Supplemental Data

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

Kun Yang, Anne Puel, Shenying Zhang, Céline Eidenschenk, Cheng-Lung Ku, Armanda Casrouge, Capucine Picard, Horst von Bernuth, Brigitte Senechal, Sabine Plancoulaine, Sami Al-Hajjar, Abdulaziz Al-Ghonaium, László Maródi, Donald Davidson, David Speert, Chaim Roifman, Ben-Zion Garty, Adrian Ozinsky, Franck J. Barrat, Robert L. Coffman, Richard L. Miller, Xiaoxia Li, Pierre Lebon, Carlos Rodriguez-Gallego, Helen Chapel, Frédéric Geissmann, Emmanuelle Jouanguy, and Jean-Laurent Casanova

Supplemental Results

IFN-α/β Induction by TLR Agonists and Viruses in Blood Cells Is Not Mediated by TNF-α

Neither IL-1β nor TNF-α induced detectable secretion of IFN-α/β and IFN-λ or IFN-β mRNA production in control and IRAK-4-deficient blood cells (data not shown). The induction of IFNs by poly(I:C) in IRAK-4-deficient patients was TNF-α independent, as TNF-α was not detectable in IRAK-4-deficient blood cells stimulated with poly(I:C) and recombinant TNF-α did not induce IFNs in control and IRAK-4-deficient blood cells (data not shown). IRAK-4-deficient blood cells stimulated with the 11 viruses selected produced similar amounts or less TNF-α than control blood cells (data not shown), but IFN induction was TNF-α independent, as suggested by the lack of induction of IFNs by recombinant TNF-α (data not shown) and demonstrated by the normal IFN induction observed in virus-stimulated control and IRAK-4-deficient cells incubated with a TNF-α-specific blocking antibody (data not shown).

Supplemental Experimental Procedures

Patients and Healthy Individuals

14 of the 18 known IRAK-4-deficient patients were enrolled in our study, corresponding to 8 of the 11 known kindreds (see Introduction). 5 of these 14 patients died before our study was initiated, and the remaining nine were tested for IFN production and/or viral infection (serological testing, P1 to P9). IRAK-4-deficient patients P1 and P2 have been described elsewhere (Picard et al., 2003). P1, a Saudi boy, died of pneumococcal meningoencephalitis shortly after this study was carried out, at the age of 7 years. P2 is a French boy of Portuguese descent, now 13 years old, with no ongoing treatment and asymptomatic chronic pansinusitis. Patient 3 (P3) is a 31-year-old British woman, currently well off all treatment despite occasional deep-seated staphylococcal abscesses (Chapel et al., 2005). Patient 4 (P4) is a 5-year-old Hungarian boy, doing well on prophylactic antibiotics and immunoglobulin substitution (C.L. Ku et al., submitted). Patient 5 (P5) is an 8-year-old Spanish boy, doing well on prophylactic antibiotics despite occasional cutaneous staphylococcal abscesses (Cardenes et al., 2005). Two IRAK-4-deficient third-degree relatives of this patient died of invasive pyogenic bacterial disease in childhood. Patient 6 (P6) is a 3-year-old Canadian child, of European descent, doing well on prophylactic antibiotics (D.S., unpublished data). His IRAK-4-deficient brother died of pneumococcal disease in childhood. Patient 7 (P7) is an 8-year-old Israeli child, who had pneumococcal sepsis and meningitis despite prophylactic treatment with amoxicillin. His sister died at the age of 2.5 months, from fulminant sepsis due to *Streptococcus milleri* (B.Z.G., unpublished data). Patient 8 (P8) is a 25-year-old Canadian of English descent who had multiple bacterial invasive infections during infancy and childhood, including meningitis, septic arthritis, and multiple abscesses (C.R., unpublished data). Patient 9 (P9) is the monozygotic twin of P8 and he too suffered from pneumococcal meningitis and upper airway abscesses. Both twins are now active and well off all treatment, but their sister died at the age of 5 months from bacterial meningitis. The patients suffered from invasive infections caused by *Streptococcus pneumoniae* (P8, P9) and *Staphylococcus aureus* (P8). The IRAK-4 mutations and clinical features of the patients will be reported elsewhere. All patients suffer from IRAK-4 deficiency, with impaired secretion of proinflammatory cytokines in response to TLRs and IL-1Rs. The patients have suffered from invasive infections caused by *Streptococcus pneumoniae* (P1-P9), *Staphylococcus aureus* (P1-2), and *Shigella sonnei* (P3). Although exposed to common viruses, such as ss(-)RNA RSV (P1-6), ds DNA human metapneumovirus (P1, P3-6), dsDNA EBV and VZV (P1-3, P5, P8-9), dsDNA HSV (P8-9), dsDNA CMV (P4-5), dsDNA HHV-6 (P1-6), ssDNA parvovirus B19 (P1, P3), and attenuated ss(+)RNA rubella virus (P1-4, P6), as shown by the positive results obtained in serological tests, they had not suffered from serious infections with these pathogens. P2, P3, P5, and P7 received live measles/mumps/rubella (MMR) vaccine with no adverse effect and tested positive for measles, mumps, and rubella viruses in serological tests. P4, P6, and P7 tested positive for mumps virus, and P1-P7 tested positive for coxsackie virus. In addition, most IRAK-4-deficient patients have probably been infected with at least other dsDNA viruses

(papillomavirus, molluscipoxvirus, adenovirus), dsRNA viruses (rotavirus), ss(+)RNA viruses (enterovirus, rhinovirus, calicivirus, coronavirus), and ss(-)RNA viruses (influenza virus, paramyxovirus), which are almost ubiquitous and cause common childhood infections. Not all the patients in our study were tested, and we did not apply all assays to all the patients investigated. We also tested 11 healthy volunteers as controls: nine adults (aged 25-45 years) and two children (aged 9 and 13 years).

Viral Stimulations

We treated 5 x 10^5 PBMC in RPMI supplemented with 10% FCS with 10 μg/ml polymyxin B for 30 min. We then added VSV (5 x 10^5 particles, moi = 1), Sindbis virus (10^5 particles, moi = 0.2), measles virus (2 x 10^3 particles, moi = 0.004), Sendai virus (6.25 x 10^6 particles, moi = 12.5), mumps virus (2 x 10^4 particles, moi = 0.04), Para-III (2 x 10^4 particles, moi = 0.04), NDV (2.5 x 10^5 particles, moi = 0.5), EMCV (5 x 10^4 particles, moi = 0.1), CVB1 (2.5 x 10^5 particles opsonized with an equal volume of 5 mg/ml IgG for 5 min, moi = 0.5), HSV-1 (5 x 10^5 particles, moi = 1), or BK (10^4 particles, moi = 0.02). The cells were incubated for 24 hr. Supernatants were then recovered and subjected to ELISA tests for IFN-α/β and IFN-λ1. We extracted RNA from the cell pellets for real-time quantitative RT-PCR for IFN-β. For experiments with SV-40-transformed fibroblasts, we seeded each well of a 24-well plate with 10^5 cells in DMEM supplemented with 10% FCS. We added VSV (10^5 particles, moi = 1), Sindbis virus (10^5 particles, moi = 1), measles virus (2 x 10^3 particles, moi = 0.02), Sendai virus (1.25 x 10^6 particles, moi = 12.5), mumps virus (4 x 10^3 particles, moi = 0.04), Para-III (4 x 10^3 particles, moi = 0.04), NDV (5 x 10^4 particles, moi = 0.5), EMCV (10^4 particles, moi = 0.1), CVB1 (2.5 x 10^5 particles opsonized with an equal volume of 5 mg/ml IgG for 5 min, moi = 2.5), HSV-1 (10^5 particles, moi = 1), or BK (5 x 10^3 particles, moi = 0.02). Cells were incubated for 24 hr and we then recovered supernatants and cell pellets for ELISA and real-time RT-PCR tests for IFN-β. Viruses were inactivated by UV irradiation for 7 min. Inactivation was documented by titrating the viruses on VERO cells (with a decrease in viral load of at least four orders of magnitude).

Cytokines and TLR Agonists

A synthetic analog of dsRNA, polyinosine-polycytidylic acid (poly(I:C), a TLR-3 agonist), and lipopolysaccharide (LPS Re 595 from *Salmonella minnesota*, a TLR-4 agonist) were purchased from InvivoGen (San Diego, CA) and Sigma-Aldrich (Lyon, France), respectively. Flagellin (a TLR-5 agonist) was provided by K.D. Smith, Institute for Systems Biology, Seattle, WA. R-848, resiquimod hydrochloride (a TLR-7/8 agonist) was purchased from GLSynthesis Inc. (Worcester, MA), whereas 3M-2 (a TLR-8 agonist) and 3M-13 (a TLR-7 agonist) were provided by Richard L. Miller of 3M Center (3M Pharmaceuticals, St Paul, MN). Unmethylated CpG DNA CpG-C (C274): 5′-TCGTCGAACGTTCGAGATGAT-3′ was provided by Robert Coffman and Frank Barrat from Dynavax Technologies (Berkeley, CA). Single-stranded RNAs (TLR-7/8 agonists), ssRNAs 33 and 40 were purchased from IBA GmBH (Göttingen, Germany), and IL-1β and TNF-α were obtained from R&D Systems Inc. (Minneapolis, MN). PAM_3CSK_4 bacterial triacetylated lipopeptide (TLR-1 and -2 agonist) and PAM_2CSK_4 bacterial diacetylated lipopeptide (TLR-2 and -6 agonist) were obtained from EMC Microcollections (Tübingen, Germany), Zymosan (TLR-2 and -6 agonist) was obtained from Molecular Probes (Invitrogen, Cergy Pontoise Cedex, France). *S. aureus* lipoteichoic acid (LTA) (TLR-2 agonist) was supplied by Sigma-Aldrich (Lyon, France), and polymyxin B was supplied by Sigma-Aldrich (Lyon, France). In some experiments, cells were first treated with IFN-α2b (Schering-Plough, Brussels, Belgium), IFN-β (Schering-Plough, Brussels, Belgium) or anti-TNF-α mAb (R&D Systems Inc., Minneapolis, MN).

Antibodies for Flow Cytometry

PBMCs were stained with antibodies against HLA-DR (biotinylated, clone L243) plus Cascade Blue-streptavidin (Molecular Probes, Invitrogen, Cergy Pontoise, France), CD14 (PE, clone M5E2), CD16 (PE/FITC, clone 3G8), from Becton Dickinson Biosciences Pharmingen, San Diego, CA, CD20 (PE, clone 2H7), from Bioscience, San Diego, CA, CD123 (APC, cloneAC145), BDCA-1 (FITC, cloneAD5-8E7) and BDCA-2 (FITC, clone AC144), from Miltenyi Biotech GmbH, Bergisch Gladbach, Germany. For the study of the induction of costimulatory molecules, after 24 hr of incubation, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD40 (clone 5C3), anti-CD80 (clone L307.4), and anti-CD86 (clone 2331, FUN-1) antibodies, phycoerythrin (PE)-conjugated anti-CD14 (clone M5E2) antibody and PE-conjugated anti-CD19 (clone HIB19) antibody (BD Biosciences Pharmingen), and relevant isotype control antibodies: FITC-conjugated mouse $IgG_{1,\kappa}$ mAb (clone MOPC-21), PE-conjugated mouse $IgG_{1,\kappa}$ mAb (clone MOPC-21), PE-conjugated mouse $IgG_{2a,\kappa}$ mAb (clone 20102.1), and were analyzed by flow cytometry.

Determination of mRNA Levels by Q-PCR

Glucuronidase (GUS) was used as the calibrator gene. Quantitative real-time PCR (Q-PCR) was performed with Applied Biosystems Assays-on-Demand probe/primer combinations and 2X universal reaction mixture, in an ABI PRISM 7700 Sequence Detection System. Results are expressed using the ΔΔCt method, as described by the manufacturer.

Supplemental References

Cardenes, M., von Bernuth, H., Garcia-Saavedra, A., Santiago, E., Puel, A., Ku, C.L., Picard, C., Casanova, J.L., Colino, E., Bordes, A., et al. (2005). Invasive bacterial disease in fourth-degree relatives with autosomal recessive IRAK-4 deficiency. J. Pediatr., in press.

Chapel, H., Puel, A., von Bernuth, H., Picard, C., and Casanova, J.L. (2005). *Shigella sonnei* meningitis due to interleukin-1 receptor-associated kinase-4 deficiency. Clin. Infect. Dis. *40*, 1227-1231.

Picard, C., Puel, A., Bonnet, M., Ku, C.L., Bustamante, J., Yang, K., Soudais, C., Dupuis, S., Feinberg, J., Fieschi, C., et al. (2003). Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science *299*, 2076–2079.

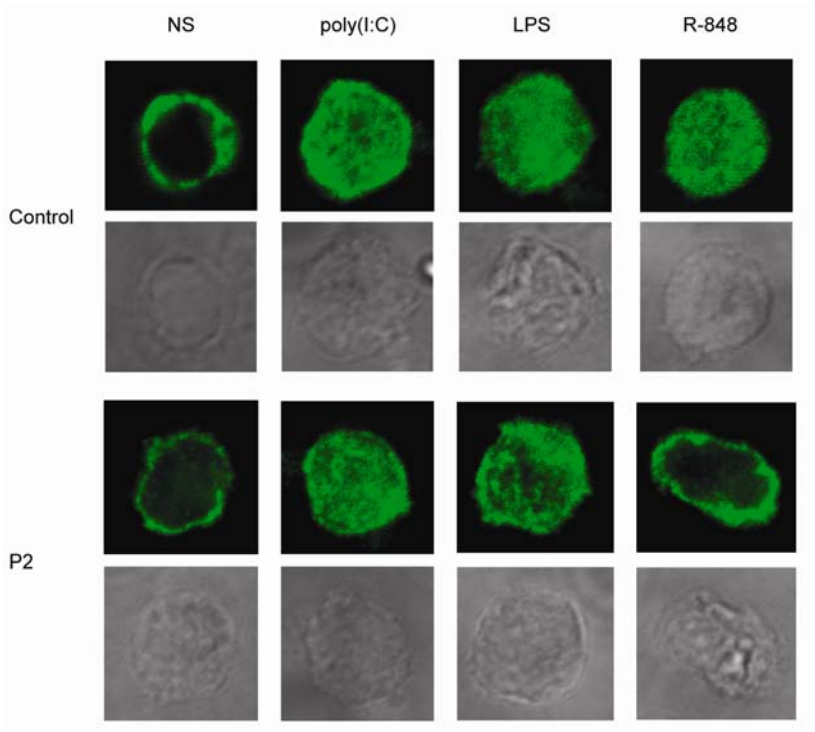

Figure S1. Immunofluorescence Analysis of NF-κB p65 Subunit Subcellular Localization in Monocytes from Control and IRAK-4-Deficient Peripheral Blood Monocytes Stimulated with Poly(I:C), LPS, and R-484

NF-κB p65 subunit subcellular localization in monocytes from peripheral blood mononuclear cells left unstimulated or stimulated with poly(I:C) (50 μg/ml), LPS (1 μg/ml), or R-848 (5 μg/ml) for 2 hr, as determined by immunofluorescence analysis (top) and phase contrast microscopy (bottom) for detection of cell nuclei.