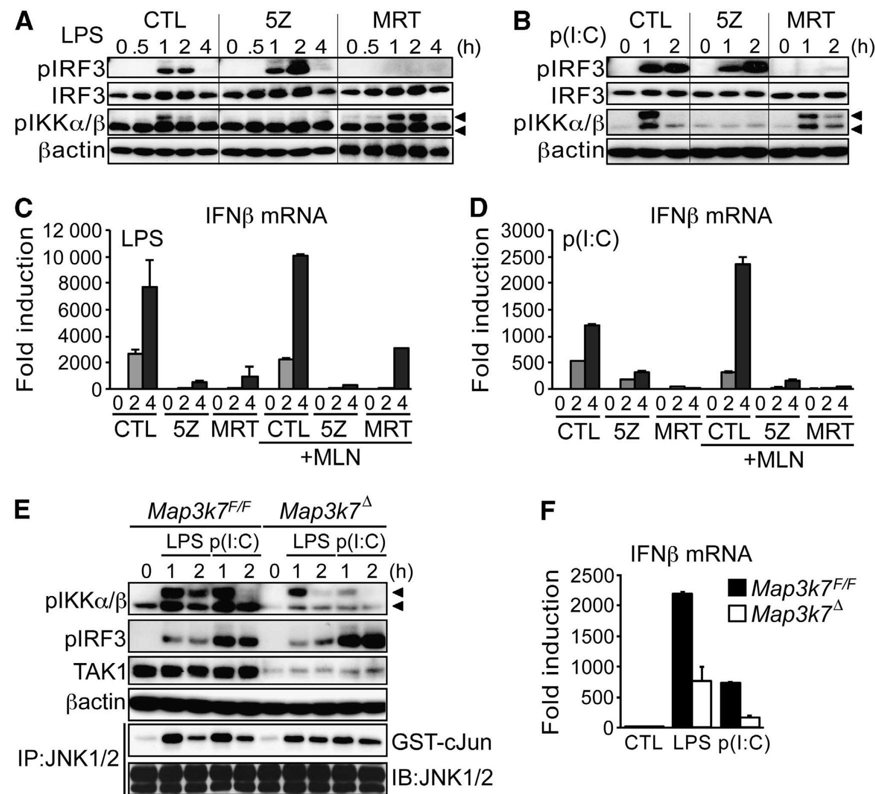

Figure 5 TAK1-mediated IKKα/β activation is required for IRF3-dependent IFNβ expression in DC. BMDC were stimulated with LPS or dsRNA (p(I:C); 25 μg/ml) in the presence of 1 μM 5Z-7-oxozeaenol (5Z), 2 μM MRT67307 (MRT), or vehicle alone (CTL). (**A**, **B**) Immunoblot (IB) analysis of IKKα/β (pIKKα/β; 85 kDa/89 kDa) and IRF3 phosphorylation (Ser396; pIRF3), βactin and IRF3 expression were used as loading controls. (**C**, **D**) BMDC were treated as above in the presence or absence of 1 μM MLN4926 (MLN), RNA was extracted at the indicated time points for qRT–PCR analysis of IFNβ mRNA expression; data are represented as fold induction over control, normalized to cyclophillin (CPH) mRNA. (**E**, **F**) BMDC were generated from *Map3k7$^{F/F}$* and *Map3k7$^{F/F}$.CreER* mice and treated with 4 μM tamoxifen (OHT) for 48 h, before stimulation with LPS or dsRNA as described above. Protein extracts were prepared at the indicated time points and phosphorylation of IKKα/β and IRF3 was measured by IB analysis; JNK activity was measured by IP kinase assay using recombinant GST-cJun as a substrate (**E**). In parallel experiments, RNA was extracted at 4 h and IFNβ expression measured by qRT–PCR (**F**). Representative data from at least two independent experiments are shown. qRT–PCR data are presented as mean ± s.e.m. of three replicates. Source data for this figure is available on the online supplementary information page.

phosphorylation of IRF3 (Figure 5E), recapitulating the effects of the TAK1 inhibitor (Figure 5A and B). Furthermore, TAK1 deletion in BMDC inhibited both LPS and dsRNA-induced IFNβ expression (Figure 5F), again in keeping with the results of the TAK1 inhibitor (Figure 5C and D). Collectively, these data suggest a requirement for TAK1-mediated IKKα activation in IRF3-dependent IFNβ expression in BMDC.

IKKα regulates IRF3-mediated transcription downstream of IKKε/TBK1

To further explore the role of IKKα in IRF3-mediated IFNβ expression, we established an IFNβ-promoter reporter assay in mouse embryonic fibroblasts (MEF). In keeping with data from *Ikkα$^{AA/AA}$* BMDC, endogenous IFNβ expression in response to dsRNA transfection, which triggers IKKε/TBK1-mediated IRF3 activation (Zhao *et al*, 2007), was blocked in IKKα-deficient cells (*Ikkα$^{-/-}$*) but was unaffected by IKKβ deletion (*Ikkβ$^{-/-}$*; Figure 6A). IFNβ-promoter activity was also IKKα and IRF3 dependent, but unaffected by IKKβ or RelA deletion (Figure 6B), demonstrating that IFNβ induction in MEF was not dependent on NF-κB activation. Furthermore, IKKε/TBK1-mediated Ser396 phosphorylation of IRF3 was not significantly impaired in *Ikkα$^{-/-}$* cells (Figure 6C), suggesting that IKKα may regulate IRF3-mediated IFNβ expression downstream of IKKε/TBK1 activation.

To further explore the role of IKKα in regulation of IRF3 activity, we performed co-transfection experiments in HEK293T cells that stably express TLR3. Co-expression of wild-type IKKα (IKKα^{WT}) and IRF3 resulted in a strong interaction detected by co-immunoprecipitation (co-IP) (Figure 7A), this interaction was significantly reduced when the IKKα^{AA} mutant was co-expressed with IRF3. Furthermore, the co-expression of IKKα, but not the IKKα^{AA} mutant, increased the association of IRF3 with the transcriptional co-activator CBP (Figure 7A). The increased recruitment of CBP after overexpression of IKKα was also associated with increased transcription of endogenous IFNβ mRNA (Figure 7B). The inability of IKKα^{AA} to promote CBP recruitment or increased IFNβ transcription indicates the requirement for IKKα kinase activity.

The rate-limiting step in IRF3 activation is the inducible phosphorylation of Ser396 in the C-terminal regulatory domain by IKKε/TBK1 (Lin *et al*, 1999). However, this domain (aa 380–427) contains a total of seven residues that are targets for inducible phosphorylation and contribute to IRF3 activity (Lin *et al*, 1999; Mori *et al*, 2004) (Figure 7C). To test if the regulatory domain of IRF3 was a target for

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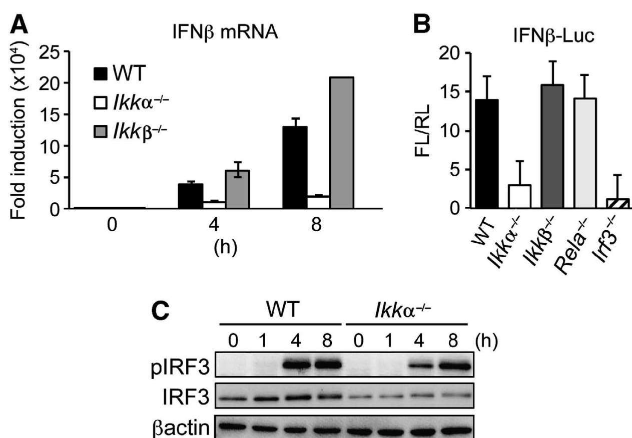

Figure 6 IFNβ-promoter activity is IKKα dependent. (**A**) WT, *Ikkα*$^{-/-}$ and *Ikkβ*$^{-/-}$ MEF cells were transfected with 1 μg/ml dsRNA (p(I:C)) and IFNβ mRNA expression measured at the indicated time points by qRT–PCR, data are expressed as fold induction over control normalized to CPH expression. (**B**) WT, *Ikkα*$^{-/-}$, *Ikkβ*$^{-/-}$, *Rela*$^{-/-}$ and *Irf3*$^{-/-}$ MEF cells were co-transfected with an IFNβ-promoter reporter vector expressing firefly luciferase (IFNβ-Luc; FL) and a constitutive renilla-luciferase reporter (RL); IFNβ-promoter activity was measured by dual luciferase assay 6 h after p(I:C) stimulation, data are expressed as fold induction of FL activity normalized to RL (FL/RL). Experiments were performed in triplicate and data represented as mean ± s.e.m. of three independent experiments. (**C**) IB analysis of IRF3 Ser396 phosphorylation (pIRF3) in WT and *Ikkα*$^{-/-}$ MEF cells after p(I:C) stimulation at the indicated time points. Representative data from three independent experiments are shown. Source data for this figure is available on the online supplementary information page.

IKKα-mediated phosphorylation, we prepared recombinant GST-IRF3 fusion protein encompassing the C-terminal regulatory domain (GST-IRF3$^{380–427}$) and various Ser-Ala point mutants, and performed *in vitro* kinase assays with active IKKα. IRF3$^{380–427}$ was strongly phosphorylated by IKKα *in vitro* and this phosphorylation was significantly reduced by mutation of Ser402/404/405 to Ala (Figure 7D), however, mutation of the other putative target residues including Ser396 and Ser386 did not affect IKKα-mediated phosphorylation. These data suggest that direct phosphorylation of IRF3 by IKKα could regulate IRF3-mediated transcription through enhanced CBP recruitment.

Discussion

Here, we describe a new role for the kinase IKKα (CHUK) in DC required for priming antigen-specific T cells. Bone marrow chimeras generated from transgenic mice that express a mutant form of IKKα (*Ikkα*$^{AA/AA}$) showed impaired DTH *in vivo*, which is mediated by antigen-specific T_H1 cells. Further experiments demonstrated impaired T_H1 cell priming in *Ikkα*$^{AA/AA}$ mice. This is distinguished from the function of IKKα in lymphoid organogenesis, which is intrinsic to radiation-resistant stromal cells through the alternative NF-κB pathway (Senftleben *et al*, 2001; Bonizzi *et al*, 2004).

We show that IKKα is required for acquired immunity to the facultative intracellular pathogen *Listeria monocytogenes* (Lm). Innate immunity to primary Lm infection was enhanced in *Ikkα*$^{AA/AA}$ mice; however, acquired immunity to secondary infection was severely impaired, which is dependent on the development of protective CD8$^+$ memory T cells (Pamer, 2004). Usually the innate immune response is tightly coupled to development of acquired immunity, these data suggest that IKKα has the unusual property of uncoupling innate and adaptive immunity; inhibiting innate non-specific immunity while enhancing antigen-specific acquired immunity.

It was previously shown that IKKα has anti-inflammatory activity by attenuating NF-κB activation in macrophages (Lawrence *et al*, 2005; Li *et al*, 2005; Liu *et al*, 2007). In addition, macrophages from *Ikkα*$^{AA/AA}$ mice have increased resistance to apoptosis, and consequently enhanced anti-microbial activity, upon infection with *Streptococcus agalactiae* (Lawrence *et al*, 2005). Macrophage apoptosis also plays an important role in resistance to primary Lm infection in mice (Stockinger *et al*, 2009). The anti-inflammatory and pro-apoptotic functions of IKKα in macrophages are likely to explain the increased clearance of Lm after primary infection in *Ikkα*$^{AA/AA}$ mice.

In contrast, acquired immunity to Lm in mice requires the development of CD8$^+$ memory T cells. Expansion of Lm-specific CD8$^+$ T cells was normal upon primary infection of *Ikkα*$^{AA/AA}$ mice. However, Lm-specific CD8$^+$ T cells in *Ikkα*$^{AA/AA}$ mice failed to respond to secondary infection, suggesting impaired development or survival of CD8$^+$ memory T cells. The expansion of pathogen-specific CD8$^+$ T cells upon primary Lm infection is independent of CD4$^+$ T-cell help, but the survival of protective CD8$^+$ memory T cells critically requires MHC II-restricted CD4$^+$ T cells (Sun and Bevan, 2003). We show that CD4$^+$ T-cell priming is defective in *Ikkα*$^{AA/AA}$ mice, which may result in defective CD8$^+$ T-cell memory. Other factors that directly influence the programming and survival of CD8$^+$ memory T cells include; antigen dose, co-stimulation and cytokine production (Pamer, 2004). However, further experiments are required to establish the role of IKKα in these additional processes that may contribute to the development and maintenance of T-cell memory.

We present several lines of evidence that show IKKα activation in DC is required for T-cell priming; DC derived from *Ikkα*$^{AA/AA}$ mice cannot prime naïve CD4$^+$ T cells *in vitro* to produce IFNγ, and antigen-loaded *Ikkα*$^{AA/AA}$ DC adoptively transferred to naïve mice show similar defects in T_H1 cell priming *in vivo*. Furthermore, targeted deletion of IKKα in CD11c$^+$ DC impairs priming of both CD4$^+$ and CD8$^+$ T cells after immunization *in vivo*. Naïve T-cell priming by DC requires three signals (Steinman, 2008); antigen presentation, co-stimulation and cytokine production. DC derived from *Ikkα*$^{AA/AA}$ mice showed no defects in antigen-presentation or co-stimulatory molecule expression, but significantly reduced IFNβ and IL-12 expression in response to TLR stimulation. Other cytokines such as TNF-α and IL-10 are expressed normally. TLR-mediated cytokine production by DC is required for T-cell priming in the context of infection, in the case of T_H1-mediated IFNγ responses the critical cytokine is IL-12 (Manicassamy and Pulendran, 2009). Type I IFN has also been shown to be an important cytokine for DC maturation and function (Honda *et al*, 2003), at least in part through promoting the increased expression of IL-12 in DC (Gautier *et al*, 2005). Thus, the ability of adjuvants to induce IFNα/β is an important facet for priming T_H1 immunity (Sugiyama *et al*, 2008; Manicassamy and Pulendran, 2009).

IKKα was previously shown to regulate MyD88-dependent type I IFN production by pDC and conventional CD8$^+$ DC

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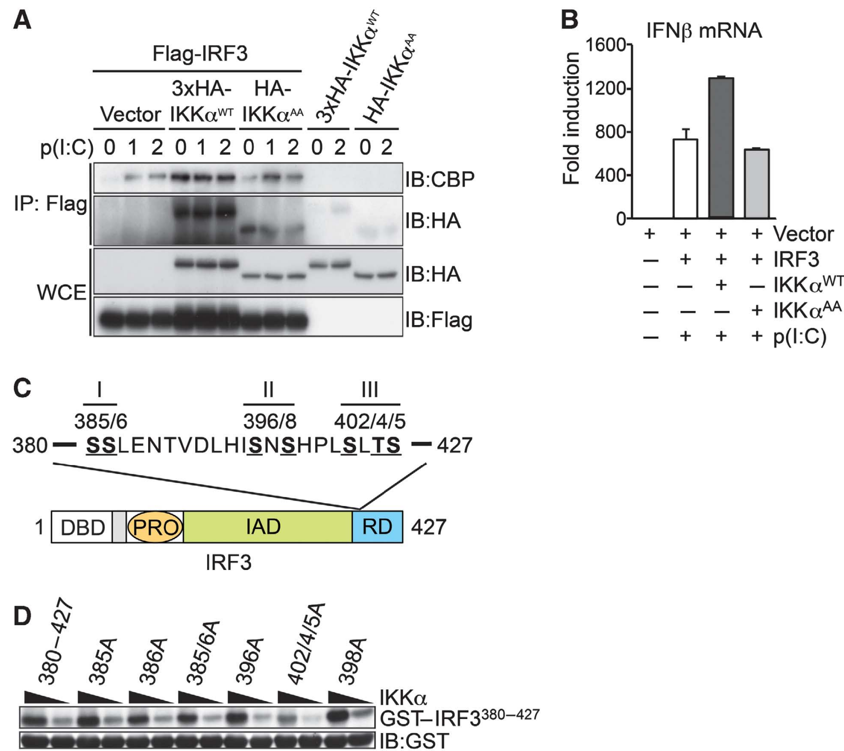

Figure 7 IKKα regulates IRF3-medaited transcription downstream of IKKε/TBK1-mediated IRF3 phosphorylation. (**A**) HEK293-TLR3 cells were transfected with cDNA vectors expressing Flag-IRF3, HA-IKKα^{WT} and HA-IKKα^{AA}, in the presence of absence of p(I:C) as indicated. Protein extracts were prepared and immunoprecipitation (IP) of IRF3 was performed with anti-Flag antibody, co-IP of HA-IKKα and endogenous CBP was detected by IB analysis. Expression of Flag-IRF3, HA-IKKα^{WT} and HA-IKKα^{AA} was measured in total protein extracts (WCE) by IB. (**B**) In parallel experiments, RNA was extracted at 4 h and IFNβ expression measured by qRT–PCR. Representative data from at least two independent experiments are shown; qRT–PCR data are presented as mean ± s.e.m. of three replicates. (**C**) Schematic representation of the seven potential phosphorylation sites in the C-terminal regulatory domain of IRF3 organized into three clusters: I—Ser385/Ser386; II—Ser396/Ser398; III—Ser402/Thr404/Ser405. DBD, DNA-binding domain; PRO, proline-rich domain; IAD, auto-inhibitory domain; RD, regulatory domain. (**D**) GST-IRF3$^{380–427}$ and various mutant peptides were expressed and purified from bacteria and incubated with recombinant active IKKα in the presence of γ-^{32}P-ATP, IKKα-mediated phosphorylation of GST-IRF3 peptides was quantified by autoradiography. IB analysis of GST was used a loading control for IRF3 substrate. Representative data from two independent experiments are shown. Source data for this figure is available on the online supplementary information page.

(cDC), in response to TLR7 and TLR9 ligands (Hoshino *et al*, 2006, 2010). TLR4 and TLR3-mediated IFNβ expression is dependent on TRIF and IRF3 activation (Yamamoto *et al*, 2003). IRF3 is also critical for IL-12 expression in response to TLR4 stimulation (Ramirez-Carrozzi *et al*, 2009). However, the role of IRF3 in DC maturation and function has not been established. We showed that IRF3-deficient DC have profoundly impaired ability to prime naïve CD4$^+$ T cells in the presence of LPS, which was associated with severely reduced IFNβ and IL-12 expression. Thus, the roles of IRF3 and IKKα in DC phenotype show a striking correlation. Interestingly, LPS-induced IFNβ expression in DC was independent of NIK, suggesting another upstream kinase regulates IKKα activation in this context. TAK1 mediates IKKβ activation in response to MyD88 signalling (Wang *et al*, 2001), and TRIF has also been shown to recruit TAK1 (Jiang *et al*, 2004). Therefore, TAK1 is a likely candidate to trigger IKKα activation in TRIF-mediated TLR signalling. We showed that TRIF-mediated IKKα/β phosphorylation and IFNβ expression in DC was blocked by a TAK1 inhibitor (Ninomiya-Tsuji *et al*, 2003), or targeted deletion of the TAK1 gene (*Map3k7*), however, IFNβ induction was not dependent on IKKβ-mediated NF-κB activation. This suggested that TAK1-mediated IKKα activation might regulate IRF3 activity independently of NF-κB.

IRF3 activation is triggered by IKKε/TBK1-mediated phosphorylation on Ser396 (Sharma *et al*, 2003); however, Ser396 phosphorylation was not affected by TAK1 inhibition. These data suggested that TAK1-mediated IKKα activation may regulate IRF3-dependent IFNβ expression downstream of IKKε/TBK1 activation in DC. Although Ser396 phosphorylation is required, it is not sufficient for full IRF3 activity (Mori *et al*, 2004). There are in fact 7 C-terminal Ser residues in IRF3 that are targets for inducible phosphorylation and regulate IRF3 activation (Hiscott and Lin, 2005), but it is not clear if IKKε/TBK1 is responsible for subsequent phosphorylation events or other kinase activities are required. To further explore the role of IKKα in IRF3 activity, we performed IFNβ-promoter activity assays in embryonic fibroblasts (MEF) from various knockout mice; *Ikkα*$^{-/-}$ MEF showed substantially reduced ability to drive IFNβ-promoter activity in response to dsRNA, despite little effect on Ser396 phosphorylation, implying that an IKKα-dependent signal is required for IRF3-mediated transcription downstream of IKKε/TBK1 activation.

In co-transfection experiments, we showed a direct interaction between IKKα and IRF3, which was associated with the increased recruitment of the transcriptional co-activator CBP, suggesting that IKKα directly regulates IRF3 transcriptional activity. We also showed IKKα strongly

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phosphorylates Ser402/404/405 in the C-terminal regulatory domain of IRF3 *in vitro*, phosphorylation of this cluster of residues has previously been shown to be important for IRF3 activation and IFNβ expression in response to viral infection (Lin *et al*, 1998). These data suggest that IKKα-mediated phosphorylation of IRF3 promotes CBP recruitment and transcriptional activity. Interestingly, IKKγ has been shown to be required for IKKε/TBK1-mediated Ser396 phosphorylation of IRF3 in response to viral infection (Zhao *et al*, 2007), this would also provide a platform to recruit both TAK1 and IKKα to the signalling complex and give access to IKKε/TBK1-phosphorylated IRF3.

In summary, IKKα has been shown to have anti-inflammatory activity through a variety of mechanisms (Lawrence *et al*, 2005; Li *et al*, 2005; Liu *et al*, 2007; Shembade *et al*, 2011); however, here we describe an important role for IKKα in priming adaptive immunity. This presents a unique role for IKKα in bridging the innate and adaptive immune systems; driving the resolution of inflammation while promoting acquired immunity. These properties of IKKα could be exploited therapeutically in several contexts; the pro-inflammatory side effects of adjuvants are a major barrier to vaccine development, adjuvants that promote IKKα activation may confer immuno-stimulatory functions without promoting excessive inflammation. On the other hand; inhibition of IKKα could have therapeutic potential in autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS), where targeting IKKα may block auto-antigen driven inflammation without compromising innate immunity and increasing susceptibility to opportunistic infections, the major limitation of current therapeutic approaches.

Materials and methods

Mice

Ikkα$^{AA/AA}$ (Chuk^{tm2Mka}), *Relb*$^{-/-}$ (Relb^{tm1Brv}), *Nfkb2*$^{-/-}$ (Nfkb2^{tm2Brv}), *Nfkb1*Δ/Δ (Nfkb2^{tm1Brv}) and *Map3k7*$^{F/F}$*.CreER* (Map3k7^{tm1Aki}/Tg(Rosa26-creERT2)) mice have been previously described (Ishikawa *et al*, 1997; Caamano *et al*, 1998, 1999; Cao *et al*, 2001; Wang *et al*, 2012). *Ikkα*$^{F/F}$ mice (Chuk^{tm1Yhu}) (Liu *et al*, 2008) were provided by Y Hu (NICR, USA) and CD11c-Cre mice (Tg(Itgax-cre)1-1Reiz) (Caton *et al*, 2007) were provided by B Reizis (Columbia University, USA). *Nik*$^{-/-}$ mice (Map3k14^{tm1Rds}) (Yin *et al*, 2001) were provided by R Schreiber (Washington University, USA). *Irf3*$^{-/-}$ (Bcl2l12/Irf3^{tm1Ttg}), *Irf3/7*$^{-/-}$ (Bcl2l12/Irf3^{tm1Ttg}/Irf7^{tm1Ttg}) (Sato *et al*, 2000) mice were kindly provided by M Albert (Institute Pasteur, Paris, France). B6.SJL-*Ptprca Pep3b*/BoyJ (C57Bl6/Ly5.1), B6.129S7-*Rag1*^{tm1Mom}Tg(TcraTcrb) (OT-I) and B6.129S7-*Rag1*^{tm1Mom}Tg(TcraTcrb) (OT-II) mice were purchased from Taconic. OT-I.EGFP mice have been previously described (Bajenoff *et al*, 2010). All mice were housed under specific pathogen-free conditions and animal experimentation was conducted in strict accordance with good animal practice as defined by the French animal welfare bodies relative to European Convention (EEC Directive 86/609) and approved by the Direction Départmentale des Services Vétérinaires des Bouches du Rhônes.

Cell lines

Murine embryonic fibroblast (MEF) cell lines; *Ikkβ*$^{-/-}$ and *Ikkα*$^{-/-}$ have been previously described (Hu *et al*, 1997; Li *et al*, 1999). *Nik*$^{-/-}$ MEF cells were kindly provided by R Schreiber (Washington University, USA), *Rela*$^{-/-}$ MEF cells were provided by N Perkins (University of Newcastle, UK) and the *Irf3*$^{-/-}$ MEF cells were a gift from T Taniguchi (University of Tokyo, Japan).

Dendritic cells

BMDC: bone marrow cells were cultured for 7 days in DMEM containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin/streptomycin, 2 mM glutamine and 20 ng/ml murine GM-CSF (Peprotech). After 7 days, cells were collected and checked for CD11c and MHC II expression by FACS, these conditions routinely generated >97% CD11c^{+} MHC II^{+} cells. For splenic DC isolation, single-cell suspensions were prepared by enzymatic digestion of spleens with 50 μg/ml collagenase D, CD11c^{+} cells were purified by positive selection using MACS separation columns (Miltenyi Biotec) according to manufacturer's instructions. The purity of DC populations was routinely between 96 and 99%.

T-cell priming assays

In vitro priming assay. Naïve CD4^{+} T cells isolated from LNs and spleens of OT-II mice using MACS negative selection columns were co-cultured with immature or LPS-activated BMDC (1:8/DC:T ratio) in presence or absence of chicken ovalbumin (OVA) peptide (OVA$^{323–339}$). After 3 days, T-cell priming was measured by cytokine production in culture supernatants by ELISA.

DC adoptive transfer. BMDC were loaded with OVA$^{323–339}$ (OVAp) in the presence or absence of LPS for 24 h, 2.5×10^5 OVAp-loaded DC were injected subcutaneously (s.c.) into the paw of naïve C57Bl6 mice, after 7 days popliteal LNs and spleens were collected and single-cell suspensions prepared. Cells were cultured in the presence or absence of OVA$^{323–339}$ for 72 h and IFNγ production measured in culture supernatants by ELISA.

In vivo T-cell priming assay. 10^6 naïve CD4^{+} or CD8^{+} T cells, isolated by MACS selection from LN and spleen of OT-II or OT-I mice, respectively, were adoptively transferred to naïve mice 24 h before immunization with an emulsion of 400 μg heat-killed *Mycobacterium tuberculosis* H37RA (Difco, BD Bioscience) in Freunds' incomplete adjuvant (CFA) and 50 μg OVA, by i.d. injection at the base of the tail. Five days later, inguinal LN was collected and single-cell suspension prepared, cells were cultured in the presence or absence of OT-II (OVA$^{323–339}$) or OT-I (OVA$^{257–264}$)-specific peptide for 72 h and IFNγ production measured in culture supernatants by ELISA.

Delayed-type (IV) hypersensitivity

Mice were immunized with 1 mg methylated bovine serum albumin (mBSA; Sigma) in CFA i.d. at the base of the tail, 14 days later 0.5 mg mBSA was injected s.c. in the right paw, and PBS alone was injected in the left paw as control. Paw swelling was measured at various time points by plethysmography. For *ex vivo* antigen re-call assays, splenocytes from immunized mice were stimulated with 25 μg/ml mBSA or 2.5 μg/ml ConA (Sigma).

Listeria monocytogenes infection

Recombinant *L. monocytogenes* expressing chicken ovalbumin (Lm-OVA) (Bajenoff *et al*, 2010) was grown in Brain Heart Infusion (BHI) broth to a density of 0.4 $OD_{600\,nm}$, equivalent to 10^8 CFU per ml. Bacteria in log-phase growth were washed in PBS and adjusted to the desired concentration before use. 10^4 naïve CD8^{+} T cells, purified from LN/spleen of OT-I.EGFP mice, were injected i.v. into naïve mice 24 h before primary infection with 10^4 CFU Lm-OVA i.v. 5 days later, liver and spleens were collected and homogenized in PBS 0.1% Triton X-100, CFU was determined by serial dilution on BHI agar plates. OT-I cell expansion was measured by FACS in the spleen 7 days after primary infection. Secondary infection with 10^6 CFU Lm-OVA was performed 35 days after primary infection. CFU in liver and spleen, and OT-I cell expansion was determined as described above. Re-call assays were performed with total splenocytes in the presence of 1 μM OVA$^{257–264}$ and GolgiStop (BD Bioscience) for 6 h, intracellular IFNγ was measured in OT-I.EGFP cells by FACS.

Kinase assay, immunoblotting and immunoprecipitation

IKK kinase activity was measured in whole cell lysates after IP with anti-IKKγ antibody (BD Bioscience) as described previously (Lawrence *et al*, 2005). IKKα recovery was determined by immunoblotting (IB). IKKα kinase activity was measured with purified recombinant IKKα (Upstate Biotechnology). IB was

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performed on SDS–PAGE gel separated whole cell lysates. Co-IP experiments were performed on whole cell extracts prepared in cold lysis buffer containing10 mM Tris–HCl pH 8, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5 mM PMSF and 5 μg/ml leupeptin, pepstatin and aprotinin, antibodies were pre-incubated with protein A-sepharose beads for 3 h at 4°C before incubation with protein extracts overnight.

Transfection and reporter assay

MEF or HEK293T-TLR3 (Invivogen) cells were plated in 12-well plates at 80–90% confluency and transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. Luciferase activity was determined with the Dual-Luciferase Reporter Assay System according to manufacturer's instructions (Promega).

Statistical analysis

At least three independent experiments were performed, and representative data are shown unless otherwise indicated. Data are represented as mean ± s.e.m. Student's *t* test or Mann–Whitney test was performed where appropriate to test the statistical significance between data sets and *P*-values indicated.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: AM performed most of the experiments with significant contributions from MH, LL, MB and EJ; MH contributed data on Lm infection, EJ contributed data on DTH, LL and MB contributed data for Figures 5 and 7. CF and SM performed specific experiments. XW, MK, JC, HC and MBa contributed reagents. TL, AM, MH, EJ, MB and LL analysed the data. TL and AM wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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