Interferon regulatory factors (IRFs) are critical components of virus-induced immune activation and type I interferon regulation. IRF3 and IRF7 are activated in response to a variety of viruses or after engagement of Toll-like receptor (TLR) 3 and TLR4 by double-stranded RNA and lipopolysaccharide, respectively. The activation of IRF5, is much more restricted. Here we show that in contrast to IRF3 and IRF7, IRF5 is not a target of the TLR3 signaling pathway but is activated by TLR7 or TLR8 signaling. We also demonstrate that MyD88, interleukin 1 receptor-associated kinase 1, and tumor necrosis factor receptor-associated factor 6 are required for the activation of IRF5 and IRF7 in the TLR7 signaling pathway. Moreover, ectopic expression of IRF5 enabled type I interferon production in response to TLR7 signaling, whereas knockdown of IRF5 by small interfering RNA reduced type I interferon induction in response to the TLR7 ligand, R-848. IRF5 and IRF7, therefore, emerge from these studies as critical mediators of TLR7 signaling.

Members of the Toll-like receptor family are essential recognition and signaling components of mammalian anti-viral host defense (1). TLR3, TLR7, TLR8, and TLR9 recognize viral nucleic acids and induce type I IFNs. TLR7 and TLR8 are similar in sequence and together with TLR9 form an evolutionarily related subgroup within the TLR superfamily (2, 3).

Whereas unmethylated CpG DNA (4), herpes simplex virus (HSV) type 1 (5), and HSV type 2 genomic DNA (6) specifically stimulate TLR9 (7, 8), TLR7 is activated by infections with single-stranded RNA viruses, including influenza virus and vesicular stomatitis virus (VSV) (7, 9). Consequently, plasmacytoid dendritic cells (pDCs) from TLR7-deficient mice fail to produce type I IFNs upon infection with influenza virus or VSV (7, 10). In addition to single-stranded RNA, the synthetic imidazoquinoline, imiquimod, a low molecular weight immune response modifier, activates TLR7 in both humans and mice, whereas its derivative resiquimod (R-848) activates TLR7 and TLR8 in humans but only TLR7 in mice (10, 11). Both imiquimod and R-848 elicit robust anti-viral and anti-tumor immune responses in vivo, which correlate with a strong induction of type I IFNs (12–14). As a consequence of this activity, imiquimod is used for the treatment of external genital warts caused by human Papillomavirus (15).

Interferon regulatory factors (IRFs) coordinate the expression of type I IFNs (16–19) as well as chemokines such as IP-10 and RANTES (regulated on activation normal T cell expressed and secreted) (20–22). Viral infections, dsRNA, or LPS signaling can activate IRF3 and IRF7 (23–25). In contrast, the activation of IRF5, another member of the IRF family, is much more restricted. Only certain viruses, including Newcastle disease virus (NDV), VSV, and herpes simplex virus type 1, have been shown to activate IRF5 (22), whereas Sendai virus (SeV) and dsRNA poly(I)poly(C) (pI Cp), which activate IRF3 and IRF7, do not activate IRF5 (22). These observations suggest that IRF5 is activated by distinct signaling mechanisms to those regulating IRF3. In unstimulated cells IRFs reside in the cytoplasm. The activation of these factors requires phosphorylation on the C terminus, leading to dimerization, nuclear translocation, and binding to promoters containing IRF binding elements (26–28). The IKK-related kinases, IκB kinase ε (IKKe) (also called IκKε) (29, 30) and TANK binding kinase 1 (TBK1) (also called T2K or NAK) (31–33)) can directly phosphorylate IRF3 and IRF7 at the C terminus and control the expression of type I IFNs in response to SeV infection and TLR3 or TLR4 stimulation (34–36). The role of these two non-canonical IKKs in the regulation of IFN gene expression resulting from TLR7, TLR8, or TLR9 ligation or their ability to phosphorylate IRF5 has not yet been addressed.

TLR3 and TLR4 are known to induce IFNβ gene expression. This induction requires IRF3 and/or IRF7. TLR3 recruits the TIR domain containing adapter-inducing IFNβ, TRIF (37–39), whereas TLR4 signaling utilizes TRIF and the adapter molecule, TRIF-related adapter molecule, TRAM (23, 40). The adapter molecules MyD88 and Mal/TIRAP are not involved in type I IFN induction by the TLR3 or TLR4 signaling pathway. In contrast, studies with MyD88-deficient mice revealed that...
TLR7 and TLR9 signaling to IFNα is dependent on MyD88 (10, 41).

Unlike IRF3, which is expressed constitutively in all cell types, the expression of IRF5 and IRF7 is restricted to B cells and dendritic cells, although their expression is inducible in other cell types by type I IFNs (19, 42). The predominant source of type I IFNs in human blood is the pDC. pDCs release large amounts of type I IFN upon viral infection or stimulation with either R-S84 or CpG DNA (43, 44). Consistent with these observations, pDCs express high levels of TLR7 and TLR9, whereas the expression of other TLRs is either very low or absent (45, 46). The molecular mechanisms responsible for the induction of IFNs by TLR7, TLR8, and TLR9 signaling are unclear at present. Because pDCs express high constitutive levels of IRF5 as well as IRF7, we were prompted to investigate the functional importance of these two IRFs in the regulation of type I IFNs by these TLRs.

We focused the present study on the TLR7 and TLR8 signaling pathway. We demonstrate that TLR7 and TLR8 activate both IRF5 and IRF7 and do not appear to activate IRF3. We also show using reconstitution experiments and siRNA silencing approaches that IRF5 is a critical mediator of TLR7 signaling. Both IRF5 and IRF7 are regulated in a MyD88-, IRAK1-, and TRAF6-dependent manner, in contrast to IRF3, which is regulated via TRIF in TLR3 or TLR4 signaling.

EXPERIMENTAL PROCEDURES

Reagents—pFLAG-CMV1-TLR3 was cloned by PCR from a full-length cDNA clone. pFLAG-CMV1-TLR7 was cloned by PCR from THP-1 genomic DNA. The plasmid pDNA3.1-TLR3 was a gift from the Eisai Research Institute (Andover, MA). Dominant negative IRF5 (DN IRF5) is a DNA binding domain deletion mutant of wild type IRF5 (variant 3, GenBankTM accession number AY504946) and is missing the first N-terminal 137 amino acids. The pDNA3-MyD88-TIR-AU1 dominant negative (DN) plasmid was generated as described (47). The Gal4 upstream activation sequence (UAS (Gal4))-driven luciferase reporter gene and Gal4-IRF3 were from T. Fujita (Tokyo, Japan (48)). Gal4-IRF5 (19), Gal4-IRF7 (49), FLAG-IRF5 (19), IRF7-GFP, and IRF5-GFP (19) were described previously. The IFNA1 and IFNB-secreted alkaline gene and Gal4-IRF3 were from T. Fujita (Tokyo, Japan (48)). Gal4-IRF5, and Gal4-DBD plus 40 ng of the UAS(GAL)-driven luciferase reporter gene. CMV1-TLR7, pcDNA3.1-TLR8, pFLAG-CMV1-TLR3, or empty vector transfected with 50 ng of the pFLAG-CMV1-TLR7. 24 h later cells were subjected to reverse transcription-PCR analysis.

RESULTS

TLR7 and TLR8 Activate IRF5 and IRF7 but Not IRF3—Double-stranded RNA and LPS induce type I IFN gene expression via the adapter molecule TRIF and the transcriptional regulator IRF3 (37–39). The molecular mechanisms responsible for the induction of type I IFNs by TLR7 or TLR8 signaling is unclear at present. We were interested in elucidating the role of the transcription factors IRF3, IRF5, and IRF7 in signaling by TLR7 and TLR8. To this end we monitored the activation of these factors individually. We employed an "in vitro" reporter assay that utilizes hybrid proteins consisting of the yeast Gal4 DNA binding domain fused either to IRF3, IRF5, or IRF7, lacking its own DNA binding domain (19, 49, 56). In this assay the UAS (Gal4) reporter gene expression requires activation of the corresponding IRF fusion protein (56). The basal level of all three IRF fusion proteins was similar in untreated cells. TLR7- or TLR8-expressing HEK293 cells transfected with Gal4-IRF3, Gal4-IRF5, or Gal4-IRF7 plasmids were stimulated with R-S84, TLR7 or -8 signaling activated IRF5 and IRF7 but not IRF3 (Fig. 1A, upper panel). Stimulation of TLR3-expressing HEK293 with pLc did not activate IRF5 (Fig. 1A, bottom left panel), although IRF3 and IRF7 were induced in a robust manner. Furthermore, infection of HEK293 cells with SeV, a well characterized activator of IRF3 and IRF7,
also failed to activate IRF5 (Fig. 1A, bottom right panel), in agreement with published reports (19). Thus, TLR7 and TLR8 activate IRF5 and IRF7 and do not appear to activate IRF3. Neither Sendai virus nor TLR3 signaling activated IRF5.

As a second independent methodology, we examined the nuclear translocation of these three IRFs in RAW264.7 macrophage-like cells transfected with IRFGFP fusion constructs. The IRF5-GFP or IRF7-GFP fusion proteins were expressed in the cytoplasm of unstimulated cells (Fig. 1, B and C). Stimulation of these cells with R-848 resulted in the nuclear translocation of both IRF5-GFP and IRF7-GFP (Fig. 1, B and C, middle panels) but did not induce nuclear translocation of IRF3-GFP (data not shown). In agreement with the Gal4-IRF5 assay, IRF5-GFP did not translocate to the nucleus in cells stimulated with pI:C (Fig. 1B, lower panel). IRF7-GFP did, however, translocate in response to pI:C, in agreement with previous reports (Fig. 1C, lower panel). These nuclear translocation data are representative of several fields analyzed. We also looked at IRF3 phosphorylation using phospho-specific IRF3 antibodies in R-848- and LPS-stimulated cells. There was no detectable phosphorylation of IRF3 with R-848 seen (data not shown), in contrast to LPS, which did induce IRF3 phosphorylation. Taken together, these observations establish that unlike TLR3, TLR7 and TLR8 can activate IRF5 but not IRF3. Consistent with these observations, R-848 did not induce IRF3-DNA binding activity to an ISG-15 probe (which binds activated IRF3) under conditions where NF-κB was activated (data not shown), further supporting the idea that IRF3 is not a mediator of the TLR7 signaling pathway. While this manuscript was in preparation, two independent reports from the Akira and Taniguchi laboratories (57, 58) demonstrated that IRF3 was not activated in either the TLR7 or TLR9 signaling pathway.

**TLR7 Can Induce Type I IFNs via IRF5**—IRF5 and IRF7 have been shown to regulate the expression of overlapping as well as distinct IFNα subsets, which are encoded by at least 13 IFNA genes in humans (17, 19, 49, 54). IRF3 alone is sufficient for the induction of IFNα (16, 18, 59). Given the specific activation of IRF5 by the TLR7 pathway, we were prompted to evaluate its contribution to the induction of type I IFNs by R-848. We, therefore, monitored the activation of IFNB and IFNA1 promoter reporter genes in 2fTGH cells, which transiently expressed TLR7 in addition to FLAG-tagged IRF5. We compared this response to the parental IRF5-null cell line, which transiently expressed only TLR7. Cells either expressing or lacking IRF5 were stimulated with R-848 or infected with NDV or VSV. The IFNB reporter was not induced in parental TLR7-expressing 2fTGH cells lacking IRF5 after R-848 stimulation (Fig. 2A). IFNB reporter gene activity was, however, induced when IRF5 was expressed in these cells (Fig. 2A). Similarly, VSV, a type I IFN-inducing virus known to signal via TLR7 (9), also required IRF5 to activate the IFNB promoter. In contrast, NDV induced the IFNB reporter via IRF5 and did not require TLR7 (Fig. 2A). Similar results were obtained in all cases using the IFNA1 reporter (Fig. 2B). Noteworthy, although 2fTGH cells constitutively express IRF3, there was no induction of the IFNB promoter in response to TLR7 engagement by R-848, further supporting the observation that TLR7 signaling does not activate IRF3 (Fig. 2A).

To further evaluate the importance of IRF5 in mediating these responses, we generated a dominant negative mutant of IRF5 lacking the DNA binding domain. As shown in Fig. 2C, overexpression of this IRF5 mutant inhibited the induction of the IFNA1 promoter after R-848 stimulation. We also measured the synthesis of endogenous biologically active type I IFNs in TLR7-expressing 2fTGH cell lines in the presence or absence of IRF5. Stimulation of TLR7-expressing 2fTGH cells with R-848 did not result in the induction of endogenous type I IFNs. In contrast, ectopic expression of FLAG-tagged IRF5 conferred on these cells the ability to induce type I IFNs upon R-848 stimulation (Fig. 2D). Similar results were obtained using HEK293T cells expressing TLR7 and IRF5 (Fig. 2D). In
HEK293T cells, the induced type I IFN response consisted primarily of IFNβ, whereas in 2fTGH cells both IFNβ and IFNb were induced.

**IRF5 siRNA Impairs R-848-induced IFNA Induction**—Having shown that IRF5 enabled TLR7-expressing cells to produce type I IFNs upon R-848 stimulation, we next analyzed the requirement for IRF5 in the TLR7 pathway in a more physiologically relevant setting by using siRNA silencing technology to knock down endogenous IRF5. It is difficult to find an appropriate system to perform these studies since few cell lines express endogenous TLR7 and IRF5 and respond to R-848 to induce type I IFNs. pDCs would be the ideal cell type to study, but these cells are not amenable to siRNA silencing. However, the human monocytic cell line, THP-1, fulfilled all the criteria required, including the capability of being transfected with siRNA. As seen in Fig. 3A, THP-1 cells constitutively express IRF5 (Fig. 3A, lane 1). These cells also express IRF7, although the expression of IRF7 is lower than that of IRF5. Stimulation of these cells with R-848 further increased IRF5 and IRF7 expression (Fig. 3A, lane 4) and strongly induced both IFNB and IFNA mRNA expression (Fig. 3A), which correlated with the synthesis of endogenous biologically active IFNs. R-848 was
a much more potent inducer of IFNB and IFNA mRNA and stimulated higher levels of interferon synthesis than either NDV or VSV.

We next monitored the effect of IRF5 siRNA silencing on the TLR7-mediated IFN response in THP-1 cells. Transfection of THP-1 cells with IRF5 siRNA decreased endogenous IRF5 mRNA, whereas the LacZ siRNA control had no effect on its expression (Fig. 3B). The analysis of IFNA expression monitored by reverse transcription-PCR revealed that the induction of IFNA by R-848 was strongly reduced in cells transfected with IRF5 siRNA but not in untransfected THP-1 cells or cells transfected with the LacZ control siRNA (Fig. 3B). Taken together, these data provide strong evidence that IRF5 is an essential transducer of the TLR7-dependent induction of type I IFNs.

MyD88, IRAK1, and TRAF6 Activate IRF5 in the TLR7 Signaling Pathway—Neither LPS nor dsRNA require the adaptor MyD88 to activate the type I IFN signaling pathway. In contrast, TLR7 signaling is completely dependent on the adapter molecule MyD88. R-848 or influenza virus or VSV infection fail to induce IFNα in cells deficient in TLR7 or MyD88 (7, 10). Because the induction of type I IFNs by the TLR7 and TLR9 subgroup is dependent on MyD88, this pathway clearly differs from that induced by the TLR3 and TLR4 pathways.

We next addressed the question of whether MyD88 could couple to IRF5 and/or IRF7 activation. Consistent with this idea, the activation of the Gal4-IRF5 and Gal4-IRF7 reporter gene by R-848 in TLR7 or TLR8-expressing HEK293 cells was inhibited in a dose-dependent manner by a dominant negative mutant of MyD88 (MyD88-TIR), suggesting that MyD88 acts upstream of IRF5 and IRF7 (Fig. 4A, data not shown). In contrast, activation of the Gal4-IRF3 and Gal4-IRF7 reporter constructs in TLR3-expressing cells by pIC was unaffected by expression of the dominant negative MyD88 (Fig. 4A, data not shown).

The association of MyD88 with the TIR domains of IL-1R/TLR family members recruits the serine/threonine kinase IRAK1 (60), a critical event leading to the activation of NF-κB. Neither IRAK1 (61, 62) nor the related serine/threonine kinase, IRAK4 (63), appears to be required for the activation of IRF3 by MyD88 independent signaling pathways. We examined if IRAK1 was also important for TLR7 signaling to IRF5 using a mutant 11A-HEK293 cell line, which is deficient in IRAK1 and is defective in IL-1 signaling to NF-κB (52). Activation of IRF3 and NF-κB in TLR3 signaling has previously been shown to be intact in these cells (61). We transfected wild type or mutant 11A-HEK293 cells with either TLR7 or TLR8 and monitored the effect of R-848 on the activation of IRF5. In contrast to wild type HEK293 parental cells, TLR7- or TLR8-expressing 11A-HEK293 cells did not activate IRF5 (Fig. 4B). Similar results were obtained for IRF7 (data not shown). Although activation of the NF-κB reporter gene was also abrogated in these cells, tumor necrosis factor α induced the NF-κB reporter in both parental HEK293 and 11A-HEK293 cells (data not shown).

TRAF6 is critical for NF-κB activation by IL-1R/TLR family members; however, activation of IRF3 in TLR3 and TLR4 signaling is independent of TRAF6 (61, 64). Because IRAK1 was required for IRF5 and IRF7 activation, we wondered if TRAF6 might also function downstream of IRAK1 in this pathway. Activation of the Gal4-IRF5 reporter gene by TLR7 and TLR8 after R-848 stimulation was inhibited in a dose-dependent manner by a dominant negative mutant of TRAF6 (Fig. 4C, right panels). Similar results were obtained with IRF7 (data not shown). In contrast, a dominant negative mutant of TRAF2 did not inhibit the activation of IRF5 (Fig. 4C, left panels). These novel observations demonstrate that TRAF6 is a critical transducer in the IRF5 activation pathway and broaden our understanding of TRAF6 as a protein that participates in signaling pathways other than those leading to the activation of NF-κB and AP-1.

TBK1 and IKKε Phosphorylate IRF5 in Vitro—The IKK-related kinases, IKKε and TBK1, are essential regulators of the IFN response (31–33). They both phosphorylate and activate IRF3 and IRF7 (34, 36). Their role in IRF5 activation has not yet been addressed. We, therefore, investigated if IRF5 was also a target of TBK1 kinase activity in vitro. As seen in Fig. 5A, TBK1, but not the related kinase IKKε, efficiently phos-
phosphorylated an IRF5-GST fusion protein in vitro. Similar results were obtained with a GST-IRF3 substrate, whereas a GST alone construct was not phosphorylated by either kinase. Re-combinant IKKε also efficiently phosphorylated GST-IRF3 and IRF5. Although IKKβ failed to phosphorylate IRF5 and IRF3, it efficiently phosphorylated IκBa. Consistent with a role for IKKε and TBK1 in the regulation of the IRF5 signaling pathway, TLR7-induced activation of Gal4-IRF5 was inhibited in a dose-dependent manner by the TBK1K38A and IKKε constructs (Fig. 5B). Similar results were seen in TLR8-expressing cells (not shown). R-848 signaling via TLR7 and TLR8 also induced NF-κB activation; however, this response was unaffected by expressing TBK1K38A or IKKεK38A kinase inactive mutants (Fig. 5B). Similar results were seen in TLR8-expressing cells (not shown). R-848 signaling via TLR7 and TLR8 also induced NF-κB activation; however, this response was unaffected by expressing TBK1K38A or IKKεK38A (data not shown). To further analyze the role of TBK1 in the TLR7 signaling pathway, we attempted to monitor IFNA induction by enzyme-linked immunosorbent assay and quantitative PCR analysis in embryonic fibroblasts derived from TBK1-deficient mice. Wild type embryonic fibroblasts did not induce type I IFN after R-848 stimulation, eliminating their usefulness for this approach. Indeed, embryonic fibroblasts were found to lack IRF5 and IRF7 expression, which may explain these observations.2

**DISCUSSION**

The innate immune system has evolved several distinct viral recognition systems that integrate complex networks of signaling pathways, leading to the activation of pathway-specific transcription factors and the induction of immune response genes. TLRs together with their associated downstream signaling molecules constitute key viral recognition systems in the innate immune response. Considerable work over the last few years has revealed that a subset of TLRs (TLR3, TLR7, TLR8, and TLR9) recognize viral nucleic acids and induce type I IFNs. It is clear that the signaling mechanisms involved in the induction of IFNs differ depending on the receptor system activated. TLR3 and TLR4 signaling have been most extensively characterized, whereas much less is known about how the TLR7, TLR8, and TLR9 subfamily regulate these responses. Understanding the molecular mechanisms regulating the induction of type I IFNs by these TLRs is likely to reveal novel therapeutic and immune-modulation strategies aimed at eliminating acute and chronic viral infections.

We report here for the first time the novel finding that engagement of TLR7 and TLR8 by R-848 activates IRF5 as well as IRF7 and does not appear to activate IRF3. These observations led us to focus the present study on IRF5, since its role in TLR signaling had not been addressed previously. We discovered that IRF5 is a central mediator of TLR7 and TLR8 signaling. IRF5 contributes to IFN induction in human cells. We were surprised to find that IRF5 was important not only for IFNα but also for IFNβ induction. However, in agreement with these observations, we have recently shown that NDV-activated IRF5 induced high levels of endogenous IFNβ in B cells (65). Small interfering RNA silencing of IFNβ attenuated IFN induction in response to R-848. Although significantly reduced, this response was not completely impaired. Although there are several possibilities to explain this observation, we propose that IRF7 mediates this residual IFN response, since IRF7 is also activated in the TLR7 and TLR8 pathway in a similar manner as IRF5 (17, 66). IRF5 has previously been shown to form both homodimers as well as heterodimers with IRF3 or IRF7 in response to virus infection (67). Under these conditions, the formation of IRF5/IRF7 heterodimers can modulate the assembly of the IFNA enhancosome and alter the profile of IFNA subtypes induced. In the case of TLR7 signaling, where IRF3 is not activated, IRF5 and IRF7 may function cooperatively to regulate IFNA gene transcription. The relative roles of IRF5 and IRF7 in TLR7 signaling can best be studied when IRF5- and IRF7-deficient mice become available.

The adapter molecule TRIF was discovered based on its role in TLR3 signaling to IRF3 (37, 38). A TRAM-TRIF module
functions in the TLR4 pathway, since TRIF is not directly recruited to the TLR4-TIR domain (23, 68). Here, we report that TLR7 and TLR8 rely on MyD88, IRAK1, and TRAF6 to activate IRF5 (and IRF7), suggesting that the IKK-related kinases, IKKe (IKKi (29, 30)) and TBK1 (T2KKNAK (31–33)), phosphorylate IRF3 and IRF7 in response to certain viruses, dsRNA, or LPS signaling (34–36). In TLR7 signaling, however, it is IRF5 and IRF7 rather than IRF3 that are important for mediating the IFN response. Although we have focused this study on the role of IRF5 in IFN regulation, a key question that arises from these studies is whether IRF5 might also contribute to the regulation of additional responses, since the MyD88-TRAF6 module is critical for proinflammatory cytokine expression.

Together these studies suggest that the activation of IRF5 and IRF7 is functionally important in mediating the MyD88-dependent IFN response. Although we have focused this study on the role of IRF5 in IFN regulation, a key question that arises from these studies is whether IRF5 might also contribute to the regulation of additional responses, since the MyD88-TRAF6 module is critical for proinflammatory cytokine expression.

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MyD88- and TRAF6-dependent Activation of IRF5 and IRF7

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