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Human TLR-7-, -8-, and -9-Mediated Induction of IFN-α/β and -λ Is IRAK-4 Dependent and Redundant for Protective Immunity to Viruses

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Summary

Five TLRs are thought to play an important role in antiviral immunity, sensing viral products and inducing IFN-α/β and -λ. Surprisingly, patients with a defect of IRAK-4, a critical kinase downstream from TLRs, are resistant to common viruses. We show here that IFN-α/β and -λ induction via TLR-7, TLR-8, and TLR-9 was abolished in IRAK-4-deficient blood cells. In contrast, IFN-α/β and -λ were induced normally by TLR-3 and TLR-4 agonists. Moreover, IFN-β and -λ were normally induced by TLR-3 agonists and viruses in IRAK-4-deficient fibroblasts. We further show that IFN-α/β and -λ production in response to 9 of 11 viruses tested was normal or weakly affected in IRAK-4-deficient blood cells. Thus, IRAK-4-deficient patients may control viral infections by TLR-3- and TLR-4-dependent and/or TLR-independent production of IFNs. The TLR-7-, TLR-8-, and TLR-9-dependent induction of IFN-α/β and -λ is strictly IRAK-4 dependent and paradoxically redundant for protective immunity to most viruses in humans.

Introduction

IL-1R-associated kinase (IRAK)-4 deficiency is the first reported inherited defect in the common Toll/IL-1 receptor (TIR) signaling pathway in humans (Ku et al., 2005; Picard et al., 2003). Since the original description of three patients (Picard et al., 2003), IRAK-4 deficiency has been identified in 18 individuals with a consistent cellular phenotype (Cardenes et al., 2005; Chapel et al., 2005; Currie et al., 2004; Day et al., 2004; Enders et al., 2004; Medvedev et al., 2003; Picard et al., 2003; this report). IRAK-4-deficient blood cells have been shown not
to produce the following proinflammatory cytokines: interferleukin-1β (IL-1β), IL-6, IL-12, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), in response to IL-1β, IL-18, and all Toll-like receptor (TLR) agonists tested, including PAM3CSK4 (an agonist of TLR-1 and -2), PAM2CSK4 and Zymosan (TLR-2 and -6), Staphylococcus aureus lipoteichoic acid (LTA) (TLR-2), poly(I:C) (TLR-3), lipopolysaccharide (LPS) (TLR-4), flagellin (TLR-5), and unmethylated CpG DNA (TLR-9). Recently described TLR-7 and -8 agonists, such as imidazoquinoline compounds (imiquimod and R-848), and GU-rich single-stranded (ss) RNAs have not been tested in IRAK-4-deficient patients. Fibroblasts are the only cells from these other patients than unselected blood cells to have been tested; they do not produce IL-6 in response to IL-1β (Picard et al., 2003).

The 18 individuals with IRAK-4 deficiency originate from 11 unrelated families from Hungary (C.L. Ku et al., submitted), Portugal (Picard et al., 2003), Spain (Cardenes et al., 2005), the United Kingdom (Chapel et al., 2005), Israel (J.-L.C. and B.-Z.G., this report), Saudi Arabia (Picard et al., 2003), Turkey (Enders et al., 2004), Canada (Currie et al., 2004; C.R. and D.S., this report), and the United States (Day et al., 2004; Medvedev et al., 2003; Picard et al., 2003). IRAK-4-deficient patients suffer from pyogenic bacterial infections. Gram-positive bacteria are the most common pathogens identified in these patients, with Streptococcus pneumoniae found in most patients and Staphylococcus aureus in approximately half the patients. In contrast, only three invasive infections caused by gram-negative bacteria have been reported (Cardenes et al., 2005; Chapel et al., 2005; Medvedev et al., 2003). Interestingly, the clinical features associated with this condition seem to improve with age, as eight of the patients died in early childhood but the four oldest patients, now aged 22, 25 (monozygous twins), and 31, are well with no treatment (Chapel et al., 2005; Medvedev et al., 2003; C.R., this report). The most surprising observation remains that IRAK-4-deficient patients present such a narrow range of susceptibility to infection, with apparently normal resistance to the various infections commonly encountered in childhood, including viral illnesses in particular.

Five TLRs (TLR-3, -4, -7, -8, -9) have been shown to induce IFN-α/β and IFN-λ production (Akira and Takeda, 2004; Coccia et al., 2004; Katze et al., 2002; Pestka et al., 2004). Four (TLR-3, -7, -8, -9) are intracellular TLRs stimulated by nucleic acids that may be produced in the course of viral infections, leading to IFN-α/β and -λ production. TLR-3 can be stimulated by double-stranded RNA (Alexopoulou et al., 2001); TLR-7 and -8 (in humans only) by antiviral derivatives of nucleoside-like imidazoquinoline (Hemmi et al., 2002; Jurk et al., 2002) and loxoribine (Heil et al., 2003) and GU-rich ssRNAs (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004); and TLR-9 by unmethylated double-stranded (ds) CpG-rich DNA (Bauer et al., 2001; Hemmi et al., 2000). Cell-surface TLR-4 induces IFN-α/β and -λ after stimulation with LPS (Coccia et al., 2004; Kato et al., 2004; Toshchakov et al., 2002). However, TLR-3-deficient mice are no more susceptible than wild-type (wt) mice to vesicular stomatitis virus (VSV) and reovirus (Edelmann et al., 2004), and TLR-4-deficient mice are no more susceptible than wt mice to respiratory syncytial virus (RSV) (Ehl et al., 2004) and the related Sendai virus (van der Sluijs et al., 2003). TLR-3-deficient mice are more susceptible to mouse cytomegalovirus (MCMV) in some (Tabeta et al., 2004), but not all (Edelmann et al., 2004), conditions. TLR-3-deficient mice are paradoxically more resistant than wt mice to coxsackievirus (Fairweather et al., 2003) and West Nile virus (Wang et al., 2004). The susceptibility of TLR-9-deficient mice to MCMV provides the best evidence that TLR-induced IFNs are involved in antiviral immunity in vivo (Krug et al., 2004; Tabeta et al., 2004).

The pathways leading to IFN-α/β production have begun to be unraveled in recent years (Akira and Takeda, 2004; Coccia et al., 2004). TLRs activate a “canonical,” inflammatory signaling pathway via the TIR domain-containing molecule MyD88 (which binds to all TLRs, with the possible exception of TLR-3) and TIRAP (which binds only TLR-2 and -4) and the IRAK complex, leading to the activation of NF-κB and MAPK. “Alternative” pathways result in the production of IFN-α/β in response to the stimulation of TLR-3, -4, -7, -8, and -9. TLR-3 and TLR-4 can signal independently of MyD88, via TRIF (Hoebe et al., 2003a; Yamamoto et al., 2003), which induces IFN-β gene transcription, partly through IRF-3 activation (Servant et al., 2002). IKKε, TBK1, and NAP1 have been shown to activate IRF-3 in response to TLR-3 and -4 (Fitzgerald et al., 2003; Sasai et al., 2005; Sharma et al., 2003). TLR-7, -8, and -9 seem to activate some IFN-λ genes directly, via the formation of a MyD88-TRAF6-IRF-7 complex (Honda et al., 2004; Kawasaki et al., 2004). In mice, IRAK-1 was shown to phosphorylate IRF-7 in this process (Uematsu et al., 2005). In IRAK-4-deficient mouse cells, IFN-β is induced by TLR-4 (Suzuki et al., 2003), but the responses to stimulation of the intracellular receptors TLR-3, -7, -8, and -9 have not been examined. We investigated the production of IFN-α/β and IFN-λ in response to TLR and IL-1R agonists in IRAK-4-deficient human patients to determine why these patients display normal resistance to viruses and to define the role of human IRAK-4 and TLRs in IFN induction.

Results

IFN-α/β and -λ Induction via TLRs in Control Blood Cells

We stimulated PBMCs from healthy individuals (9 adults and 2 children) with various TLR agonists (see Supplemental Data available with this article online) and with HSV-1 and VSV as controls of IFN-α/β and IFN-λ induction. Viruses (Supplemental Data) and agonists of TLR-3 (poly(I:C)), TLR-7 (3M-13, R-848), TLR-8 (3M-2, R-848), and TLR-9 (CpG-C274, selected from various CpG oligonucleotides based on potent IFN-α/β-inducing capacity) induced detectable levels of IFN-α (Figure 1A), IFN-β (Figure 1B), and IFN-λ1 (also known as IL-29, referred to hereafter as IFN-λ; data not shown) in the supernatant, as measured by ELISA after 24 hr of stimulation. Similar results were obtained with the TLR-7 and -8 agonists GU-rich ssRNA 33 and 40 (data not shown). We did not determine experimentally whether poly(I:C), which activates various pathways in human cells (Balachandran et al., 2004; Kato et al., 2005; Maggi et al., 2000), induced IFN-α/β via TLR-3 in our assay. No such
induction was observed with agonists of TLR-1/2 (PAM3CSK4), TLR-2/6 (PAM2CSK4, Zymosan) (data not shown), TLR-4 (LPS), and TLR-5 (flagellin) (Figures 1A and 1B and data not shown). LPS, an agonist of TLR-4, also induced IFN-β mRNA production, as shown by reverse transcription and real-time quantitative PCR, 2 hr after stimulation for TLR-3, -4, -5, -7, and -8, 6 hr after stimulation for TLR-9, and 24 hr after infection for HSV-1 and VSV, corresponding to the respective peaks of induction. Means and standard deviations (SD) were calculated for each patient from two experiments, and for the controls from 11 independent individuals, each tested once. (D) PBMCs from a healthy control and from IRAK-4-deficient patient P7 were left unstimulated or stimulated with poly(I:C) (50 μg/ml), IFN-β (8 x 10^4 U/ml), or both for 36 hr. IFN-α was measured by ELISA. The experiment shown is representative of two independent experiments.

Figure 1. Production of IFN-α/β in Response to TLR Agonists, HSV-1, and VSV in Blood Cells from Healthy Controls and IRAK-4-Deficient Patients

PBMCs from nine healthy adults and two healthy children used as controls and from IRAK-4-deficient patients P1, P2, and P3 were left unstimulated, stimulated with TLR agonists, or infected with HSV-1 and VSV in the following conditions: medium (Med), poly(I:C) (50 μg/ml, an agonist of TLR-3), LPS (100 ng/ml, TLR-4), flagellin (10 ng/ml, TLR-5), 3M-13 (3 μg/ml, TLR-7), 3M-2 (3 μg/ml, TLR-8), R-848 (5 μg/ml, TLR-7/8), CpG-C274 (5 μg/ml, TLR-9), HSV-1 (MOI: 1), VSV (MOI: 1). Experiments were carried out twice for each patient.

(A and B) IFN-α secretion (A) and IFN-β secretion (B) were measured by ELISA after 24 hr of stimulation.

(C) IFN-β mRNA levels were analyzed by reverse transcription and real-time quantitative PCR, 2 hr after stimulation for TLR-3, -4, -5, -7, and -8, 6 hr after stimulation for TLR-9, and 24 hr after infection for HSV-1 and VSV, corresponding to the respective peaks of induction. Means and standard deviations (SD) were calculated for each patient from two experiments, and for the controls from 11 independent individuals, each tested once.

(D) PBMCs from a healthy control and from IRAK-4-deficient patient P7 were left unstimulated or stimulated with poly(I:C) (50 μg/ml), IFN-β (8 x 10^4 U/ml), or both for 36 hr. IFN-α was measured by ELISA. The experiment shown is representative of two independent experiments.
IFN-α/β and IFN-λ Induction via TLRs in IRAK-4-Deficient Blood Cells

PBMCs from three IRAK-4-deficient patients (P1, P2, P3) (Supplemental Data) did not respond to TLR-7, -8, and -9 stimulation by producing IFN-β mRNA (Figure 1C) and protein (Figure 1B), but did respond normally to stimulation with poly(I:C). Similar results were obtained for IFN-α (Figure 1A) and IFN-λ (data not shown) levels determined by ELISA, with normal induction by poly(I:C) and a lack of induction by TLR-7, -8, and -9 agonists, including GU-rich ssRNA (data not shown). The TLR-4-mediated induction of IFN-β mRNA in response to LPS was detected in the patients (Figure 1C). The mean levels of IFN-α/β and IFN-λ mRNA or protein produced in response to poly(I:C) and LPS in patients and controls were not statistically different (t test, p values > 0.05). Moreover, IFN-α production was normal in IRAK-4-deficient blood cells from P7 costimulated with IFN-β to increase TLR-3 expression (Siren et al., 2005) and with poly(I:C) (Figure 1D). As in two other patients tested (P1 and P7; data not shown), IFN-α/β and IFN-λ responses to TLR-3 and -4 agonists were normal in four IRAK-4-deficient patients, but no induction of IL-6 was observed in response to IL-6 was observed on ELISA (Picard et al., 2003; data not shown). Monocyte subsets, plasmacytoid dendritic cells (PDCs), and myeloid dendritic cells (MDCs) were present in normal numbers in the peripheral blood of two IRAK-4-deficient patients (P2 and P3) (Figure 2; Supplemental Data). The lack of induction of IFN-α/β and IFN-λ upon TLR-7-9 stimulation in IRAK-4-deficient patients therefore did not result from the lack of PDCs (Coccia et al., 2004; Kadowaki et al., 2001). Moreover, the normal induction of IFN-α/β and IFN-λ upon stimulation by viruses and poly(I:C) indicated that the lack of induction of IFN-α/β and IFN-λ upon TLR-7-9 stimulation in IRAK-4-deficient patients was due to impaired TLR signaling rather than deficiencies in the IFN-α/β and IFN-λ machinery.

Investigation of IFN-α/β- and -λ-Inducible Genes in IRAK-4-Deficient Blood Cells

IFN-α/β and IFN-λ induce the expression of numerous genes exerting early, innate antiviral immunity—as is the case for the myxovirus-resistance protein gene (MX1), also induced by IFN-λ (Kotenko et al., 2003)—or late, adaptive antiviral immunity—as for the genes encoding CD40, CD80, and CD86 (Akira and Takeda, 2004; Beutler, 2004; Hoebe et al., 2003b; Pestka et al., 2004). We first investigated the expression of CD40, CD80, and CD86 on the surface of blood monocytes and B cells from healthy controls and four IRAK-4-deficient patients (P2, P3, P4, and P7) after 24 hr of stimulation of monocytes and PBMCs with agonists of TLR-3, -4, -7/8 (stimulating monocytes), and -9 (B cells) (Supplemental Data). TLR-7/8 and -9 agonists did not induce the upregulation of CD40, CD80, and CD86 (Aikira and Takeda, 2004; Beutler, 2004; Hoebe et al., 2003b; Pestka et al., 2004). We then investigated the expression of CD40, CD80, and CD86 on the surface of blood monocytes and B cells from healthy controls and four IRAK-4-deficient patients (P2, P3, P4, and P7) after 24 hr of stimulation of monocytes and PBMCs with agonists of TLR-3, -4, -7/8 (stimulating monocytes), and -9 (B cells) (Supplemental Data). TLR-7/8 and -9 agonists did not induce the upregulation of CD40, CD80, and CD86 in the IRAK-4-deficient patients studied. Thus, the TLR-7-, -8-, and -9-mediated
pathway of IFN-α/β and IFN-λ induction was completely abolished in blood cells from IRAK-4-deficient patients, as shown by direct IFN-α/β and IFN-λ determination or analysis of the level of expression of IFN-α/β- and IFN-λ-inducible genes. In contrast, the TLR-3/4-IFN-α/β/λ pathway was apparently intact.

Viral Induction of IFN-α/β and IFN-λ in IRAK-4-Deficient Blood Cells

We assessed the induction of IFN-α/β and IFN-λ by 11 viruses, including ss(+)RNA (VSV, NDV, measles virus, Sendai virus, mumps virus, parainfluenza virus-III), ss(-)RNA (Sindbis virus, EMCV, coxsackievirus B1 [CVB1]), and dsDNA (HSV-1, BK) viruses (Supplemental Data). The induction of IFN-α/β by nine viruses in three patients (P2, P3, and P7) was clearly detectable, similar to (HSV-1, BK, Sendai, EMCV; t test, p values > 0.05) or weaker than (VSV, measles, Para III, NDV, Sindbis; t test, p values < 0.05) that in 14 healthy controls, as shown by Q-PCR for IFN-β (Figure 4A) and by ELISA for IFN-β (data not shown) and IFN-λ (Figure 4B). The pattern of induction of IFN-λ by these nine viruses in one patient (P7) was
similar, with the possible exceptions of NDV (high inducer) and Sindbis virus (low inducer) (Figure 4C and data not shown). Control blood cell stimulation with UV-inactivated aliquots of the nine viruses resulted in the normal induction of IFN-α (Figure 4D), IFN-β (data not shown), and IFN-λ (data not shown), suggesting that the parental viral strains did not require replication to induce IFNs. Similar results were obtained in three patients (P2, P5, and P7). We did not determine whether these nine viruses actually infected and replicated within blood cells. In contrast, P2, P3, and P7 showed little or no induction of IFN-α/β and IFN-λ in response to the remaining two viruses tested, mumps virus and CVB1 virus (Figures 4A–4C). The production of IL-6 and TNF-α in response to these two viruses was normal (mumps) or subnormal (CVB1) in the two patients tested (P2 and P3) (Figure 4E and data not shown). Moreover, the stimulation of control blood cells with UV-inactivated CVB1 virus gave impaired induction of IFN-α (Figure 4D), IFN-β (data not shown), and IFN-λ (data not shown), whereas this was not the case for the mumps virus, suggesting that infection was required for IFN production for at least one of the two IRAK-4-dependent viruses. However, neither of the viruses seemed to replicate in control and IRAK-4-deficient blood cells, as shown by a decrease in supernatant viral titers in 48 hr (data not shown).
shown). IRAK-4 may therefore be required for the induction of IFNs by CVB1 and mumps virus via its involvement in the viral cycle (Avota et al., 2001), or via its role as a critical component of the cellular IFN pathway, or both. The induction of IFN-α/β and IFN-λ by 9 of the 11 viruses tested in blood cells was nevertheless detectable, suggesting that TLR-7-9 are partly redundant in sensing these viruses in blood cells in vitro.

**IFN-β and IFN-λ Induction by Poly(I:C) and Viruses in IRAK-4-Deficient Fibroblasts**

We studied SV-40-transformed fibroblast cell lines from three of our patients (P1, P2, and P4) and from two healthy controls. Human fibroblasts express a single TLR, TLR-3, respond to only one known TLR agonist, poly(I:C), and secrete IFN-β and IFN-λ (this report). IL-6, IL-8, IFN-β, and IFN-λ were determined by ELISA in the supernatant of fibroblasts stimulated with poly(I:C), IL-1β, TNF-α, and viral stimulations in IRAK-4-deficient (P1, P2, or P4) and control fibroblasts.

![Image](image-url)

**Figure 5. IFN-β, IFN-λ, and IL-6 Induction in IRAK-4-Deficient Fibroblasts upon Stimulation by Poly(I:C), IL-1β, TNF-α, and Viruses**

Induction of IFN-β, IFN-λ, and IL-6 in response to poly(I:C) (50 μg/ml), IL-1β (20 ng/ml), TNF-α (10 ng/ml), and viral stimulations in IRAK-4-deficient (P1, P2, or P4) and control fibroblasts.

- **(A and B)** IFN-β mRNA induction, as determined by Q-PCR 2 hr after stimulation (A) and IFN-β, as determined by ELISA 24 hr after stimulation (B).
- **(C)** IFN-β and IFN-λ as determined by ELISA 24 hr after stimulation by poly(I:C), following prior treatment with IFN-α2b (10^5 U/ml for 12 hr). This experiment is representative of two independent experiments.
- **(D)** IL-6, as determined by ELISA 24 hr after stimulation. Means and standard deviations (SD) were calculated for each patient from two experiments, and for the controls from two independent individuals, each tested twice.
- **(E)** IFN-β mRNA induction 24 hr after stimulation by seven intact viruses (see Supplemental Data for the multiplicities of infection, moi). Means and standard deviations (SD) were calculated for each patient from two experiments, and for the controls from two independent individuals, each tested twice.
P1, P2, and P4. Only 7 of the 11 viruses tested induced IFN-β in two control fibroblastic cell lines (HSV-1, BK, VSV, measles, Para-III, Sindbis, EMCV); these viruses also induced IFN-λ (Figure 5E and data not shown). The stimulation of IRAK-4-deficient fibroblasts with all the viruses that activated control fibroblasts, including HSV-1 and VSV, led to normal IFN-β (data not shown) induction in the patients tested (Figure 5E). No IFN-β induction was found with five of seven UV-inactivated viruses in both controls and patients (not BK virus or HSV-1, data not shown). The induction of IFN-λ in the patients by the intact viruses was normal (data not shown). Thus, TNF-α, poly(I:C), and virus-induced, but not IL-1β-induced, IFN-β and IFN-λ production occurred normally in IRAK-4-deficient fibroblasts.

IFN-β- and IL-6-Inducing Pathways in IRAK-4-Deficient Fibroblasts Stimulated with Poly(I:C) and Viruses

IFN-β can be induced by poly(I:C) via IRF-3, NF-κB, and AP-1 (Taniuchi and Takaoka, 2002). The poly(I:C)-responsive TLR-3 signaling pathway in human cells has been shown to be IRAK-1 independent (Jiang et al., 2003). We first assessed the activation of NF-κB in IRAK-4-deficient fibroblasts stimulated with poly(I:C), IL-1β, and TNF-α. We observed normal DNA binding activity of NF-κB dimers (consisting of p50 and p65, data not shown) in response to poly(I:C) and TNF-α, in contrast to the impaired response to IL-1β observed in cell lines from patients (Figure 6A). We then investigated IRF-3 dimerization in response to poly(I:C) and found that IRF-3 dimers formed normally in the patients' fibroblast cell lines (Figure 6B). Finally, we tested the activation of p38 and JNK MAP-kinases (MAPK) by Western blotting to assess the phosphorylation of these kinases. MAPKs were activated equally strongly in response to poly(I:C) and TNF-α, in the patients' and control cell lines, but not in response to IL-1β (Figure 6C). Our findings thus indicate that the activation of IRF-3, NF-κB, and MAPK is IRAK-4 independent in human fibroblasts in response to poly(I:C) stimulation. This accounts for the normal induction of both IFN-β and IL-6 by poly(I:C) in IRAK-4-deficient fibroblasts (Figures 5B and 5D). We then assessed the activation of NF-κB and IRF-3 in response to stimulation with HSV-1 and VSV. IRAK-4-deficient fibroblasts showed normal activation of NF-κB (Figure 6D) and IRF-3 (Figure 6E). Thus, the pathways leading to IFN-β production in response to HSV-1 and VSV were intact in IRAK-4-deficient fibroblasts. This finding accounts at least partly for the normal induction of IFN-β and IFN-λ in IRAK-4-deficient fibroblasts upon stimulation with the seven viruses capable of inducing these IFNs in control cells.

TLR-Mediated IFN-Inducing Pathways in Immortalized and Fresh Blood Cells

We attempted to characterize the signaling pathways leading to IFN-α/β and IFN-λ secretion in blood cells from IRAK-4-deficient and control individuals upon stimulation by TLR agonists and intact viruses. We used EBV-transformed B cells (EBV-B cells) to study the signaling pathway downstream from TLR-7/8, as control EBV-B cells were found to respond to R-848, 3M-2, and 3M-13, but not to any other known TLR agonists (data not shown). IRAK-1 was degraded in control EBV-B cells stimulated with R-848, but not in IRAK-4-deficient cells, as shown by Western blotting (Figure 7A). TNF-α was not induced by 3M-13, 3M-2, and R-848 in IRAK-4-deficient EBV-B cells from P2 and P3, in contrast to control cells, as measured by ELISA (Figure 7B). These results confirm that IRAK-4 is a critical component of TLR-7/8 signaling, not only in freshly prepared blood cells, but also in EBV-B cell lines, in which IRAK-4 is located upstream from IRAK-1 in this signaling pathway. No IFN-α/β or IFN-λ was produced upon stimulation of control EBV-B cells with TLR-7/8 agonists or HSV-1 (data not shown). We then used fresh peripheral blood mononuclear cells to investigate the signaling pathways triggered by poly(I:C), LPS, and R-848. NF-κB p65 was translocated normally to the nucleus of monocytes from control and IRAK-4-deficient individual P2 in response to poly(I:C) and LPS, but not in response to R-848 in IRAK-4-deficient monocytes, as shown by immunofluorescence (Figure 7C). Nuclei were visualized by DAPI staining (data not shown) or phase contrast microscopy (Supplemental Data). Moreover, IRAK-1 was degraded upon R-848 stimulation in control monocytes, but not in IRAK-4-deficient monocytes, as shown by Western blotting with a specific antibody (Figure 7D). Thus, the signaling pathway leading to IFN-α/β secretion by blood cells stimulated with poly(I:C) and LPS involves NF-κB but does not require IRAK-4; conversely, the pathway leading to IFN-α/β secretion in blood cells stimulated with R-848 involves NF-κB and requires IRAK-4 and IRAK-1.

Discussion

IRAK-4 deficiency in humans abolishes the induction of IFN-α/β and IFN-λ via TLR-7, -8, and -9 in blood cells. We demonstrated this by measuring IFN-α, IFN-β, and IFN-λ levels and by studying the expression of IFN-α/β-inducible target genes, such as MX1 and the genes encoding the costimulatory molecules CD40, CD80, and CD86. As TLR-7 and TLR-8 agonists, we used the imidazoquinoline compounds R-848, 3M-2, and 3M-13 and GU-rich ssRNAs 33 and 40 and, as a TLR-9 agonist, the potent IFN-α/β inducer CpG-C. We also showed that IRAK-4-deficient monocytes and EBV-B cells did not activate IRAK-1 in response to TLR-7/8 stimulation. This suggests that IRAK-4 and IRAK-1 are both involved in TLR-7/9 signaling and that human IRAK-1, downstream from IRAK-4, may be critical for IRF-7 phosphorylation, as recently shown in the mouse (Uematsu et al., 2005). Indeed, a complex formed between MyD88, TRAF-6, and IRF-7 has been shown to mediate signaling via TLR-7/8 to induce IFN-λ (Honda et al., 2004; Kawai et al., 2004). Our data thus demonstrate that human IRAK-4 and, probably, IRAK-1 are critical, nonredundant components for IFN-α/β induction by blood cells in response to TLR-7, TLR-8, and TLR-9. Our study indicates that the regulation of IFN-λ by TLRs follows that of IFN-α/β, with IRAK-4 being critical for the TLR-7/9-mediated induction of both IFN-α/β and IFN-λ.

In contrast, human IRAK-4 is largely dispensable for the induction of IFN-α/β and IFN-λ in response to TLR-3 and -4 agonists in blood cells, even upon coinduction of these TLRs by exogenous IFN-β. IRAK-4-deficient
Figure 6. IFN-β-Inducing Signaling Pathways in Control and IRAK-4-Deficient Fibroblasts Stimulated with Poly(I:C) and Viruses

SV40-transformed fibroblast lines from a control and from patients P1 and P2 were left unstimulated (labeled “NS”) or stimulated with poly(I:C) (50 μg/ml), IL-1β (20 ng/ml), TNF-α (10 ng/ml), and viruses (see Supplemental Data for the multiplicities of infection).

(A) DNA binding activity of nuclear extracts from fibroblasts left unstimulated or stimulated with poly(I:C) for 2 hr, IL-1β for 20 min, and TNF-α for 40 min was assessed by EMSA, using an NF-κB-specific DNA probe.

(B) IRF-3 dimers and monomers detected by Western blotting in whole-cell extracts from fibroblasts left unstimulated or stimulated with poly(I:C) for 2 hr.

(C) Phospho-p38, phospho-JNK, and p38 detected by Western blotting in cytoplasmic extracts from fibroblasts left unstimulated or stimulated with poly(I:C) for 1 hr and IL-1β and TNF-α for 20 min.

(D) DNA binding activity of nuclear extracts from fibroblasts left unstimulated or stimulated with HSV-1 or VSV for 4, 8, and 12 hr was assessed by EMSA, via an NF-κB-specific DNA probe.

(E) IRF-3 dimers and monomers detected by Western blotting in whole-cell extracts from fibroblasts left unstimulated or stimulated with VSV for 12 hr. These experiments are each representative of two experiments.
fibroblasts also responded normally to poly(I:C) in terms of MAPK, NF-κB, and IRF-3 activation, resulting in normal IFN-β induction, even upon coinduction of TLR-3 by exogenous IFN-α. Thus, the TLR-3-IRF-3/NF-κB/MAPK-IFN-β and the less well-known TLR-3-IFN-λ signaling pathways in fibroblasts do not require IRAK-4 to be operational. Our study also suggests that IRAK-4 is redundant for other TLR-3-mediated signals, as the induction of IL-6 by poly(I:C) was normal in IRAK-4-deficient fibroblasts. However, blood cells produced little IL-6 upon stimulation by poly(I:C) (Picard et al., 2003). A human cell line bearing a somatic mutation in IRAK-1 was previously shown to respond normally to TLR-3 stimulation by poly(I:C) (Jiang et al., 2003). In mice, the TLR-4-mediated induction of IFN-α/β has been shown to be independent of IRAK-4 (Suzuki et al., 2003). Our findings show that the induction, in blood cells, of both IFN-α/β and IFN-λ by poly(I:C) is IRAK-4 independent. The induction of IFN-α/β in response to LPS was less intense in humans (this report) than in mice (Suzuki et al., 2003; Toshchakov et al., 2002).

Blood cell subsets expressing these five TLRs were present in IRAK-4-deficient patients: neutrophils (which normally express TLR-4), eosinophils (TLR-7), B cells (TLR-7 and TLR-9), NK cells (TLR-3 and TLR-9), and the main producers of IFN-α/β and IFN-λ—monocytes (TLR-4 and TLR-8), MDCs (TLR-3), and PDCs (TLR-7 and -9) (Bekeredjian-Ding et al., 2005; Bourke et al., 2003; Coccia et al., 2004; Diebold et al., 2003; Kadowaki et al., 2001; Nagase et al., 2003; Sabroe et al., 2002; Sivori et al., 2004). The results obtained in healthy individuals suggest that PDCs (expressing TLR-7 and TLR-9, triggered by 3M-13 and CpG, respectively) and monocytes (TLR-8, 3M-2) produce similar, large amounts of IFN-α/β. TLR-3-expressing MDCs were found to produce IFN-α/β in response to poly(I:C), but in smaller amounts. We did not test the IFN response of individual cell subsets to TLR-3/4 and TLR-7/9 stimulation. However, TLR-7-expressing eosinophils and B cells (Bekeredjian-Ding et al., 2005; Nagase et al., 2003) produce little, if any, IFN-α/β, and TLR-4-expressing neutrophils (Sabroe et al., 2002) were not present in PBMCs. We did not test other cell types shown to express both TLR-3 and IFN-α/β, such as mast cells (Kulka et al., 2004) and epithelial cells (Schaefer et al., 2005), or TLR-4 and IFN-β, such as microglial cells (Jung et al., 2005).
Human IRAK-4 is thus probably a critical component of the TLR-7/9 pathway leading to IFN-α/β and IFN-λ production by monocytes and PDCs and a redundant component of the TLR-3/4-IFN pathway in monocytes and MDCs.

At least 20 common viruses infect children worldwide, and most children have been infected with these viruses by the age of 18 years. These viruses include dsDNA, ssDNA, ss(+)RNA, ss(−)RNA, and dsRNA viruses (Casanova and Abel, 2004). Several of the 18 known IRAK-4 patients have been infected with some of these viruses, as shown by serological testing: HSV-1, CMV, EBV, VZV, HHV-6, parvovirus B19, rubella virus, RSV, and human metapneumovirus (Supplemental Data). Many patients have also probably been infected by many other widespread viruses, such as adenoavirus, influenza virus, papillomavirus, enterovirus, and coronavirus. As IRAK-4-deficient patients are naturally resistant to most human-tropic viruses, in natural infection conditions in vivo (Casanova and Abel, 2004), our results suggest that the TLR-7/9 induction of IFN-α/β and IFN-λ in humans is redundant in protective immunity to viruses. This in turn implies that the TLR-mediated production of IFN-α/β and IFN-λ by human PDCs is redundant. Resistance in vivo correlates with our observation that 9 of 11 viruses tested induced normal or low, but not nil, levels of IFN-α/β and IFN-λ in IRAK-4-deficient blood cells in vitro. The induction of IFN-α and IFN-λ by fibroblasts was also normal, implying that viral induction of IFNs in human cells may be TLR dependent (Boehme and Compton, 2004; Finberg and Kurt-Jones, 2004) but IRAK-4 independent. IRAK-4 appears to be redundant for protective immunity to most viruses in humans.

However, as only 18 patients with IRAK-4 deficiency are known, we cannot exclude the possibility of ascertainment bias. Patients with fatal viral infections, involving mumps virus and coxsackie viruses in particular, which did not induce IFN-α/β and IFN-λ in IRAK-4-deficient blood cells in vitro, may have remained undiagnosed (Casanova and Abel, 2004; Ku et al., 2005). Nevertheless, four patients were vaccinated with attenuated mumps virus with no adverse effect and mounted a specific antibody response, whereas three others had tested positive in serological tests for mumps virus. Moreover, six patients had high titers of serum antibodies against coxsackie viruses. Careful and prolonged follow-up of the known patients is also warranted. The observation that imiquimod, an agonist of TLR-7/8, is active against warts caused by human papillomaviruses (HPV) suggests that the patients should be carefully followed up for HPV-induced mucocutaneous lesions. More patients with IRAK-4 deficiency, from diverse ethnic groups and living in various geographical areas, thus need to be diagnosed and followed up before we can draw firm conclusions regarding their exact infectious phenotype, particularly as concerns viral susceptibility and resistance. Nevertheless, this study strongly suggests that IRAK-4 deficiency is associated with resistance to most common viruses.

There is currently little evidence that IRAK-4 and TLR-7/9 play an important role in antiviral immunity, even in the mouse model of experimental infection. TLR-9-deficient mice are susceptible to the only virus tested, MCMV (Krug et al., 2004; Tabeta et al., 2004). In one study, IRAK-4-deficient mice showed impaired IFN-γ production in response to lymphocytic choriomeningitis virus (LCMV), but the outcome of infection was not determined (Suzuki et al., 2002). The human model of natural infections offers the advantage of defining the ecologically relevant functions of immune genes in host defense (Casanova and Abel, 2004). The TLR-7/9-IRAK-4-IFN pathway appears to be redundant in protective immunity against natural infections with common viruses. Our previous observation of lethal viral diseases in patients with complete STAT-1 deficiency and impaired cellular responses to IFN-α/β (Dupuis et al., 2003) provides conclusive evidence that human IFN-α/β is crucial for antiviral immunity. The production of protective levels of IFN-α/β and IFN-λ in IRAK-4-deficient and proficient patients may be dependent on TLR-3 and TLR-4. Alternatively, this production may be TLR independent, mediated by other sensors such as PKR (Maggi et al., 2000), FADD (Balachandran et al., 2004), and RIG-I (Kato et al., 2005). It is possible that multiple sensors, including TLRs, operate collectively, certain sensors being of particular importance for some viruses and not others. The best approach to resolving this important question will be to search for novel germline mutations in human pathways leading to IFN-α/β and IFN-λ production (Casanova and Abel, 2005).

**Experimental Procedures**

**Viral Stimulations**

We stimulated PBMCs from the IRAK-4-deficient patients with various intact viruses: ss(−)RNA (vesicular stomatitis virus [VSV, strain Indiana], Newcastle disease virus [NDV, strain BR24 444], measles virus [strain Edmonton], Sendai virus [strain E92], mumps virus [vaccine strain Uraue], parainfluenzae virus III [Para-III, strain EA102], ss(+)RNA [Sindbis virus [strain VR1248 ATCC], encephalomyocarditis virus [EMCV], coxsackievirus B1 [CVB1, strain Conn-5]), and dsDNA ([herpes simplex virus-1 [HSV-1], strain KOS-1; BK, an isolate from a patient provided by Pierre Lebon, Paris) viruses (Supplemental Data).

**Cytokines, TLR Agonists, and ELISA**

PBMCs isolated by Ficoll-Paque density gradient centrifugation or SV-40-transformed fibroblasts were treated with 10 μg/ml of polymyxin B for 30 min (except in the case of LPS stimulation) and stimulated with various agonists of TLRs, IL-1β, and viruses. The supernatant was recovered after 24 hr of stimulation. ELISA tests for IFN-α (AbCys SA, Paris, France) and IFN-γ (TFB, Fujirebio, Inc., Tokyo, Japan) were performed according to the kit manufacturer’s instructions. The IFN-λ ELISA was developed in the laboratory. Plates were coated with 1 μg/ml of anti-human IFN-λ mAb (AF1959, R&D Systems) overnight at 4°C, and the IFN-λ levels in the supernatant were estimated with a secondary biotinylated anti-human IFN-λ mAb (BAF1598, R&D Systems) used at a concentration of 400 μg/ml (Supplemental Data).

**Antibodies and Flow Cytometry**

Peripheral blood mononuclear cells (PBMCs) were stained with antibodies and were analyzed by flow cytometry, using a CyanADP machine and Summit 4.1 software (DakoCytoformation). Monocytes isolated from PBMCs by negative selection (CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A) with a monocyte isolation kit (Miltenyi Biotech GmbH) were left unstimulated or were stimulated with poly(I:C), LPS, and R-848. For B cell studies, PBMCs were left unstimulated or were stimulated with CpG-C (Supplemental Data).

**Determination of mRNA Levels by Q-PCR**

Total RNA was extracted with Trizol reagent (Invitrogen) from unstimulated PBMCs or from PBMCs stimulated with agonists of TLRs and viruses. RNA was reverse transcribed directly with Oligo-dT.
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assays (EMSA) were performed with an NF-

line at http://www.immunity.com/cgi/content/full/23/5/465/DC1/. and Experimental Procedures and can be found with this article on-

confocal microscope.

lecular Probes, Interchim, Montluc¸on, France) for 30 min. Nuclei
followed by Alexa Fluor 488 goat anti-rabbit antibody (A-11034; Mo-
clonal antibody against p65 (C-20, sc-372, Santa Cruz) for 30 min,

lized with 0.1% Triton X-100 (Sigma), and incubated with rabbit poly-

LPS, and R-848, were fixed with 3% paraformaldehyde, permeabi-

Monocytes, unstimulated or stimulated for 1 or 2 hr, with poly(I:C),

Intracellular Staining

Monocytes, unstimulated or stimulated for 1 or 2 hr, with poly(l:C), LPS, and R-848, were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma), and incubated with rabbit polyclonal antibody against p65 (C-20, sc-372, Santa Cruz) for 30 min, followed by Alexa Fluor 488 goat anti-rabbit antibody (A-11034; Molecular Probes, Interchim, Montluçon, France) for 30 min. Nuclei were stained with DAPI or observed by phase contrast microscopy. The slides were analyzed by confocal microscopy on an LSM 510 confocal microscope.

Supplemental Data

Supplemental Data include one figure and Supplemental Results and Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/23/5/465/DC1/.

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