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Two Phases of Inflammatory Mediator Production Defined by the Study of IRAK2 and IRAK1 Knock-in Mice

Eduardo Pauls,*†,1 Sambit K. Nanda,*† Hilary Smith,* Rachel Toth,* J. Simon C. Arthur,*† and Philip Cohen*†

The roles of IL-1R–associated kinase (IRAK)2 and IRAK1 in cytokine production were investigated using immune cells from knock-in mice expressing the TNFR-associated factor 6 (TRAF6) binding-defective mutant IRAK2[E525A] or the catalytically inactive IRAK1[D359A] mutant. In bone marrow–derived macrophages (BMDMs), the IRAK2–TRAF6 interaction was required for the late (2–8 h) but not the early phase (0–2 h) of Il6 and Tnfa mRNA production, and hence for IL-6 and TNF-α secretion by TLR agonists that signal via MyD88. Loss of the IRAK2–TRAF6 interaction had little effect on the MyD88-dependent production of anti-inflammatory molecules produced during the early phase, such as Dual Specificity Phosphatase 1, and a modest effect on IL-10 secretion. The LPS/TLR4-stimulated production of Il6 and Tnfa mRNA and IL-6 and TNF-α secretion was hardly affected, because the Toll/IL-1R domain–containing adapter-inducing IFN-β (TRIF) signaling pathway was used instead of the IRAK2–TRAF6 interaction to sustain late-phase mRNA production. IRAK1 catalytic activity was not rate limiting for Il6, Tnfa, or Il10 mRNA production or the secretion of these cytokines by BMDMs, but IFN-β mRNA induction by TLR7 and TLR9 agonists was greatly delayed in plasmacytoid dendritic cells (pDCs) from IRAK1[D359A] mice. In contrast, IFN-β mRNA production was little affected in pDCs from IRAK2[E525A] mice, but subsequent IFN-α mRNA production and IFN-α secretion were reduced. IFN-β and IFN-α production were abolished in pDCs from IRAK1[D359A] × IRAK2[E525A] double knock-in mice. Our results establish that the IRAK2–TRAF6 interaction is rate limiting for the late, but not the early phase of cytokine production in BMDM and pDCs, and that the IRAK2–TRAF6 interaction is needed to sustain IκB-inducing kinase β activity during prolonged activation of the MyD88 signaling. The Journal of Immunology, 2013, 191: 2717–2730.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; DC, dendritic cell; DD, death domain; DUSP1, Dual Specificity Phosphatase 1; ES, embryonic stem; HEK293, human embryonic kidney 293; IKK, IκB-inducing kinase; IRAK, IL-1R–associated kinase; LTA, lipoteichoic acid; MALP2, macrophage-activating lipopeptide 2; MSK1/MSK2, mitogen- and stress-activated protein kinases 1 and 2; pDC, plasmacytoid dendritic cell; poly(dU), poly(deoxyuridylic acid); TAB, TGFβ–activated kinase 1 binding protein; TAK1, TGFβ–activated kinase 1; TRAF6, TNFR-associated factor 6; TRIF, Toll/IL-1R domain–containing adapter-inducing IFN-β; USP2, ubiquitin-specific protease 2; WT, wild type.

The death domain (DD) of IRAK4 then interacts with the DD of IRAK1 and IRAK2 to form a structure termed the “Myddosome” (4, 5). IRAK1 and IRAK2 undergo covalent modification by phosphorylation and ubiquitylation, and interact with TNFR-associated factor 6 (TRAF6) via their C-terminal TRAF-binding domains (6). This increases the E3 ubiquitin ligase activity of TRAF6, which is thought to produce Lys63-linked polyubiquitin chains in the presence of UBE1 and the E2 conjugating complex Ubc13-Uev1a. Lys63-linked polyubiquitin chains can interact with the TGFβ-activated kinase 1 (TAK1) binding protein (TAB)2 and TAB3 components of the TAK1 complex, which has been suggested to induce a conformational change that leads to the autoactivation of TAK1 (7, 8). TAK1 can then initiate the activation of the MAPK kinases that switch on p38 MAPK and JNKs. TAK1 may also initiate the activation of IκB-inducing kinase α (IKKα) and IKKβ provided that linear polyubiquitin chains produced by the E3 ubiquitin ligase linear ubiquitin assembly complex are bound to NEMO (9, 10), which is an essential regulatory component of the canonical IκK complex. Together, the canonical IκKks and MAPks, and other protein kinases that they activate, catalyze many phosphorylation events that stimulate transcriptional and posttranscriptional events that culminate in the production of inflammatory mediators. For example, IKKβ not only induces the activation of the transcription factor NF-κB, but also activates the protein kinase Tpl2 (11, 12), which leads to the activation of the MAPks ERK1 and ERK2 (13).

Much of our knowledge about the physiological roles of IRAK1 and IRAK2 has been obtained by studying knockout mice. Studies with macrophages from mice deficient in either IRAK1 or IRAK2 showed that they produced reduced amounts of the mRNAs encoding a number of proinflammatory cytokines after stimulation with the TLR2 agonist macrophage-activating lipopeptide 2 (MALP2), but mRNA production was impaired much more severely in...
macrophages from double knockout mice that do not express either of these proteins (14). Consistent with these findings, IRAK1- (15) and IRAK2-deficient mice (14, 16) were found to be more resistant to septic shock than wild type (WT) mice, whereas the double-knockout mice were far more resistant. Taken together, these results indicated that IRAK1 and IRAK2 are both required for maximal production of proinflammatory cytokine mRNAs. However, a much more drastic reduction in the secretion of proinflammatory cytokins was observed in macrophages from IRAK2-deficient mice than from IRAK1-deficient mice (14).

Plasmacytoid dendritic cells (pDCs) are a subset of dendritic cells (DCs) that produce high levels of type 1 IFNs in response to viral nucleic acids that activate TLR7 and TLR9, probably because they express high levels of IFN regulatory factor 7 constitutively (17). The production of type 1 IFNs by these cells plays an important role in defense against viral infection (reviewed in Ref. 18), but because TLR7 and TLR9 have the potential to trigger immune responses if they are recognized by immune complexes consisting of autoantibodies bound to self-RNA and self-DNA (19), they may also contribute to the development of autoimmune diseases, such as systemic lupus erythematosus (20). TLR7 and TLR9 signal via MyD88, and IKKβ and IKKα are both required for the production of type 1 IFNs by pDCs and conventional DCs (21, 22). However, in these pathways, IKKα and IKKβ exert their effects by mechanisms that are at least partially independent of NF-κB (23, 24). Interestingly, the TLR7/TLR9-stimulated production of IFN-α by pDCs was reported to be greatly impaired in IRAK1-deficient mice (25) but enhanced in pDCs from IRAK2-deficient mice (26). Based on the latter observation it was suggested that IRAK2 may function as a negative regulator of IFN-α production in these cells (26).

IRAK1 and IRAK2 are both multimdomain proteins, the DD being followed by a region rich in proline, serine, and threonine residues, then the kinase domain, and finally the C-terminal TRAF6 interacting domain (6, 27). Although IRAK1, like IRAK4, is catalytically active, the physiological roles of the protein kinase activity of IRAK1 are poorly understood. In contrast, the kinase-like domain of IRAK2 lacks amino acid residues that are essential for catalysis by most protein kinases, and when expressed in and purified from transfected mammalian cells, it displays negligible kinase catalytic activity in vitro (28). It was therefore assumed that IRAK2 is an inactive “pseudokinase,” 1 of some 40 “pseudokinases” encoded by the human genome. However, subsequently, IRAK2 was reported to undergo phosphorylation when it was immunoprecipitated from the extracts of MALP2-stimulated WT macrophages and incubated with Mg-ATP. Because this did not occur when IRAK2 was immunoprecipitated from IRAK4-deficient macrophages, it was suggested that IRAK2 was catalytically active and able to undergo autophosphorylation after it had been phosphorylated by IRAK4 (14). However, because IRAK2 and IRAK4 interact via their DD the possibility that the protein kinase activity associated with IRAK2 immunoprecipitates was IRAK4, or another protein kinase whose activity was dependent on IRAK4 expression, was not excluded by these studies (14). Therefore, whether IRAK2 is catalytically active is still an unresolved issue.

The gene encoding IRAK2 can create four different splice variants, termed IRAK2a, IRAK2b, IRAK2c, and IRAK2d (29). IRAK2a and IRAK2b both possess the DD, the pseudokinase domain, and TRAF6-binding motifs and were reported to potentiate LPS-stimulated NF-κB-dependent gene expression in overexpression studies. In contrast, IRAK2c and IRAK2d lack the DD and are presumably unable to interact with IRAK4. Moreover, they inhibited NF-κB-dependent gene transcription in overexpression experiments, suggesting a potential role in the feedback control of the MyD88 signaling network (29). The IRAK2c variant was not expressed in the wild-derived mouse strain MOLF/En, which might explain why this mouse line produced higher levels of IL-6 in response to TLR agonists compared with the inbred strain C57BL/6J (30).

A full understanding of the physiological roles played by each IRAK family member in the MyD88 signaling network requires knowledge of the separate roles of each functional domain. This cannot be evaluated by studying cells from knockout mice because the observed phenotypes may arise from the loss of any or all of the functional domains. In addition, ablating the expression of one IRAK family member may affect the way in which other IRAK family members interact and signal within the Myddosome. One approach to address this complex problem is to study knock-in mice carrying mutations that inactivate a single functional domain of each protein. In this article, we investigated how the MyD88 signaling network is affected in bone marrow–derived macrophages (BMDMs) and pDCs from knock-in mice that express the catalytically inactive mutant IRAK1[D359A] instead of the WT protein and in knock-in mice that express IRAK2[E525A], a mutant that is unable to interact with TRAF6. These and other experiments have revealed that the IRAK2-TRAF6 interaction is needed to sustain IKKβ activity during prolonged TLR stimulation, and we show that this is critical for the late surge in il6 and tnfα mRNA production in BMDMs, and for IRAK2 to stimulate IFN-α production by TLR9 agonists by pDCs. In contrast, IRAK1 catalytic activity is critical for type 1 IFN production by pDCs but is not rate limiting for the production of il6 and tnfα mRNA by BMDMs.

Materials and Methods

Materials

The TLR agonists Pam3CSK4, lipoteichoic acid (LTA), R848 (also called Imiquimod), CpG type B (ODN1826), and CpG type A (ODN1585) were from Invivogen; LPS (E. coli O55:B5) was from Alexius Biochemicals, and the TLR7 agonist poly(deoxyuridylic acid) [poly(dU)] was from Sigma-Aldrich. The poly(dU) was added to the culture medium conjugated with Lipofectamine 2000 (Invitrogen) (22). The phage A phosphatase was purchased from New England Biolabs, and ubiquitin-specific protease 2 (USP2) was provided by Dr. Axel Knebel, Medical Research Council Protein Phosphorylation and Ubiquitylation Unit. Actinomycin D was purchased from Sigma. B605906 (31) was synthesized by Dr. Natalia Shpiro, Medical Research Council Protein Phosphorylation and Ubiquitylation Unit.

Abs

Abs that recognize IFN-α phosphorylated at Ser77 and Ser180 and IKKβ phosphorylated at Ser217 and Ser183, p105/NF-κB phosphorylated at Ser325, p38 MAPK phosphorylated at the TGY motif, ERK1/ERK2 phosphorylated at the TGY motif, Abs that recognize all forms of ERK1/ERK2, JNK1/2, and p38 MAPK, as well as Abs that recognize IRAK1 (clone number D31G7) and Abs that recognize all forms of STAT1 or STAT1 phosphorylated at Tyr705 were from Cell Signaling Technology. An Ab recognizing JNK phosphorylated at the TPY motif was obtained from Invitrogen, anti-TRAF6 for immunoblotting was from Santa Cruz, and anti-α-tubulin was from Sigma. A rabbit polyclonal Ab for immunoblotting of mouse IRAK2 (raised against a C-terminal peptide that is common to all four spliced variants of mouse IRAK2) was purchased from Abcam, whereas a rabbit secondary Ab conjugated to HRP was from Pierce. The HRP-conjugated anti-HA Ab was from Roche, and HRP-conjugated anti-FLAG Ab was from Sigma. Abs against human IRAK2 (sheep number 8479C, third bleed), mouse IRAK1 (sheep number S690C, third bleed), and TRAF6 (sheep number S691C, third bleed) for immunoprecipitation experiments were produced by the Ab Production Team of the Division of Signal Transduction Therapy, Medical Research Council Protein Phosphorylation Unit, University of Dundee, coordinated by Dr. James Hastie.

Generation of IRAK2[E525A] knock-in mice

To generate knock-in mice expressing the IRAK2[E525A] mutant (numbering refers to the IRAK2a variant) instead of the WT protein, we mutated the GAA codon for Glu at this position to the GCA codon for Ala. Glu525 is encoded in exon 12 of the mouse Ira2 gene, and a targeting
vector was constructed to introduce the required mutation. This vector was designed to introduce IoxP sites on either side of exon 12 in addition to mutating Glu252 to Ala. Neomycin and thymidine kinase casettes were included to allow for positive and negative selection, respectively, in the embryonic stem (ES) cells. The neomycin marker consisted of a G418 promoter, the neomycin-resistance gene open reading frame, an internal ribosome entry site sequence, an in-frame start codon, and the splice donor site from exon 12 of IRAK2. This selection cassette acts as a polyaA trap and also allows a reverse transcription–based RNA screen for homologous recombination.

The three arms of homology to the IRAK2 locus were generated by PCR for an appropriate murine BAC clone using the primers listed in Supplemental Table I. All PCR products were fully sequenced to ensure the absence of PCR-generated mutations.

The targeting vector was used to generate mutant ES cells as described previously. ES cells were transformed into embryonic stem (ES) cells and positive colonies were selected via blastocyst injection. Chimeric mice giving germline transmission were bred to C57/Bl6 Flp transgenic mice (TaconicArtemis) to excise the selection cassette. All PCR products were fully sequenced to ensure the absence of PCR-generated mutations.

Generation of IRAK1[D359A] knock-in mice

The aspartyl residue present in the “DFG” motif of most protein kinases is essential for their activity because it interacts with the magnesium ion of the Mg−ATP complex. To check that this amino acid residue is also critical for the kinase activity of IRAK1, we mutated the aspartic acid residue at position 358 of human IRAK1 to alanine. The normal human IRAK1 underwent autophosphorylation when it was overexpressed in IL-1R cells, as shown by a decrease in its electrophoretic mobility, which could be reversed by treatment with a protein phosphatase. In contrast, the human IRAK1[D358A] mutant did not undergo this band shift, and its mobility was unaffected by phosphatase treatment, demonstrating that it was indeed inactive (Supplemental Fig. 1A). The mutation of the aspartic acid residue of the “DFG” motif has also been found to not alter overall kinase conformation significantly (33). We therefore generated knock-in mice in which WT IRAK1 was replaced by the equivalent IRAK1[D359A] mutant as detailed previously (34).

Generation and culture of BMDMs

BMDMs were obtained by differentiating bone marrow obtained from the femur and tibia with M-CSF (R&D Systems) or L929 preconditioned medium as the source of M-CSF. Cells were maintained on bacterial-grade plates for 1 wk in DMEM supplemented with 10% (v/v) heat-inactivated FBS (Biosea), 2 mm l-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, and 10 ng/ml M-CSF. When L929 preconditioned medium was used to prepare BMDM, bone marrow cells were differentiated in DMEM containing 20% L929-preconditioned medium, 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mm sodium pyruvate, 0.2 mm 2-ME, and nonessential amino acids at the concentrations recommended by the manufacturer (Life Technologies). Adherent BMDMs were then replated into 100-mm tissue culture grade plates (5 × 106 cells) using fresh culture medium as the source of M-CSF. Cells were maintained on bacterial-grade plates for 1 wk in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 50 μg/ml amphotericin, and 10 ng/ml M-CSF.

Measurement of cytokine concentrations

After stimulation with ligands, the cell culture medium was removed, clarified by centrifugation for 10 min at 14,000 × g, and the levels of IL-6, IL-10, TNF-α, MIP1α, and MIP1β were measured either by end-point ELISA Development Kits from PeproTech or the Bio-Plex Pro Assay multiplex system from Bio-Rad.

Measurement of IFNs

A total of 3.5 × 105 Flt3-DCs were incubated for 1 h in 96-well plates, then stimulated with 0.05 μM CpG type B or 1 μM CpG type A. A total of 25 μg/ml poly(dU) was conjugated with LipoFectamine 2000 to stimulate Flt3-DCs. After stimulation for 12 h, the cell culture supernatants were collected, clarified by centrifugation, and frozen at −80˚C until IFN-α levels were analyzed. The concentrations of IFN-α in the cell culture supernatant was measured by ELISA using the Verikine Mouse IFNα kit (PBL, IFN Source).

Quantitative real-time PCR

After stimulation with ligands, the cell culture medium was removed and total RNA was extracted from cells using the RNeasy Micro kit (Qiagen). A total of 1 μg RNA was reverse transcribed using random and oligo(dt) primers, iScript reverse transcriptase, and the accompanying reagents (Bio-Rad or Quanta Biosciences), according to the manufacturer’s instructions. PCRs were performed using the PerfeCTa Syber Green Fast mix (Quanta Biosciences) in the BioRad iCycler (BioRad). The primers used for measuring mRNA encoding mouse il6, tnfα, and il10 mRNA (37) and ifnb, ifna4, and ifna6 (23) were described previously. Normalization and quantitation were performed using 18S RNA and the ΔΔCt method.

Expression vectors and cell transfection

Human IRAK1 (National Center for Biotechnology Information NP 001560.2) was amplified from IMAGE EST 6164719 using KOD Hot Start DNA Polymerase (Merrick) and inserted into the Not1 site of pCMVHA-2. Mouse IRAK2 (National Center for Biotechnology Information NP 751893.3) was amplified from IMAGE EST 30733777 and cloned into the BamH1 Not1 sites of pCMVFLAG-1. Mutations were created using the QuikChange method (Stratagene) but using KOD Hot Start DNA Polymerase. Human embryonic kidney 293 (HEK293) cells stably expressing the IL-1R, termed IL-1R cells and IRAK1-null IL-1R cells (a generous gift from Xiaoxia Li, Cleveland Clinic Foundation), were cultured in DMEM supplemented with 10% FBS, 2 mm glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell transfections were performed using Lipofectamine 2000 (Invitrogen) using 5 μg expression vector DNA and 15 μl transfection reagent per 10 ml cell culture medium. However, for cotransfection of TRAF6 and IRAK2, 4.5 μg HA-TRAF6 DNA and 0.5 μgFLAG-IRAK2 DNA were used.

Statistical analysis

Data were analyzed with the PRISM statistical package and, if not stated otherwise, was distributed normally and expressed as the mean ± SEM/SD.
Statistical significance was calculated using the unpaired, two-tailed Student t test.

Results
Generation of IRAK2 knock-in mice

We generated knock-in mice in which WT IRAK2 was replaced by IRAK2[E525A] as described in Materials and Methods (Fig. 1A).

The mice were born at normal Mendelian frequencies and were of normal size and weight (results not shown). The mutant protein was expressed at similar levels to WT IRAK2 in BMDMs (Fig. 1B). Both the WT and mutant forms of IRAK2 migrated as a major, more slowly migrating band of apparent molecular mass 75 kDa and a minor, more rapidly migrating component of 65 kDa. The molecular masses are consistent with the more slowly migrating

FIGURE 1. Generation of IRAK2[E525A] knock-in mice. (A) Mice were generated in which Glu525 of IRAK2 was mutated to Ala using the strategy detailed in Materials and Methods. The polyA-trapping neomycin cassette was used for positive selection, which allowed ES cell colonies to be screened by reverse transcription PCR of mRNA using the primers indicated (p1 and p2). This generated a 317-bp band in correctly targeted clones. The presence of the 5' loxP site was confirmed by PCR of genomic DNA using primers p2 and p3. Correctly targeted ES cell clones were used to generate chimeric mice, and germline transmitting chimeric mice crossed to mice containing an Flp transgene to excise the neomycin gene. Excision of this gene was confirmed by PCR using primers p5 and p6. (B) BMDM lysates (10 μg protein) from WT or IRAK2[E525A] (E/A) mice were immunoblotted with the Abs indicated. (C) IRAK1-null IL-1R HEK293 cells were cotransfected with plasmids encoding HA-tagged mouse TRAF6 (HA-TRAF6) or an empty vector (vector), and either FLAG-mouse WT IRAK2 (FL-WT) or FLAG-mouse IRAK2[E525A] (FL-E/A). After 24 h, the cells were lysed and cell extracts subjected to SDS-PAGE and immunoblotting with anti-HA or anti-FLAG to monitor the expression of TRAF6 and IRAK2 (top two panels). The FLAG-tagged IRAK2 (middle two panels) or HA-tagged TRAF6 (bottom two panels) were immunoprecipitated from 0.25 mg cell extract protein, then denatured in SDS subjected to SDS-PAGE and immunoblotted with either anti-HA or anti-FLAG Abs. (D) The experiment was carried out as in (C), except that HA-TRAF6 was omitted from all transfections. In the middle two panels, the endogenous human TRAF6 in the cells was immunoprecipitated from 0.5 mg cell extract protein, and the presence of FLAG-IRAK2 (FL-WT) or FLAG-IRAK2[E525A] (FL-E/A) in the immunoprecipitates was detected by immunoblotting with anti-FLAG. In the bottom two panels, FLAG-IRAK2[WT] (FL-WT) or FLAG-IRAK2[E525A] (FL-E/A) was immunoprecipitated, and the presence of the endogenous human TRAF6 was detected by immunoblotting. (E) Primary BMDMs were stimulated with 1.0 μg/ml R848 for the times indicated. The cells were lysed and TRAF6 immunoprecipitated from the cell extracts and treated with USP2 and phage λ phosphatase to deubiquitylate and dephosphorylate IRAK2 (see Materials and Methods). Proteins were released from the Ab-Sepharose conjugate by denaturation in SDS, and the supernatants were subjected to SDS-PAGE and immunoblotting with the Abs indicated.
band being the IRAK2a and/or IRAK2b alternatively spliced variants and the more rapidly migrating band being the IRAK2c and/or IRAK2d, variants that lack the DD (see the introduction).

Glu-525 of murine IRAK2 is equivalent to Glu-528 of human IRAK2, which lies in one of the two putative TRAF6-binding motifs of IRAK2 (Pro-X-Glu-X-A), where A is in an aromatic or acidic residue (6). The mutation of Glu-525 to Ala in human IRAK2 has been reported to prevent the activation of TRAF6 E3 ligase activity in overexpression studies (38). To investigate whether the interaction between mouse IRAK2 and mouse TRAF6 was affected, we cotransfected DNA vectors encoding tagged versions of these proteins into IRAK1-null human HEK293 cells that stably express the IL-1R, termed in this article IL-1R cells (39). We found that WT mouse IRAK2 interacted with TRAF6 as expected, but the mouse IRAK2[E525A] mutant did not (Fig. 1C). The mouse IRAK2[E525A] mutant was also unable to interact with the endogenous human TRAF6 in IRAK1-null IL-1R cells, in contrast with WT mouse IRAK2 (Fig. 1D). We further showed that, in contrast with the WT mouse IRAK2, the endogenous mouse IRAK2[E525A] mutant did not interact with the endogenous TRAF6 in extracts from R848-stimulated BMDMs from 15 min to 4 h after stimulation (Fig. 1E). Thus, IRAK2[E525A] is a mutation that prevents the interaction of IRAK2 and TRAF6.

Decreased MyD88-dependent activation of the canonical IKK complex and MAPKs after prolonged TLR stimulation in BMDMs from IRAK2[E525A] mice

It is well documented that the stimulation of macrophages with TLR agonists is followed by a strong activation of several MAPK cascades and the canonical IKK complex within minutes, but activation is transient and returns to much lower levels after 30–60 min of stimulation. These low levels of activation are maintained for a couple of hours before beginning to rise again. In this study, we monitored the activation of IKKα, IKKβ, and MAPKs for a prolonged period using Abs that recognize amino acid residues whose phosphorylation is required for the activation of these protein kinases. We observed that the activation of the MAPKs JNK, p38 MAPK, and ERK1/ERK2 induced by R848, a TLR7 agonist, or Pam3CSK4, an activator of the TLR1/2 heterodimer, were similar in BMDMs from IRAK2[E525A] and WT mice, for up to 1 h after stimulation, but the activation of IKKα/β and the phosphorylation of the IKKβ substrate p105 (11, 40) were partially reduced in BMDMs from the IRAK2[E525A] mice (Fig. 2A, 2B). The activation of IKKβ and the phosphorylation of p105 was virtually abolished in BMDMs from the IRAK2[E525A] knock-in mice after stimulation for 2–4 h (highlighted by the arrows in Fig. 2A, 2B), and the activation of MAPKs was also partially reduced at these later times.

Phosphorylation, ubiquitylation, and expression of IRAK1 and IRAK2

To investigate why IRAK2 became rate limiting for the activation of IKKβ, after prolonged stimulation, we studied the covalent modification and expression of IRAK1 and IRAK2 after stimulation with R848. The unmodified form of IRAK1 largely disappeared from the cell extracts when BMDM from WT mice was stimulated for 15 min with R848, but could be recovered by incubation with a combination of the protein phosphatase from phage λ and the deubiquitylase USP2 (Fig. 2C). This demonstrated that the disappearance of unmodified IRAK1 had not resulted from its degradation, but from conversion to a variety of more slowly migrating phosphorylated and ubiquitylated species. However, after stimulation with R848 for 2 h, the unmodified form of IRAK1 could be recovered only partially by phosphatase/deubiquitylase treatment, and expression was greatly reduced after 4 h of stimulation. These experiments indicated that IRAK1 undergoes degradation after stimulation with R848 for >2 h.

Like IRAK1, IRAK2 also became extensively modified by phosphorylation and ubiquitylation in response to R848 (Fig. 2D), which was similar in BMDMs from WT mice or IRAK2[E525A] knock-in mice (Fig. 2E). The broad “smear” of slowly migrating species was abolished by deubiquitylase treatment, whereas the most prominent slowly migrating form of IRAK2 was recovered to the unmodified form by phosphatase treatment (Fig. 2F). IRAK2 was fully recovered to the unmodified form at all time points by treatment with deubiquitylase plus phosphatase. The expression of IRAK2 did not decrease, but started to increase after 3–4 h (Fig. 2D). IRAK1 and IRAK2 may therefore function redundantly in the activation of TRAF6 initially, the IRAK2–TRAF6 activation becoming critical only for the activation of the canonical IKK complex when IRAK1 is degraded or inactivated, or both.

Decreased production of il6 and tnfα mRNA via the MyD88 signaling pathway after prolonged TLR stimulation in BMDMs from IRAK2[E525A] mice

To investigate the consequence of the loss of IKK activation after prolonged stimulation, we followed the time course of production of the mRNA encoding il6 and tnfα. The production of these mRNAs was similar in BMDMs from IRAK2[E525A] mice and WT mice for up to 2 h after stimulation with R848 and partially reduced after stimulation with Pam3CSK4 (Fig. 3A, 3B). However, the further 100- to 1000-fold increase in il6 mRNA and more modest increase in tnfα mRNA observed between 3 and 8 h after stimulation of WT BMDMs was greatly reduced in BMDMs from IRAK2[E525A] mice (Fig. 3A, 3B, note that the ordinate for il6 mRNA production is plotted on a log scale). Consequently, there was virtually no secretion of IL-6 or TNF-α into the cell culture medium after stimulation with R848 or Pam3CSK4, or with the TLR9 agonist ODN1826 (hereafter called CpG-B) or the TLR2,6 agonist LTA (Fig. 3C). The secretion of MIP1α and MIP1β (Fig. 3C), as well as IL-12p40 (results not shown), was also virtually abolished.

To investigate whether the IRAK2–TRAF6 interaction was required for the stimulation of production of il6 and tnfα mRNA, WT mice could account for these results, we added BI605906, an exquisitely specific inhibitor of IKKβ (31), to the cell culture medium 2 h after stimulation with R848 and then monitored subsequent production of il6 and tnfα mRNA over the next few hours. These experiments demonstrated that BI605906 suppressed the R848-stimulated production of il6 and tnfα mRNA (Fig. 4A) and the secretion of these cytokines (Fig. 4B) in BMDMs from WT mice, similar to the observations made in BMDMs from the IRAK2[E525A] mice in the absence of BI605906 (Fig. 3C). Thus, the failure to sustain the activation of IKKβ in BMDMs from the IRAK2[E525A] mice can explain why significant amounts of IL-6 and TNF-α were not produced.

To investigate whether the IRAK2–TRAF6 interaction was required to stimulate gene transcription or enhance mRNA stability, we added actinomycin D to the cell culture medium 90 min after stimulation to inhibit further transcription. The production of il6 and tnfα mRNA over the next few hours was dramatically reduced (Fig. 3C), as well as the rate at which these proinflammatory cytokine mRNAs declined was similar in BMDMs from WT mice or IRAK2[E525A] knock-in mice (Fig. 3C, 4A, 4B), and the secretion of these cytokines (Fig. 4B) in BMDMs from WT mice, similar to the observations made in BMDMs from the IRAK2[E525A] mice in the absence of BI605906 (Fig. 3C). Thus, the failure to sustain the activation of IKKβ in BMDMs from the IRAK2[E525A] mice can explain why significant amounts of IL-6 and TNF-α were not produced.

Effect of IRAK2 on the production of some anti-inflammatory molecules

The MyD88 signaling network induces not only the production of proinflammatory cytokines, but also molecules that restrict the
MyD88 signaling network to prevent the overproduction of inflammatory mediators, such as A20 and Dual Specificity Phosphatase 1 (DUSP1), and anti-inflammatory cytokines, such as IL-10. A20 is the product of the \textit{tnfaip3} gene, which is dependent on NF-κB (41), and its synthesis therefore requires the activation of the canonical IKK complex, whereas the induction of DUSP1 is dependent on the activation of mitogen and stress-activated protein kinases 1 and 2 (MSK1/MSK2), which are themselves activated by \textit{p38} MAPK and \textit{ERK1/ERK2} (32, 42).

The R848-stimulated induction of the \textit{tnfaip3} mRNA in BMDMs was maximal after an hour, and was similar in BMDMs from WT mice and IRAK2[E525A] mice (Fig. 5A, right panel). The level of \textit{tnfaip3} mRNA then declined to half the maximal level, which was sustained from 2 and 8 h in WT BMDMs. In
contrast, the level of *tnfαp3* mRNA declined drastically over this period in BMDMs from IRAK2[E525A] mice (Fig. 5A, right panel), consistent with the essential role of the IRAK2–TRAF6 interaction in sustaining IKKβ activity during prolonged activation of the MyD88 signaling network. The R848-stimulated induction of *dusp1* mRNA in BMDMs also peaked after an hour and was similar in BMDMs from WT or IRAK2[E525A] mice (Fig. 5A, left panel). This is consistent with similar activation of p38α MAPK and ERK1/ERK2 in BMDMs from the WT and mutant mice during the first hour of R848 stimulation. The *dusp1* mRNA levels declined rapidly after 1 h, and the decline was even more marked in BMDMs from the IRAK2[E525A] mice than WT mice (Fig. 5A, left panel), consistent with reduced MAPK activation after prolonged stimulation with R848 (Fig. 2A).

MSK1/MSK2 are not only required for the transcription of the gene encoding DUSP1, but are also important for the transcription of the gene encoding IL-10. MSK1/MSK2 stimulate transcription of the *il10* gene by phosphorylating and activating the transcription factor CREB (32, 42), explaining why, similar to *dusp1* mRNA, the R848 or Pam3CSK4-stimulated production of *il10* mRNA peaked after 1 h and then declined over the next few hours (Fig. 5B). However, the level of *il10* mRNA increased again from 6 h onward, which may reflect the time at which CRTCC3, a key coactivator of CREB in BMDMs, undergoes dephosphorylation and activation because of inactivation of the protein kinase SIK2 by autocrine factors, such as PGE2 (43). We found that the early phase of *il10* mRNA production was partially reduced in BMDMs from IRAK2[E525A] mice, but the late phase from 6–8 h was not and was even enhanced in Pam3CSK4-stimulated macrophages (Fig. 5B). Taken together, these findings can explain why the IL-10 secreted into the culture medium was reduced only modesty in BMDMs from IRAK2[E525A] mice when measured after prolonged stimulation with TLR agonists (Fig. 5C).

The TRIF signaling pathway is used instead of IRAK2 to sustain the late phase of *il6* and *tnfα* mRNA production by the TLR4 agonist LPS

The LPS-stimulated activation of IKKβ and MAPKs (Fig. 6A) was similar at all time points in BMDMs from IRAK2[E525A] and WT mice. Moreover, the late, as well as the early, phase of LPS-stimulated *il6*, *tnfα*, and *il10* mRNA production (Fig. 6B) and the secretion of these molecules (Fig. 3C) was reduced only modestly in BMDMs from IRAK2[E525A] mice.

In contrast with other TLRs, which signal solely via MyD88, TLR4 signals via TRIF and MyD88, and signaling via both adaptors is required for significant amounts of proinflammatory cytokines to be secreted into the culture medium (2, 44). We therefore investigated whether the requirement for the IRAK2–TRAF6 interaction was being replaced by the TRIF signaling pathway in LPS-stimulated macrophages. We found that the activation of IKKβ (as judged by p105 phosphorylation) and MAPKs (Fig. 6C), as well as proinflammatory cytokine mRNA production up to 2 h (Fig. 6D), was similar in BMDMs from TRIF−/− and WT mice. In contrast, the late phase of proinflammatory cytokine mRNA production (Fig. 6D), and hence cytokine secretion (Fig. 6E), was drastically reduced in BMDMs from the TRIF−/− mice. Thus, the LPS-stimulated production of proinflammatory cytokines in BMDMs from TRIF−/− mice was strikingly similar to the situation seen in BMDMs from IRAK2[E525A] mice after stimulation with R848 or Pam3CSK4 (Fig. 3).

The LPS-stimulated production of *il10* mRNA in BMDMs from WT and TRIF−/− mice was similar to that observed after stimulation with R848 or Pam3CSK4, with a peak at 1 h, followed by a decline up to 4 h and an increase after 6 h (Fig. 6D, bottom panel). The decline after 4 h was more marked in BMDMs from TRIF−/− mice similar to the observations made in BMDMs from IRAK2[D525A] mice (Fig. 5C). The total amount of IL-10 secreted into the cell culture medium was critically dependent on the times at which this was measured, being decreased after 8 h (Fig. 6E) but enhanced after 24 h because of the continued increase in *il10* mRNA between 8 and 24 h (results not shown). This might be explained by the inactivation of SIK2 by autocrine factors (43), as discussed earlier.

**IRAK1 catalytic activity is not required for cytokine production in BMDMs**

To investigate the role of IRAK1 catalytic activity in the production of inflammatory mediators, we used BMDMs from knock-in mice...
cells, we crossed the IRAK2[E525A] mice with the knock-in mice that express the catalytically inactive IRAK1[D359A] mutant and studied IFN production in double knock-in mice, as well as the single knock-in mice.

We found that the production of ifnb, ifna4, and ifnα6 mRNA induced by the TLR9 agonist CpG-B (Fig. 7A) was virtually abolished in pDCs from the IRAK2[E525A] × IRAK1[D359A] double knock-in mice, and consistent with these findings, the secretion of IFN-α induced by CpG-B, CpG-A, and the TLR7 agonist poly(dU) was barely detectable (Fig. 8A). In contrast, the CpG-B–stimulated production of ifnb mRNA was only reduced slightly in pDCs from the IRAK2[E525A] mice, whereas the production of ifna4 was partially reduced and the production of ifnα6 mRNA severely reduced (Fig. 7B). Consistent with these findings, there was a partial reduction in the CpG-B, CpG-A, and poly(dU)–stimulated secretion of IFN-α (Fig. 8B). In pDCs from the IRAK1[D359A] mice, the CpG-B–stimulated production of ifnb, ifna4, and ifnα6 mRNA was greatly delayed (Fig. 7C) and IFN-α secretion measured after 12 h was reduced (Fig. 8C).

IRAK1 and IRAK2 are required to activate IKKβ in pDCs

We have reported previously that IKKβ activity plays an essential role in the production of IFN-β in the human pDC line Gen2.2, as well as in Flt3-derived DCs, and that IFN-β, as well as IKKβ activity, is required for the production of IFN-α by these cells, as judged by both siRNA “knock-down” experiments and studies with the specific IKKβ inhibitor BI605906 (23). We therefore investigated the activation of IKKβ in pDCs from IRAK1[D359A], IRAK2[E525A], and the IRAK1[D359A] × IRAK2[E525A] double knock-in mice by monitoring IKKβ phosphorylation at Ser177 and Ser181, the amino acid residues whose phosphorylation is required for activation. We found that the activation of IKKβ for the first 3 h after stimulation with CpG-B was unaffected in pDCs from IRAK2[E525A] mice (Fig. 8D), delayed considerably in pDCs from IRAK1[D359A] mice (Fig. 8E), and abolished in pDCs from IRAK2[E525A] × IRAK1[D359A] mice (Fig. 8F), correlating with the observed effects on IFN-β secretion.

The first traces of IFN-β secreted activate the type 1 IFNR, stimulating the activation of members of the Janus family of protein kinases (JAKs), which then phosphorylate and activate STAT1 and STAT2, enabling these transcription factors to stimulate the transcription of IFN-stimulated genes, including the different species of IFN-α. We found that the CpG-B–stimulated phosphorylation of STAT1 at Tyr701 was delayed in pDCs from IRAK1[D359A] mice (Fig. 9), consistent with our previous report that IKKβ activation is required for the production of IFN-β in pDCs and that IKKβ and IFNβ are both required for the production of IFN-α (Fig. 9) (23).

We also observed that the expression of STAT1 was reduced significantly in pDCs from the IRAK1[D359A] and IRAK2 [E525A] knock-in mice (Fig. 8F–H). This may contribute to the reduced production of phospho-STAT1 and type 1 IFNs, but cannot entirely explain this finding because STAT1 phosphorylation at Tyr701 and type 1 IFN production was abolished in pDCs from the IRAK1[D359A] × IRAK2[E525A] double knock-in mice, even though the expression of STAT1 was not reduced any further than in pDCs from the single knock-in mice.

Discussion

In this study, we generated knock-in mice that express the IRAK2 [E525A] mutant that is unable to interact with TRAF6 (Fig. 1C–E) and investigated how the MyD88 signaling network was affected...
in BMDMs from these animals. The results allowed the MyD88 signaling network to be divided into two phases: an initial phase lasting 1–2 h during which the IRAK2–TRAF6 interaction was not rate limiting, and which was characterized by strong, but transient, activation of the canonical IKK complex and MAPKs; and a second phase from 2–8 h during which the IRAK2–TRAF6 interaction plays a critical role in sustaining a low level of activation of the IKK complex (Fig. 9A). Although the production of mRNAs encoding proinflammatory cytokines, such as IL-6 and TNF-α, is initiated during the first 1–2 h after stimulation, a key role for the initial phase is to rapidly recruit or induce molecules such as A20, ABIN1, DUSP1, and IL-10 that restrict the activation of the MyD88 signaling network (45–47) and its binding to Lys 63-linked and linear polyubiquitin chains that are unique in signaling via TRIF, as well as via MyD88. The IRAK2 interaction is not rate limiting during the first phase because it operates redundantly with IRAK1 in the activation of TRAF6 during this period. We showed that the IRAK2–TRAF6 interaction was critical during the second phase to sustain a low level of IKKβ (Fig. 2A, 2B) without which the surge in il6 mRNA levels failed to occur and ifna mRNA levels could not be sustained (Fig. 3A, 3B). These results explain why the secretion of IL-6, TNF-α, and other proinflammatory cytokines was virtually abolished in BMDMs from the IRAK2[E525A] mice (Fig. 3). The activation of JNKs, p38 MAPKs, and ERK1/2 during the second phase was also reduced in BMDMs from IRAK2[E525A] mice (Fig. 2A, 2B), and the IRAK2–TRAF6 interaction is likely to have additional roles during the second phase that have yet to be identified. Importantly, loss of the IRAK2–TRAF6 interaction had relatively little effect on the production of the anti-inflammatory molecule DUSP1, which was produced during the first phase when the IRAK2–TRAF6 interaction failed to occur and its production was virtually abolished, whereas overall IL-10 secretion was much less affected in BMDMs from the IRAK2[E525A] mice. The finding the IL-6 and TNF-α secretion was virtually abolished, whereas overall IL-10 secretion was much less affected in BMDMs from the IRAK2[E525A] mice raises the possibility that IRAK2 could be an interesting target for the development of an anti-inflammatory drug.

A striking finding made during this study was that the LPS-stimulated production of il6 and ifna mRNA (Fig. 6B) and the secretion of these cytokines (Fig. 3C) were reduced only modestly in BMDMs from IRAK2[E525A] mice. LPS activates TLR4, which is unique in signaling via TRIF, as well as via MyD88. The
TRIF-dependent signaling pathway does not appear to require members of the IRAK family (14, 51), but instead signals via RIP1 (52), explaining why the LPS-stimulated production of il6 and tnfα mRNA (Fig. 6B) was little affected in BMDMs from IRAK2[E525A] mice. However, interestingly, the second but not the first phase of il6 and tnfα mRNA production induced by LPS was greatly reduced in BMDMs from TRIF−/− mice (Fig. 6D), mimicking the situation seen when BMDMs from the IRAK2[E525A] mice were stimulated with TLR agonists that signal only via MyD88 (Fig. 3A–C). Taken together, our findings suggest that
The TRIF-dependent signaling network is used instead of IRAK2-TRAF6 to sustain the LPS-stimulated *il6* and *tnfa* mRNA production during the second phase (Fig. 9B). IRAK2 and the TRIF-dependent pathway clearly do not function redundantly during this stage; otherwise, IRAK2 should have been able to compensate for the loss of TRIF in BMDMs from TRIF<sup>−/−</sup> mice, which was not the case. The translocation of TLR4 to the endosomes after prolonged stimulation with LPS, from where it can signal only via TRIF during the second phase (Figs. 7B, 8B). The production of IFN-*α* was abolished in pDCs from mice lacking both a catalytically inactive IRAK1 and a functionally inactive IRAK2 (Figs. 7A, 8A).

We have reported previously that IKKβ activity is essential for *ifnb* mRNA production in response to TLR7 and TLR9 agonists in both mouse pDCs and the human Gen2.2 pDC line (23), and that IFN-β and IKKβ were both required for the subsequent production of IFN-α. In the present study, we again found a striking correlation between the degree of activation of IKKβ and the
production of $\text{ifn}_b$ mRNA in CpG-B–stimulated pDCs. Thus, the activation of IKK$\beta$ and $\text{ifn}_b$ mRNA production were delayed in pDCs from IRAK1[D359A] mice, little unaffected in IRAK2[E525A] mice and abolished in pDCs from IRAK2[E525A] mice (Figs. 7, 8). The molecular mechanism by which IKK$\beta$ stimulates IFN production has yet to be defined but is at least partially independent of NF-κB (23, 24). In summary, a key role for the catalytic activity of IRAK1 and the IRAK2–TRAF6 interaction in pDCs is to activate IKK$\beta$, although other essential roles for these IRAKs in IFN production is not excluded.

FIGURE 8. Inhibition of IFN production in IRAK2[E525A] × IRAK1[D359A] mice correlates with impaired activation of IKK$\beta$. (A) pDCs from WT or IRAK2[E525A] × IRAK1[D359A] mice were not stimulated (NS) or stimulated for 12 h with 0.05 μM CpG-B, 1 μM CpG-A, or 25 μg/ml poly(dU) and the concentration of IFN-α in the cell culture medium measured by ELISA. (B) As in (A), but pDCs from WT or IRAK2[E525A] mice were compared. (C) As in (B), except WT pDCs were compared with pDCs from IRAK1[D359A] mice. Experiments were performed in 96-well plates, with three wells being used for each condition, each containing pDCs from a different mouse. Bars represent the mean ± SEM of one representative experiment. (A–C) *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. Similar results were obtained in two to three independent experiments. (D) pDCs from WT or IRAK2[E525A] mice were stimulated for the times indicated with 0.05 μM CpG-B and cell lysates subjected to SDS-PAGE followed by immunoblotting with the Abs indicated. The blot shown is representative of three independent experiments. (E) As in (D), except pDCs from WT or IRAK1[D359A] were compared. (F) As in (E), but pDCs from WT and IRAK2[E525A] × IRAK1[D359A] mice were compared. The blots shown are representative of two to three independent experiments.

FIGURE 9. Two phases of inflammatory mediator production defined by the study of IRAK2 and IRAK1 knock-in mice. (A) Inflammatory mediator production in BMDMs by TLR agonists that signal via MyD88. (B) Inflammatory mediator production in BMDMs by the TLR4 agonist LPS that signals via both MyD88 and TRIF. (C) Type 1 IFN production in pDCs by ligands that activate TLR7 and TLR9.
For example, IKK\(\alpha\) is also known to play an essential role in IFN production by pDCs (21). In summary, a key role of IRAK2 in both proinflammatory cytokine production in BMDMs and type 1 IFN production in pDCs is to sustain the activation of key signaling components, such as IKK\(\alpha\) and IKK\(\beta\), after prolonged activation of TLRs that signal via MyD88.

To our knowledge, our study provides the first genetic evidence that the catalytic activity of IRAK1 is critical for \(\text{IL-1R}}\alpha\text{-mRNA production and IFN secretion by pDCs, although a requirement for IRAK1 catalytic activity to produce IFN was suggested previously from the observation that the overexpression of a catalytically inactive mutant of IRAK1 blocked transcription from an IFN-\(\alpha\)-reporter gene induced by the cotransfection of MyD88 and IFN regulatory factor 7 into HEK293 cells (25). Because IRAK1 catalytic activity is critical for type 1 IFN production by pDCs, but is not required for cytokine production by macrophages, specific inhibitors of IRAK1 merit evaluation for the treatment of autoimmune diseases that have been linked to the overproduction of type 1 IFNs by pDCs (20).

In contrast with this study in which we have shown IRAK2 to be a positive regulator of IFN-\(\alpha\)-production in pDCs, other investigators reported that IFN-\(\alpha\)-production was enhanced in pDCs from IRAK2 knockout mice after stimulation with a TLR9 agonist or during viral infection, and concluded that IRAK2 was a negative regulator of IFN production (26). In pDCs from IRAK2 knockout mice, IFN production is presumably driven solely by the MyD88-IRAK4-IRAK1 oligomeric complex, which may stimulate IFN production more strongly than the MyD88-IRAK4-IRAK1\(\alpha\)-module, and thus enhance IFN production in pDCs from IRAK2 knockout mice. However, our results clearly show that IRAK2 is not a negative regulator but a positive regulator of IFN-\(\alpha\)-production in WT pDCs. Our findings highlight a potential difficulty in interpreting results obtained by studies on cells from knockout mice in which every functional domain of a complex multidomain protein is deleted, and demonstrate the potential power of studying knockout mice in which just a single functional domain of a protein is disabled to gain a deeper insight into the operation of innate immune signaling networks.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

9. Acknowledgments

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Corrections


In the Abstract, the word “network” was omitted from the last sentence. It should read: “Our results establish that the IRAK2–TRAF6 interaction is rate limiting for the late, but not the early phase of cytokine production in BMDM and pDCs, and that the IRAK2–TRAF6 interaction is needed to sustain IkB-inducing kinase β activity during prolonged activation of the MyD88 signaling network.”

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