

IRAK4 kinase activity controls Toll-like receptor–induced inflammation through the transcription factor IRF5 in primary human monocytes

Received for publication, May 15, 2017, and in revised form, August 22, 2017. Published, Papers in Press, September 18, 2017. DOI 10.1074/jbc.M117.796912

 Leah Cushing^{†1,2},  Aaron Winkler^{†1},  Scott A. Jelinsky[†], Katherine Lee[§], Wouter Korver^{¶3}, Rachael Hawtin^{¶4},  Vikram R. Rao[†],  Margaret Fleming^{‡5}, and  Lih-Ling Lin^{‡6}

From the Departments of [†]Inflammation and Immunology and [§]Medicinal Chemistry, Pfizer Inc., Cambridge, Massachusetts 02139 and [¶]Nodality Inc., South San Francisco, California 94080

Edited by Luke O'Neill

Interleukin-1 receptor–associated kinase 4 (IRAK4) plays a critical role in innate immune signaling by Toll-like receptors (TLRs), and loss of IRAK4 activity in mice and humans increases susceptibility to bacterial infections and causes defects in TLR and IL1 ligand sensing. However, the mechanism by which IRAK4 activity regulates the production of downstream inflammatory cytokines is unclear. Using transcriptomic and biochemical analyses of human monocytes treated with a highly potent and selective inhibitor of IRAK4, we show that IRAK4 kinase activity controls the activation of interferon regulatory factor 5 (IRF5), a transcription factor implicated in the pathogenesis of multiple autoimmune diseases. Following TLR7/8 stimulation by its agonist R848, chemical inhibition of IRAK4 abolished IRF5 translocation to the nucleus and thus prevented IRF5 binding to and activation of the promoters of inflammatory cytokines in human monocytes. We also found that IKK β , an upstream IRF5 activator, is phosphorylated in response to the agonist-induced TLR signaling. Of note, IRAK4 inhibition blocked IKK β phosphorylation but did not block the nuclear translocation of NF κ B, which was surprising, given the canonical role of IKK β in phosphorylating I κ B to allow NF κ B activation. Moreover, pharmacological inhibition of either IKK β or the serine/threonine protein kinase TAK1 in monocytes blocked TLR-induced cytokine production and IRF5 translocation to the nucleus, but not nuclear translocation of NF κ B. Taken together, our data suggest a mechanism by which IRAK4 activity regulates TAK1 and IKK β activation, leading to the nuclear translocation of IRF5 and induction of inflammatory cytokines in human monocytes.

Interleukin-1 receptor–associated kinase 4 (IRAK4)⁷ is a critical component of the Toll-like receptor/interleukin-1 receptor (TLR/IL1R) signaling pathway that plays a major role in innate immune responses and inflammation. Ablation of IRAK4 expression in mouse and in humans causes susceptibility to bacterial infections and defects in the ability to sense TLR and IL1 ligands (1, 2). Mice expressing kinase-inactive IRAK4 are resistant to multiple models of autoimmune and inflammatory disease and inhibitors of IRAK4 are currently under investigation in the clinic as therapeutics for multiple autoimmune and inflammatory disease as well as cancers (3). TLRs recognize and bind bacterial, viral, and fungal pathogen ligands, and IL1R recognizes the inflammatory IL1 family cytokines. Both TLRs and the IL1 family of receptors have Toll/IL1 receptor domains that bind the adaptor protein MyD88, which then associates with IRAK4 and IRAK1/2 via death domain interactions. The IRAKs then activate the ubiquitin E3 ligase TRAF6. Activation of the pathway induces strong inflammatory responses through nuclear factor kappa B (NF κ B), interferon regulatory factor (IRF), and activator protein-1 (AP-1) responsive genes (4). However, work by multiple investigators has shown that inactivation of IRAK4 kinase activity has minimal effects on the activation of the canonical inflammatory pathways of NF κ B and the MAP kinases in mice and human cells (5–7). These data lead to the question of how IRAK4 kinase activity controls pro-inflammatory cytokine production. Studies in murine cells suggest that IRAK4 kinase activity may control the stability of cytokine mRNA (6) but the impact of IRAK4 kinase activity on transcription factors other than NF κ B or AP-1 has not been examined.

Interferon regulatory factor family members regulate defenses against pathogens via their DNA-binding domain, which recognizes interferon-stimulated response elements (ISRE) found on many genes that encode type I interferons as well as pro-inflammatory cytokines and chemokines. Overexpression of interferon-induced genes is a characteristic of multiple autoimmune disorders, such as systemic lupus erythematosus (SLE) (8), psoriasis (9), dermatomyositis (10), and rheumatoid arthritis (RA) (11). The transcription factor IRF5

W. K. and R. H. were employees of Nodality, contracted by Pfizer to perform SCNP studies with the IRAK4 inhibitor. All other authors were Pfizer employees.

This article contains supplemental Figs. 1 and 2 and supplemental Table 1. Data are available in the Gene Expression Omnibus (GEO) database under accession no. GSE86234.

[†] Both authors contributed equally to this work.

² Present address: EMD Serono, Inc., 45 Middlesex Turnpike, Billerica, MA 01821.

³ Present address: Allakos Inc., 75 Shoreway Rd., Suite A. San Carlos, CA 94070.

⁴ Present address: Gilead Sciences, 333 Lakeside Dr., Foster City, CA 94404.

⁵ Present address: Novartis Institutes for Biomedical Research, 250 Massachusetts Ave., Cambridge, MA 02139.

⁶ To whom correspondence should be addressed: 1 Portland St., Cambridge, MA 02139. Tel.: 617-674-7304; E-mail: lih-ling.lin@pfizer.com.

⁷ The abbreviations used are: IRAK4, interleukin-1 receptor–associated kinase 4; IRF, interferon regulatory factor; TLR, Toll-like receptor; IL1R, interleukin-1 receptor; RA, rheumatoid arthritis; hPBMC, human peripheral blood mononuclear cells; SCNP, single cell network profiling.

This is an open access article under the CC BY license.

has been implicated in TLR/IL1R signaling and is activated in a MyD88-dependent manner to induce cytokines and interferon (12). In particular, IRF5 is known to be a critical mediator of TLR7 signaling that requires not only MyD88, but also IRAK1/4 and TRAF6 in this pathway. Activation requires phosphorylation, dimerization, nuclear translocation, and binding to promoters with IRF binding elements (13). IRF5 is known to cooperate with the NF κ B subunit RelA to promote transcription of pro-inflammatory cytokines in myeloid cells (14). Several groups have recently provided evidence in human myeloid cells that phosphorylation of IRF5 by IKK β results in IRF5 activation and nuclear translocation following TLR ligation (15, 16) and that this process involved TAK1 (17).

In humans, *IRF5* polymorphisms are associated with autoimmune and inflammatory conditions such as inflammatory bowel disease (18), RA (19), and systemic lupus erythematosus (20). Furthermore, it has been shown that *IRF5*^{-/-} mice are protected from development of lupus in the *Fcgr2b*^{-/-} Yaa, *Fcgr2b*^{-/-} (21), and pristane-induced (22) models of the disease. In addition, mice deficient in *IRF5* have decreased pro-inflammatory cytokines, such as IL6 and TNF, in their serum. They are therefore more susceptible to viral infections. After stimulation with unmethylated DNA and lipopolysaccharide, IRF5-deficient mice are more resistant to lethal shock. However, activation of NF κ B is comparable to wild type in these mice (23). This is similar to what is seen in immune cells from IRAK4 kinase-inactive knock-in mice. Given the importance of IRF5 in regulating TLR-mediated cytokine and type I interferon production as described above, we set out to investigate the role of IRAK4 kinase in regulating IRF5 activation. Our data, generated using a highly potent and selective IRAK4 inhibitor (24), led us to propose that activation of the transcription factor IRF5 via TAK1 and IKK β is the central mechanism by which IRAK4 kinase inhibitors mediate their anti-inflammatory effects. Thus we uncovered a novel role of IRAK4 in activation of the TAK1-IKK β -IRF5 axis that leads to the induction of cytokines and interferons following TLR7/8 stimulation.

Results

IRAK4 kinase is essential for inflammatory mRNA and cytokine production

To determine the effect of IRAK4 kinase inhibition on TLR-induced cytokines we employed a recently developed potent and selective IRAK4 inhibitor PF-06426779 (compound 38 in Ref. 24). This compound has an IC₅₀ of 1 nM against full-length IRAK4 kinase, a cell-based IC₅₀ of 12 nM, and excellent selectivity against the human kinome and is >100 \times selective for IRAK4 over IRAK1 (24). PF-06426779 was assessed in a KiNativTM panel (ActivX Biosciences Inc.) in hPBMC cell lysates at 10, 1, and 0.1 μ M. Estimated IC₅₀s were more than 1 μ M for all kinases assayed except for IRAK4 (0.01–0.026 μ M) and IRAK3 (0.73 μ M) (supplemental Table 1).

Primary human monocytes from healthy donors were pre-treated with 1 μ M compound for 30 min and then stimulated with the TLR7/8 ligand R848. Robust induction of IL1, IL6, and TNF cytokine protein production occurred with R848 treatment alone, whereas R848-induced IL1, IL6, and TNF cytokine

Human Monocytes

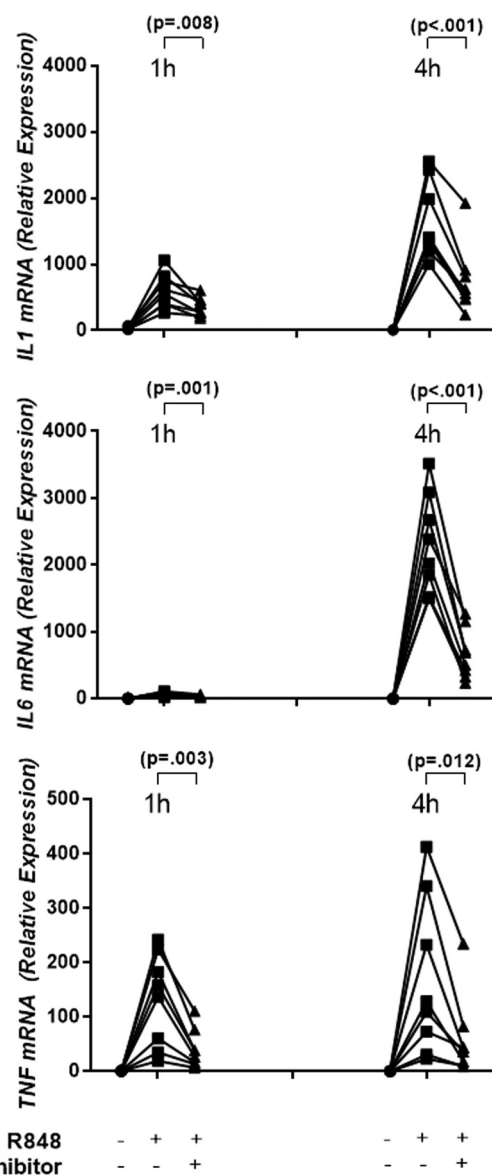


Figure 1. IRAK4 kinase is essential for inflammatory cytokine mRNA expression. Primary human monocytes were stimulated with R848 in the presence and/or absence of the IRAK4 kinase inhibitor. *IL1*, *IL6*, and *TNF* mRNA expression was measured 1 and 4 h after R848 stimulation ($n = 8$ donors).

production was significantly reduced by the inhibitor (5). Furthermore, mRNA levels of *IL1*, *IL6*, and *TNF* were induced by R848 by at least 10-fold at 1 h and by at least 100-fold at 4 h in all donors (Fig. 1). IRAK4 kinase inhibition reduced R848-induced *IL1* mRNA levels by an average of 1.7-fold at 1 h ($p = 0.008$) and 2.6-fold at 4 h ($p < 0.001$); *IL6* mRNA by 2.3-fold at 1 h ($p = 0.001$) and 4.2-fold at 4 h ($p < 0.001$); and *TNF* mRNA by 3.5- and 3.6-fold at 1 h ($p = 0.003$) and 4 h ($p = 0.012$) ($n = 8$ donors, Student's t test, Fig. 1), respectively. We had previously determined that despite the ability of IRAK inhibitors to decrease cytokine production, there are minimal effects on the activation status of the NF κ B pathway (5). Because we observed here that regulation of cytokine production occurred at the mRNA level,

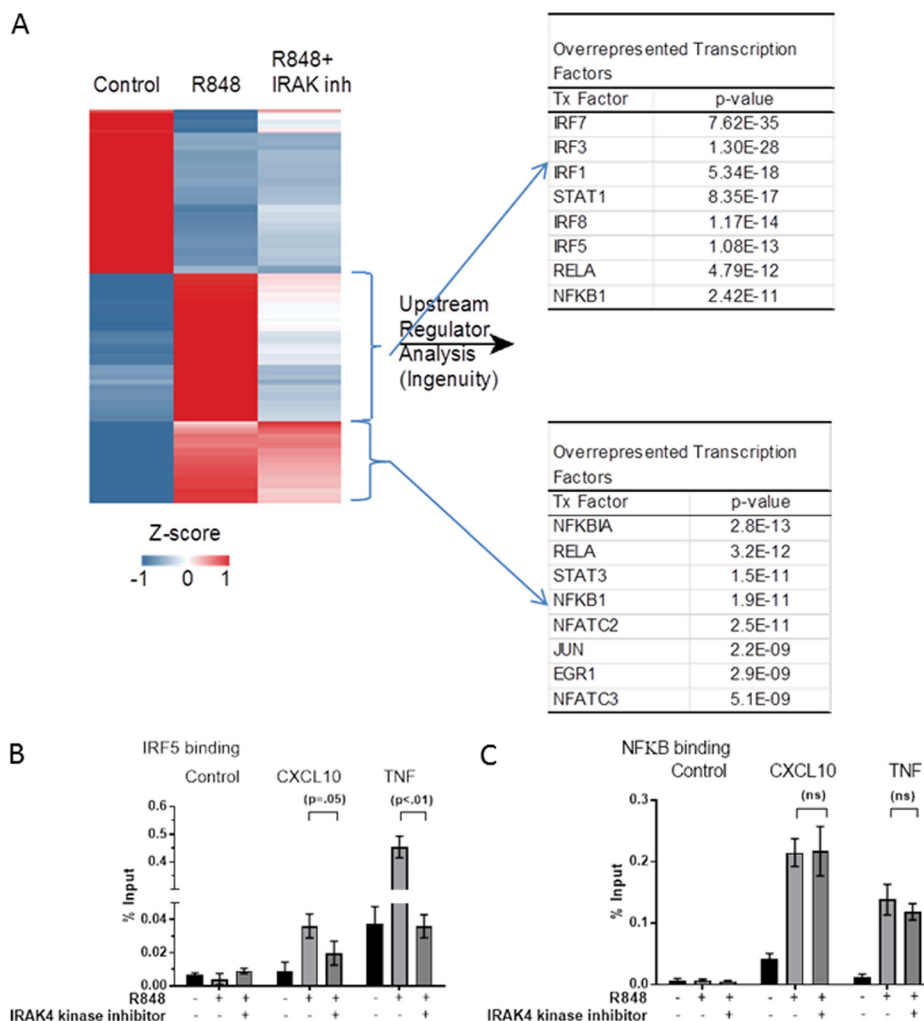


Figure 2. IRAK4 kinase inhibition results in differential regulation of genes in monocytes. A, 860 probes that showed a significant difference in expression ($n = 8$ donors, Welch's p value < 0.01 , -fold difference > 4.0) following treatment with R848 for 1 h were visualized in TIBCO Spotfire 3.3.2. Each row was z-score normalized and then subjected to hierarchical clustering. The middle bracketed cluster represents 203 genes that were down-regulated following treatment with IRAK4, the lower bracketed cluster represents 185 genes that remained elevated despite IRAK4 inhibitor treatment. Ingenuity Pathway Analysis was used to identify the transcriptional regulators significantly associated with these two clusters of genes and the top are presented here. B and C, ChIP-qPCR demonstrates that IRAK4 kinase controls IRF5 binding to promoters of target genes but not NF κ B. Human monocytes were pretreated with DMSO or IRAK4 kinase inhibitor for 30 min followed by treatment with 1 μ g/ml R848 or untreated for 30 min. Increased binding of both IRF5 (B) and NF κ B (C) were seen upon R848 treatment as compared with untreated conditions. Binding of IRF5 to promoters of target genes CXCL10 and TNF α was reduced with IRAK4 kinase inhibition (B), whereas no reduction of NF κ B binding was observed with IRAK4 kinase inhibition (C). Error bars are standard deviation from the mean.

we chose to query the mechanism of cytokine inhibition through genome-wide transcriptome analysis.

Transcriptional analysis suggests IRAK4 kinase regulates IRF5-mediated transcripts

Our goal was to determine what genes, groups of genes, and pathways were affected when IRAK4 kinase is inhibited. To answer this question, human monocytes were isolated from nine healthy donors. They were treated with and without IRAK4 kinase inhibitor and with and without R848, and RNA was collected at 1 h post stimulation. Global transcriptional analysis using Affymetrix genome-wide U133 2.0 plus arrays (GEO accession no. GSE86234) identified 860 genes that were differentially regulated based on a Welch's t test ($p < 0.01$, -fold change ≥ 4.0) by R848 stimulation. Hierarchical clustering of the regulated genes identified three main patterns of expression (Fig. 2A). One group was induced by R848 but unaffected by IRAK4 kinase inhibition. Thus,

this group contains genes that work through an IRAK4 kinase-independent pathway that may utilize the scaffold function of IRAK4. A second group was induced by R848 and reversed by IRAK4 kinase inhibition. This group contains genes that are regulated in an IRAK4 kinase-dependent manner. The final group contained genes that were inhibited by R848 but were not significantly affected by IRAK4 inhibition.

To determine the role of IRAK4 kinase activity in TLR-induced cytokine production, we focused on the second group, containing IRAK4 kinase-dependent genes (Fig. 2A, middle bracket). Upstream analysis in Ingenuity Pathway Analysis (IPA) of the 203 genes in this cluster identified the IRF family of transcription factors (IRF1, 3, 5, 7, and 8) as being five of six of the top predicted transcriptional regulators of genes induced by R848 and reversed by IRAK4 kinase inhibition. On the other hand, the genes not significantly affected by IRAK4 inhibition tended to be regulated by non-IRF transcription factors such as

NF κ B, Jun, and NFAT (Fig. 2A). This demonstrates that IRAK4 kinase regulates a narrow selection of TLR-induced transcription factors, mainly within the IRF family.

Mice expressing kinase-inactive IRAK4 have normal NF κ B and MAP kinase activation but are deficient in cytokine responses when challenged with TLR ligands (6, 7). Similarly, mice deficient in *Irf5* expression have a nearly identical phenotype, exhibiting normal activation of NF κ B and MAP kinases but deficiencies in the production of multiple inflammatory cytokines in response to TLR ligands (23). IRF5 is the only member of the IRF family of transcription factors reported to control all MyD88-dependent effects on cytokines and interferon (25). In addition, human monocyte expression of IRF5 is significantly higher as compared with other IRFs (IRF3, 7) (26). We therefore focused first on IRF5 and investigated the binding of IRF5 to the promoters of IRAK4 target genes. We chose two genes identified as highly induced by R848 and reversed by IRAK4 kinase inhibition from our profiling data that had both IRF5 and NF κ B binding sites in their promoters, *CXCL10* and *TNF*. ChIP-quantitative PCR (qPCR) (Active Motif, Carlsbad, CA) confirmed increased IRF5 binding in the upstream promoter of *CXCL10* and *TNF* upon R848 treatment. Binding of IRF5 is reduced by IRAK4 kinase inhibition in both of these genes, suggesting IRF5 as a downstream target of IRAK4 activity (Fig. 2B). In contrast, although R848 increased NF κ B binding to the promoters of *CXCL10* and *TNF*, IRAK4 kinase inhibition did not significantly reduce NF κ B binding to the promoters of these genes (Fig. 2C). Combined with previous data that IRAK4 kinase minimally affects phosphorylation of p65 (5), this suggests that IRAK4 kinase activity does not regulate NF κ B activity. Therefore, regulation of cytokine production in myeloid cells by IRAK4 kinase activity occurs through the regulation of IRF5 but not NF κ B.

IRAK4 kinase controls IRF5 nuclear translocation

To determine the mechanism by which IRAK4 inhibition is blocking IRF5-mediated transcription, we investigated IRF5 translocation to the nucleus of primary human monocytes stimulated with R848. As measured by Amnis imaging cytometry in CD14⁺ human monocytes and depicted in Fig. 3, A–C, upon stimulation with R848, IRF5 translocates to the nucleus (86.7 ± 1.1 versus $10.2 \pm 0.5\%$ similarity score percent positive, $p < 0.0001$). This R848-induced IRF5 nuclear translocation was blocked by the IRAK4 inhibitor (Fig. 3C, 86.7 ± 1.1 versus $21.8 \pm 2.9\%$ similarity score percent positive, $p < 0.0001$). By contrast (Fig. 3C), NF κ B p65 translocation was also induced by R848 (86.5 ± 3.6 versus $11.0 \pm 0.9\%$ similarity score percent positive, $p = 0.0002$), but was not significantly inhibited by the IRAK4 inhibitor (86.5 ± 3.6 versus $77.9 \pm 8.4\%$ positive, $p = 0.2$). To confirm that the lack of NF κ B inhibition was not specific to the IRAK4 inhibitor or species, we performed the same experiment using bone marrow–derived macrophages from *Irak4* knock-in mice, which confirmed that IRAK4 kinase activity was required for IRF5 but not NF κ B p65 nuclear translocation (supplemental Fig. 1). Finally, an independent flow cytometry platform was used to measure NF κ B pathway activation by R848 and inhibition by the IRAK4 inhibitor (Fig. 3D) using I κ B degradation as a readout. In PBMC isolated from 59 healthy

human volunteers, R848 induced I κ B degradation, and this was minimally affected by pretreatment with the IRAK4 inhibitor. The median control I κ B expression (L2DiffAF) was 10.48, whereas those for R848 stimulated and R848 stimulated in the presence of IRAK4 inhibitor were significantly lower, indicating I κ B degradation at 8.31 and 8.85, respectively ($n = 59$, two-sided paired Wilcoxon exact form (“signed rank test”), $p < 2.2 \times 10^{-16}$). Because we determined that IRAK4 kinase activates transcription of inflammatory cytokines through IRF5, we next sought to investigate the mechanism by which IRF5 is activated downstream of IRAK4 kinase.

IRAK4 kinase acts through IKK β to activate IRF5

The mechanism by which TLRs activate IRF5 is currently unclear. Recently, it has been shown that IKK β can phosphorylate IRF5 *in vitro* and induce dimerization and nuclear translocation in human monocytic and dendritic cell lines (15,16). Additionally, Bergstrom *et al.* (17) have shown that TLR activation of IFN β and IL12 in primary human monocytes involves activation of IRF5 through TAK1 and IKK β . Therefore, we investigated whether IRAK4 kinase acts through IKK β and TAK1 to control IRF5 activation. We treated human monocytes with IRAK4 kinase inhibitor and then stimulated for 15 and 30 min with R848. Fig. 4A (densitometry in supplemental Fig. 2) shows that the IRAK4 inhibitor blocks IRAK4 kinase autophosphorylation under the same conditions that block IRF5 nuclear translocation (Fig. 3C). Similar to IRAK4 kinase inhibition, we found that pharmacologically inhibiting IKK β (27) or TAK1 (28) in human monocytes blocks inflammatory cytokine production (Fig. 4B) and IRF5 translocation to the nucleus (Fig. 4C), but does not impact NF κ B p65 nuclear translocation (Fig. 4C). The IRAK4 inhibitor also blocks the phosphorylation of IKK β at Ser-177 but has no effect on the phosphorylation and degradation of I κ B (Fig. 4A). Inhibition of IKK β blocks phosphorylation and degradation of I κ B but appears to enhance the phosphorylation of Ser-177 on IKK β . This increase in phosphorylated IKK upon its inhibition has been observed previously but the mechanism is not known (29). A TAK1 inhibitor blocks IKK β and I κ B phosphorylation but not IRAK4 phosphorylation following R848 stimulation (Fig. 4A), demonstrating that TAK1 lies between IRAK4 and IKK β in the signaling cascade. Taken together these data delineate a pathway by which IRAK4 kinase activates TAK1 to phosphorylate and activate IKK β which in turn phosphorylates IRF5 to induce the translocation of IRF5 to the nucleus and the transcription of pro-inflammatory cytokines (Fig. 5). Therefore, inhibition of IRAK4 kinase activity is sufficient to block this signaling cascade and cytokine production without impacting NF κ B activation.

Discussion

We show that IRAK4 kinase inhibition in R848-treated human monocytes blocks inflammatory cytokine mRNA and protein production, including IL1, IL6, and TNF. Transcriptional pathway analysis suggests that this block in mRNA and cytokine production is mediated by IRF5, and this is confirmed by demonstrating the reduction of IRF5 nuclear translocation and IRF5 binding to the promoters of target

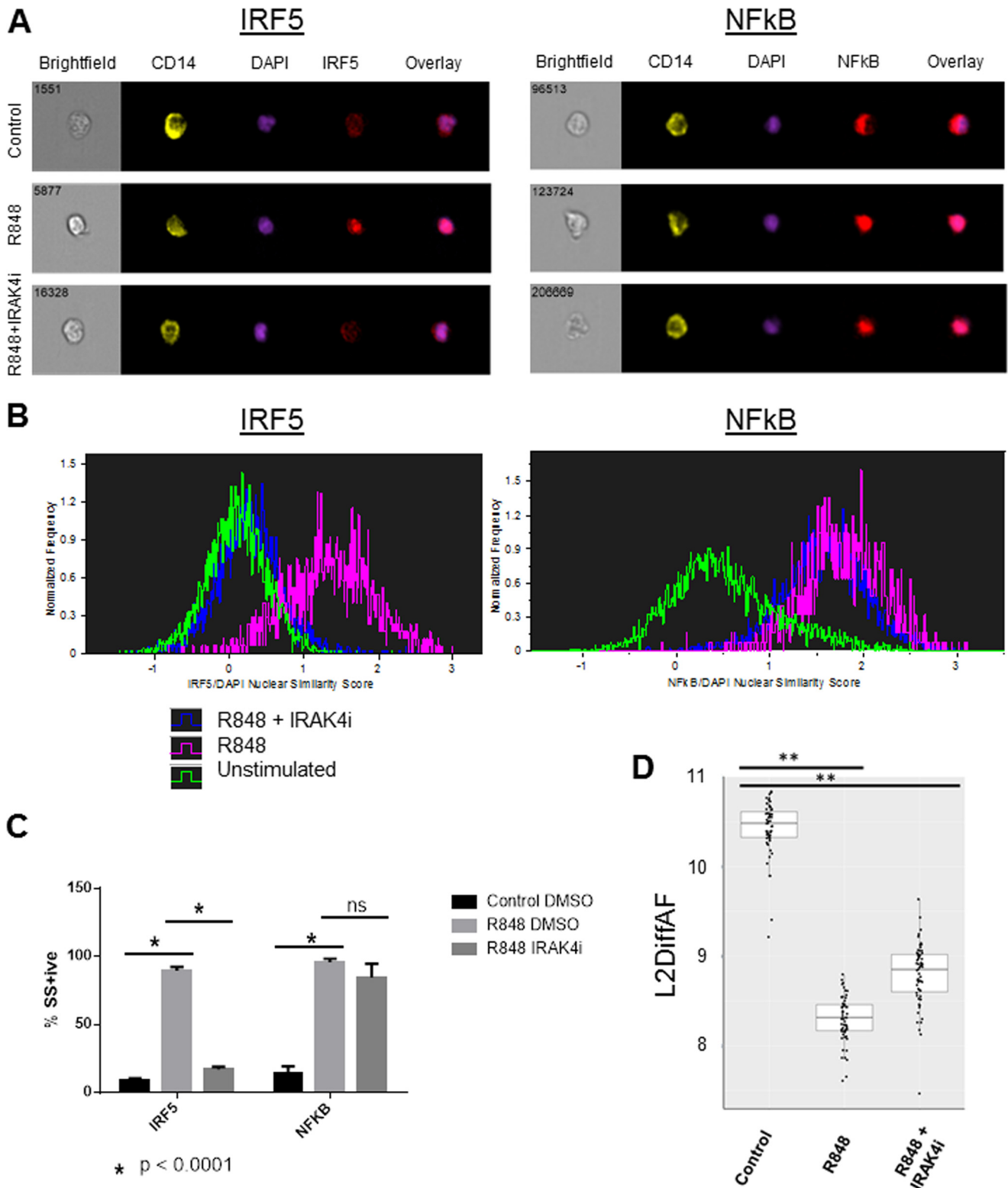


Figure 3. IRAK4 kinase controls IRF5 but not NFκB activation and translocation to the nucleus. ImageStream files of human PBMC were gated for single, focused cells and CD14⁺ monocytes, then analyzed for nuclear translocation using similarity score (SS). **A**, Brightfield, CD14, DAPI, IRF5, and NFκB images and overlays from samples with and without R848 stimulation, with and without IRAK4 inhibitor treatment. **B**, single parameter histogram overlays depicting the distribution of monocyte nuclear similarity scores with and without R848 stimulation, with and without IRAK4 inhibitor treatment. **C**, quantification of IRF5 and NFκB nuclear translocation by similarity score percent positive events. $n = 4$ human PBMC samples (mean \pm S.E.; *, $p < 0.0001$). **D**, analysis of IRAK4 kinase inhibitor-mediated inhibition of IκB degradation by SCNP flow cytometry analysis in PBMC from $n = 59$ healthy human volunteer blood donors. $L2DiffAF = \log_2(MFI - MFI_{AF})$, where MFI = mean fluorescence intensity and AF = autofluorescence (**, $p < 2.2 \times 10^{-16}$).

IRAK4 activity controls TLR inflammation through IRF5 in monocytes

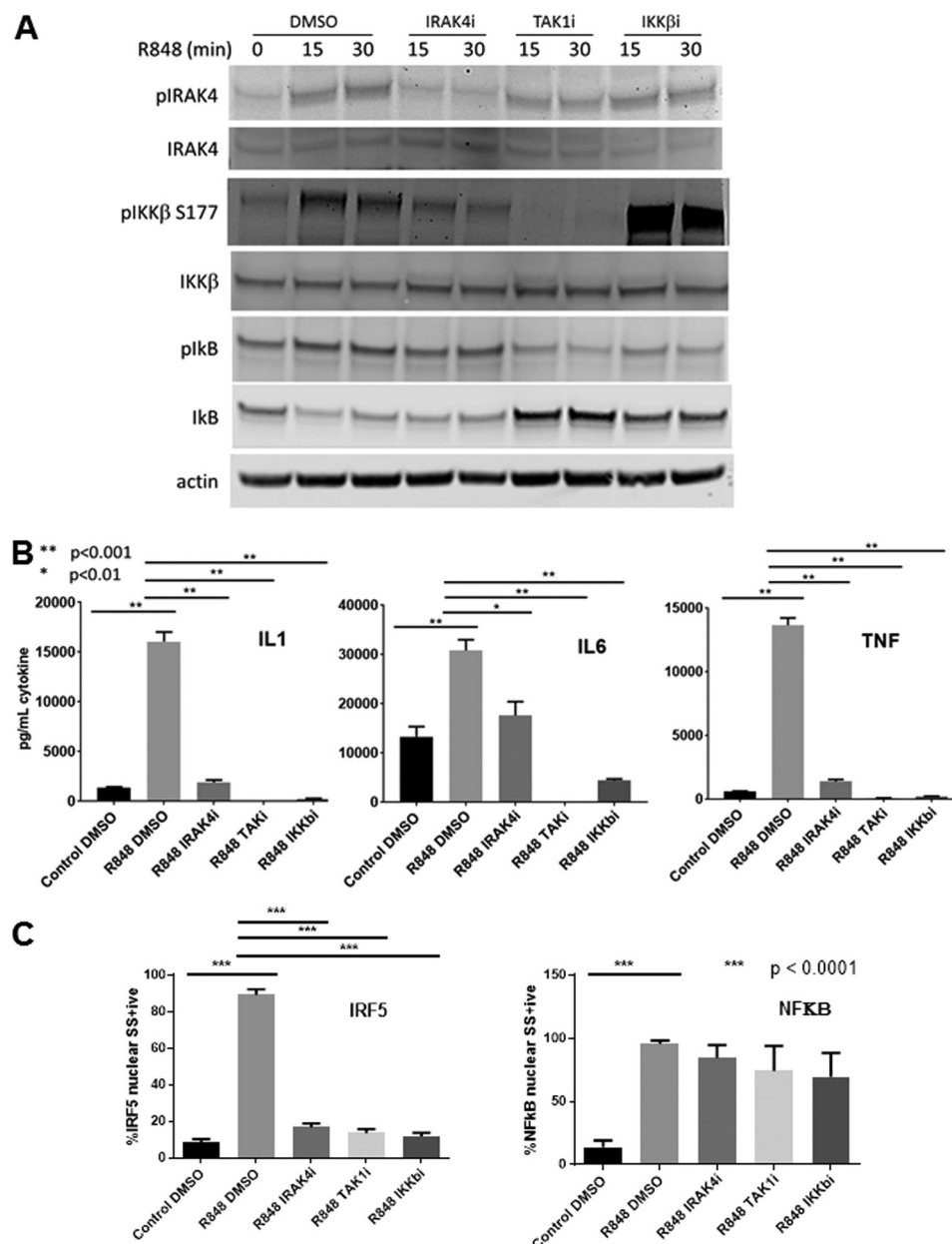


Figure 4. IRAK4 kinase acts through IKK β to activate IRF5. A and B, 10 million monocytes from healthy human blood donors were reconstituted in 2 ml of RPMI with PenStepGln/HEPES/0.5% heat-inactivated fetal bovine serum (Thermo Fisher) and incubated with 1 μ M IRAK4i (IRAK4 inhibitor), 1 μ M TAK1i (TAK1 inhibitor 5Z-7 oxozeanol, Tocris), 1 μ M IKK β i (IKK β inhibitor TPCA, Sigma), or 0.1% DMSO for 30 min prior to stimulation with 0.5 μ g/ml R848 for 15 or 30 min prior to analysis of cell lysates by Western blot (A) or 20 h prior to analysis of supernatant cytokines by ELISA (B). A, Western blot image representative of one of three donors. Quantitation by LI-COR image analysis software shown in [supplemental Fig. S2](#). B, cytokines represent mean \pm S.E. for $n = 3$ donors, comparison by one-way analysis of variance (ANOVA). C, 2-ml cultures of freshly isolated human PBMC were stimulated 30 min with 1 μ g/ml R848 after incubation with or without inhibitors for 30 min and then subjected to Amnis ImageStream analysis of IRF5 and NF κ B nuclear localization (mean \pm S.E.; $n = 4$ donors).

genes following IRAK4 inhibition. Interestingly, and in agreement with previous reports from our lab, the effect of an IRAK4 kinase inhibitor on the NF κ B nuclear translocation and binding to promoters of target genes is minimal. Finally, we find that IRAK4 kinase acts through IKK β and TAK1 to activate IRF5.

Activation of IRF5

Exactly which kinases phosphorylate and activate IRF5 is not known, and there are likely different mechanisms depending on cell type and stimulation. *In vitro* studies have shown that TBK1

and RIP2 can phosphorylate IRF5 (30). Overexpression studies also identified IKKE and TBK1 as activators of IRF5 (31). However *Tbk1* deficiency in mouse macrophages had no effect on the TLR7/8/9-induced cytokine production, in contrast to *Irak4*- and *Irif5*-deficient cells (32). These data suggest that TBK1 is not involved in IRAK4 regulation of the IRF5 pathway. Most recently, IKK β was identified as the kinase that phosphorylates IRF5 on Ser-462 and causes its dimerization and activation in myeloid cells (16, 33). Here we show that inhibition of IRAK4 blocks IKK β phosphorylation and IRF5 nuclear translocation, similar to the inhibition of TAK1 or IKK β . Our data

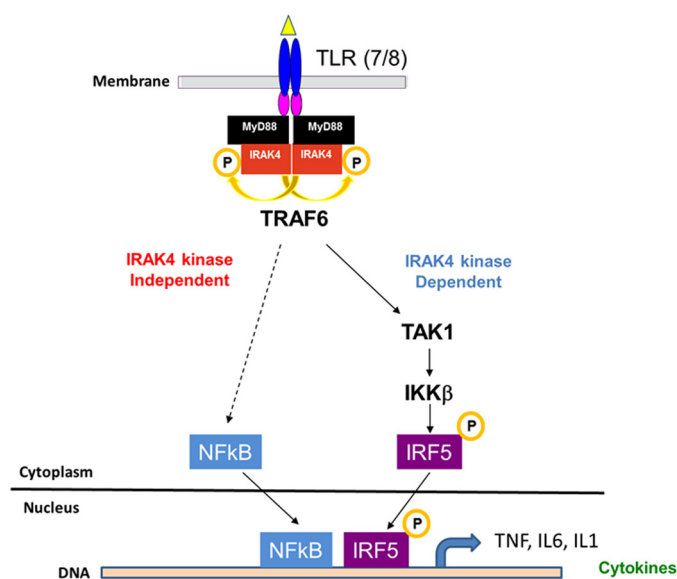


Figure 5. A proposed model of IRAK4 regulation of TLR7/8 signaling pathway in human monocytes is shown. Ligation of endosomal TLR7 and/or TLR8 by R848 leads to cytoplasmic assembly of the myddosome and autophosphorylation of IRAK4. Inflammatory cytokine production is induced by a signaling cascade including TAK1 and IKK β that results in phosphorylation, nuclear translocation, and transcription mediated by IRF5 (right). At the same time, an as yet unidentified signaling cascade (left, dashed line) that requires IRAK4 scaffolding activity but not IRAK4 kinase activity but is independent of TAK1 and IKK β , induces translocation of NF κ B to the nucleus. Binding of both NF κ B and IRF5 to the promoters of inflammatory cytokines is required for transcription.

suggest IRAK4 kinase activity regulates inflammatory cytokine production in human monocytes via an IRAK4-TAK1-IKK-IRF5 axis (17).

IKK β activates IRF5 but not NF κ B in TLR signaling

Our data show that IRAK4 controls the activation of IKK β to activate IRF5 but not NF κ B. Of note, and entirely consistent with our results, Dudhgaonkar *et al.* (34) recently demonstrated inhibition of IKK β phosphorylation and cytokine release from TLR ligand stimulated human PBMC using an undisclosed highly potent and selective IRAK4 inhibitor, but did not present data regarding NF κ B. IKK β is thought to be the canonical activator of NF κ B, yet our data demonstrate that NF κ B is minimally affected with IRAK4 kinase inhibition in R848-treated human monocytes, as is phosphorylation and degradation of I κ B. This phenomenon has been observed previously as TLR/IL1-induced NF κ B activation was shown to occur through a TAK1/IKK β -independent pathway (35, 36). These studies found that IRAK4 kinase activity led to TAK1-dependent IKK α/β phosphorylation and IKK β activation as TAK1 and IKK β were neither phosphorylated nor activated in cells from IRAK4 kinase-inactive mice. Interestingly, our data show that NF κ B was activated by R848 in the absence of IRAK4 kinase activity in human monocytes treated with an IRAK4 expression was ablated (1), indicating that a TAK1/IKK β -independent pathway of NF κ B activation is controlled by IRAK4 scaffolding activity. This IRAK4 scaffold-dependent activation of NF κ B was ablated when *Mekk3* expression was eliminated, suggesting that MEKK3 is the kinase mediating activation of NF κ B through the IRAK4 scaffold (37). Furthermore the authors

found that the MEKK3-dependent pathway utilized IKK γ phosphorylation and IKK α activation. Yamazaki *et al.* (38) further confirmed and added to this knowledge, showing that TAK1-dependent signaling required a complex for signaling that included TRAF6, MEKK3, and TAK1. After TAK1 initially activates NF κ B, MEKK3 subsequently activated NF κ B independently of TAK1 for continuous NF κ B activation and cytokine production. Thus it is tempting to speculate that inhibiting IRAK4 kinase blocks the TAK1-dependent pathway through IKK β with a resultant inhibition of IRF5 activation, but the MEKK3-dependent pathway is untouched, allowing near normal NF κ B activity. Further studies need to be carried out to prove this hypothesis and further clarify the mechanisms involved. Indeed, multiple kinases have been shown to have the ability to phosphorylate and activate NF κ B (39). In addition, post-transcriptional modifications other than phosphorylation, such as ubiquitination, and their role in this signaling cascade need further exploration.

Proposed model for IRAK4 regulation of IRF5

Based on our data reported here and published previously, we propose the following model for the regulation of TLR responses by IRAK4 in human monocytes. IRAK4 regulates the TLR response through both IRAK4 kinase activity-dependent and -independent (scaffold function-dependent) pathways. In response to TLR7/8 stimulation by ligands such as R848, IRAK4 is activated by recruitment into the receptor complex via interaction with MyD88. The resulting myddosome formation promotes autophosphorylation of IRAK4 and increases the kinase activity of IRAK4. IRAK4 kinase activity is responsible for the activation of TAK1 and IKK β , likely mediated through IRAK1 and TRAF6. The activated IKK β can then phosphorylate IRF5 and induce nuclear translocation of IRF5, leading to the expression of inflammatory cytokines. In addition to the IRAK4 kinase activity-dependent pathway, the scaffolding function of IRAK4, independent of kinase activity, is essential for the activation of the MAP kinase and NF κ B pathways. Future studies are required to understand the signaling components in both pathways. Our data indicate that an IRAK4 inhibitor will only affect the IRAK4 kinase-dependent pathways in human myeloid cells while preserving the scaffolding function in both myeloid and structural cells (5). The effect on the kinase-dependent pathway appears sufficient to provide a protective effect in mouse models of autoimmune diseases such as RA or multiple sclerosis based on mouse studies with kinase-inactive knock-in mice (42, 43) or IRAK4 inhibitor studies.⁸ Whether inhibition of the IRAK4 kinase-dependent pathway is sufficient to provide efficacy will await data from ongoing clinical trials.

Experimental procedures

Monocyte enrichment and treatment

Leukopaks from healthy donors (discarded byproducts of blood component donation, Massachusetts General Hospital, Boston) were incubated with RosetteSep Monocyte

⁸ L. Cushing, A. Winkler, S. A. Jelinsky, K. Lee, V. R. Rao, M. Fleming, and L.-L. Lin, unpublished data.

IRAK4 activity controls TLR inflammation through IRF5 in monocytes

Enrichment Cocktail (STEMCELL Technologies) and purified according to the manufacturer. The isolated monocytes were then resuspended for culture in RPMI plus 0.5% FBS (Thermo Fisher), pretreated with 1 μ M inhibitor compounds or DMSO (Sigma) for 30 min and then stimulated with 0.5 μ g/ml R848 (InvivoGen) for 0, 15, 30 min (phosphoprotein) or 1 and 4 h (RNA and cytokines).

ELISA

Culture supernatants were transferred to 4-plex pro-inflammatory II plates (Mesoscale Discovery) and developed according to the manufacturer's instructions. Statistical significance was determined by Student's *t*-tests.

RNA isolation and qRT-PCR

RNA was isolated utilizing the RNeasy Mini Kit (Qiagen) and quality and concentration assessed utilizing an Agilent 2100 bioanalyzer (Santa Clara, CA) and NanoDrop ND-1000 (Wilmington, DE), respectively. Total RNA (10 ng) was then processed using TaqMan Gene Expression Assays (Applied Biosystems) and qScript 1-Step qRT-PCR Kit, with ROX (Quanta Biosciences) according to the manufacturer's protocol. qRT-PCR and analysis were performed on the Viia7 Real-Time PCR system (Applied Biosystems). Statistical significance was determined by Student's *t*-tests.

Affymetrix array

Total RNA was processed following the NuGen (San Carlos, CA) RNA amplification protocol to produce double-stranded cDNA. 3.75 μ g of the cDNA was fragmented to between 35 and 200 bases in length and used as a template to generate biotin-targeted cDNA following the manufacturer's recommendations. Biotin-labeled cDNA was hybridized onto the Affymetrix (Santa Clara, CA) GeneChipTM genome array HG-U133 Plus overnight in the GeneChip Hybridization Oven 640. The following day the arrays were washed and then stained in a GeneChip Fluidics Station 450. Scanning was carried out with the GeneChip Scanner 3000 and image analysis was performed using the GeneChip Operating Software.

Bioinformatics analysis

Signal values were determined using GeneChip Operating System 1.0 (GCOS) (Affymetrix). For each array, all probe sets were normalized to a mean signal intensity value of 100. The default GeneChip Operating System statistical values were used for all analysis. Signal values and absolute detection calls were imported into Expressionist (GeneData, Lexington, MA) for analysis. A gene was considered detectable if at least 80% of the samples within a group had an expression greater than 20 signal units. Two independent Welch's *t* test analyses on the normalized signal values were performed between the R848-stimulated samples and the control samples and between the samples pretreated with 1 μ M inhibitor compounds with R848 stimulation and the R848-stimulated samples. The 860 probes with a difference of at least 4.0-fold ($p < 0.01$) in the R848 stimulated *versus* control comparison were used for further analysis. For some computations, the expression values for each qualifier were normalized to a mean of zero and a standard

deviation of 1 (z-score normalization). This allowed a direct comparison of patterns within the data without respect to absolute expression levels.

ChIP-qPCR

50 million cells per sample of primary human monocytes were pretreated with IRAK4 kinase inhibitor or DMSO for 30 min and stimulated with R848 or media control for 30 min. The cells were then fixed according to the cell fixation protocol recommended by Active Motif. Briefly, the cells were fixed in formaldehyde for cross-linking of proteins to DNA, and fixation was stopped with glycine. Cells were then washed in PBS (Life Technologies), IGEPAL (Sigma), and PMSF (Sigma) at 1 mM. After a final centrifugation, cell pellets were snap frozen on dry ice and shipped to Active Motif for ChIP-qPCR processing and analysis. Antibodies used for immunoprecipitation were IRF5 (Abcam, ab2932) and p65 (Santa Cruz Biotechnology, sc-109). Binding was tested for negative control regions (Untr12) and positive control regions (*IRF2BP2* and *IFNGR2*), as well as binding sites for IRF5 and NF κ B in *CXCL10* and *TNF*. The primers used for IRF5 binding *CXCL10* were TGTGGGAGGAGAGGAAGAAG and TTGCACCATCATC-CAATCAG; for *IRF5* binding *TNF*, TCATACAACCTC-CGAAACTGAG and GAAGGGTAAGACTGGGCTGAG; for p65 binding *CXCL10*, GAGTCTGCAACATGGGACTTC and TTTCCCTCCCTAATTCTGATTG; and for p65 binding *TNF*, TGTGAGGGGTATCCTTGATG and GCACCTTCTGTC-TCGGTTTC. Quantitative PCR analyses were done in triplicate and represented as percent input after normalization for primer efficiency and starting material per manufacturer's instructions.

Transcription factor nuclear translocation

Peripheral blood mononuclear cells were prepared from fresh heparinized peripheral whole blood (Pfizer volunteer donation program, Institutional Review Board-approved protocol) using Ficoll-Paque Plus (GE Lifesciences), suspended in complete RPMI (RPMI 1640 supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, penicillin-streptomycin, and 10% heat-inactivated fetal bovine serum (Thermo Fisher)). Aliquots of 2×10^6 cells were rested in a 96-well polypropylene deep-well plate (Axygen) at 37 °C in a 5% CO₂ incubator for 1 h, incubated with 1 μ M compounds for 1 h, and stimulated 30 min with 1 μ g/ml R848 (InvivoGen). After centrifugation, cells were fixed 15 min with Cytofix (BD Biosciences), washed, and permeabilized with phosphate-buffered saline containing 2% FBS and 0.1% Triton X-100 (Sigma). Cells were incubated with phycoerythrin-labeled CD14 antibody (Beckman Coulter) and unlabeled rabbit antibodies to IRF5 (Abcam) or NF κ B p65 (Cell Signaling Technology) for 30 min. After washing, secondary Alexa 647-labeled goat anti-rabbit IgG and the DNA dye DAPI were incubated with cells for 20 min, and fixation with Cytofix was repeated for 10 min after washing. At least 100,000 cellular events were collected on an Amnis ImageStream Mark II imaging cytometer. Single fluorophore controls were used to construct a compensation matrix for each experiment, monocytes were gated by CD14 staining, and nuclear localization was quantified using pixel similarity

scoring with a nuclear mask generated from the DAPI image. A gate was set on the 10% highest similarity scores in the unstimulated sample and applied to the remaining samples to quantify changes in nuclear translocation of the target molecule. The identical experiment was performed on blood from four volunteers on three different days, and compared with a Student's *t* test.

Analysis of intracellular signaling by SCNP technology

Single cell network profiling (SCNP) was carried out as described previously (40). Briefly, 59 healthy donor PBMC were plated and incubated at 100,000 cells/well in 96-well plates. The IRAK4 inhibitor PF-06426779 at 500 nM was applied for 1 h prior to TLR7/8 modulation (R848, InvivoGen, catalog no. tlrl-r848, 2 µg/ml) for 15 min after which all wells were fixed, permeabilized, and incubated with a mixture of fluorochrome-conjugated antibodies that recognize extracellular lineage markers (CD20 (BD Biosciences, catalog no. 558021, 1:4 from neat), CD14 (Beckman Coulter, catalog no. B01175, 1:16 from neat)) and intracellular epitopes (cPARP (BD Biosciences, catalog no. 560640, 1:8 from neat), IκBα (Cell Signaling Technology, catalog no. 7523BC, 1:4 from neat)). Cleaved PARP was examined in every well to enable assessment of cell health and to allow gating on cPARP negative (*i.e.* nonapoptotic) monocytes (41). Data were acquired using FACS Diva software (BD Biosciences) on a Canto II (BD) flow cytometer. Flow Cytometry Standard Data (FCS) files were gated using WinList (Verity House Software, Topsham, ME) and all data were stored in a MySQL database for access and querying.

Western blotting

Cells were lysed in 200 µl of 1× RIPA lysis buffer (Cell Signaling Technology) according to the manufacturer's instructions. They were then heated to 95 °C for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The antibodies phospho-IRAK4 (Thr-345/Ser-346) rabbit monoclonal (5), IRAK4 (mouse, Abcam, catalog no. 119942), phospho-IκBα (mouse mAb, Cell Signaling Technology, catalog no. 9246), IκBα (rabbit, Cell Signaling Technology, catalog no. 4812), phospho-IKKβ Ser-177 (rabbit, Cell Signaling Technology, catalog no. 2078), and actin (mouse, Cell Signaling Technology, catalog no. 3700) were incubated with membranes blocking in Odyssey Blocking Buffer (LI-COR) for 1 h at room temperature and then incubated overnight in primary antibody at 4 °C. Membranes were washed four times for 5 min each in 1× PBS-T, incubated in secondary antibody for 1 h at room temperature, washed again four times for 5 min each in PBS-T, rinsed in PBS, and imaged on the Odyssey CLx.

Author contributions—L. C., A. W., V. R. R., M. F., and L.-L. L. contributed to experimental design. L. C. and A. W. wrote the manuscript. L. C. performed monocyte stimulations and analyzed protein and mRNA expression as well as ChIP-Seq. A. W. performed nuclear translocation assays. S. A. J. performed bioinformatics analyses. K. L. designed IRAK4 inhibitors. W. K. and R. H. designed and supervised SCNP experiments. V. R. R. performed cell signaling phosphoprotein analysis. L.-L. L. and V. R. R. edited the manuscript. All authors reviewed the manuscript.

Acknowledgments—We are thankful for the following contributions: Erik Evensen and Andrew Conroy at Nodality performed and analyzed SCNP assays and John Cheng at Pfizer assisted in establishing IRF5 nuclear translocation assay. We thank Shizuo Akira of the Japan Science and Technology Agency, Osaka, Japan, for the generous gift of Irak4 knock-in mice.

References

- Picard, C., Puel, A., Bonnet, M., Ku, C. L., Bustamante, J., Yang, K., Soudais, C., Dupuis, S., Feinberg, J., Fieschi, C., Elbm, C., Hitchcock, R., Lammas, D., Davies, G., Al-Ghonaim, A., *et al.* (2003) Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science* **299**, 2076–2079
- Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W., and Yeh, W.-C. (2002) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* **416**, 750–756
- Seganish, W. M. (2016) Inhibitors of interleukin-1 receptor-associated kinase 4 (IRAK4): A patent review. (2012–2015). *Expert Opin. Ther. Pat.* **26**, 917–932
- Flannery, S., and Bowie, A. G. (2010) The interleukin-1 receptor-associated kinases: Critical regulators of innate immune signalling. *Biochem. Pharmacol.* **80**, 1981–1991
- Cushing, L., Stochaj, W., Siegel, M., Czerwinski, R., Dower, K., Wright, Q., Hirschfield, M., Casanova, J. L., Picard, C., Puel, A., Lin, L. L., and Rao, V. R. (2014) Interleukin 1/Toll-like receptor-induced autophosphorylation activates interleukin 1 receptor-associated kinase 4 and controls cytokine induction in a cell type-specific manner. *J. Biol. Chem.* **289**, 10865–10875
- Kim, T. W., Staschke, K., Bulek, K., Yao, J., Peters, K., Oh, K. H., Vandenberg, Y., Xiao, H., Qian, W., Hamilton, T., Min, B., Sen, G., Gilmour, R., and Li, X. (2007) A critical role for IRAK4 kinase activity in Toll-like receptor-mediated innate immunity. *J. Exp. Med.* **204**, 1025–1036
- Kawagoe, T., Sato, S., Jung, A., Yamamoto, M., Matsui, K., Kato, H., Uematsu, S., Takeuchi, O., and Akira, S. (2007) Essential role of IRAK-4 protein and its kinase activity in Toll-like receptor-mediated immune responses but not in TCR signaling. *J. Exp. Med.* **204**, 1013–1024
- Baechler, E. C., Batliwalla, F. M., Karypis, G., Gaffney, P. M., Ortmann, W. A., Espe, K. J., Shark, K. B., Grande, W. J., Hughes, K. M., Kapur, V., Gregersen, P. K., and Behrens, T. W. (2003) Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2610–2615
- Yao, Y., Richman, L., Morehouse, C., de los Reyes, M., Higgs, B. W., Boutrin, A., White, B., Coyle, A., Krueger, J., Kiener, P. A., and Jallat, B. (2008) Type I interferon: Potential therapeutic target for psoriasis? *PLoS One* **3**, e2737
- Baechler, E. C., Bilgic, H., and Reed, A. M. (2011) Type I interferon pathway in adult and juvenile dermatomyositis. *Arthritis Res. Ther.* **13**, 249
- Wright, H. L., Thomas, H. B., Moots, R. J., and Edwards, S. W. (2015) Interferon gene expression signature in rheumatoid arthritis neutrophils correlates with a good response to TNFi therapy. *Rheumatology (Oxf.)* **54**, 188–193
- Barnes, B. J., Moore, P. A., and Pitha, P. M. (2001) Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon α genes. *J. Biol. Chem.* **276**, 23382–23390
- Schoenemeyer, A., Barnes, B. J., Mancl, M. E., Latz, E., Goutagny, N., Pitha, P. M., Fitzgerald, K. A., and Golenbock, D. T. (2005) The interferon regulatory factor, IRF5, is a central mediator of Toll-like receptor 7 signaling. *J. Biol. Chem.* **280**, 17005–17012
- Saliba, D. G., Heger, A., Eames, H. L., Oikonomopoulos, S., Teixeira, A., Blazek, K., Androulidaki, A., Wong, D., Goh, F. G., Weiss, M., Byrne, A., Pasparakis, M., Ragoussis, J., and Udalova, I. A. (2014) IRF5:RelA interaction targets inflammatory genes in macrophages. *Cell Rep.* **8**, 1308–1317
- Hayden, M. S., and Ghosh, S. (2014) Innate sense of purpose for IKKβ. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 17348–17349

16. Lopez-Pelaez, M., Lamont, D. J., Pegg, M., Shpiro, N., Gray, N. S., and Cohen, P. (2014) Protein kinase IKK β -catalyzed phosphorylation of IRF5 at Ser462 induces its dimerization and nuclear translocation in myeloid cells. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 17432–17437
17. Bergström, B., Aune, M. H., Awuh, J. A., Kojen, J. F., Blix, K. J., Ryan, L., Flo, T. H., Mollnes, T. E., Espevik, T., and Stenvik, J. (2015) TLR8 senses *Staphylococcus aureus* RNA in human primary monocytes and macrophages and induces IFN- β production via a TAK1- $\text{IKK}\beta$ -IRF5 signaling pathway. *J. Immunol.* **195**, 1100–1111
18. Dideberg, V., Kristjansdottir, G., Milani, L., Libioulle, C., Sigurdsson, S., Louis, E., Wiman, A. C., Vermeire, S., Rutgeerts, P., Belaiche, J., Franchimont, D., Van Gossum, A., Bours, V., and Syvänen, A. C. (2007) An insertion-deletion polymorphism in the interferon regulatory factor 5 (IRF5) gene confers risk of inflammatory bowel diseases. *Hum. Mol. Genet.* **16**, 3008–3016
19. Dieguez-Gonzalez, R., Calaza, M., Perez-Pampin, E., de la Serna, A. R., Fernandez-Gutierrez, B., Castañeda, S., Largo, R., Joven, B., Narvaez, J., Navarro, F., Marenco, J. L., Vicario, J. L., Blanco, F. J., Fernandez-Lopez, J. C., Caliz, R., et al. (2008) Association of interferon regulatory factor 5 haplotypes, similar to that found in systemic lupus erythematosus, in a large subgroup of patients with rheumatoid arthritis. *Arthritis Rheum.* **58**, 1264–1274
20. Sigurdsson, S., Nordmark, G., Göring, H. H., Lindroos, K., Wiman, A. C., Sturfelt, G., Jönsen, A., Rantapää-Dahlqvist, S., Möller, B., Kere, J., Koskenmies, S., Widén, E., Eloranta, M. L., Julkunen, H., Kristjansdottir, H., Steinsson, K., Alm, G., Rönnblom, L., and Syvänen, A. C. (2005) Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. *Am. J. Hum. Genet.* **76**, 528–537
21. Richez, C., Barnette, T., Miceli-Richard, C., Blanco, P., Moreau, J. F., Rifkin, I., and Schaeffer, T. (2010) Role for interferon regulatory factors in autoimmunity. *Joint Bone Spine* **77**, 525–531
22. Feng, D., Yang, L., Bi, X., Stone, R. C., Patel, P., and Barnes, B. J. (2012) *Irfs*-deficient mice are protected from pristane-induced lupus via increased Th2 cytokines and altered IgG class switching. *Eur. J. Immunol.* **42**, 1477–1487
23. Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T. W., and Taniguchi, T. (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* **434**, 243–249
24. Lee, K. L., Ambler, C. M., Anderson, D. R., Boscoe, B. P., Bree, A. G., Brodfuehrer, J. I., Chang, J. S., Choi, C., Chung, S., Curran, K. J., Day, J. E., Dehnhardt, C. M., Dower, K., Drozda, S. E., Frisbie, R. K., et al. (2017) Discovery of clinical candidate 1-[(2S,3S,4S)-3-ethyl-4-fluoro-5-oxopyrrolidin-2-yl]methoxy-7-methoxyisoquinoline-6-carboxamide (PF-06650833), a potent, selective inhibitor of interleukin-1 receptor associated kinase 4 (IRAK4), by fragment-based drug design. *J. Med. Chem.* **60**, 5521–5542
25. Honda, K., and Taniguchi, T. (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat. Rev. Immunol.* **6**, 644–658
26. Rieckmann, J. C., Geiger, R., Hornburg, D., Wolf, T., Kveler, K., Jarrossay, D., Sallusto, F., Shen-Orr, S. S., Lanzavecchia, A., Mann, M., and Meissner, F. (2017) Social network architecture of human immune cells unveiled by quantitative proteomics. *Nat. Immunol.* **18**, 583–593
27. Podolin, P. L., Callahan, J. F., Bolognese, B. J., Li, Y. H., Carlson, K., Davis, T. G., Mellor, G. W., Evans, C., and Roshak, A. K. (2005) Attenuation of murine collagen-induced arthritis by a novel, potent, selective small molecule inhibitor of I κ B kinase 2, TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), occurs via reduction of proinflammatory cytokines and antigen-induced t cell proliferation. *J. Pharmacol. Exper. Ther.* **312**, 373–381
28. Ninomiya-Tsuji, J., Kajino, T., Ono, K., Ohtomo, T., Matsumoto, M., Shiina, M., Mihara, M., Tsuchiya, M., and Matsumoto, K. (2003) A resorcylic acid lactone, 5Z-7-oxozeanol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase. *J. Biol. Chem.* **278**, 18485–18490
29. Zhang, J., Clark, K., Lawrence, T., Pegg, M. W., and Cohen, P. (2014) An unexpected twist to the activation of IKK β : TAK1 primes IKK β for activation by autophosphorylation. *Biochem. J.* **461**, 531–537
30. Chang Foreman, H. C., Van Scoy, S., Cheng, T. F., and Reich, N. C. (2012) Activation of interferon regulatory factor 5 by site specific phosphorylation. *PLoS One* **7**, e33098
31. Cheng, T. F., Brzostek, S., Ando, O., Van Scoy, S., Kumar, K. P., and Reich, N. C. (2006) Differential activation of IFN regulatory factor (IRF)-3 and IRF-5 transcription factors during viral infection. *J. Immunol.* **176**, 7462–7470
32. Marchlik, E., Thakker, P., Carlson, T., Jiang, Z., Ryan, M., Marusic, S., Goutagny, N., Kuang, W., Askew, G. R., Roberts, V., Benoit, S., Zhou, T., Ling, V., Pfeifer, R., Stedman, N., Fitzgerald, K. A., Lin, L. L., and Hall, J. P. (2010) Mice lacking Tbx1 activity exhibit immune cell infiltrates in multiple tissues and increased susceptibility to LPS-induced lethality. *J. Leukocyte Biol.* **88**, 1171–1180
33. Ren, J., Chen, X., and Chen, Z. J. (2014) IKK β is an IRF5 kinase that instigates inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 17438–17443
34. Dudhgaonkar, S., Ranade, S., Nagar, J., Subramani, S., Prasad, D. S., Karunanithi, P., Srivastava, R., Venkatesh, K., Selvam, S., Krishnamurthy, P., Mariappan, T. T., Saxena, A., Fan, L., Stetsko, D. K., Holloway, D. A., Li, X., et al. (2017) Selective IRAK4 inhibition attenuates disease in murine lupus models and demonstrates steroid sparing activity. *J. Immunol.* **198**, 1308–1319
35. Fraczek, J., Kim, T. W., Xiao, H., Yao, J., Wen, Q., Li, Y., Casanova, J. L., Pryjma, J., and Li, X. (2008) The kinase activity of IL-1 receptor-associated kinase 4 is required for interleukin-1 receptor/Toll-like receptor-induced TAK1-dependent NF κ B activation. *J. Biol. Chem.* **283**, 31697–31705
36. Qin, J., Yao, J., Cui, G., Xiao, H., Kim, T. W., Fraczek, J., Wightman, P., Sato, S., Akira, S., Puel, A., Casanova, J. L., Su, B., and Li, X. (2006) TLR8-mediated NF- κ B and JNK activation are TAK1-independent and MEKK3-dependent. *J. Biol. Chem.* **281**, 21013–21021
37. Yao, J., Kim, T. W., Qin, J., Jiang, Z., Qian, Y., Xiao, H., Lu, Y., Qian, W., Gulen, M. F., Sizemore, N., DiDonato, J., Sato, S., Akira, S., Su, B., and Li, X. (2007) Interleukin-1 (IL-1)-induced TAK1-dependent versus MEKK3-dependent NF κ B activation pathways bifurcate at IL-1 receptor-associated kinase modification. *J. Biol. Chem.* **282**, 6075–6089
38. Yamazaki, K., Gohda, J., Kanayama, A., Miyamoto, Y., Sakurai, H., Yamamoto, M., Akira, S., Hayashi, H., Su, B., and Inoue, J. (2009) Two mechanistically and temporally distinct NF- κ B activation pathways in IL-1 signaling. *Sci. Signal.* **93**, ra66
39. Buss, H., Dörrie, A., Schmitz, M. L., Hoffmann, E., Resch, K., and Kracht, M. (2004) Constitutive and interleukin-1-inducible phosphorylation of p65 NF- κ B at serine 536 is mediated by multiple protein kinases including I κ B kinase (IKK- α , IKK β , IKK ϵ , TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. *J. Biol. Chem.* **279**, 55633–55643
40. Cesano, A., Rosen, D. B., O'Meara, P., Putta, S., Gayko, U., Spellmeyer, D. C., Cripe, L. D., Sun, Z., Uno, H., Litzow, M. R., Tallman, M. S., and Paietta, E. (2012) Functional pathway analysis in acute myeloid leukemia using single cell network profiling assay: Effect of specimen source (bone marrow or peripheral blood) on assay readouts. *Cytometry B Clin. Cytom.* **82**, 158–172
41. Cesano, A., Putta, S., Rosen, D. B., Cohen, A. C., Gayko, U., Mathi, K., Woronicz, J., Hawtin, R. E., Cripe, L., Sun, Z., Tallman, M. S., and Paietta, E. (2013) Functional pathway analysis using SCNP of FLT3 receptor pathway deregulation in AML provides prognostic information independent from mutational status. *PLoS One* **8**, e56714
42. Koziczak-Holbro, M., Littlewood-Evans, A., Pöllinger, B., Kovarik, J., Dawson, J., Zenke, G., Burkhardt, C., Müller, M., and Gram, H. (2009) The critical role of kinase activity of interleukin-1 receptor-associated kinase 4 in animal models of joint inflammation. *Arthritis Rheum.* **60**, 1661–1671
43. Staschke, K. A., Dong, S., Saha, J., Zhao, J., Brooks, N. A., Hepburn, D. L., Xia, J., Gulen, M. F., Kang, Z., Altuntas, C. Z., Tuohy, V. K., Gilmour, R., Li, X., and Na, S. (2009) IRAK4 kinase activity is required for Th17 differentiation and Th17-mediated disease. *J. Immunol.* **183**, 568–577